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REVIEW

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Metabolic rewiring of microbial cell factories for improved production of succinic acid

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Abstract

Succinic acid (SA) is a valuable platform chemical with diverse applications. It serves as a precursor for synthesizing tetrahydrofuran, γ-butyrolactone, 1,4-butanediol, and biodegradable polymers. However, conventional chemical synthesis of SA relies solely on petroleum-derived feedstocks, raising concerns about environmental pollution and resource depletion. Microbial fermentation using renewable feedstocks offers a sustainable and environmentally friendly alternative. This review explores the current state of microbial SA production, associated bottlenecks, and recent advancements in strain improvement techniques and utilization of agro-industrial feedstocks for cost-effective bio-based SA manufacturing.

Keywords Succinic acid, Microbial cells, Fermentation, Metabolic engineering, Renewable feedstock

Introduction

In the current scenario, the production of chemicals is completely reliant on fossil fuels which have an immensely negative impact on nature and several environmental policy issues. Increasing demands on the development of chemicals through sustainable processes pose a serious question and challenge for the biotechnologist [1]. Microbial conversion of renewable biomass into a value-added product is getting a colossal response from the scientific community as it can be a game-changing alternative for petroleum-based biorefineries which is the major cause of environmental pollution [2, 3]. Succinic acid (SA) ($C_4H_6O_4$) has been listed as one of the top 12 platform chemicals by the U.S. Department of Energy (US-DOE). Its spectacle is an inclusive array of applications in industries related to pharmaceuticals, food, biopolymers, plasticizers, and green solvents [4]. SA comprises of saturated linear structure with two carboxyl groups which serve as a predecessor molecule for the production of chemical commodities such as tetrahydrofuran, 1,4-butanediol, γ-butyrolactone, adipic acid, and aliphatic esters [5, 6]. The market demand for SA was about 50,000 metric tons in 2016 and was expected to double by the year 2025 [7]. Generally, the SA is chemically synthesized by oxidation of paraffin or by the catalytic reduction of maleic anhydride. The chemical process generates different dicarboxylic acids which are recovered by distillation which is often tedious and time-consuming. Currently, petroleum-based SA is assumed to have a market price of 2.0 USD/kg [8].

In recent years, due to the finite nature of fossil fuels and the drastic increase in environmental pollution, there is an urgent requirement for green technology to overcome these barriers. More interest is being developed for bio-based SA production via microbial fermentation. In this regard, several microbial cell factories were used as potential hosts to produce SA [9]. A plethora of literature

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suggests that *A. succinogenes* is one of the prominent producers of the SA [10]. The main drawbacks of bacterial fermentation are high susceptibility to low pH and are requirement of a large amount of neutralizing agent. Further, the SA appears as a succinate salt at neutral pH, which again needs to be acidified resulting in the generation of gypsum as a major by-product making the separation process tedious [9]. Since the bacteria produces SA through the reductive cycle, supplementing CO_2 is very essential, this in turn enhances the cost of the upstream processing.

Currently, the market value of bio-based SA is approximately 2.94 USD/kg, whereas the SA produced through petrochemical routes is around 2.5 USD/kg. In the year 2013–2014, approximately 38,000 tons of SA was produced through the biological process which constituted around 49% of the total market. It was estimated that by the end of 2020, bio-based SA production is expected to reach 600,000 tons with a market size of USD 539 million. Nevertheless, the existing manufacturing cost was pretty much higher as the expected production cost should be well under 1 USD/kg [11, 12] owing to the cost of the pure substrates, glucose, and sucrose used in the fermentation. The extensive use of bio-SA led to projected growth in the global succinic acid market, from USD 131.7 million in 2018 to USD 282.8 million by 2023 as predicted by Newark, 2020. This growth was expected to occur at a compound annual growth rate (CAGR) of 6.8% during the forecast period of 2019–2023. Alternatively, renewable feedstock generated from agro-industrial waste can be considered for the production of bio-chemicals. Lignocellulosic biomass (LCB) is one of the striking feedstocks for biochemical production due to its abundant nature. The LCB comprises three major fractions namely cellulose (30–60%), hemicellulose (25–30%), and lignin (15–20%). Most of the studies are focused on utilizing the cellulose part of the LCB, whereas the hemicellulose part is underutilized because the majority of the microorganisms are deficient in pentose utilizing pathway, but from the economic feasibility point of view, efficient bioconversion of both fractions is prerequisite. Hence more attention is paid to the upstream metabolic engineering to simultaneously utilize multiple carbon sources from the feedstock which improves the economic viability of biochemical production via the microbial route by engineering the native strain for effective carbon utilization [13, 14].

Furthermore, enzyme engineering demonstrates an extensive approach for maximizing the synthesis of SA through the incorporation of advanced techniques that aim to alter key enzymes like phosphoenolpyruvate carboxylase (PEPC), pyruvate carboxylase (PYC), fumarase (FUM), fumarate reductase (FRD), and malate dehydrogenase (MDH), to raise their catalytic activity, stability, and efficiency to boost productivity of SA [15]. Metabolic pathway optimization is a critical component of enzyme engineering for succinic acid synthesis. This entails changing the entire metabolic cascade to boost the flow of SA [16]. Methods like gene knockouts, which block the production of byproducts, and gene overexpression, which increases the amounts of important enzymes, are frequently used. For instance, it has been demonstrated that overexpressing *pepC*, *pyc*, and *frd* genes in conjunction with the removal of competing pathways for the synthesis of lactate and ethanol greatly increases the yields of SA in modified strains of *Escherichia coli* [17, 18]. Directed evolution is an alternative approach that replicates natural selection by repeatedly modifying, selecting, as well as amplifying enzyme variants exhibiting desired characteristics. Common methods in directed evolution include site-directed mutagenesis, which targets particular amino acid residues, and random mutagenesis, which introduces modifications across the enzyme's gene [19]. One effective use of directed evolution has been utilized to increase the thermal stability and catalytic efficiency of PYC and PEPC, which has led to increased synthesis of SA in microbial strains that have been modified [20, 21]. Carboxylase enzymes with potential are typically part of the phosphoenolpyruvate carboxylase (PEPC) or pyruvate carboxylase (PYC) families. These demonstrate significantly higher catalytic efficiencies compared to the average enzymes present in natural CO_2 fixation cycles, such as RuBisCO. In the presence of these enzymes, pathways are devised to determine a series of chemical reactions that effectively generate an organic output molecule, like succinic acid, that can be further metabolized or utilized [22]. Using structural and functional data, rational design is a further vital strategy that is used to precisely modify enzymes. This can include active site engineering to improve substrate binding and catalytic activity, as well as stability engineering to increase enzyme robustness in demanding industrial circumstances [23]. Developments in structural biology, including X-ray crystallography and cryo-electron microscopy, have given precise insights into the structures of enzymes, allowing for more efficient and rational design. These methods, for instance, have been applied in recent research to develop more stable variants of MDH and FRD, that are essential for the efficient synthesis of SA [24].

Recent advancements in genome editing technologies, notably CRISPR-Cas9, have significantly transformed enzyme and metabolic pathway engineering. Microbial genomes may be precisely and effectively modified with CRISPR-Cas9, allowing for the addition or deletion of certain genes necessary for the production of SA. This

approach makes it easier to swiftly recognize and optimize metabolic pathways and variations of enzymes when used with high-throughput screening techniques [25]. Additionally, adaptive laboratory evolution (ALE) is another potent approach that may promote the evolution of microbial strains with improved SA production capacities by culturing them under selection pressures [26]. More productivity and succinic acid output may be achieved by using ALE to improve microbial strains' redox balance and metabolic fluxes, as indicated by recent research [27].

