

Review

Insights on the Advancements of In Silico Metabolic Studies of Succinic Acid Producing Microorganisms: A Review with Emphasis on *Actinobacillus succinogenes*

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Abstract: Succinic acid (SA) is one of the top candidate value-added chemicals that can be produced from biomass via microbial fermentation. A considerable number of cell factories have been proposed in the past two decades as native as well as non-native SA producers. *Actinobacillus succinogenes* is among the best and earliest known natural SA producers. However, its industrial application has not yet been realized due to various underlying challenges. Previous studies revealed that the optimization of environmental conditions alone could not entirely resolve these critical problems. On the other hand, microbial in silico metabolic modeling approaches have lately been the center of attention and have been applied for the efficient production of valuable commodities including SA. Then again, literature survey results indicated the absence of up-to-date reviews assessing this issue, specifically concerning SA production. Hence, this review was designed to discuss accomplishments and future perspectives of in silico studies on the metabolic capabilities of SA producers. Herein, research progress on SA and *A. succinogenes*, pathways involved in SA production, metabolic models of SA-producing microorganisms, and status, limitations and prospects on in silico studies of *A. succinogenes* were elaborated. All in all, this review is believed to provide insights to understand the current scenario and to develop efficient mathematical models for designing robust SA-producing microbial strains.

Keywords: in silico metabolic modeling; succinic acid; *Actinobacillus succinogenes*; pathways

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1. Introduction

In the past few years, tremendous attempts and successes have been witnessed in the development of the green economy through the production of chemicals, fuels, materials, etc., from bio-based sources [1]. To begin with, succinic acid (SA) is one of the top potential value-added chemicals that can be produced biotechnologically from biomass resources [2,3]. More interestingly, SA as a high-value platform chemical can be co-produced with high-yield products such as biofuels via integrated biorefinery approaches [4,5] that could offset the process cost [6] and also alleviate waste management issues. In fact, the application of SA ranges from being a specialty chemical in pharmaceutical, food and agricultural areas to being a precursor for industrially important bulk chemicals [7,8]. Among the well-known SA producers are natural host rumen bacteria, model microorganisms and non-conventional microbial cell factories. To this end, *Actinobacillus succinogenes*, being one of the best natural SA producers, has been given more emphasis in the current review.

From the pre-genomic era to date, the metabolic control and optimization of environmental conditions have been implemented in the microbial production of value-added commodities. However, obviously, there are other complicated genotypic traits that are

beyond the appliance of these strategies. In addition, traditional mutagenesis and screening methods have also been employed in the development of improved strains to produce desired products [9]. The success stories of these traditional methods in strain improvement are still undeniable. However, despite being time- and resource-consuming, they could also pose some problems such as the possession of undesired mutations [10], uncontrolled multiple traits, and irreversible damage to the host cell [11]. Furthermore, Lee et al. explained that it could be challenging to recognize which genes have to be manipulated from many lists of genes of a given organism to generate the desired phenotype [12]. On the other hand, taking advantage of technological advancements in high-throughput (HT) techniques and the availability of vast genomic data provides a new paradigm for the rational design of cell factories via the development of predictive mathematical models [9,13]. Hence, these mathematical models would assist in predicting *in silico* outcomes of genetic and phenotypic traits rather than choosing desired strains resulting from tedious random mutagenesis [9]. Besides, *in silico* metabolic models combined with other computational, evolutionary and comparative genomic analyses could serve as proof-of-concept for the successful metabolic engineering of cell factories for the synthesis of desired products such as SA [12,14].

Despite the availability of numerous studies and huge potential applications, there is a lack of up-to-date comprehensive reviews covering the implications of *in silico* metabolic modeling in SA production. The only claimed review in this regard was first published online at the end of 2016 [14]. Therefore, the current study included topics that were not discussed in the previous review, viz., *A. succinogenes*, new advancements and prospective in this area. This review presented highlights on advancements of research and development (R&D), metabolic pathways and *in silico* studies generally on SA production and specifically regarding *A. succinogenes*. Finally, the status quo and future perspectives of *in silico* metabolic modeling in SA production were summarized. We believe this review can be taken as the first step (from the lists below) in the quest towards the development of robust SA-producing hosts by applying computational methods: (1) understand, summarize the current scenario, identify gaps and suggest possible prospects, (2) design and employ state-of-the-art models, (3) validate via experimentation and literature, and (4) implement an *in silico* model *in vitro* and *in vivo*.

2. Major R&D Advancements on SA and *A. succinogenes*

When we look back to timelines of SA, its discovery, application and production should be assessed. Figure 1 demonstrates major milestones in the history of SA and *A. succinogenes*. To begin with, documents revealed that SA was first purified in 1546 by distillation from amber (SA is also known as amber acid), and since then it has been used for various applications [15,16]. However, until recently, the majority of SA has been derived from petroleum-based sources. Encouragingly, SA was proposed twice as “top value-added chemicals from biomass” in 2004 [2] and in the revised 2010 [3] studies. Following this, the first commercial bio-based SA production plants were launched and it was reported that these companies contributed half of the annual global SA production [17,18]. As far as *A. succinogenes* is concerned, a patent for the production of SA using this strain was registered in 1996 [19], and the first strain isolated from bovine rumen was published in 1999 [20].

