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Succinate production in *Escherichia coli*

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Abstract

Succinate has been recognized as an important platform chemical that can be produced from biomass. While a number of organisms are capable of succinate production naturally, this review focuses on the engineering of *Escherichia coli* for production of the four-carbon dicarboxylic acid. Important features of a succinate production system are to achieve optimal balance of reducing equivalents generated by consumption of the feedstock, while maximizing the amount of carbon that is channeled to the product. Aerobic and anaerobic production strains have been developed and applied to production from glucose as well as other abundant carbon sources. Metabolic engineering methods and strain evolution have been used and supplemented by the recent application of systems biology and in silico modeling tools to construct optimal production strains. The metabolic capacity of the production strain, as well as the requirement for efficient recovery of succinate and the reliability of the performance under scale-up are important in the overall process. The costs of the overall biorefinery compatible process will determine the economical commercialization of succinate and its impact in larger chemical markets.

Keywords

Metabolic engineering; Industrial biotechnology; Succinate; Genetic engineering; Biomass feedstock

Introduction

The role of microbial production of useful chemicals from biomass has become of considerable interest to companies, governments, and downstream users as environmental concerns and high petroleum prices provoke assessment of long-term strategies. The higher value of chemicals vs biofuel molecules has also led to heightened interest on the part of biofuel companies as production of such chemicals in a biorefinery could contribute significantly to the overall value generated by the operation. This review will focus on succinate and strategies for engineering its economic production.

Succinic acid has been considered a valuable molecule that could be produced by microorganisms, however the placement of this molecule on the DOE list of twelve platform chemicals from biomass [1] spurred widespread attention to its potential [2]. Among the

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molecules that can be derived from succinic acid by known chemical processes are 1,4-butanediol, maleic anhydride, succinimide, 2-pyrrolidinone and tetrahydrofuran, all of which have large markets and can be converted to a wide range of other valuable molecules for use in polymers [3], industrial solvents, and specialty chemicals e.g. biodegradable succinate esters [4, 5]. The use of biodegradable polymers in tissue engineering applications is also of interest in the biomedical community [6].

Succinate is used in foods as a flavoring agent as it contributes a somewhat sour and astringent component to umami taste (e.g. in shellfish) and is often added to foods [7]. It is widely available as the mono or disodium salt for adding flavor to meats, soups etc. Succinate is used as a counter ion in many pharmaceutical formulations. Various derivatives of succinate such have found uses for their innocuous properties in food. Esterification with various small alcohols produces additives for the food processing industry. Octenyl succinate modified starch is used as an emulsifier and thickener in many foods requiring an appropriate texture or consistency in the product. Sodium dioctyl sulphosuccinate is used as a surfactant and wetting agent in textile & printing products and was a component of the Corexit dispersant used in the recent Gulf oil spill cleanup operations.

Zeikus *et al.* [1999] reported a relatively small succinic acid market at 15,000 ton/year worldwide. Recent estimates of the market potential for succinic acid and its immediate derivatives were projected to be as much as 245×10^3 ton/year, with a market size for succinic acid-derived polymers up to 25×10^6 ton/year [8]. While succinate is produced petrochemically from butane through maleic anhydride, fermentation process costs have become more competitive at about $0.55–1.10 per kg [9], and further reductions in cost are anticipated. Several companies and affiliates such as Mitsubishi Chemical, Japan; Myriant Technologies, USA; Diversified Natural Products, USA; Michigan Biotechnology Institute International, USA; BASF, Germany; DSM, The Netherlands and Roquette, France; BioAmber, France are setting up commercial biobased succinate production plants and planning for additional production in Asia.

Recent reviews of succinate production [10] and recovery of succinate from fermentation broth [11] provide background of general interest. The recent development of a succinate production process with *E. coli* via engineering and strain evolution has been covered in recent articles with attention to genetic methods and culture optimization [12–14] and some earlier articles have discussed the strategies and efforts in succinate production in engineered *E. coli* [15–17].