Metabolic pathway for succinic acid production in microbial cell factories

In microbes, the SA production will be preceded by the reduction, oxidization, and glyoxylate pathways of the tricarboxylic acid (TCA) cycle (Fig. 1). In an anaerobic environment, the succinate acts as the proton acceptor and thereby follows the reductive route for SA. The succinate will be derived through phosphoenolpyruvate (PEP) with intermediates such as oxaloacetate (OAA), malate, and fumarate. This kind of reaction is most common in bacterial systems such as *A. succinogenes*, which is known to be a prominent producer of SA. In this pathway, the P + -EP is converted to SA with the consumption of 2 mol of NADH per mol of SA [28]. The equation for the anaerobic pathway can be given as:

$$\mathrm{PEP} + \mathrm{CO_2} + 2\mathrm{NADH} \rightarrow \mathrm{Succinate} + 2\mathrm{NAD^+}$$

In this pathway, the maximum possible yield of SA will be 2 mol/mol of glucose, and limitation of NADH will hinder the productivity as 1 mol of glucose can produce 2 mol of NADH via glycolytic pathway and hence the molar yield of SA will be limited to 1 mol/mol of glucose molecule under oxygen-deprived condition. Alternatively, the microbes also switch to the glyoxylate pathway which is active under aerobic conditions, when the carbon substrate is mainly acetate. The glyoxylate pathway is a modified version of the TCA cycle, containing some characteristic enzymes like isocitrate lyase and malate synthase which convert isocitrate and acetyl CoA into succinate and malate. In this reaction, 2 mol of acetyl CoA is converted to 1 mol of

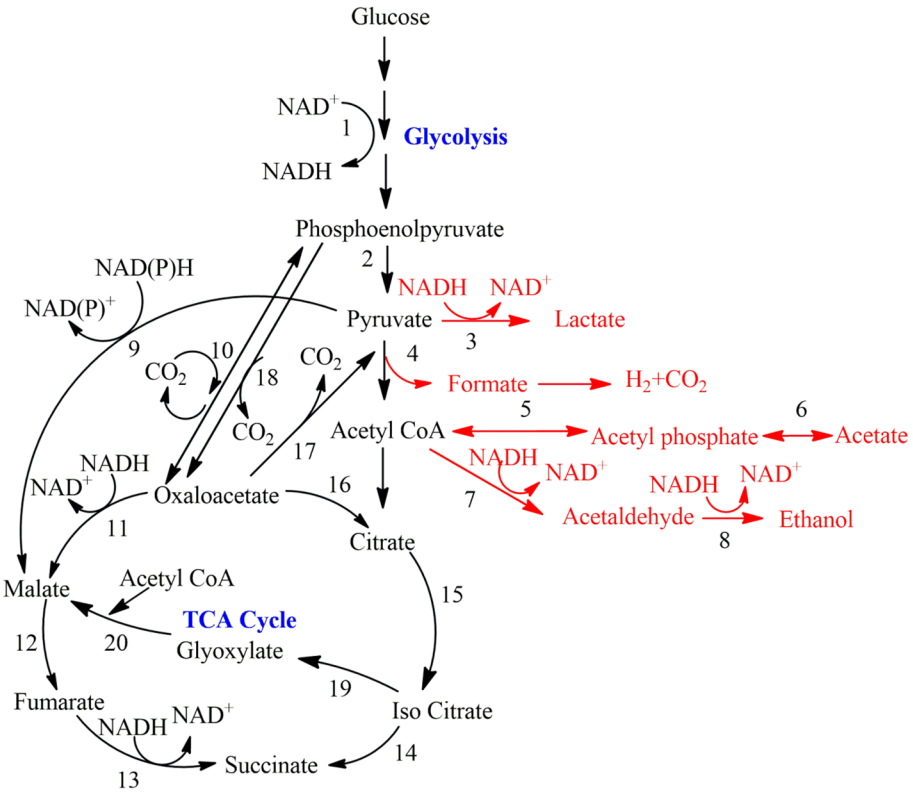

Fig. 1 Metabolic pathway of succinic acid production in bacteria (simplified, based on [23]) (1) Embden–Meyerhof pathway enzymes; (2) pyruvate kinase; (3) lactate dehydrogenase; (4) pyruvate–formate lyase; (5) phospho-transacetylase; (6) acetate kinase; (7) acetaldehyde dehydrogenase; (8) alcohol dehydrogenase; (9) malic enzyme; (10) phosphoenolpyruvate carboxykinase; (11) malate dehydrogenase; (12) fumarase; (13) fumarate reductase; (14) isocitrate lyase; (15) aconitase; (16) citrate synthase; (17) oxaloacetate decarboxylase; (18) PEP carboxylase; (19) isocitrate lyase; (20) malate synthase

SA per turn of the cycle [29]. The reaction for succinate generation from the glyoxylate pathway can be given as

$$2\ \text{Acetyl CoA} \rightarrow 1\ \text{Succinate}$$

The NADH generated via glyoxylate shunt will not be sufficient to carry out electron balance. However, the NADH produced here aids as an electron donor under anaerobic conditions and enhances the succinate production. In eukaryotic systems such as yeast the oxidative TCA is hyperactive under aerobic conditions; the SA will be generated using acetyl-CoA as a substrate. The pathway involves the conversion of acetyl CoA to SA via intermediates such as citrate, isocitrate, and succinyl-CoA. The formed SA will be converted to fumarate via succinate dehydrogenase enzyme. In practical situations, the production of SA under aerobic conditions is hampered as SA is a precursor of fumaric acid. Hence deletion of *sdh* (succinate dehydrogenase) is required to accumulate a substantial amount of SA [30]. The oxidative TCA cycle for SA production can be given as

$$2\ \text{Pyruvate} + 2H_2O + 3NAD^+ \rightarrow \text{Succinate} + 3NADH + 2CO_2$$

The microbial world harbors a diverse collection of different groups of microbes, and some other instances have also been observed where succinate is formed by various microbial species during different metabolic processes. In the members of the *Propionibacteria* genus, the pyruvate produced from glycolysis enters the Wood-Werkman cycle by forming oxaloacetate. By the expenditure of 1 mol of reducing equivalent NADH, the oxaloacetate is converted to succinate in a three step pathway. The succinate then goes along to form propionate via epimerization and transcarboxylation [29, 31, 32]. Most organisms are known to utilize the ubiquitous NAD^+-dependent 2-oxoglutarate dehydrogenase enzyme for the conversion of oxoglutarate to succinyl-CoA, while some others are known to utilize a ferredoxin dependent 2-oxoglutarate synthase [33]. The conversion of succinyl-CoA to succinate has been observed to have significant variability. Different enzymes have been identified in different organisms which catalyze the step, like succinyl-CoA:acetate CoA-transferase in *Acetobacter aceti* [34], and succinyl-CoA:acetoacetate CoA-transferase in *Helicobacter pylori* [35]. Adding more diversity to the entire succinate production scenario, obligately autotrophic and methanotrophic bacteria and archaea, directly convert 2-oxoglutarate to succinate via an intermediate succinate semialdehyde, using 2-oxoglutarate decarboxylase and succinate-semialdehyde dehydrogenase enzymes, completely bypassing the generation of succinyl-CoA [36].

Wild-type SA producers

SA is the intermediate product of the citric acid cycle and can be amassed as the end product of the fermentation process by numerous bacterial species such as *Actinobacillus succinogenes*, *Mannheimia succiniciproducens,* and *Anaerobiospirillum succiniciproducens*. Some of the fungal strains such as *Paecilomyces varioti*, *Aspergillus niger*, and *Penicillium simplicissimum* also showed some glimpse of succinate production under aerobic/anaerobic conditions, however, the productivity is very less compared with that of the bacterial strains as the SA in fungal strains are produced in mitochondria and has to cross the cellular and mitochondrial membrane [37]. Among the wild-type SA producers, *A. succinogenes,* a capnophilic bacterium isolated from the gut of the rumen is a widely studied organism and a promising host for SA production and is amenable to consuming a wide range of substrates. The strain is categorized under biosafety level type 1 by DSMZ and ATCC [11]. *A. succinogenes* 130Z strain can secrete a SA titer of 66.4 g/L with the yield of 1.02 mol/mol using glucose as a carbon source [38] The mutant strain of *A. succinogenes* FZ53 showed a SA titer of 105. 8 g/L with 0.82 g/g yield. the mutant strain was developed from *A. succinogenes* 130Z strain by increased exposure to fluoroacetate, and thereafter selection. The variants resulting from the exposure, i.e., the FZ53 strain showed higher SA yield and lower AA and formic acid (FA) yields as compared to the original strain [39]. Even though *A. succinogenes* has been reported to be one of the most efficient succinic acid-producing strains [28], limited studies have been carried out on engineering the strain as the genetic tool for this strain is not well established. Park et al., 1999 [40] deleted pyruvate-formate lyase and formate dehydrogenase gene in *A. succinogenes,* but the resultant recombinant strain did not show much improvement in succinic acid production. However, when the culture is supplemented with electrical neutral red or hydrogen, it can uptake formate as the sole carbon substrate for succinic acid production, where the reductive pathway will be active. A recent study reported that three single gene overexpressions (*pck, fum, mdh*) in *A. succinogenes* were compared and the strain with MDH overexpression showed the highest SA production titer of 34.2 g/L, having a net 11.8% titer enhancement. However, these strains also expressed higher yields of the main byproduct acetic acid in comparison to the wild-type strain [41].