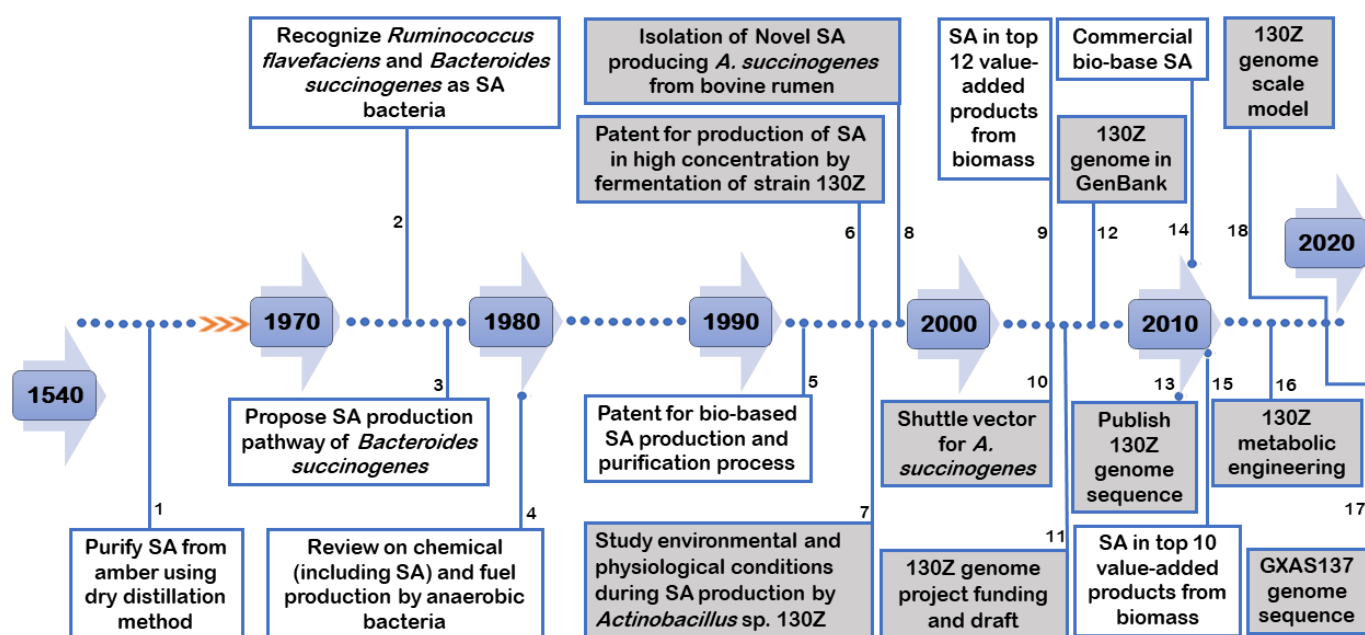


Figure 1. Major timelines of succinic acid and *A. succinogenes*. The shaded boxes represent *A. succinogenes*. The numbers represent the sources of the studies: 1 [15,16], 2 [21], 3 [22], 4 [23], 5 [24], 6 [19], 7 [25], 8 [20], 9 [2], 10 [26], 11 [27], 12 [27], 13 [8], 14 [18], 15 [3], 16 [28], 17 [29], 18 [30].

A literature survey was conducted using the Web of Science database against the terms “succinic acid” and “*Actinobacillus succinogenes*” separately, employing the “Basic Search” option. According to the search results, there were 19,338 and 426 studies about SA and *A. succinogenes*, respectively. Figure 2 shows the number of these publications filtered from 1999 to 2020. The research trends of SA revealed that the number of publications has increased chronologically with slight exceptions. For instance, the number of studies in 2018 (the highest) was increased almost 5-fold compared with the 1999 counterpart. This is, in fact, one indication of the attraction of the subject matter to the scientific community. Unlike SA, the number of studies on *A. succinogenes* indicated yearly fluctuations, with only one published study in 2000 and the absence of publications in the next two consecutive years. Additionally, the number of publications has increased dramatically since 2007, which coincided with the release of *A. succinogenes*’ genome sequence to GenBank (Figure 1). As a matter of fact, from the total number of publications regarding *A. succinogenes*, more than 96% of them have been disseminated since the aforementioned year. This shows that the aftermath of the availability of the organism’s genomic data has led to a significant growth of interest and opened a new avenue in research. It is worth mentioning that genomic data are also a stepping stone for in silico metabolic modeling, which is the core theme of this review. Moreover, the highest number of publications for both SA and *A. succinogenes* was recorded in 2018 (Figure 2).

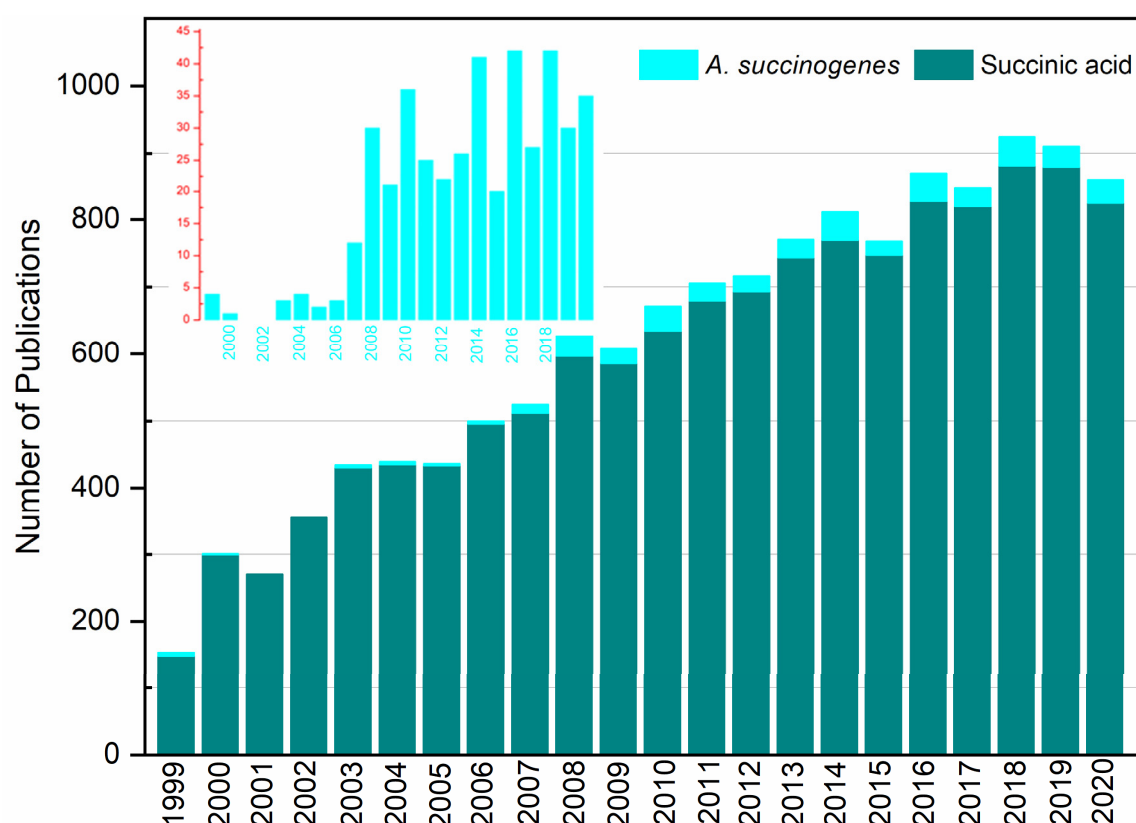


Figure 2. Literature survey results on succinic acid and *A. succinogenes*. The inset graph represents *A. succinogenes*.