While many organisms produce succinate at low levels the analysis of natural isolates that produce a high proportion of succinate as a final metabolite gives a broader context for the engineering efforts. The anaerobic environment of cattle rumen has provided a rich source of such organisms with several widely studied microorganisms capable of high natural production of succinic acid being isolated from rumen. These anaerobic organisms have been studied extensively and key aspects of their specialized metabolism relating to redox and formation of key intermediates such as oxaloacetate have been analyzed. Work with *Actinobacillus succinogenes* [18] has been recently reviewed [19–21] and it worthwhile to note its early use in electrochemical stimulation of succinate production [22]. Methods to obtain variants of this organisms have also been reported [23]. Recent work with this organism has analyzed the metabolic fluxes [24, 25] and has also focused on the organisms ability to use a number of low cost carbon sources for succinate production [26–29]. Another rumen organism used for succinate production has been *Anaerobiospirillum succiniciproducens* and examined in terms of its use of various carbon feedstocks and high yield production [30–32] and culture innovations [33]. The organism *Mannheimia succiniciproducens* was isolated [34], sequenced [35] and genetic tools developed were

rapidly generated for the organism [36, 37]. Workers on the organism have studied the metabolism and its novel features have provided an illustration of the application of systems biology and computational approaches to engineering a new organism for production [37–40]. A genome-scale in silico metabolic model [41, 42] and "omics" has given better understanding of cellular physiology and metabolism [39, 43] and development of genetic tools [36, 39, 44] allowed genome-based metabolic engineering [39, 40]. *Corynebacterium glutamicum* strains [45, 46] and *Zymomonas mobilis* ZM4 [47], have also been studied. BASF in collaboration with CSM announced production in a bio-succinate facility located in Spain utilizing a newly isolated strain of rumen bacteria, *Basfia succiniproducens* [48]. A continuous cultivation culture of this bacterium has been studied by Scholten *et al.* [49] to produce 5.21 g/L succinate from 5.1 g/L glycerol with productivities and yields of 0.094 g/L/h and 1.02 g/g, respectively. Yeasts have also been explored for succinate production as the highly acid and osmotolerance are advantages for succinic acid production [50]. In glucose-grown shake flask cultures, the quadruple deletion strain *Δsdh1Δsdh2Δidh1Δidp1* produced succinic acid at a titer of 3.62 g/L at a yield of 0.11 mol/mol glucose. A new strategy employed *Yarrowia lipolytica* with a deletion in the gene coding one of succinate dehydrogenase subunits was reported [51] and accumulated succinate at the level of 45 g/L in shaking flasks with $CaCO_3$ buffering. DSM and Roquette have jointly developed a process using engineered *S. cerevisiae* SUC-297 that produced 43 g/L succinic acid in 95 h along with 16.4 g/L ethanol and 14.9 g/L glycerol [52]. The major advantage is that it operates at low pH which prevents bacterial contamination and aids the downstream purification process. These and other organisms have provided a context for the engineering of *E. coli* for succinate production and this will be the main focus for subsequent sections of the short review. *E. coli* remains a preferred organism for testing new succinate technologies due to the extensive knowledge of its genome, proteome, availability of genetic tools, simple nutrient requirements and facile cultivation.

The ability to grow easily on a variety of abundant feedstocks is an important practical advantage in developing a commercially viable process. The above organisms as well as some engineered *E. coli* strains have been examined for their ability to use waste agricultural material and increasingly a focus has been on the use of lignocellulosic derived biomass as a feedstock [53–55]. The processing of such material will not be covered here, only some features needed for efficient use of the mixture of hexoses and pentoses without severe inhibition due to residual acids. Several articles have focused on the use of xylose from hemicellulose found in baggasse [26], straw [56] or corn cobs [29] since this sugar is more readily released upon limited acid hydrolysis. These studies have shown promise in generating useful yields from the pentose available from such biomass sources.

Metabolic engineering strategies

Many microorganisms are natural producers of a wide variety of compounds of industrial interest, e.g. antioxidants, polymers, amino acids, hydroxyacids and chiral alcohols, among others. However, in many cases the production processes are not economically feasible due to a low product yield, low productivity, and/or difficulties on cultivating the native producers. Product yield and productivity are affected by a variety of factors. Reducing equivalents, and the metabolic pathway used by the cells for product synthesis is critical, different pathways leading to the same product could require different precursors and, have different reducing equivalents and energy requirements and theoretical yields [57].

Metabolic engineering (ME) is generally defined as the rational redesign of biological system using genetic engineering techniques by modifying existing or introducing new metabolic pathways to improve production of certain valuable compounds. Therefore, ME principles are applied to the design and construction of more efficient metabolic pathways to

increase product yield and productivity, either in the native producer organism or a in a more suitable host [58]. Genes can be deleted to eliminate competing pathways [59–62], overexpressed to increase enzyme pool [61, 63] or introduced from a different organism into a suitable host for industrial purposes [64, 65]. The current availability of genomic information for many organisms has allowed a rapid increase in ME research.