Mannheimia succiniciproducens, is another strain isolated from the ruminant gut in the 1990s, which is

widely explored for the production of SA. This strain is gram-negative, facultative anaerobe, non spore-forming, mesophilic, CO_2 fixing, and capnophilic in nature and can consume a wide range of carbon substrates [42]. The wild-type strain *M. succiniciproducens* MBEL55E displayed a succinic acid titer of 10.50 g/L with the yield and productivity of 0.45 g/g and 1.17 g/L/h, respectively using pure glucose under anaerobic conditions [43]. The strain also produced by-products such as acetic acid (AA), formic acid (FA), and lactic acid (LA) at the following titers of 4.96 g/L, 4.1 g/L, and 3.47 g/L, respectively, which severely hampered the SA production. To improve SA production it is very important to eliminate the by-product formation avoiding the redistribution of carbon flux. Lee et al., 2006 [44] have sequentially deleted genes encoding pyruvate formate lyase (*pflB*), lactate dehydrogenase (*ldhA*), phosphate acetyltransferase (*pta*), and acetate kinase (*ackA*) and developed a mutant strain LPK-7. The mutant strain displayed a drastic reduction in by-product formation and improved SA titer of 52.4 g/L with a yield of 1.16 mol/mol under fed-batch cultivation. Another study reports a final productivity of 38.6 g/L/h by a metabolically engineered *M. succiniproducens* lacking the *ldhA, pta, ackA,* and *fruA* genes for deregulation of catabolite repression and employing a membrane cell recycle bioreactor [45].

A. succiniciproducens, a gram-negative bacterium used as a potential candidate for SA production. The wild-type strain DD1 has shown a high similarity with *M. succiniciproducens* MBEL55E [46, 47]. Becker et al., 2013 [48] have identified intracellular fluxes based on ^{13}C metabolic flux analysis, and based on the result they have knocked out *pflA* and *ldhA*, which abolished FA and LA formation and improved SA yield to 1.08 mol/mol as compared to the wild type DD1 strain with yield of 0.75 mol/mol using glucose as carbon substrate. However, the mutant strain displayed high pyruvic acid accumulation.

Heterologous host for SA production

Despite of high SA production by wild-type strains such as *A. succinogenes* and *M. succiniciproducens*, the strains require a complex medium for their optimal growth making the process cost-intensive. With the development of genetic tools model strains such as *Escherichia coli, Saccharomyces cerevisiae* are being explored for the production of SA with high titer values.

Heterologous SA production in prokaryotic systems

Escherichia coli

Escherichia coli is a gram-negative organism from the Enterobacteriaceae family, facultative anaerobic, and mesophilic. It prefers commonly available six-carbon sugars as an energy source but can metabolize other forms also depending upon the bacterial strains. As it is exhaustively well-researched, numerous tools are available for its genetic manipulation. It is one of the primary choices for any possible engineering due to the above-listed features and its remarkably efficient expression [49].

A plethora of literature is available on metabolic engineering on *Escherichia coli* using various approaches. One of the methods involves overexpression of the genes coding for the enzymes utilized in the pathway for succinic acid formation. One of the initial studies was done by Millard in 1996, demonstrating the overexpression of the genes PEP carboxykinase (*pck)* and PEP carboxylase (*ppc*) to check for higher succinic acid titer in *E. coli* [50]. The overexpression of *pck* alone was incapable since gluconeogenesis is the major characteristic of PEP carboxykinase enzyme. When PEP carboxylase enzyme was overexpressed by introducing a *tac* promoter-based expression vector pJF118EH, a 3.5-fold elevation of the concentration of succinic acid was reported. The strain JCL1208 possessing a chromosomal *lacI* gene, and lacking a *lac* operon was used as a host for the process. Under suitable induction conditions (IPTG and glucose), upregulation of pCP201 was witnessed in the JCL1208 using the pJF118EH plasmid strain under anaerobic conditions leading to the desired outcome [51]. Alternatively, the introduction of non-native genes can significantly improve the flux on the desired pathway or alternate pathways for the preferred outcome. *E. coli* with deficient pyruvate carboxylase gene (*pyc*), can divert the additional pyruvate supply to form oxaloacetate utilizing alternate pathways. Therefore, *pyc* was cloned from *Rhizobium etli* by Gokarn et al., 1998 [52] to a pUC18 expression vector and introduced into *E. coli* MG1655 strain. The succinic acid production was improved from 1.18 g/L to 1.77 g/L, whereas the acetic acid production was reduced from 2.33 g/L to 1.88 g/L in the *E. coli* MG1655 strain with *pyc* expression. The reason for this refinement is the ability of pyruvate carboxylase to reroute more carbon to produce oxaloacetic acid [53]. Guo et al., 2022, engineered *E. coli* to enhance succinic acid production by introducing a one-carbon dissimilation pathway and redirecting pyruvate metabolism, and the yield was enhanced by 4% with methanol and formate as substrates. Additionally, metabolic engineering incorporating CO_2 fixation potentially improved efficiency. Finally, immobilization on a specialized membrane enhanced cell viability and further boosted the final succinic acid yield to 0.98 g/g [54].

Since SA is one of the fermentation products; its yield was still compromised by the formation of other metabolites. Hence, the approach was to inactivate or delete the genes responsible for the formation of other metabolites competing with succinic acid yield. The idea is to redirect the metabolic flow of carbon flux towards the SA pathway

[55]. The lactate dehydrogenase (*ldh)* gene catalyzes the reaction from pyruvate to lactate in *E. coli* [56]. Similarly, pyruvate-formate lyase enzyme (*pfl* gene) directs the flow of pyruvate to formate. Inactivating mutation in these *ldh* and *pfl* genes leads to the formation of NZN111 strain, to block the production of lactate, and formate byproducts. However, NZN111 possessed relatively very sluggish cell growth owing to the disruption in pathways for pyruvate dissimilation. A widely accepted view suggests that the deactivation of NADH-dependent LDH restricts the replenishment of NAD + and thus impedes growth in anaerobic conditions [57]. With the increase in NADH accumulation, the rate of cell growth decreased. Nevertheless, in-silico analysis by Jian et al., 2016 [58] reported that the flux-sum value of NADH exhibited a gradual decline as the accumulated NADH was utilized to facilitate the regeneration of q8h2 (ubiquinol-8), thereby enhancing succinate production. According to reports, reducing the intracellular redox ratio (NADH/NAD +) in NZN111 resulted in enhanced growth and increased succinate production [59]. In addition, the overexpression of ATP-forming PEPCK from *A. succinogenes* in a quadruple mutant strain lacking *ldhA*, *pflB*, *ptsG*, and *ppc* genes led to a 60% augmentation in both biomass and succinate synthesis [60]. *E. coli* harbors a NAD-dependent malic enzyme (*sfcA*), responsible for pyruvate conversion to malate, eventually generating SA [61]. In NZN111, there was a limitation of sufficient regeneration of NAD, as well as the enzyme concentration was not sufficient for converting enough pyruvate to malate. When the *sfcA* gene encoding malic acid production was upregulated in *E coli*, the SA increased from 1.80 to 12.8 g/L. In a previous study, Zhang et al., 1995 [62] genetically modified an *E. coli* strain to produce succinic acid and utilize methanol as a carbon source. A methanol assimilating module containing NAD + dependent methanol dehydrogenase (Mdh) from *Bacillus methanolicus* and the ribulose monophosphate pathway from either *B. methanolicus* or *B. subtilis* was introduced [17]. To further enhance its ability to produce succinic acid, the metabolic pathways of methanol and formate were incorporated to generate additional NADH. The introduction of pyruvate carboxylase from *Lactococcus lactis* enhanced its ability to fix CO_2 [63] Ultimately, a glycosylated membrane was used to immobilize the cells in place and enhance their resistance towards the toxic C1-substrates [64].

Initially, a spontaneous mutation in the cell culture of NZN111 led to efficient glucose utilization, and later on the mutated strain was renamed AFP111. It produced succinic acid along with ethanol and acetic acid in a ratio of 2:1:1. In AFP111, the cell growth was reinstated with a productivity of 0.87 g/L/h. The mutation blamed to cause the emergence of AFP111 was recognized in a section of the phosphotransferase (*ptsG*) gene, which encodes a permease specific to glucose, bound on the cell membrane. Repression of glucose was found to be absent in mutant strains harboring functional loss of *ptsG* gene product either due to insertional inactivation or spontaneous mutation by Chatterjee et al. 2001 [65]. Also, null mutations generated in *ptsG* region of other already glucose-fermenting *E coli* strains resulted in enhanced concentrations of succinic acid. To further increase succinic acid yield, *E coli* AFP111 was engineered to harbor a plasmid (pTrc99A-*pyc*) containing pyruvate carboxylase gene (*pyc).* The *pyc* gene for the same purpose was isolated from *Rhizobium etli* and overexpressed in the bacterium by Vemuri et al., 2002 [66]. The productivity increased to 1.3 g/L/h whereas the concentration increased to 99.2 g/L when AFP111/pTrc99A-*pyc* was cultured in the presence of oxygen in the initial phase, followed by anaerobic conditions in a fed-batch mode [53].

Research also demonstrates process optimization techniques for the production of succinate from metabolically engineered strains. The productivity and mass yields were reported to increase via two-stage fermentation with recovered engineered cells. The two-stage fermentation process resulted in an overall productivity of 1.19 g/L/h, and a mass yield of 0.82 g/g, with the time and substrate consumed in the aerobic stage included. Andersson et al., 2010 reported that the average productivity for succinic acid production was 1.77 g/L/h, with an average mass yield of 0.77 g/g over three resuspensions [67]. Upon comparison, it was observed that there was no reduction in succinic acid productivity and mass yield when the recovered cells were used. The average productivity was 1.81 g/L/h and the mass yield was 0.85 g/g over three recycling rounds.