3. Succinic Acid Production Pathways

It is important to have an unequivocal understanding about SA production pathways before directly moving to the topic of metabolic modeling. This is partly because finding appropriate biochemical pathways from the metabolic network represents the most challenging task in computational metabolic modeling [31]. Therefore, the realization of the participating pathways in SA production is a crucial step to develop plausible metabolic models and implement the existing ones accordingly. In line with this, applying accumulated knowledge of metabolic pathways combined with the optimization of fermentation processes and other advanced techniques has enabled the development of efficient strains capable of producing target products with high efficiency [32,33].

The tricarboxylic acid (TCA) cycle is among the major biochemical hubs with inevitable functions in the cell mainly for energy generation and precursor synthesis [34]. Obviously, being an intermediate compound of the TCA cycle [35], it is possible to infer that almost all living things can produce SA, whereas the choice of host strain relies on SA being the major end product and, so far, rumen microbes such as *A. succinogenes* are potentially considered as the best natural SA producers [36]. In general, based on the downstream metabolites of glycolysis and the TCA cycle, there are three pathways that lead to SA production, namely the reductive and oxidative branches of the TCA cycle and the glyoxylate shunt (GS) (Figure 3). Accordingly, various organisms follow one or more of these routes to produce SA. This diversity may arise due to the absence and/or inactivation of certain enzymes, environmental conditions (e.g., mode of fermentation) or any other reasons. However, interestingly, it is possible to manipulate biochemical pathways by adding novel routes, redirecting existing pathways or removing unnecessary ones through genetic engineering to construct efficient tailor-made cell factories. What is more fascinating is if the TCA cycle has to be exploited for chemical production, SA is the best candidate to be produced with maximum possible routes, whereby the two branches of the TCA cycle are linked via the GS [34] (Figure 3).

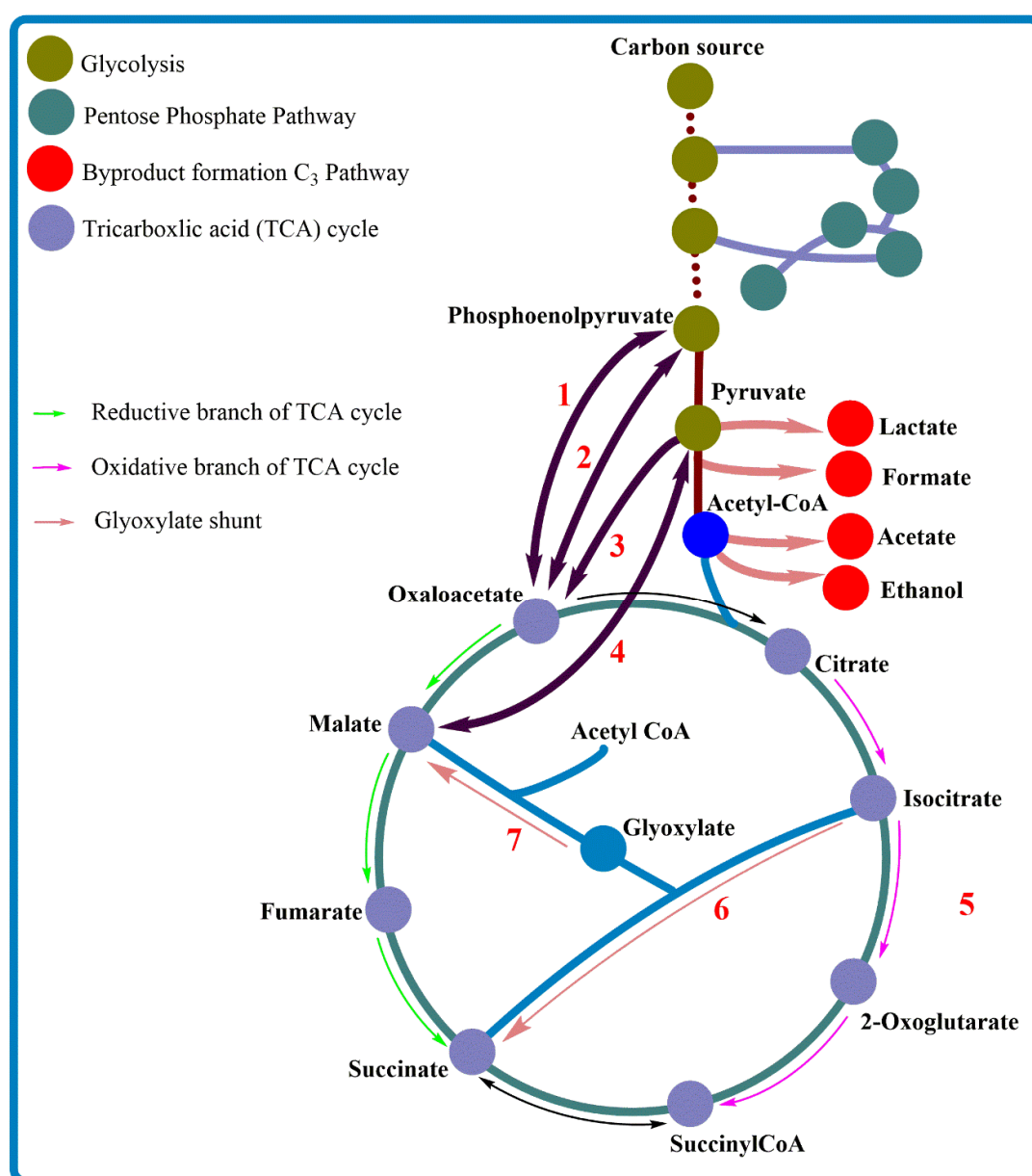


Figure 3. Major metabolic pathways of succinic acid production. The numbers represent alternative routes towards succinic acid.