The selected substrate together with the metabolic pathway used by the microorganism will determine the maximum theoretical yield of the product of interest according to carbon and redox balances as well as energy requirements. Substrates with higher reduction states (e.g. glycerol) favor the synthesis of the more reduced products [66–69]. Industrially, the relative cost of the feedstock is an important component of production and this as well as environmental factors need to be considered in the commercial context. The increasing genetic information available together with the development of computational tools has led to the development of different methodologies and models to explain or describe cell metabolic networks attempting to interconnect cell genotype to the corresponding phenotype [70].

An important tool used in ME is the metabolic flux analysis (MFA); this technique studies the effect of genetic changes in the distribution of pathway fluxes. Unknown metabolic fluxes are calculated using experimentally measured metabolite concentrations together with a stoichoimetric model that includes all the important reactions in the network, where the metabolic fluxes are determined for a specific culture condition. In recent years, ^{13}C labeled substrates together with NMR or GC-MS techniques and complex computational schemes have been applied to solve the unknown metabolic flux vector [71]. Information obtained from MFA can serve to identify potential “bottleneck” (rate-limiting) reactions and to understand the effect of the genetic alterations in cell metabolism [72]. Further genetic modifications designed based on the MFA results have demonstrated whole-cell biocatalysis improvement [73, 74].

Other important methodologies to analyze cell metabolic fluxes are the flux balance analysis (FBA) and the metabolic pathway analysis (MPA). FBA uses linear programming which main difference with MFA is the use of an objective function, e.g. maximize cell growth or product formation, under defined constrains such as a determined substrate uptake rate and thermodynamic constrains. It can be used when not enough information is available for MFA. The result of this analysis is one optimal set of fluxes for a specific growth condition, but there are potentially many sets that would satisfy the system. Some examples of application of FBA have included the identification of the gene products essential for *E. coli* aerobic or anaerobic growth [75]. On the other hand, the MPA determines all the metabolic flux vectors possible in a specific metabolic network without the need of an objective function or previously defined flux rates [70]. All three methods, MFA, FBA and MPA assume no changes in internal metabolites concentrations at steady state.

The elementary mode analysis, a type of MPA, where the elementary modes are defined as a the minimum sub-set of reactions that allows the metabolic network to operate at steady state [76], has been applied to identify potential gene deletions or overexpression that would increase the synthesis of a desired product. This analysis has been applied to evaluate the potential use of glycerol for succinate production under either aerobic or anaerobic conditions [77]. Several other computational tools have been developed that include the methodologies mentioned above or a combination of them [78–80].

Engineering *E. coli* for succinate production

Under anaerobic conditions, wild type *E. coli* produces a mixture of fermentation products including acetate, formate, ethanol and minor amounts of succinate, while under aerobic

conditions succinate is formed only as an intermediate of the TCA cycle unless the glyoxylate bypass is operating (Figure 1). In wild type *E. coli* the maximum theoretical yield of succinate is 1 mol/mol glucose in anaerobic conditions; this yield is limited by reducing equivalents availability (NADH). Strategies to improve succinate production in *E. coli* have included the elimination of competing pathways; the activation of pathways with lower reducing equivalents requirement, and the evolution of strains with improved succinate producing capabilities. Several mutant and recombinant strains have been constructed and tested under aerobic [81–83] anaerobic [12, 84] or two-stage fermentation, where the first stage is aerobic for biomass accumulation and the second stage is anaerobic for succinate production [85, 86]. A summary of metabolic engineered strains and conditions for succinate production was presented recently by Jantama and collaborators [84]. Some of the approaches are described below and the general pathway strategy is shown in Figure 2.