Efforts have also been made to alter or engineer strains capable of withstanding a variety of substrate ranges. One mol of succinate is formed anaerobically with the two mols of NADH generated from one mol of glucose. Hence, efforts are made to divert the NADH flux from all other non-essential possible directions to succinate production. A strain SBS110MG was engineered to comply with this by inactivating genes *adhE, ldhA,* followed by the addition of a heterologous *pyc* gene from *Lactococcus lactis* via expressing through pHL413 plasmid. The lactate production was blocked by the loss of function of lactate dehydrogenase, whereas the inactivation of alcohol dehydrogenase stopped the conversion to ethanol [63]. By these specific deletions, the PFL (pyruvate formate lyase) pathway remained functional to produce acetyl-CoA for biosynthetic purposes, along with the enzymes for its recycling (phosphotransacetylase-acetate kinase). To improve the succinate-to-acetate ratio, the *ptsG* deletion was attempted along with *pyc* gene expression, and

a new strain SBS220MG was developed by Chatterjee et al., 2001 [65] Further, the strain was altered to additively utilize the conventional aerobic glyoxylate cycle in anaerobic mode through *iclR* inactivation. The *iclR gene* acts by coding for a repressor for the glyoxylate bypass, hence its deletion improved productivity with reduced needs for NADH. Accommodating this modification, along with the deletion of genes for byproduct pathways such as *adhE, ldhA,* and *ack-pta;* a new strain SBS550MG was developed. The activation of the glyoxylate shunt provided an additional route for succinate production that requires fewer reducing equivalents, resulting in a dual pathway for succinate synthesis, resulting in a maximum theoretical succinate yield of 1.6 mol/mol glucose [68].

In *E. coli*, aerobic pathways were not primarily used for succinate production as acetate is the most common byproduct during aerobic cycles, with succinate being an intermediate only. However, in anaerobic cycles, the organism displays low biomass, slow conversion, and productivity rates. Hence it is ambient to engineer the strain for producing SA under an aerobic cycle. In this regard, a penta-mutant strain was engineered to fabricate an extremely active glyoxylate cycle in the *E. coli* by the mutations of *poxB, ackA-pta* gene in acetate pathways, and *ΔsdhAB, Δicd, ΔiclR* genes in the tricarboxylic acid cycle. The varying sequential alterations of these genes yielded approximately 0.344 mol succinate/ mol of glucose. Another mutant strain was developed with mutations in sequence *ΔsdhAB, ΔpoxB, Δ(ackA-pta), Δicd, ΔiclR* providing a yield of 0.406 mol/mol of glucose. However, the maximum theoretical yield of 1 mol succinate per mol of glucose was not achieved in batch conditions [53]. Later on, this became possible by the overexpression of *Sorghum pepc* encoding phosphoenolpyruvate carboxylase and deletion of *ptsG* into the previously fabricated pentavalent mutant strains or in the same mutants lacking mutation at *icd* gene [69]. The strain HL27659 harboring *ΔsdhAB, Δ(ackA-pta), ΔpoxB, ΔiclR, ΔptsG* displayed a remarkably efficient aerobic system with an average succinate productivity of 73.66 mg/g/h [70].

Thakker et al., 2012 discussed the metabolic engineering strategies coupled with the selection of spontaneous mutations for the generation of a range of new strains [68]. These strains were broadly exposed to deleterious mutations while engineering. Genes involved with central metabolic pathways *(ldhA, adhE, ackA)* were eliminated and NADH regeneration was taken care of by the malate pathway. ATP productions in anaerobic conditions were linked with fumarate reductase and malate dehydrogenase enzymes. Two major succinate-producing strains KJ032 and KJ072 were created via engineering and cultured in media for improvement. The strain KJ073 with mutations *ΔldhA, ΔadhE, ΔackA, ΔfocA, ΔpflB, ΔmgsA, ΔpoxB,* when acquiring an advantageous mutation for improved ATP production provided a yield of 1.2 mol/mol glucose. These strains were able to generate 1.2–1.6 mol of succinate per mol of glucose, along with byproducts [60]. Various other deletions were carried out in strains like KJ073 to improve to new strains KJ122, KJ134, etc., possessing yield similar to the maximum theoretically determined succinate yields i.e., 1.7 mol/mol glucose. Metabolomics studies were also conducted to identify the potential metabolites that affect the succinate acid yield [71]. The major genes and the enzymes coded by them involved in the metabolic engineering of *E. coli* have been represented in Fig. 2.

Corynebacterium glutamicum

C. glutamicum is a gram-positive bacterium widely used in industries for the production of amino acids and value-added chemicals. The genome of the strain is well annotated and several genetic tools are available for strain engineering [72, 73]. In oxygen-deficient conditions, *C. glutamicum* can produce various organic acids (mainly succinate and lactate), and stunted growth is observed. Hence, to produce a high yield using *C. glutamicum* under oxygen deprivation supplementation of bicarbonate is necessary, which acts as a co-substrate for anaplerotic enzymes increasing glucose consumption rate. It was found that by increasing bicarbonate concentration, the yield of SA was enhanced with simultaneous reduction of lactic acid formation [74]. *C. glutamicum* was subjected to metabolic engineering by Okino et al., 2008, where *pyc* gene was over-expressed and *ldhA* was disrupted [75]. The mutant strain displayed a succinic acid production and yield of 146 g/L and 0.92 g/g, respectively with the supplementation of glucose and sodium bicarbonate in the media and AA was produced as a major byproduct. A succinate producing mutant of *C. glutamicum* (Psod:SucE-ΔldhA*)* was generated using CRISPR-Cpf1 genome editing system, where the lactate dehydrogenase 1 gene was knocked out and the succinic acid transporter was co-expressed. The strain produced 0.77 g of succinic acid/g sugar from pine softwood hydrolysate [25].

Arabinose can be used for the production of succinate because of its profusion in hemicellulosic ravages. In this study conducted by Chen et al., 2014, the operon gene *araBAD* from *E. coli* was inserted into SA-producing *C. glutamicum*, which, as a result, can produce SA under aerobic conditions using arabinose as a sole carbon source [76]. The modified strain ZX1 (pX*araBAD*, pE*acsAgltA*) can produce 74.4 mM succinate having a yield of 0.58 mol/mol arabinose. This engineered strain can also produce 110.2 mM succinate when both arabinose and glucose are used. *C. glutamicum* CS176 strain

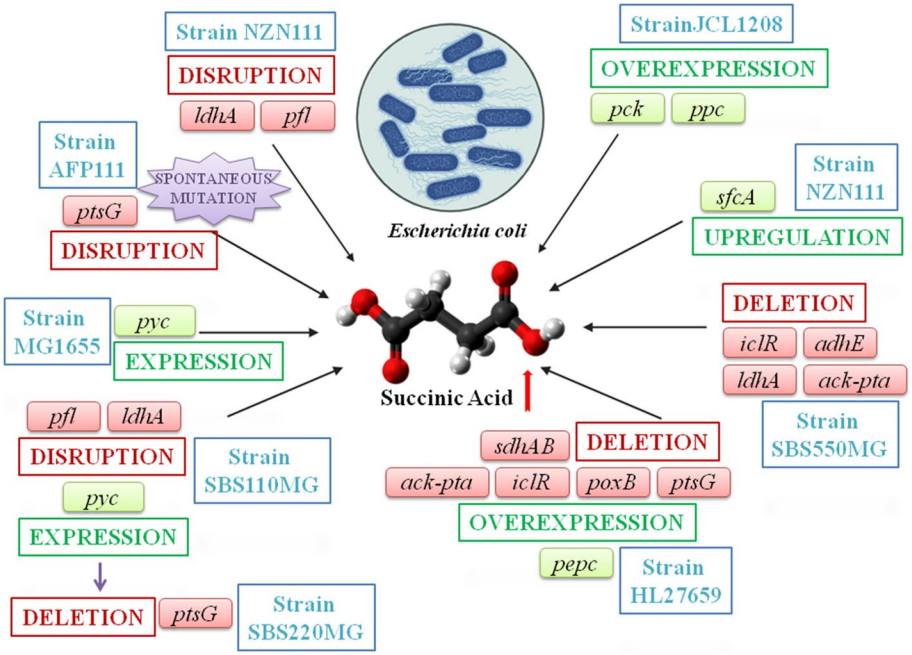

Fig. 2 Genes involved in the enhancement of SA production by engineered *E. coli* strains. *pck*- PEP phosphoenolpyruvate carboxykinase; *ppc*- PEP carboxylase; *sfcA*- NAD-dependent malate dehydrogenase; *iclR*- regulatory protein (repressor) for the *aceBAK* operon; *adhE*- aldehyde/alcohol dehydrogenase;*ldhA*- lactate dehydrogenase; *ack-pta*- phosphotransacetylase-acetate kinase; *sdh*- succinate dehydrogenase; *poxB*- pyruvate oxidase; *ptsG*- phosphotransferase; *pepc*- phosphoenolpyruvate carboxylase; *pyc*- pyruvate carboxylase; *pfl*- pyruvate formate lyase

can use L-arabinose, a pentose sugar as a fermentable substrate. Fueangbangluang and Trakulnalaemsai, 2018 tried to increase the production rate by deleting *ldhA*. The mutant strain was able to produce SA with a titer of 1.74 g/L within 2 h after adding 10.7 mM of sodium bicarbonate to the media [77].