Phosphoenolpyruvate (PEP) and pyruvate are important branching nodes for SA production. As shown in Figure 3, the reductive branch of the TCA cycle possibly splits from these nodes and is directed towards SA predominantly by carboxylation reaction. In total, four routes are shown in Figure 3, with two routes from each node represented by 1 to 4. Route 1 and 2 depict the route from PEP to oxaloacetate (OAA), catalyzed by PEP carboxylase and PEP carboxykinase. Route 3 and 4 are directed from pyruvate to OAA and malate, catalyzed by pyruvate carboxylase and malic enzyme, respectively. All in all, this pathway is active under anaerobic conditions and covers TCA cycle intermediate metabolites of OAA, malate, fumarate and SA as an end product. The detailed reactions and mechanisms have been reported in previous publications [34,37–39]. Microbes may possess one or more of these alternative pathways, and their efficiencies in SA production have been evaluated previously (Table 1). According to Kim et al., the overexpression of PEP carboxykinase from *A. succinogenes*’ genome to *Escherichia coli* improved SA production compared with the native PEP carboxylase [40]. The common byproducts of SA pro-

duction (formate, acetate, lactate and ethanol) occur at the C₃ pathway diverted from pyruvate and acetyl-CoA and cause the competition of carbon flux between the product formation C₄ pathway (reductive TCA pathway) and byproduct formation C₃ pathway (Figure 3). Researchers attempted to overexpress one or more of the above-mentioned carboxylation enzymes and could enhance SA production and reduce byproduct formations through redirecting carbon flow towards SA-producing reductive branches of the TCA cycle [38,41–43].

Table 1. A few examples of metabolic pathways employed for succinic acid production.

Production Route	Pathway Construction	Microorganism	Reference
Reductive TCA cycle (RT)	Inactivate lactate and acetate formation pathways and overexpress pyruvate carboxylase gene (<i>pyc</i>)	<i>Corynebacterium acetoacidophilum</i>	[44]
	Activate enzymes of RT	<i>Lactobacillus plantarum</i>	[45]
	Overexpress <i>pyc</i>	<i>C. glutamicum</i>	[46]
Oxidative TCA cycle (OT)	Delete succinate dehydrogenase (SDH) gene (<i>sdh</i>)	<i>Yarrowia lipolytica</i>	[47]
	Overexpress genes in the PPP for xylose utilization and delete <i>sdh</i>	<i>Y. lipolytica</i>	[48]
Glyoxylate shunt (GS)	Reverse PEP carboxylase via deletion of <i>sdh</i> and overexpress genes involved in GS	<i>E. coli</i>	[49]
	Activate GS via inactivation of SA biosynthetic byproduct (lactate, acetate, formate and ethanol) formation-encoding genes	<i>E. coli</i>	[50]
RT-OT (TCA)	Disrupt genes of aconitase, fumarate reductase, alpha ketoglutarate dehydrogenase, SDH, fumarase, isocitrate lyase and fumarate reductase	<i>S. cerevisiae</i>	[51]
	Delete <i>ptsG</i> and genes of SA biosynthetic byproducts and overexpress PEP carboxykinase	<i>E. coli</i>	[52]
RT-GS	Overexpress genes in the PPP for xylose utilization, <i>pyc</i> , citrate synthase and succinate exporter	<i>C. glutamicum</i>	[53]
	Delete genes of SDH (<i>sdh1</i> and <i>sdh2</i>) and isocitrate dehydrogenase (<i>idh1</i> and <i>idh2</i>)	<i>S. cerevisiae</i>	[54]
OT-GS	Delete genes of SDH, IDH and acetate-producing pathway	<i>E. coli</i>	[55]
	Delete genes of SDH and acetate-producing pathway and overexpression of <i>pyc</i> and PEP carboxylase	<i>C. glutamicum</i>	[56]
TCA-GS (RT-OT-GS)	Delete genes of SDH and acetyl-CoA transferase and overexpression of key enzymes of RT, OT and GS	<i>Y. lipolytica</i>	[41]
	Kinetic study including RT, OT, GS and other pathways	<i>E. coli</i>	[57]

PPP: pentose phosphate pathway.

In the oxidative TCA cycle, pyruvate enters into the TCA cycle via acetyl-CoA and is converted to citrate, isocitrate and eventually to SA under aerobic fermentation, as shown

in Figure 3 route 5. Likewise, SA production using GS, shown in Figure 3 route 6 and 7, is catalyzed by isocitrate lyase and malate synthase, respectively. This pathway also operates optimally under aerobic conditions. The central metabolic pathway of *A. succinogenes* lacks GS, and the TCA cycle is also incomplete due to the absence of oxidative TCA cycle enzymes of citrate synthase and isocitrate dehydrogenase [8] (Figure 4). Hence, the wild strain depends entirely on the reductive branch of the TCA cycle for SA production. Table 1 demonstrates SA production by various microorganisms using the aforementioned three major pathways and their combinations. It is also worth noting that each route has merits and demerits. In this regard, Raab et al. explained that the concurrent operation of oxidative and reductive routes appears to be more advantageous than exclusively oxidative or reductive, compromising the advantages and disadvantages of each pathway [58]. As a remark, the final goal of metabolic modifications should be to maximize SA production and at the same time minimize/eliminate byproduct formation to achieve a homo-SA production system.