One of the first approaches to produce succinate in *E. coli* was to eliminate competing pathways by inactivating pyruvate-formate lyase gene (*pflB*) and lactate dehydrogenase (*ldhA*). This strain, named NZN111, only produced minor amounts of succinate and showed growth impairment on glucose in anaerobic conditions [87, 88]. This result was most likely due to a redox imbalance and intermediate metabolite accumulation, especially pyruvate [86]. A spontaneous NZN111 mutant strain, named AFP111, generated succinate as the major product; where succinate, acetate and ethanol were produced in a 2:1:1 ratio and molar yields of 1.0, 0.5 and 0.5 mol/mol glucose, respectively [88]. The spontaneous mutation was in the *ptsG* gene, encoding a glucose specific phosphotransferase system. Later, inactivation of *ptsG* in several *E. coli* mutant strains was shown to change product profile, favoring succinate production with various yields [89]. On the other hand, the overexpression of the native NAD^+-dependent malate dehydrogenase or the NAD^+-dependent malic enzyme in NZN111 resulted in a restoration of the strain ability to grow anaerobically on glucose and the strain produced succinate, with a yield of 1.19 and 1.04 mol/mol glucose, respectively [61, 90]. In addition, native genes whose overexpression can mitigate growth impairment under microaerobic conditions in NZN111 have been identified. Most of these genes were involved in oxidation-reduction reactions or cofactor synthesis related to electron transport [91].

From strictly a carbon balance arising from three-carbon precursors, two moles of succinate could be produced from one mole of glucose consumed, upon appropriate carboxylation and redox availability. To increase succinate yield, competing pathways have been inactivated, the pyruvate carboxylase (*pyc*) from *Lactococcus lactis* has been overexpressed and the glyoxylate shunt has been activated. The activation of the glyoxylate shunt contributes an alternative pathway for succinate production with a lower reducing equivalent requirement, leading to a dual pathway to produce succinate. This led to a maximum theoretical succinate yield of 1.6 mol/mol glucose [92] achieved experimentally with a metabolic engineered strain, SBS550MG, carrying *ldhA adhE ackpta::Cm iclR* inactivations and overexpressing the *pyc* from *L. lactis* in a two-stage system [85]. Related strains yielded also formed succinate (58.3 g/L) under aerobic conditions with an overall yield of 0.85 mol/mol glucose [83]. *E. coli* C strains, KJ060 and KJ073, have been reported to produce 622–733 mM of succinate with molar yields of 1.2–1.6 per mol of metabolized glucose [84]. On the other hand, it has been hypothesized that other strains showing yields over 1 mol/mol glucose have a mutation that activates pyruvate dehydrogenase (PDH) complex in anaerobic conditions, with this being the source of the extra NADH available for succinate production [12].

To produce succinate, CO_2 fixation is required, one molecule of CO_2 is incorporated to phosphoenolpyruvate (PEP) to form oxaloacetate (OAA) catalyzed normally by PEP carboxylase (PPC). In succinate production processes, CO_2 is usually provided in the form

of carbonate (e.g. $MgCO_3$, $NaHCO_3$) or directly by CO_2 sparging in a bioreactor. CO_2 sparging serves a double purpose, maintaining anaerobic conditions for succinate production and supplying the required CO_2 for OAA formation from PEP. The effect of CO_2 concentration in the gas phase has been evaluated on succinate production by AFP111. Going from 0 to 50% CO_2 showed a dramatic increase in succinate specific productivity, however, the increase of CO_2 concentration above 50% did not show a significant improvement in succinate production [93].

To increase metabolic flux to succinate, several enzymes involved in CO_2 fixation have been overexpressed, including PPC and PEP carboxykinase (PEPCK, gene *ppck*), which transform PEP into OAA with the incorporation of one molecule of CO_2 and the generation of one Pi or ATP, respectively [57, 83, 94, 95]. In addition, pyruvate carboxylase (PYC) and malic enzyme, that incorporates one molecule of CO_2 into pyruvate to form OAA and malate, respectively, have also been overexpressed [12, 95]. The overexpression of PEPCK from *A. succinogenes* has an advantage over the native PPC in that it generates one molecule of ATP for each PEP converted to OAA. The ability to favor the carboxylation direction is important and specific enzymes derived from natural succinate producers or mutant PEPCK enzymes have been beneficial [57]. To increase availability of carbon dioxide for fixation, a cyanobacterial carbonic anhydrase has been cloned in a succinate producing host and improved the yield of succinate from glucose [96].