Litsanov et al., 2012b [78] observed that with the deletion of *sdh* gene, the SA titer was enhanced to 4.7 g/L along with the secretion of AA as a major by-product. Hence, targeting genes of acetate-producing pathways like *pta-ackA, pqo* & *cat* is imperative [79]. The deletion of these genes reduced acetate production to approximately 83% and increased the production of SA up to 7.8 g [80]. Furthermore, overexpression of *pyc* and *pepc* occasioned SA titer of 9.7 g/L. The same group has developed BOL-2 strain from ATCC 13032 by deleting *Δcat, Δpqo, Δpta- ackA, ΔldhA,* and overexpressing pyruvate carboxylase gene the BOL-2 strain showed reduced acetate production. Further, the strain was modified by deleting *fdh* encoding formate dehydrogenase and over-expression of the glyceraldehyde 3-phosphate dehydrogenase gene (*gapA*). The recombinant strain BOL- 3/pAN6-gap displayed SA titer of 134 g/L and yield of 1.67 mol/mol, using glucose as the sole carbon source [81, 82]. *C. glutamicum* overexpressing the glycerol utilizing gene *glpFKD* from *E. coli* produced 9.3 g/L of SA from glycerol under aerobic conditions, resulting in 42% of the maximal theoretical yield under aerobic conditions and volumetric productivity of 0.423 g/L/h [82]. Zhu et al., 2013 deleted the acetate formation pathways and over-expressed the anaplerotic pathways. The acetyl-CoA synthetase (*acs*) gene was isolated from *B. subtilis* and was expressed in *C. glutamicum*. The modified strain ZX1 (pE*acsA*) accumulated succinate having a yield of 0.50 mol/ mol glucose but did not show acetate production. To further increase the production of SA native *gltA* gene (citrate synthase) is over-expressed. The recombinant strain ZX1 (pE*acsAgltA*) depicted an increase in SA yield by 22% and a decrease in pyruvate yield by 62% in comparison with the strain ZX1 (pE*acsA*). The strain produced 241 mM SA with a yield of 0.63 mol/ mol glucose in fedbatch under aerobic conditions [83].

C. glutamicum can fabricate SA from glucose using a reductive tricarboxylic acid pathway following both microaerobic and anaerobic conditions. Fukui et al., 2011 recognized the *NCgl*2130 gene of *C. glutamicum,* succinate exporter (*sucE1*) that plays a role in SA fabrication and is expressed more strongly under microaerobic conditions as compared to well aerated environments [84]. The over-expression or deletion of *sucE1* can severely interrupt the productivity of succinate. In a microaerobic environment, the disrupted *sucE1* displayed nearly 30% less SA production by decreasing the substrate utilization rate. In anaerobic conditions, the production of SA

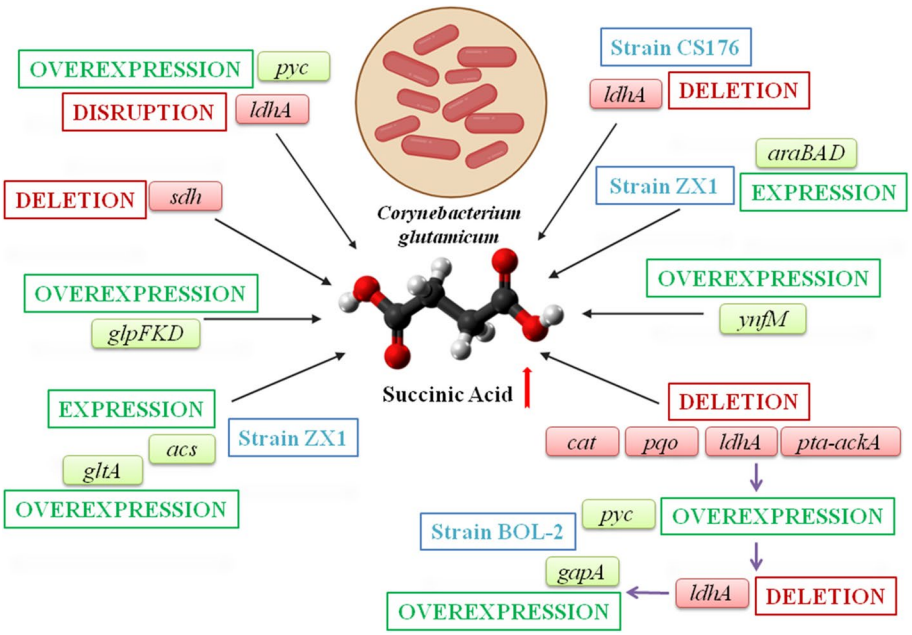

Fig. 3 Genes involved in the enhancement of SA production by engineered *C. glutamicum* strains. *pyc*- pyruvate carboxylase; *ldhA*- lactate dehydrogenase; *sdh*- succinate dehydrogenase; *araBAD*- arabinose operon; *ynfM*- inner membrane transport protein; *cat*- chloramphenicol acetyltransferase, *pqo*- pyruvate quinone oxidoreductase; *ack-pta*- phosphotransacetylase-acetate kinase; *gapA*- glyceraldehyde 3-phosphate dehydrogenase; *gltA*- citrate synthase; acs- acetyl coA synthetase; *glpFKD*- glycerol catabolism operon

is completely halted. Fukui et al., 2019 modified SDH-deficient *C. glutamicum* by overexpressing *ynfM* from *Pantoea ananatis* (*PaynfM*), a gene for dicarboxylate transporter which is homologous to the gene (*CgynfM*) found in *C. glutamicum*. This strain, when cultured in the presence of oxygen, has a notable increase in the rate of sugar consumption. SA production increased from 66 to 110 mM, and acetate and pyruvate levels decreased drastically [85]. The techniques discussed for the metabolic rewiring of *C. glutamicum* have been illustrated in Fig. 3.

Heterologous SA production in eukaryotic systems

Saccharomyces cerevisiae

Saccharomyces cerevisiae is one of the most studied organisms and the genome is well curated. Over the last few years, enormous metabolic tools have been developed including the CRISPR-Cas method to engineer the strain for the overproduction of biochemicals [86]. The strain is able to withstand variable pH or osmotic changes, implying its suitability for processes with altering conditions by product accumulation [87]. It is well adaptable to survive on a range of substrates and can grow aerobically as well as anaerobically [88]. In the process of engineering this strain for succinic acid production, one of the primary advantages was its ability to produce succinic acid into the medium rather than intracellular accumulation, which in turn, minimized the costs of product extraction and downstream processes [89].

The initial clue to the possibility of succinic acid production in *Saccharomyces cerevisiae* was hinted during the sake (Rice wine in Japan) fermentation. The major flavoring agent in sake was projected to be succinic acid. In leniently anaerobic conditions, Arikawa et al., 2000 [90] observed elevated levels of succinic acid in sake yeast strains harboring deletions in TCA cycles. However, in conditions with zero oxygen exposure, these results were found to be inconsistent. Upon disruption of the activity of the succinate dehydrogenase gene (*sdh*) by various means, a new spectrum of conclusions was driven by Kubo et al., 2000 [91]. The yield of succinic acid scantily improved in the presence of oxygen, upon disruption of *sdh1* alone. Surprisingly, the activity of this gene was not eliminated; it was being compensated by a gene *sdh2* or *sdh1b*. Hence, deletion of either *sdh2* or *sdh1b* complementary to *sdh1* deletion in strain XU-1U, displayed a remarkable 1.9-fold productivity than wild strain. However, no considerable change was observed with the same strains in static or fermentative conditions. It is not wrong to infer that, the oxidative pathway (glyoxylate cycle) instead of fermentation is most desirable for high succinic acid productivity in these yeast cells [92].