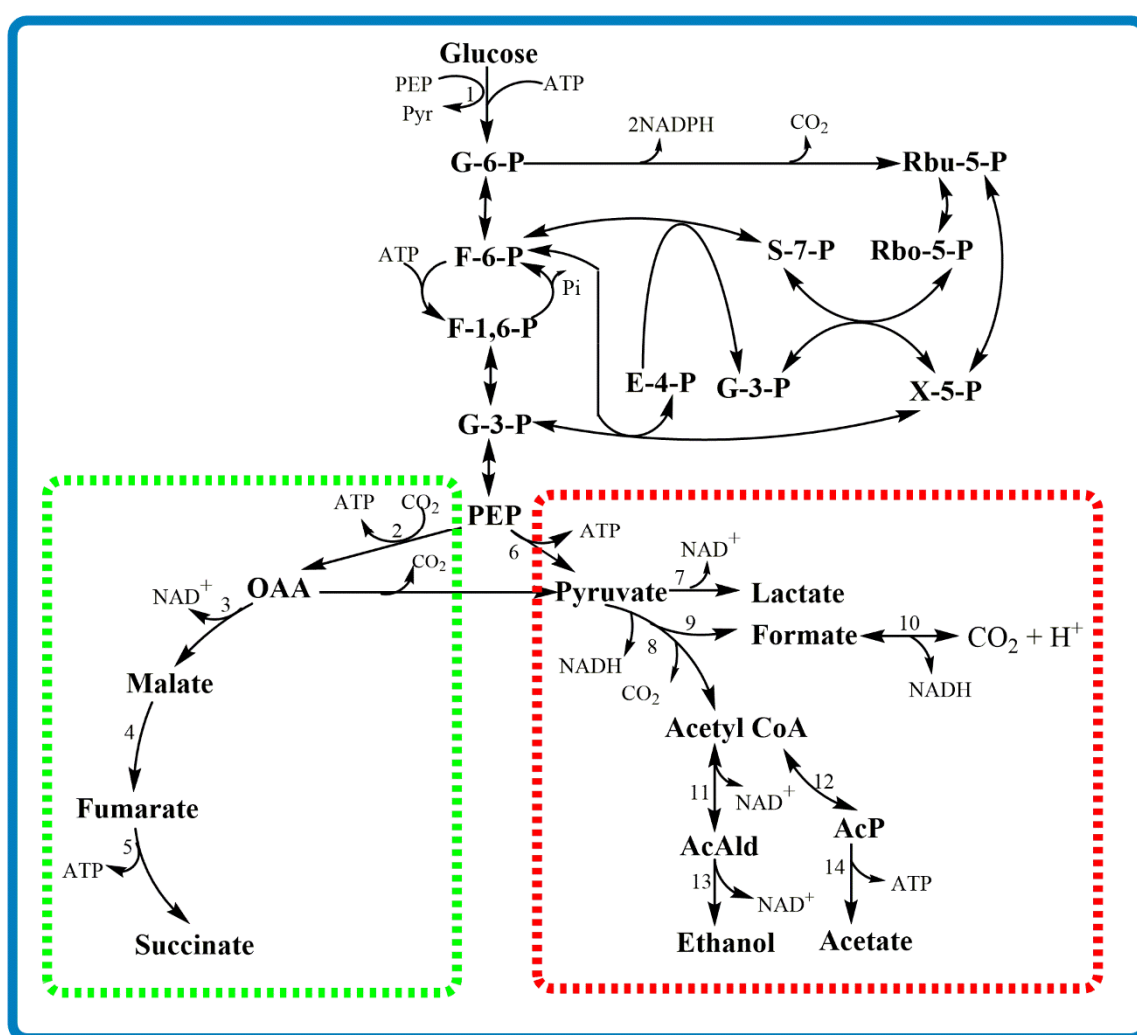


Figure 4. Central metabolic pathway of *A. succinogenes*. The green dashed box represents the reductive TCA cycle (C₄) for succinic acid production (incomplete TCA cycle) and the red dashed box represents the byproduct formation routes (C₃). Numbers represent enzymes involved in the metabolic reactions: 1, PEP: glucose phosphotransferase or hexokinase; 2, PEP carboxykinase; 3, malate dehydrogenase; 4, fumarase; 5, fumarate reductase; 6, pyruvate kinase; 7, lactate dehydrogenase; 8, pyruvate dehydrogenase; 9, pyruvate-formate lyase; 10, formate dehydrogenase; 11, phosphotransacetylase; 12, alcohol dehydrogenase; 13, acetyl-kinase; 14, acetaldehyde dehydrogenase. See the abbreviations in the designated section “Abbreviations”.

4. Metabolic Models of SA Producers

This section describes the developments of in silico metabolic modeling in selected SA-producing strains. The detailed description of various computational methods of metabolic modeling is not within the scope of this review as these concepts have been exhaustively reviewed elsewhere [9,10,14,59–64]. Scholars have proposed several metabolic modeling approaches and each method comes with intrinsic advantages and disadvantages. Hence, the application of a specific modeling strategy should consider viable parameters accordingly. In fact, various resources are publicly available for users to engage with for such purposes. For the sake of convenience, Copeland et al. [65], Jing et al. [66] and O'Shea et al. [67] listed out databases, software tools, resources, etc., that could be employed for metabolic modeling studies. Combining accumulated biological knowledge, experimental studies, genomic information and appropriate aforementioned computational inputs, one can possibly execute a metabolic model of a particular biocatalyst for target product synthesis.

Metabolic modeling studies can be generally based on either of the two broad assumptions or their combinations: dynamic/kinetic and static/stoichiometric/steady-state [9,68]. The former describes the variation of metabolites with time by applying differential equations, while the latter assumes that the production and loss of metabolites are equal, resulting in net zero production [31,61]. Kinetic models require a myriad of experimental data for enzyme rate parameters. On the contrary, constraint-based metabolic models, under the assumption of steady-state conditions, involve fundamentally fewer parameters for construction [61]. Hence, this model is commonly employed for the metabolic synthesis of desired products including SA.

Flux balance analysis (FBA) based on constraints has become a universally applicable strategy for metabolic studies [68,69]. In FBA, the flow of metabolites in the metabolic network is analyzed using mathematical approaches. In principle, in silico approaches are executed at dry labs and these simulation results could provide theoretical backgrounds to move to wet labs for the actual experimental studies. This is because of the presence of a plethora of conditions that are practically challenging or even impossible to be tested experimentally in vitro or in vivo. Metabolic models of SA-producing microbes from the very earliest study by Lee et al. [70] to the latest ones are listed chronologically in Table 2. Most of the computational studies were basically focused on non-native strains such as *E. coli* to explore the metabolic capabilities of microbes and hunt for efficient ways to produce SA. These metabolic models led to the identification and construction of alternative SA pathways (as discussed in the above section) and finally the development of model-guided experimental strategies for enhanced SA production. To begin with, in probably the first in silico study for SA production, Lee et al. constructed a metabolic pathway of *E. coli* and further conducted metabolic flux analysis (MFA) to calculate flux distributions for the maximum possible SA yield [70]. MFA results of this study revealed that the theoretical SA yield could be improved through recruiting the pyruvate carboxylation pathway (see Figure 3, route 4) rather than the native PEP carboxylation. Interestingly, this theoretical assumption was ultimately validated by experimental set ups.