Several strains have been constructed where genes involved in succinate competing pathways, among others, were inactivated and no foreign genes were expressed. This approach together with several rounds of metabolic evolution, selecting for strains with improved growth in minimal medium, using glucose as sole carbon source, have led to strains with enhanced capability to produce succinate. Engineered *E. coli* strains KJ060 (Δ*ldhA,* Δ*adhE,* Δ*ackA,* Δ*focA,* Δ*pflB*) and KJ073 (Δ*ldhA*, Δ*adhE*, Δ*ackA*, Δ*focA*, Δ*pflB*, Δ*mgsA*, Δ*poxB*) showed succinate yields of 1.2–1.6 mol/mol glucose and succinate concentrations of about 70–80 g/L. However, significant amounts of acetate and malate were also produced [84]. Further improvements, based on *E. coli* strain KJ073, were achieved by inactivating threonine decarboxylase (*tdcD*), 2-ketobutyrate formate-lyase (*tdcE*) and phosphotransacetylase (*pta*) genes, to conserve pyruvate for succinate production and decrease acetate synthesis; and inactivating aspartate aminotrasferase (*aspC*) and the NAD^+-malic enzyme (*sfcA*) to conserve oxaloacetate and fumarate for succinate production. The resulting strain, KJ134, showed a succinate yield of 1.5 mol/mol glucose and a decrease in acetate production of about 80%, compared to the parent strain KJ073 [12].

An important approach to increase succinate production has been to manipulate glucose transport and the metabolic steps related to ATP generation [84]. In these strains PEPCK (*ppck*) was found to catalyze the carboxylation of PEP into OAA, increasing ATP generation. This change was related to a promoter mutation that increased gene expression [13]. In addition, a mutation in *pstI* resulting in the inactivation of the PEP-phosphotransferase system (PTS) was identified. The PTS function was spontaneously replaced by the D-galactose transporter (*galP*) and glucokinase (*glk*) by increasing their expression. These strains have higher PEP availability for succinate production and higher ATP level, showing increased succinate production and improved growth [97]. The resulting pathway was closely related to the native pathway for succinate production in *A. succinogenes* [13].

With each engineered strain the optimal growth and production conditions need to be examined and the optimal production window and the effect of parameters such as temperature, pH, cation, feedstock concentration, level of aeration if any, needs to be defined for appropriate scale-up.

Production of succinate using different carbon sources

Various carbon sources such as glucose, sucrose, xylose, galactose, and glycerol have been examined by different research groups. Recently, efforts are being made to use renewable agriculture resources with pretreatment to release sugar molecules for succinate production. Two of the most interesting feedstocks are sucrose and glycerol due to their abundance and low cost. See Figure 2 for how these feedstocks can fit into the engineered pathways discussed above.

Recently, Wang *et al.* [98] have engineered an *E. coli* strain capable of fermenting fructose, sucrose, and glucose mixtures. Xylose has been examined as sole carbon source for succinate production using *E. coli.* In a dual-phase batch fermentation containing 100 g/L of total initial xylose, *E. coli* strain AFP184 produced succinate with a yield of 0.50 g/g [99].

Researchers have demonstrated the use of various agricultural wastes and byproducts to produce succinate. Production of succinate from orange peel and wheat straw by consolidated bioprocessing using a cellulolytic bacterium, *Fibrobacter succinogenes* S85, was studied [100] giving succinate titers of 1.9 and 2.0 g/L respectively. Wheat flour milling byproducts have been used as the sole medium for *A. succinogenes* fermentations, which led to the production of 50.6 g/L succinic acid [101]. Corncob hydrolysate [29], crop stalk wastes, [28] corn stover [102], sugarcane bagasse hemicellulose hydrolysate, corn fiber hydrolysate [27] and galactose were studied for succinate production [30]. *E. coli* engineered to use various sugars in lignocellulose hydrolysates have been discussed [55].

Glycerol has attracted attention as a feedstock for production of bio-based chemicals due to its abundance, low cost, and high degree of reduction. Lee *et al.* [103] showed that *A. succiniciproducens* can efficiently convert glycerol to succinate. Succinic acid production from glycerol yields low levels of acetate which is advantageous for downstream process recovery of succinic acid [104]. By integrating the restriction of oxygen and redox sensing/ regulatory system, elementary mode analysis was used to predict the metabolic potential of glycerol for succinate production by *E. coli* under either anaerobic or aerobic conditions [77]. Pathways and mechanisms for the utilization of glycerol by *E. coli* in minimal salts medium under microaerobic conditions have been reported and *E. coli* has been engineered for the production of succinate from glycerol [66]. Zhang *et al.* [105] reengineered fermentative metabolism of *E. coli* to convert glycerol to succinate under anaerobic conditions without the use of foreign genes. In mineral salt medium, an *E. coli* strain XZ721 (*pck** Δ*ptsI* Δ*pflB*) fermented 128 mM glycerol to 102 mM succinate with a molar yield of 0.8.