In an approach of metabolic flux redirection to the oxidative pathway, disruptions in the TCA cycle other than *sdh* were tested. One of the targets was the isocitrate dehydrogenase enzyme, responsible for the commencement of the TCA cycle by converting isocitrate to alpha-ketoglutarate. Inactivation of this enzyme can directly contribute to the flow of isocitrate to the glyoxylate cycle. But when this disruption was tested alone in fermentative sake conditions, the yield of succinic acid was further halved [93]. Efforts were made to engineer an *S. cerevisiae* strain possessing all these above-listed advantageous deletions in combination. To minimize remaining isocitrate dehydrogenase activity after *idh1* deletion, a mitochondrial origin gene *idp1* coding for *idh1* isoenzyme was also targeted for elimination. Alterations *Δsdh2, Δsdh1, Δidh1,* and *Δ idp1* were performed by homologous recombination technique on S. *cerevisiae* AH22ura3. The resulting strain produced nearly five times the concentration of wild type with productivity of 0.022 g/L/h [53, 94].

Efforts attempting strategies with a combination of multiple techniques brought about some novel strains. An alteration into a wild type/reference strain with a well-studied target through the in-silico programming methods leads to the generation of an 8D strain. This strain possessed deleted *sdh3, ser3, ser33* genes, in an approach to focus all the carbon flux to the succinate production only. The gene product of *sdh3* was the succinate dehydrogenase`s cytochrome b subunit and its deletion reduced the succinate conversion. The other two genes were responsible for serine generation and their interruption provided more carbon for improved succinate titer. This 8D strain was remarkably better and possessed nearly 13 folds more titer than the reference strain, the only issue being its requirement of glycine supplementation for growth. Later on, this issue was also swiftly dealt with by the directed evolution approach. Constant growth and selection of 8D in a controlled environment led to a mutant with no such glycine requirement and it was renamed to evolved 8D. For further refinement of succinic acid titer, a range of approaches were tried and a few other new strains were also developed. Transcriptome analysis is one of the few strategies that can also aid in deriving improvement by hinting the other possible alterations, not previously known. Out of a range of located targets, a gene for the enzyme isocitrate lyase was selected. This particular enzyme converts isocitrate to equimolar succinate and glyoxylate concentrations; making it a possibly suitable target. Plasmid pRS426CT, when engineered with *icl1* gene was interchangeably named as pICL1 upon its expression. With this specific plasmid in evolved 8D, the maximum titer became 0.90 g/L i.e., 30 folds more than the original reference strain [94, 95]. An industrial *S. cerevisiae* SUC-632 strain was reported to produce 0.61 mol/mol of succinic acid at near-zero growth rates, maintaining a stable biomass-specific SA production rate for over 500 h [96]. Some of the *S. cerevisiae* strains were engineered by deleting the pyruvate decarboxylase gene to reduce the carbon flux toward ethanol

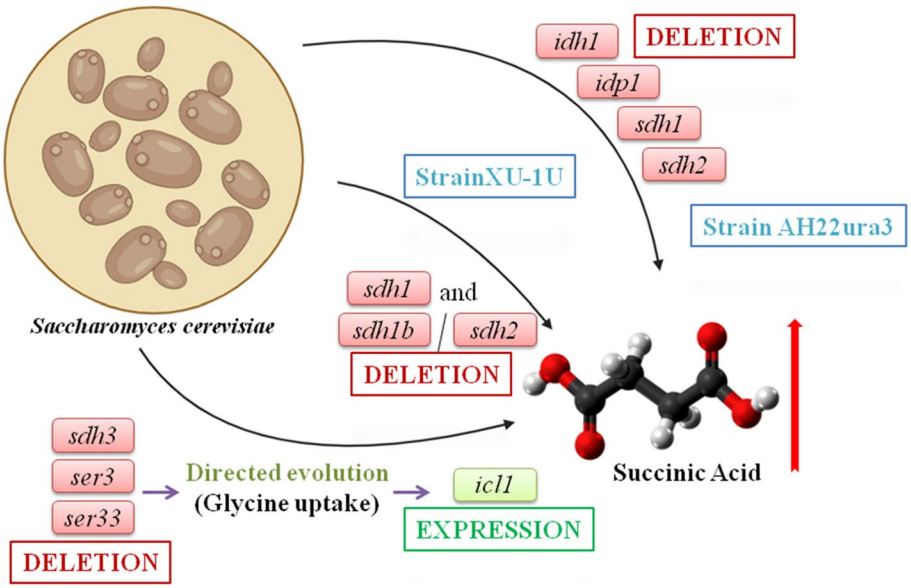

Fig. 4 Genes involved in the enhancement of SA production by engineered *S. cerevisiae* strains. *sdh*- succinate dehydrogenase (1- flavin containing subunit, 2- iron sulfur protein subunit, 3- ubiquinone cytochrome b subunit); *idh1*- isocitrate dehydrogenase; *icl1*- isocitrate lyase; *ser3*- D-3 phosphoglycerate dehydrogenase; *ser33*- phosphoglycerate dehydrogenase

production [97]. The genes involved in the techniques used for the metabolic engineering of *S. cerevisiae* for enhanced succinic acid production have been represented in Fig. 4.

Yarrowia lipolytica

Among the non-conventional yeast *Y. lipolytica* has been widely used as a workhorse for the manufacturing of biochemicals owing to its versatile characteristics such as its ability to consume a wide range of hydrophilic and hydrophobic substrates, availability of genetic tools, tolerant to extreme fermentation conditions such as osmotic stress etc., high cell density growth can be achieved and is recognized as GRAS (Generally Regarded as Safe Organism) by US-FDA [14, 98–101]. Since it can secrete numerous organic acids it is naturally predisposed to grow under low pH conditions [102]. The pKa value of the succinic acid lies between 4.5 and 5.6 at 25 °C and pH 3.5 about 80% succinic acid will be in its protonated form. Naturally yeast strains tend to accumulate a small amount of succinic acid as a by-product. *Y. lipolytica* can accumulate more than 20% of dry cell weight as lipid, this quality of producing fatty acid and lipid-based products sparked the mounting interest in developing metabolic tools such as CRISPR-Cas9 for engineering the strain to produce high titer of fatty acid-derived products. Further, plenty of literature on *Y. lipolytica* is grounded on the production of Krebs cycle intermediates such as citric acid, isocitric acid, and α-ketoglutaric acid [101, 103, 104]. This makes *Y. lipolytica* a most favorable host to produce another citric acid cycle product such as SA. Kamzolova et al., 2014 engineered *Y. lipolytica* VKMY-2412 strain which was able to secrete high titers of α-ketoglutaric acid, which is later decarboxylated using H_2O_2 to produce SA [105].

The state of art rational metabolic engineering by targeted modification in the genome has proven to enhance the productivity of the desired products. However, some modifications will not take a minute detail about metabolic reactions and complex regulatory networks, which leads to a halted growth rate and reduced metabolic activity [106, 107]. Production of citric acid generates reducing equivalents which is essential for the respiratory chain; disruption of succinate dehydrogenase (SDH) will eventually result in the accumulation of succinic acid with liberation of one carbon dioxide molecule. SDH is the only pathway for the conversion of succinate to fumarate and also leads to disruption of reducing equivalent molecule regeneration such as $FADH_2$ molecule and ultimately results in lower ATP generation in oxidative phosphorylation. Further channeling the produced succinic acid molecule is an energy-intensive process that requires additional ATP. The drain of ATP molecules in SDH mutant cells is reflected in a slower growth rate when grown on glucose-based media. Yuzbashev et al., 2016, deleted *sdh*2 gene which impaired its growth in glucose while higher productivity was observed in glycerol as it is a more reduced carbon source compared to glucose and generates 3 molecules of ATP [108]. Gao et al., 2016 deleted *sdh5* which encodes the SDH factor2 assembly in *Y. lipolytica* PO1f strain (derived from W29). The designated strain PGC01003 displayed a good amount of succinic acid titer with 160 g/L in a fed-batch cultivation condition using crude glycerol as a carbon substrate [109]. However, this strain was unable to uptake glucose and was subjected to adaptive evolution for 21 days and the resulting strain PSA02004 was able to uptake a higher concentration of glucose and produced 65.7 g/L of succinic acid with pH maintained at 6.0 [110]. Cui et al., 2017 have attempted to overexpress the crucial enzymes involved in the oxidative TCA cycle, glyoxylate shunt, and reductive carboxylation and observed its effect on SA production and by-product formation [111]. The mutant strain expressing phosphoenolpyruvate carboxykinase (ScPCK) from *S. cerevisiae* and endogenous succinyl-CoA synthase beta subunit (YlSCS2) showed 110.7 g/L of SA production with the titer of 0.53 g/L using glycerol as the carbon source in a complex medium. Another study led by Babaei et al., 2019, showed that the deletion of SDH1 reduced 77% of SDH activity but did not impair the cell growth with glucose. Further, the overexpression of PEP carboxykinase and short adaption on glucose has significantly reduced the lag phase and improved the SA production by 35.3 g/L with 0.26 g/g yield on glucose-containing mineral medium with mannitol as a major by-product [7]. Figure 5 shows the techniques used for the enhancement of non-native SA production in *Y. lipolytica*.

Table 1 summarizes some of the metabolic engineering strategies adapted for enhanced succinic acid production in heterologous hosts.