Table 2. In silico metabolic studies on succinic acid-producing microorganisms.

Organism	In Silico Operation and Purpose	Year	Reference
<i>E. coli</i>	Metabolic flux analysis (301 reactions and 294 metabolites) to attain the highest in silico SA yield	2002	[70]
<i>M. succiniciproducens</i>	Genome-scale flux analysis (373 reactions and 352 metabolites) to determine the general genome-scale metabolic characteristics	2004	[71]
<i>E. coli</i>	Metabolic flux analysis (310 reactions and 295 metabolites) to predict volumetric rates of intracellular metabolites	2004	[72]
<i>E. coli</i>	Comparative genomic analysis to estimate the relationship between the maximum biomass and SA production in metabolically modified strains	2005	[73]

<i>E. coli</i>	Genome-scale in silico aided metabolic analysis and flux comparisons to determine the in silico optimal SA production pathway	2006	[74]
<i>M. succiniciproducens</i>	Genome-scale metabolic analysis (686 reactions and 519 metabolites) for genome-scale analysis and designing efficient metabolic engineering studies	2007	[75]
<i>M. succiniciproducens</i>	Constraints-based flux analysis of genome-scale metabolic model to evaluate the production patterns of various organic acids against variable rates of glucose, CO ₂ and H ₂	2009	[76]
<i>A. niger</i>	Genome-scale stoichiometric metabolic model to identify target genes for metabolic manipulation and redirect the pathway towards SA production route	2009	[77]
<i>E. coli</i>	Genome-scale thermodynamics-based flux balance analysis to predict the maximum biomass and SA flux	2011	[52]
<i>S. cerevisiae</i>	Genome-scale metabolic model and flux balance analysis to establish SA overproduction strategies	2013	[78]
<i>S. cerevisiae</i>	Genome-scale metabolic network reconstruction to predict gene deletions that can couple enhanced biomass and SA production	2013	[79]
<i>Basfia succiniciproducens</i>	Metabolic flux analysis to identify undesired fluxes and improve SA yield	2013	[80]
<i>E. coli</i>	Metabolic network construction (65 reactions and 44 metabolites) to evaluate the effect of the carboxylation reactions on SA production	2014	[39]
<i>A. succinogenes</i>	Metabolic model (27 reactions and 28 metabolites) for SA production using a mixture of glucose and xylose substrates	2014	[81]
<i>E. coli</i>	Kinetic model prediction to predict response to multiple environmental perturbations and overproduction of SA	2015	[57]
<i>E. coli</i>	Optimization algorithm and flux balance analysis to identify a set of genes for deletion to improve SA and lactic acid productions	2015	[82]
<i>E. coli</i>	Genome-scale metabolic core model to reconstruct the metabolic fluxes and evaluate the characteristics so as to improve SA production and reduce byproduct formation	2016	[83]
<i>E. coli</i>	Genome-scale metabolic model and Minimization of Metabolic Adjustment algorithm to improve the strain and increase SA production using glucose and glycerol substrates	2016	[84]
<i>E. coli</i>	Genome-scale metabolic model to evaluate the effect of gene deletion for enhanced SA production	2016	[85]
<i>M. succiniciproducens</i>	Genome-scale metabolic simulations to identify gene targets to be engineered for enhanced nearly homo-SA production	2016	[86]
<i>M. succiniciproducens</i>	Genome-scale metabolic flux analysis, omics analyses and metabolic reconstruction to develop a high-yield homo-SA-producing strain by metabolic engineering and carbon source optimization	2016	[87]
<i>A. succinogenes</i>	Thermodynamically constrained metabolic flux analysis to demonstrate the effect of environmental conditions on metabolic fluxes	2016	[88]
<i>E. coli</i>	Simulation and reaction expression analysis to identify genetic strategies for overproduction of SA	2017	[89]
<i>E. coli</i> and <i>A. succinogenes</i>	Dynamic flux balance analysis to estimate the maximum theoretical productivity of a batch culture system	2017	[90]
<i>E. coli</i>	Metabolism–downstream coupled model for metabolic engineering of the strain to produce SA using glycerol substrate	2018	[91]
<i>E. coli</i> and <i>Z. mobilis</i>	Hybrid of differential search algorithm and flux balance analysis to identify knockout relations for enhanced SA production	2018	[92]
<i>E. coli</i>	Genome-scale metabolic model to predict gene deletion for enhanced SA production using glycerol substrate	2018	[93]

<i>E. coli</i> and <i>S. cerevisiae</i>	Hybrid of optimization algorithm and genome-scale metabolic models to predict the near-optimal set of gene deletions for overproduction of SA	2018	[94]
<i>A. succinogenes</i>	Comprehensive carbon metabolism model (375 reactions) to analyze the metabolism and predict knockout strategies for maximum SA production with maintaining the cell growth	2018	[95]
<i>A. succinogenes</i>	Genome-scale metabolic model to evaluate the metabolic capability of the strain to produce SA under various conditions	2018	[30]
<i>Zymomonas mobilis</i>	Genome-scale metabolic model to characterize SA-producing capability and comparatively identify gene deletions for enhanced SA production	2018	[96]
<i>E. coli</i>	Optimization modeling to identify near-optimal knockout genes for the maximum production of SA	2020	[97]
<i>Aspergillus niger</i>	Integration of genome-scale metabolic model with dynamic modeling and genetic algorithm to provide simplified gene deletion strategies for the complex evolutionary goals containing multiple targets	2020	[98]
<i>M. succiniciproducens</i>	Flux variability scanning using genome-scale metabolic model to identify amplification target genes for improved SA production	2020	[99]