Downstream processing factors

In order to develop competitive biotechnological process for succinate production with petrochemical process, it is necessary to minimize the production costs. A recent review provides an overview of this topic [106]. Downstream processing can contribute about 60% of the total production costs, e.g. the isolation and the purification of the product from the fermentation broth [107]. Various methods such as precipitation, sorption and ion exchange, electrodialysis, and liquid–liquid extraction have been investigated for the recovery of succinic acid from fermentation broth. The first step is separation of cell debris which is performed by ultrafiltration through a bypass crossflow, hollow-fiber ultrafiltration unit [108].

The classical industrial method for the isolation of carboxylic acids from fermentation broth is precipitation with calcium hydroxide or calcium oxide [109–111] followed by filtration. The calcium salt is then treated with sulfuric acid which results in byproduct calcium sulfate

after recovery of the succinic acid. Precipitation with ammonia has been described [112, 113]. With ammonia precipitation, succinic acid can be refined with a yield of 93.3% [113]. For some uses the ammonium salt is desirable for a specific downstream purpose such as the production of pyrrolidones [114].

The application of electrodialysis for downstream processing of succinic acid has been studied on a laboratory scale and is useful for industry. A desalting electrodialysis combined with a water-splitting electrodialysis was proposed by Glassner *et al.* [115]. A yield of 77% of succinic acid was achieved after electrodialysis. To yield a higher purity (>99%) of succinic acid the aqueous solution is subjected to anionic and cationic ion exchangers [111, 116, 117]. Disadvantages of electrodialysis are the high energy consumption, the material costs of the membranes and the low selectivity for succinic acid if other substances are present at high concentrations [106].

Liquid-liquid extraction is a commonly used method in the chemical industry. Most conventional extraction agents show very unfavorable distribution coefficients for organic acids. Reactive extraction is considered to be an effective primary separation step for the recovery of carboxylic acids [118] and long-chain aliphatic primary, secondary and tertiary amines have been proposed [119–122]. Reactive extraction with aliphatic amines depends on many factors [123]. If the separation process enables the recycling of the costly amines efficiently, then an optimized reactive extraction processes might serve a role in industrial production of bio-succinic acid although it is better suited to the processing of higher value compounds.

Summary

Metabolic engineering of *E. coli* to produce succinate has been a fruitful challenge, not only in producing higher yields of the product but as an example of the strategies and new techniques used for engineering high flux to a desired compound of medium value. The need to coordinate redox and carbon metabolism as well as consider the uptake of feedstock carbon and its partitioning at key metabolic nodes has illustrated key features that need to be addressed in other metabolic engineering efforts. Recently, the placement of such engineered strains into an appropriate industrial or biorefinery setting for implementation, has brought up the more traditional process engineering aspects of microbial production of chemicals and work on succinate has added to this broader, applied field. For the development of a highly efficient technology certain challenges such as developing high succinate tolerant strain with maximum theoretical yield and optimizing fed-batch cultivation method to increase and maintain the volumetric succinic acid productivity needs to be considered. Succinate production with no by-product formation would be desirable for the most efficient use of substrate and for less expensive downstream methods.

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Abbreviations

FBA	flux balance analysis
ME	metabolic engineering
MFA	metabolic flux analysis
MPA	metabolic pathway analysis

OAA	oxaloacetate
PEPCK	PEP carboxykinase
PEP	phosphoenol pyruvate
PPC	PEP carboxylase
PTS	PEP-phosphotransferase system
PYC	pyruvate carboxylase
PDH	pyruvate dehydrogenase