Utilization of renewable sources for the production of Succinic acid

The majority of the research interest is concentrated on utilizing waste renewable resources for the development of value-added products. As mentioned above production of SA using pure carbon sources such as glucose will severely affect the production cost. Hence feedstock plays a key role in the cost economics for the production of bulk chemicals through biological routes. Hence it is imperative to use inexpensive feedstocks for the bioproduction of SA making the process economical [112]. The biochemical route requires carbon dioxide, a primary greenhouse gas, as a co-substrate and therefore, can contribute towards curbing carbon emissions. The usage of

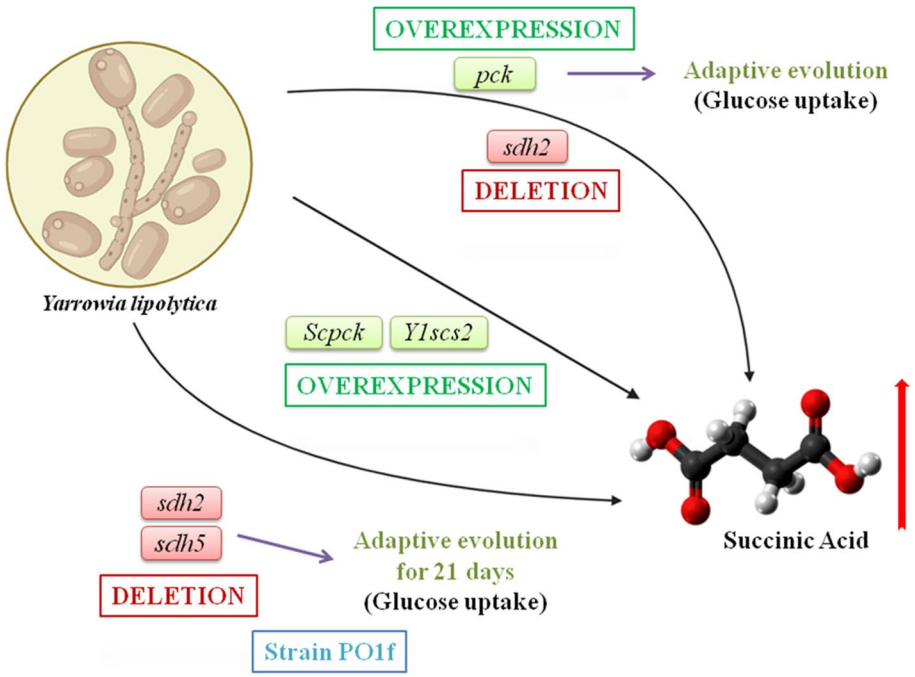

Fig. 5 Genes involved in the enhancement of SA production by engineered *Y. lipolytica* strains. *pck*- phosphoenolpyruvate carboxykinase; *Scpck*- phosphoenolpyruvate carboxykinase from *Saccharomyces cerevisiae*; *sdh2*- iron sulfur protein subunit of succinate dehydrogenase; *sdh5*- succinate dehydrogenase assembly factor; *Y1sc2*- beta subunit of endogenous succinyl-CoA synthase

renewable sources other than pure carbon source such as glucose, which is currently used in many industries significantly brings down the production cost [11].

LCB comprises abundant organic content which can be channeled towards the production of bio-based components. However, to date, much of the research attention is being paid to cellulosic components, and xylose is discarded as waste [113, 114]. Over the last few decades, enormous efforts have been made towards SA production through microbial routes using crude renewable sources. *A. succinogenes* can utilize an eclectic range of carbon sources and can efficiently utilize C5 and C6 carbon sources, hence can be cultured using LCB hydrolysates for the production of SA. Sugarcane bagasse (SB) is one of the attractive LCB feedstocks with rich cellulose and hemicellulose content, Borges and Pereira, 2011 have optimized a medium for *A. succinogenes* CIP 106512 and reported the SA production titer of 22.5 g/L using SB with sodium bicarbonate as a neutralizing agent under batch cultivation [115]. Pateraki et al., 2016a used xylose-rich spent sulfite liquor and reported the SA production of 27.4 and 26.0 g/L by *A. succinogenes* 130Z and *B. succiniciproducens* JF4016, respectively [116]. Corncob hydrolysate is another substrate rich in hemicelluloses and other sugars, thereby making it highly suitable for biorefineries. Wang et al., 2011 used corn stalk enzymatic hydrolysate as a carbon substrate and observed SA production of 57.8 g/L. The mutant *E. coli* 408 with deletion of a gene such as *pflB, ldhA, ppc, and ptsG* and overexpressed ATP-forming phosphoenolpyruvate (PEP) carboxykinase (PEPCK) was able to convert corn stalk hydrolysate to SA with the titer and yield of 23.1 g/L and 0.85 g/L, respectively [117]. *A. succinogenes* CICC 11014 was able to consume xylose-rich corncob hydrolysate and produce SA with titer 23.64 g/L [118]. The strain is also able to consume corn stover, wheat milling by-products, Duckweed, rape seeds, waste bread, cane molasses, etc., [119–122]. The *E. coli* strain possesses the xylose isomerase gene which enables the strain to consume xylose, which is the second largest fraction of LCB. Hodge et al., 2009, reported 42.2 g/L SA with a yield of 0.78 g/g using softwood dilute acid hydrolysate [123].

C. glutamicum is engineered to utilize C5 sugars along with its natural C6 sugar utilization property, as most of the bacteria lack a pentose assimilation pathway. Heterologous gene expression of *xylA* encoding xylose isomerase and *xylB* encoding xylulokinase from *E. coli* MG1655 enables the bacteria to consume both sugars efficiently [124]. Mao et al., 2018 overexpressed *xylA* and *xylB* from *Xanthomonas campestris* in *C. glutamicum* strain SAZ3 and designated the strain as CGS5, followed by endogenous expression of *tkt* and *tal* genes and the introduction of the *araE* gene from *B. subtilis*. The mutant illustrated

Table 1 Metabolic engineering of heterologous host for the production of SA

Engineering Strategy	Carbon substrate	Culture methods	Titer (g/L)	Productivity (g/L/h)	Yield (g/g)	By-product	Reference
Escherichia coli							
Overexpression of *ppc* gene expressing PEP carboxykinase	Glucose	Batch, Anaerobic	10.7	0.594	0.29	Ethanol, Lactate, Acetate, Formate	[32]
Overexpression of heterologous *pyc*	Glucose	Batch, Anaerobic	1.77	0.177	0.177	Lactate, Formate	[85]
Δldh and *Δpfl* gene, Overexpression of *sfcA* gene	Glucose	Batch, Anaerobic	12.8	0.29	0.64	Acetate Formate	[37]
Δldh and *Δpfl* gene, Mutation in *ptsG* gene	Glucose	Fed-Batch, Dual Phase Aeration	-	0.87	0.65	Acetate, Ethanol	[40]
	Glucose	Fed-Batch, Anaerobic	-	-	0.60	Acetate, Ethanol	[39]
	Glucose	Fed-Batch, Anaerobic	-	-	0.88	Acetate, Ethanol	[86]
Overexpression of *pyc*	Glucose	Fed-Batch, Dual Phase Aeration	99.2	1.3	1.1	Pyruvate, Formate	[40]
Mutation in *ptsG21* gene, followed by *Δpfl* and *ΔldhA* gene,	Glucose	Batch, Dual Phase Aeration	-	-	0.88	Acetate	[41]
	Fructose	Batch, Dual Phase Aeration	-	-	0.44	Acetate, Formate, Malate	[41]
	Glucose and Fructose (1:1)	Batch, Dual Phase Aeration	-	-	0.71	Acetate. Formate	[41]
ΔadhE, ΔldhA genes; and overexpression of heterologous *pyc* from *Lactococcus lactis*	Glucose	Batch, Anaerobic	15.6	0.65	0.83	Formate. Acetate	[42]
ΔptsG system	Glucose	Batch, Anaerobic	-	-	0.91	Formate. Acetate	[42]
ΔadhE, ΔldhA, and *Δackpta, ΔiclR* gene inactivation; overexpression of heterologous *pyc* from *Lactococcus lactis*	Glucose	Fed-batch, anaerobic	40	0.42	1.06	Formate. Acetate	[43]
ΔadhE, ΔldhA, and *Δackpta;* overexpression of heterologous *pyc* from *Lactococcus lactis*	Glucose	Batch, Anaerobic	15.9	0.64	1.07	Formate	[87]
ΔiclR, ΔsdhAB, Δicd, Δ (ackA-pta), ΔpoxB	Glucose	Batch, Aerobic	4.61	0.06	0.43	Pyruvate, Acetate	[44]

Table 1 (continued)