Likewise, other potential microbes were also evaluated for their metabolic capability of SA production. The fundamental difference in these in silico studies, besides the algorithms employed, is the scope of metabolic network coverage. The scope could range from central carbon, intermediate, to genome-scale metabolic models. Genome-scale metabolic models (GEMs) are by far the most inclusive models that can help us to predict system-wide phenotypic and genotypic traits so as to facilitate the manipulation of the metabolic network of an organism [13]. Herewith, we have included GEMs of the most commonly employed SA-producing microorganisms (Table 3). As it can be seen in Table 3, each version (if any) of GEM of a given microbe evolves with the incorporation of more metabolites, metabolic reactions, genes and so forth (in reference to the previous version) in the quest towards the construction of the most comprehensive model. In this regard, recent reviews pointed out that *E. coli*'s GEM appears to be the most complete [59] and the best validated [100] so far. On top of other advantages, one beauty of GEM construction is that it can be applied not only for specific target products like SA, but it can also be used for any aspects of studies of an organism. The GEMs shown in Table 3 may not be necessarily constructed for the purpose of SA production. Therefore, the public availability of the GEMs can be used as a reference (1) for the construction of their latest version, (2) to manipulate the organism's metabolism for tailored studies and (3) to design GEMs for other related stains and purposes. Moreover, the availability of multiple GEMs of an organism may assist in picking the most suitable model based on specific interest. For instance, Agren et al. [78] considered the earliest GEM (*i*FF708) [101] to metabolically engineer *S. cerevisiae* for SA production, despite the availability of other latest models. The authors explained that they chose this model because it focuses on central carbon metabolism and includes relatively small subcellular compartments, which favors it for SA production studies. The power of in silico metabolic modeling has also been seen in a recent SA production study using *M. succiniciproducens* as a biocatalyst. In this research, GEM [75] was analyzed to characterize malate dehydrogenase (MDH) and finally the overexpression of genes encoding MDH led to the production of the highest overall SA reported to date [99]. This is one of the latest tangible pieces of evidence of applications of model-guided in silico studies in the journey of developing industrially compatible SA-producing strains.

Table 3. Genome-scale metabolic models of *A. succinogenes* and other selected succinic acid producers.

	Genome Size (bp)	Total #Genes	Model Version	Year	#Genes in the Model	#Metabolites	#Reactions	ORF Coverage (%)	Reference
<i>A. succinogenes</i>	2,319,663	2210	iBP722	2018	722	713	1072	35.00	[30]
<i>M. succiniciproducens</i>	2,314,078	2384	-	2004	335	352	373	14.05	[71]
			-	2007	425	519	686	17.83	[75]
<i>C. glutamicum</i>	3,282,708	3002	Model _c g1	2008	247	411	446	8.23	[102]
	3,282,708	3002	Model _c g2	2009	277	423	502	9.23	[103]
	3,292,392	3015	iJM658	2015	658	984	1065	21.82	[104]
	3,282,708	3002	iC773	2017	773	950	1207	25.57	[32]
<i>E. coli</i>	4,641,652	4453	iJR904	2003	904	625	931	20.30	[105]
	4,641,652	4453	iAF1260	2007	1260	1039	2077	28.30	[106]
	4,639,675	4325	iJO1366	2011	1366	1136	2251	31.58	[107]
	4,639,675	4420	iOL1650-ME	2013	1541	6563	12009	34.86	[108]
<i>S. cerevisiae</i>	12,261,038	6183	iFF708	2003	708	733	1175	16.00	[101]
			iN800	2008	800	1013	1446	17.20	[109]
			iMM904	2009	904	1228	1412	19.65	[110]
			iTO977	2013	977	1353	1566	21.24	[111]

#number; ORF: open reading frame.

5. Attempts at Metabolic Modeling of *A. succinogenes*

Despite the fact that excess studies have been published on the optimization of up-stream, midstream and downstream processing steps of SA production using *A. succinogenes*, the application of in silico study on this microorganism has not yet been covered as expected. Additionally, one of the core objectives of this study is to expose the current status of computational studies on the development of this biocatalyst and inspire scholars to engage with the topic straightaway. In our previous review [11], we identified six major bottlenecks for industrial application of this strain: (1) byproduct formation, (2) auxotrophy, (3) pH sensitivity, (4) dearth of metabolic engineering tools, (5) redox imbalance (NADH limitations) and (6) product inhibition. Several attempts have been assessed to overcome these challenges. However, almost all of these efforts were fragmented and tedious in a way to solve these grand challenges with commonly employed traditional methods. Therefore, developing well-organized systematic strategies through computational approaches is crucial to alleviate these situations.

As shown in Tables 2 and 3, there are very few in silico studies in general and only one GEM specifically regarding *A. succinogenes*. The majority of modeling studies were based on glucose metabolism focusing on the central carbon (specifically glycolysis and TCA cycle) pathways. This is most probably because glucose is the most preferable carbon source of the strain and the two pathways harbor the most important steps in the SA production process. Examples of non-glucose-based metabolic modeling studies include xylose [112,113], sugar mixture [113] and glycerol [88,114]. In general, the metabolic modeling study timeline of *A. succinogenes* could be seen by dividing it into two major separate eras as pre- and post-genomic. The fundamental research during the pre-genomic [115–117] and genomic [8] metabolic modeling studies of *A. succinogenes* was led by McKinlay et al. In the pre-genomic era, metabolic studies were essentially based on isotope labeling experiments. In the first study, a chemically defined medium was created to evaluate the intracellular metabolic flux and predict the SA production metabolic map of *A. succinogenes* using ¹³C labeling experiments [115]. Later on, the strain's metabolic pathways and fluxes were determined by spectrometry (gas chromatography–mass spectrometry and nuclear magnetic resonance) through C-labeled product isotopes [116]. In the subsequent study, MFA was performed to estimate the influence of carbon dioxide and reductant concentrations on the strain's metabolism [117]. All the above three experiments were based on C-labeled glucose substrate, in which the first one provided insights on the gen-

eral SA production metabolism, the second focused on the product/byproduct metabolism and the last one emphasized the intermediate metabolites. Obviously, the results of these in vitro experiments could be viable assets for the construction and validation of the upcoming in silico metabolic studies after them.