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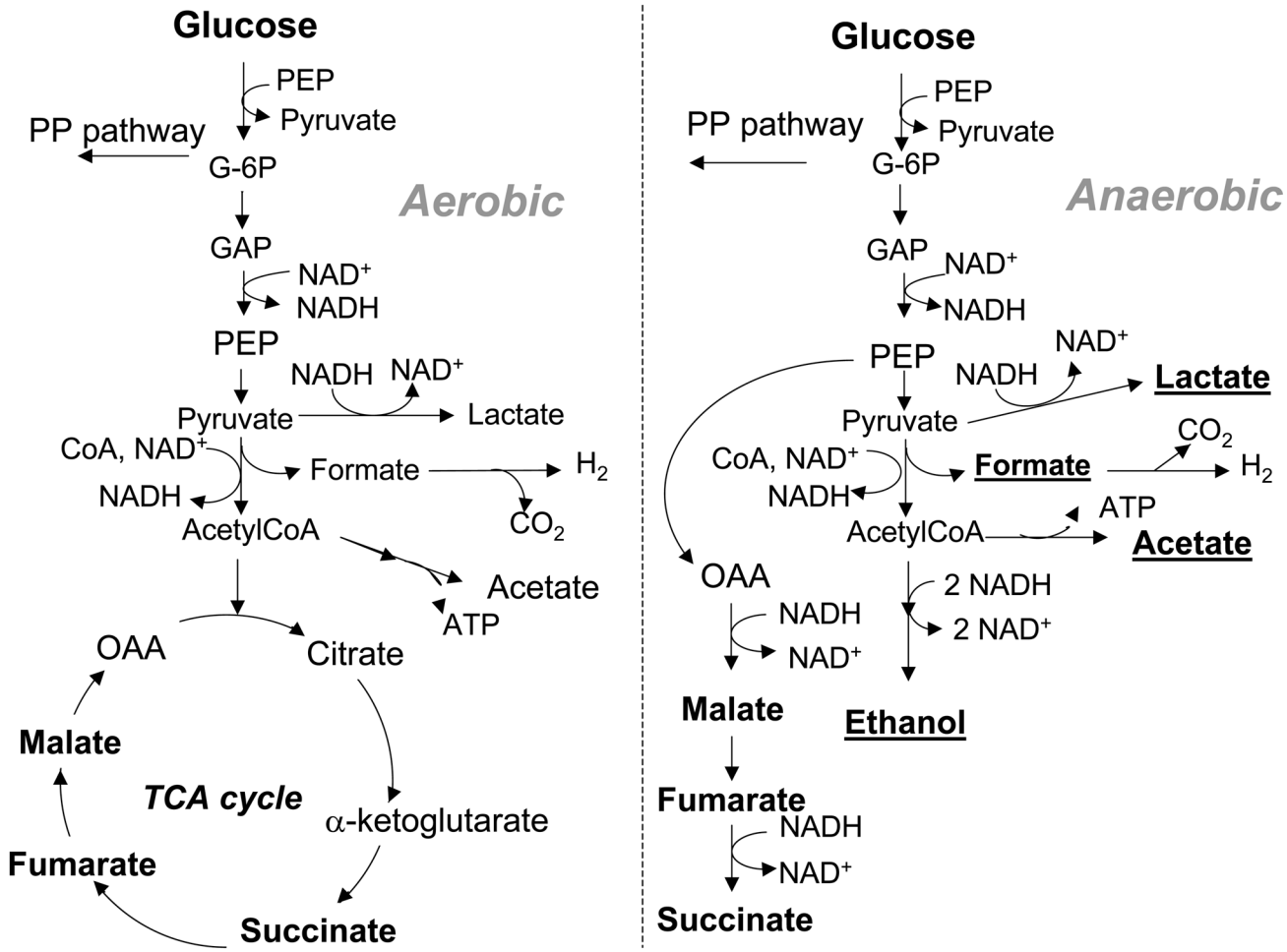

Figure 1.

Normal metabolic routes to succinate in wild type *E. coli*. The aerobic metabolism does not produce succinate as a final product unless the glyoxylate bypass is in operation. The anaerobic metabolism produces the mixed acid fermentation products, ethanol, acetate, formate, lactate and succinate, while the TCA cycle does not function.