Engineering Strategy	Carbon substrate	Culture methods	Titer (g/L)	Productivity (g/L/h)	Yield (g/g)	By-product	Reference
Overexpression of sorghum *pepc*	Glucose	Batch, Aerobic	9	0.14	0.72	Pyruvate, Acetate	[44]
ΔldhA, ΔadhE, ΔackA, ΔfocA-pflB	Glucose	Fed-batch, Anaerobic	86.6	0.9	0.92	Malate, acetate	[46]
ΔldhA, ΔadhE, ΔackA, ΔfocA-pflB, ΔmgsA, ΔpoxB	Glucose	Fed-batch, Anaerobic	78.8	0.82	0.79	Acetate, Pyruvate, Malate	[46]
Δpta-ackA in KJ122(*ΔldhA, ΔadhE, ΔfocA-pflB, ΔmgsA, ΔpoxB, ΔtdcDE, ΔcitF, ΔaspC, ΔsfcA*)	Glucose	Fed-batch, Anaerobic	71.6	0.75	1	Acetate, Pyruvate, Malate	[47]
Corynebacterium glutamicum							
ΔldhA	Glucose	Micro-aerobic, fed-batch with membrane for cell recycling	23	3.83	0.19	Lactate	[51]
ΔldhA, Over-expressed *pyc*	Glucose	Two stages – aerobic growth / Anaerobic fed-batch	146	3.17	0.92	Acetate	[52]
ΔldhA Δpta Δpqo ΔcatP$_{sod}$-*ppcP*$_{sod}$-*pyc* Expression of *xylA, xylB, araE* over- expression of tkt and tal	Corn stalk	Two stage, aerobic culture of biomass and anaerobic batch fermentation, minimal medium	98.6	4.29	0.87	-	[88]
araBAD from *Escherichia coli* was inserted	Arabinose	Aerobic	74.4	0.36	0.58	Acetate Pyruvate	[53]
	Glucose and arabinose		110.2	0.28	0.56 (mol substrate)$^{-1}$	Acetate Pyruvate	
ΔldhA gene	Arabinose	Two-stage fermentation system in screw-cap tube	1.74	0.87	0.89	-	[54]
	Glucose and arabinose		1.31	0.22	0.63	-	
	Glucose and Sucrose		3.31	0.41	1.48	-	
	Sucrose and Arabinose		2.85	0.36	1.28	-	
	Glucose, Sucrose and Arabinose		2.84	0.36	1.17	-	

Table 1 (continued)

Engineering Strategy	Carbon substrate	Culture methods	Titer (g/L)	Productivity (g/L/h)	Yield (g/g)	By-product	Reference
Δcat, Δpqo, Δpta-ackA, ΔldhA, over-expression of native *pyc* and *Mycobacterium vaccae fdh*	Glucose	Two stages – aerobic culture/ anaerobic fed- batch production	82	1.60	0.36	Acetate	[55]
Δcat Δpqo Δpta- ackA ΔldhA over-expression of native pyc and *Mycobacterium vaccae fdh*	Glucose	Two stages – aerobic culture/ anaerobic fed- batch production	134	2.48	1.67	-	[89]
Expression of *E. coli glpFKD* operon	Glycerol	Fed-batch, aerobic culture	9.3	0.423	0.42	-	[56]
ΔldhA derivative with additional in-frame deletion of NCgl2130	Glucose	Fermentation culture under microaerobic and anaerobic conditions,	8.1	3.24	0.56	-	[58]
Corynebacterium glutamicum AJ13869DsdhA/ pVK9::PmsrA-CgynfM AJ110655	Glucose	Aerobic condition	110	15	0.33	-	[59]
Corynebacterium glutamicum FK948/pVK9::PmsrA-CgynfM (deleted sucE1 gene)	Glucose	Anaerobic condition	270	15.3	-	-	[59]
Δldh, Δpta-ackA, ΔactA, ΔpoxB, pycP458, Δpck_ Ptuf::Ms.pckG, Ptuf::ppc, ΔptsG Over-expression of NCgl0275	Glucose	One stage – aerobic culture/anaerobic fed- batch production	152.2	1.11	1.10	-	[90]
ΔaceE Δpqo ΔldhA ΔC-T ilvN ΔalaT ΔavtA ΔPDHC, ΔPQO	Glucose	One stage fed-batch fermentation	38.9	0.66	0.67	Pyruvate	[91]
BL-1 harboring pBbEB1c-torA-SbAmyA	Micro-algal starch	Minimal medium Sequential aerobic fermentations of CO_2-grown microalgae and engineered *C. glutamicum*	0.5	0.021	0.28	-	[92]
ΔldhA chromosomal integration into the *pta-ackA* locus of *xylA* and *xylB* gene from *E. coli*	Corn cob Hydrolysate (Xylose + Glucose), bicarbonate	Two-stage, aerobic culture of biomass and anaerobic batch fermentation, minimal medium	40.8	0.85	0.69 (mol/mol)	Acetate, Pyruvate	[93]
Expression of acetyl-CoA synthetase Over-expression of gltA gene (citrate synthase)	Glucose	Minimal medium Aerobic growth Fed-batch culture	241 (mM)	3.55 ($mM\ h^{-1}$)	0.62 (mol/mol)	-	[57]

Table 1 (continued)

Engineering Strategy	Carbon substrate	Culture methods	Titer (g/L)	Productivity (g/L/h)	Yield (g/g)	By-product	Reference
Saccharomyces cerevisiae							
Δsdh2, Δsdh1, Δidh1, Δidp1	Glucose	Batch, Aerobic	3.62	0.022	0.072	Ethanol, Glycerol, Acetate	[65]
Δsdh3, Δser3, Δser33	Glucose	Batch, Aerobic	0.60	-	0.03	-	[69]
Evolved 8D with *icl1*	Glucose	Batch, Aerobic	0.90	-	0.05	-	[69]
Yarrowia lipolytica							
Δsdh5	Crude glycerol	Aerobic Fed-batch	160	0.4	0.4	Acetic acid	[94]
sΔsdh5, overexpression of *ScPCK, YlSCS2*	Glycerol	Aerobic Fed-batch	110.7	-	0.53	Mannitol	[84]
Δsdh5, overexpression of *XR, XDH, XK*	Xylose	Aerobic Fed-Batch	22.3	-	0.15	Acetic acid	[95]

an exceptional capability to manufacture SA in two-stage fermentation by utilizing both glucose and xylose in a mixture in an anaerobic process [125]. The succinate titer was about 98.6 g/L from corn stalk hydrolysate with a yield of 0.87 g/g total substrates and a productivity of 4.29 g/L/h during the anaerobic stage. Wang et al., 2014 designed a xylose metabolic pathway in *C. glutamicum* by expressing the *xylA* and *xylB* genes from *Escherichia coli* in a heterologous manner. Engineered *C. glutamicum* NC-2 utilized both glucose and xylose fractions obtained from dilute-acid hydrolysates of corn cobs. To overcome the inhibitory effect of fermentative inhibitors such as furfural and hydroxymethyl furfural in succinate production the corn cob hydrolysate was pretreated with activated charcoal. The result showed succinate production of 40.8 g/L and 0.69 g/g from corn cob hydrolysates (containing 4 g/L glucose and 55 g/L xylose) under oxygen deficit conditions with the addition of sodium carbonate [126].

The yeast *Y. lipolytica* has been explored for SA production using different carbon sources such as glycerol and glucose [111, 127–130]. A recent report by Ong et al., 2019 attempted SA production from recombinant *Y. lipolytica* (*Δsdh*) using a glucose and xylose mixture obtained from the sugarcane bagasse. However, as expected the strain showed a preference towards glucose rather than xylose, and the consumption of xylose was witnessed after the complete uptake of glucose. However, a major portion of the xylose fraction was unutilized (50–70%) depicting the absence of the xylose metabolizing capability of strain [131]. Genome studies of *Y. lipolytica* have shown weak XDH expression in the xylose metabolizing pathway, which is the main limiting factor of C5 carbon uptake [132]. Prabhu et al., 2020b overexpressed endogenous *XR* (xylose reductase), *XDH* (xylitol dehydrogenase), and *XK* (xylulose kinase) in *Y. lipolytica* PSA02004. The recombinant strain was able to take up xylose as the sole carbon source and able to produce 5.6 g/L of SA with 0.14 g/g yield using xylose-rich sugarcane bagasse without pH control [133].

Conclusion

Succinic acid is a valuable platform chemical with a multitude of applications. The microbial route of SA production is directed toward sustainability and avoiding dependence on fossil fuels. However, there are several bottlenecks associated with the production of SA using microbial cell factories. With the advancement in genetic tools, the barriers to SA production can be resolved using rational engineering. Several non-SA-producing hosts such as *E. coli, S. cerevisiae*, *C. glutamicum,* and *Y. lipolytica* have been engineered to improve the secretion of SA. Further, the comprehension of bio-based SA production is extremely reliant on the exploitation of inexpensive renewable resources. The biological SA manufactured from LCB and other waste sources can be a potential competitor of the petrochemical route. Strain engineering has significantly improved the potential of the strain to valorize the waste and convert it to value-added products such as SA.

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