The release of the complete genome sequence of *A. succinogenes* 130Z [8] was a phenomenal motivation for researchers to undergo progressive experiments and attempt a few in silico analysis studies as well. Remarkably, the output has been further stretched beyond this strain in a way that powerful genes of *A. succinogenes* were transferred into other microorganisms to develop efficient SA-producing cell factories. Besides, these sequence data could be applied in comparative genomic studies of related microbes. The post-genomic metabolic modeling studies are shown in Table 2. Rafieenia developed the metabolic model of *A. succinogenes* composed of 27 reactions and 28 metabolites with a sugar mixture of glucose and xylose by applying MFA via a computational technique [81]. Furthermore, constrained [88] and dynamic [90] MFA methods were employed to determine flux ranges and maximum productivity in *A. succinogenes* metabolism, respectively. Clearly, previously discussed modeling studies were all limited at the central carbon metabolism boundaries. Hence, the development of models with more inclusive and higher predictive power was needed. Nag et al. developed an extended intermediate model that contained nucleic acid, amino acid, lipid and glycogen metabolisms in addition to the central carbon metabolism [95]. However, this model still did not explicitly incorporate all the known metabolic pathways of *A. succinogenes* to have a comprehensive understanding of the strain's metabolism. At last, the first and only GEM (iBP722) of *A. succinogenes* was published [30] two decades after the first GEM (i.e., *Haemophilus influenzae*) was released [118]. To date, to the best of our knowledge, there is no experimental study based on this GEM. All in all, advancements of in silico metabolic modeling of *A. succinogenes* upgraded from the first 27 reactions to 375 and finally a GEM with 1072 reactions (Tables 2 and 3). The next model is expected to consider these achievements as frameworks and will be designed thereof.

6. Perspectives and Conclusions

Beholding the magnificent characteristics of computing such as the speed, versatility, accuracy and more, in silico study of metabolic modeling is generally priceless. We have listed some of the advancements, existing gaps and future outlooks in the development of computational methods for effective SA production (Table 4). The accumulated experimental data can be principally helpful to construct and validate in silico metabolic models. In the case of *A. succinogenes*, the majority of the modeling study relied on isotope labeling on a glucose substrate. The limitation of this approach is its restriction to only core metabolic networks so that it is unable to move forward to a GEM [119]. Besides, in such studies, non-glucose substrates were overlooked and this could impact the notion to exploit all available bioresources for SA production. Bear in mind that one of the fascinating advantages of *A. succinogenes* as an SA-producing biocatalyst is its utilization of a wide range of substrates.

Table 4. Opportunities, gaps/challenges and perspectives for in silico metabolic modeling of succinic acid production.

Opportunities/Advancements	Gaps/Challenges	Perspectives/Recommendations
<ul style="list-style-type: none"> Availability of sufficient experimental data * 	<ul style="list-style-type: none"> Limited study on non-glucose substrates 	<ul style="list-style-type: none"> Modeling more non-glucose-based metabolism
<ul style="list-style-type: none"> Sequence and omics data 	<ul style="list-style-type: none"> Confined at theoretical stage 	<ul style="list-style-type: none"> More GEMs and other models

• Resources, database and software tools	• Limited number of metabolic models	• Model-guided metabolic engineering
• Advantages of the strain over the other related organisms*	• Auxotrophy and other drawbacks*	• Explore metabolic pathways to avoid auxotrophy *
• Lessons learned from existing models	• Appropriate computational method	• Adopt genomic modeling studies
• High-throughput (HT) techniques	• Operation skill for some HT techniques	• New computational tools and strategies
• High-performance computing (HPC)	• Cost and operation skill for HPC	• Collaboration from various disciplines
		• Develop user-friendly and cost-effective instruments

* more emphasis on *A. succinogenes*.

Progress in in silico modeling is related to advancements in biotechnological HT techniques and access to omics data, bioinformatics tools and resources and literatures integrated with high-performance computing (HPC) systems. This is an interdisciplinary approach that needs collaboration from various experts from life, chemical and physical sciences and computational, mathematical and other backgrounds. In case technical gaps in HT and HPC and other issues are encountered, this could apparently be addressed by these collaborations and lessons learned from previous models. It is worth mentioning that the final goal of in silico modeling is to develop metabolically capable organisms for the large-scale production of target products. Nonetheless, most of the previous computational studies, particularly on SA production, remained at the theoretical stage. Evidently, a review by Valderrama-Gomez et al. [14] verified that only approximately 38% of the reported studies in the temporal years between 2002 and 2016 could be experimentally tested from the total computational studies. Unfortunately, none of them were the exact applications of their corresponding in silico predictions. It is recommended to construct highly predictive models, and this model should guide metabolic engineering strategy to achieve the outline goal: in silico → in vitro → in vivo.

As far as *A. succinogenes* is concerned, taking overwhelming advantages of this strain over other SA-producing strains and research advancements discussed in our previous review [11] and points raised here, it is crucial at this point to design an industrially robust biocatalyst of its kind.

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Abbreviations

AcAld	acetaldehyde
ATP	adenosine triphosphate
C ₄	4 carbon
e.g.,	example
F-6-P	fructose-6-phosphate
G-3-P	glyceraldehyde-3-phosphate
GEM	genome-scale metabolic model
HPC	high-performance computing
IDH	isocitrate dehydrogenase
NADH	nicotinamide adenine dinucleotide
OAA	oxaloacetate
PEP	phosphoenolpyruvate
PPP	pentose phosphate pathway
<i>pyc</i>	pyruvate carboxylase gene
R&D	research and development
Ru-5-P	ribulose-5-phosphate
SA	succinic acid
TCA	tricarboxylic acid
AcP	acetylphosphate
C ₃	3 carbon
CO ₂	carbon dioxide
F-1,6-P	fructose-1,6-bisphosphate
FBA	flux balance analysis
G-6-P	glucose-6-phosphate
GS	glyoxylate shunt
HT	high throughput
MFA	metabolic flux analysis
NADPH	nicotinamide adenine dinucleotide phosphate
OPR	open reading frame
Pi	inorganic phosphate
<i>ptsG</i>	PEP-dependent phosphotransferase system glucose-specific gene
Pyr	pyruvate
Rbo-5-P	ribose-5-phosphate
S-7-P	sedoheptulose-7-phosphate
SDH	succinate dehydrogenase
X-5-P	xylulose-5-phosphate

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