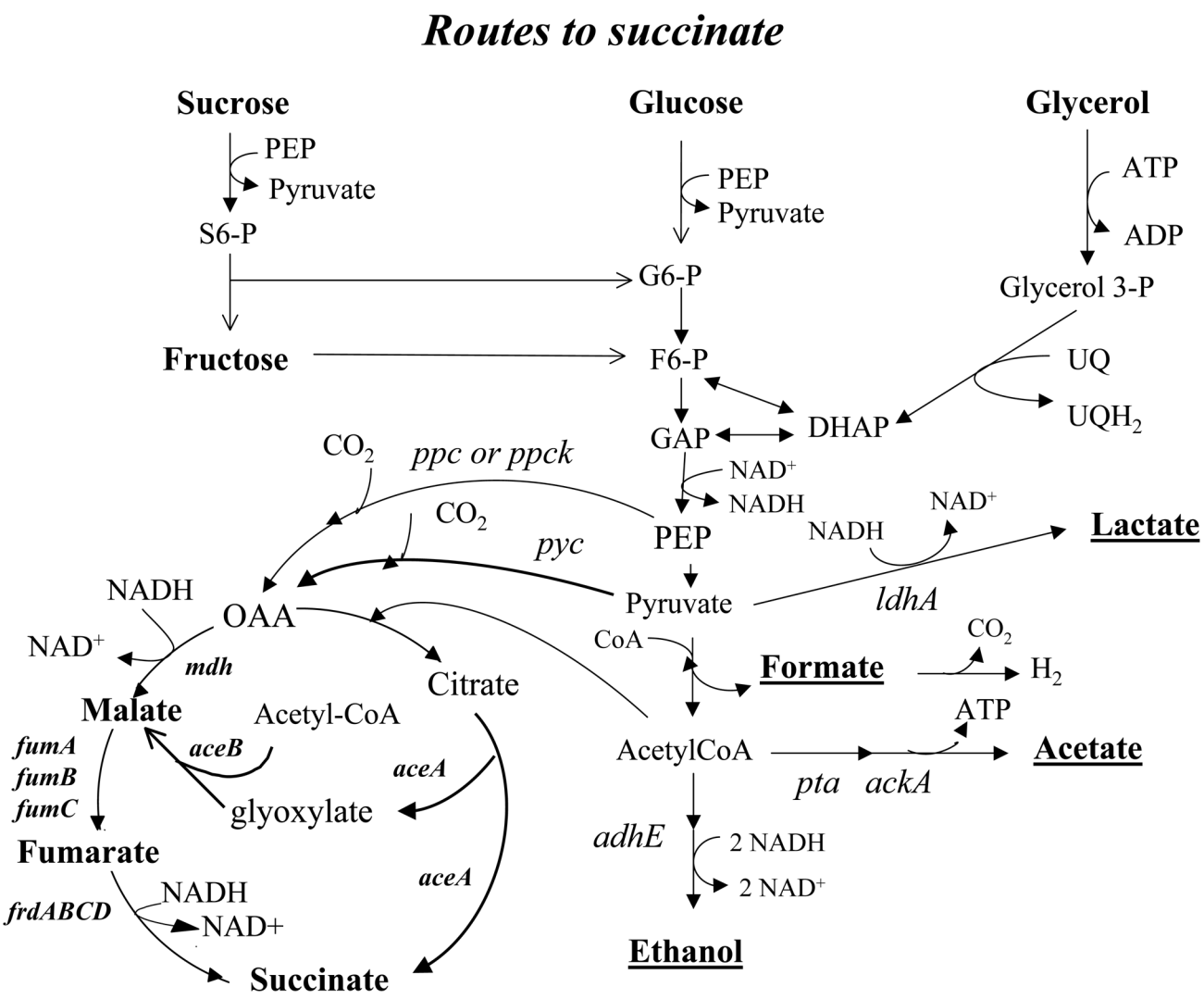

Figure 2.

Routes for overproduction of succinate in engineered *E. coli*. The routes from glucose, glycerol and sucrose to succinate are shown. The glycolytic reactions form PEP and pyruvate. Typically the routes to the other fermentation products, ethanol, lactate and acetate would be inactivated by disruption of genes *ldhA*, *pta* and/or *ackA*, and *adhE*. The glyoxylate bypass enzymes encoded by *aceA* and *aceB* would be expressed to convert the citrate via isocitrate to glyoxylate and succinate without requiring reductant. In the glyoxylate bypass, malate is formed by condensation of glyoxylate with acetyl-CoA formed from pyruvate. OAA is formed from PEP by either phosphoenolpyruvate carboxylase (*ppc*) or by phosphoenolpyruvate carboxykinase (*ppck*). OAA is reduced to malate by malate dehydrogenase (*mdh*) and the fumarase genes *fumA*, *fumB*, and *fumC* encode isoenzymes, with FumB appearing to play the major role. Fumarate is then reduced by fumarate reductase encoded by the *frdABCD* operon.