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Technological advancements in valorization of second generation (2G) feedstocks for bio-based succinic acid production

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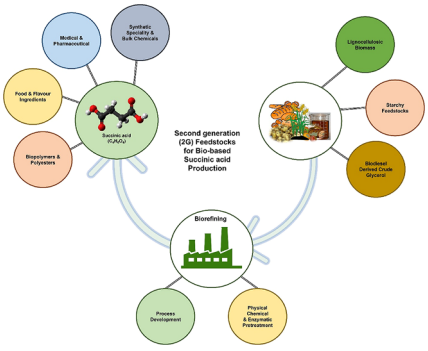
HIGHLIGHTS

- Biobased production SA from 2G feedstocks can be cost-effective.
- Process engineering approaches can improve substrate utilization and SA synthesis.
- Reduced by-products formation by gene knock out can increase the flux towards SA accumulation.
- Integrating various processes aid in cost-competitive SA fermentation processes.
- Techno-economic analysis helps to understand risks in commercialization.

GRAPHICAL ABSTRACT



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ABSTRACT

Succinic acid (SA) is used as a commodity chemical and as a precursor in chemical industry to produce other derivatives such as 1,4-butaneidol, tetrahydrofuran, fumaric acid, and bio-polyesters. The production of bio-based SA from renewable feedstocks has always been in the limelight owing to the advantages of renewability, abundance and reducing climate change by CO_2 capture. Considering this, the current review focuses on various 2G feedstocks such as lignocellulosic biomass, crude glycerol, and food waste for cost-effective SA production. It also highlights the importance of producing SA via separate enzymatic hydrolysis and fermentation, simultaneous saccharification and fermentation, and consolidated bioprocessing. Furthermore, recent advances in genetic engineering, and downstream SA processing are thoroughly discussed. It also elaborates on the techno-economic analysis and life cycle assessment (LCA) studies carried out to understand the economics and environmental effects of bio-based SA synthesis.

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1. Introduction

Global climate change and other environmental issues associated with the petrochemical routes (which use fossil-based resources) such as toxic catalysts, high temperature and pressure operation conditions, and dangers from excessive CO_2 levels in exhausts have been highlighted in the literature (Pinazo et al., 2015). To prevent this global warming and deleterious impacts on the environment, policies were developed with suggestions on the generation of biofuels and bio-based chemicals. The global chemical market is advancing and the transition to a bio-based production of chemicals is being considered highly sustainable for a low carbon economy (E4 Tech, 2017). By 2025, it is estimated that over 15% out of 3 trillion chemicals required around the World would be derived from bio-based sources (Vaswani, 2010). Among these chemicals, succinic acid (SA) ($C_4H_6O_4$) also called as amber or butanedioic acid with molecular weight of 118.09 g/mol is a platform chemical with numerous applications in the food, polymer, paints and pharmaceutical industries. SA and its derivatives are applied in the production of green solvents, surfactants, detergents, pigments, biodegradable polymers and plasticizers (Fig. 1) (McKinlay et al., 2010; Vaswani, 2010; Zeikus et al., 1999) and the market has been divided accordingly (industrial – 57.1%; pharmaceutical – 15.91%; food and beverages – 13.07% and others – 13.92%). In food industries, it is mostly used as an additive in beverages, pH regulator, flavouring enhancer and as an acidulating agent (Ahn et al., 2016; Saxena et al., 2016). Its largest market is from the surfactant, foaming and detergent industries. It is used in the prevention of corrosion (acting as an ion chelator) in the industrial sector. In the pharmaceutical industry, it is used to produce antibiotics, amino acids, and vitamins. Currently, antimicrobial resistance (AMR) is a huge risk, where opportunistic fungi like *Candida* sp. have become a major health concern. These organisms form a biofilm on the tissues or bones, and are hard to treat as they are more resistant than the planktonic cells. However it was understood that organic acids are effective antimicrobial metabolites. Jäger and associates developed biocompatible SA based polyesters like polyethylene succinate (PES), polypropylene succinate (PPS), and polybutylene succinate (PBS), where PBS was observed to have strong anti-fungal activity and PES could inhibit the biofilm formation of *C. albicans* and *C. tropicalis* (Jäger et al., 2015; Mohan and Purohit, 2020) Conventionally SA is synthesized via catalytic hydrogenation, electrolytic reduction, or paraffin oxidation of maleic anhydride sourced from crude benzene (McKinlay et al., 2007; Zeikus et al., 1999).

The US Department of Energy (US, DOE) recognised SA as one of the twelve high-value platform chemicals which are obtainable from biomass (Chinthapalli et al., 2018; Werpy and Petersen, 2004). The global bio-based SA market is expected to hit $235.02 million by 2030 (Bio-Succinic Acid Market) making SA a high contending bio-based chemical. SA is an important metabolite in the biological metabolism of plants, humans, and microorganisms, but the maximum concentration was accumulated during anaerobic fermentation.

With the concept of biorefinery, utilization of renewable feedstocks for bio-based SA production should alleviate the current energy crisis associated high carbon emissions in traditional SA synthesis (Eurostat, 2015; Hellenic Biogas Association, 2018). The production costs for petrochemical SA are estimated to be €2554/MT that is expensive compared to bio-based SA synthesis (€1045/MT) (Pinazo et al., 2015). Furthermore, SA derived from biomass-based feedstocks may lead to the reduction of more than 60% of greenhouse gases (GHG) emissions when compared to the carbon footprints from petrochemical-based SA production (Musonda et al., 2020; Stegmann et al., 2020), since 1 mol of CO_2 is fixed for every mol of SA produced (Almqvist et al., 2016). Despite these advantages, the commercial implementation of bio-based SA production is still hindered due to various reasons. During bio-based SA production, 60–80% of the cost could be attributed to the downstream processing and purification, 20–25% is linked to fermentation and the remaining 10–15% can be related to the cost of the feedstock (Morales et al., 2016). This suggests that improvements with respect to fermentation and purification of bio-based SA could decrease the overall production costs (Morales et al., 2016). Furthermore, the major challenges in the biological upstream process include the cost of the feedstock, low productivity and yield as well as formation of other acid by-products. Numerous studies have been carried out to overcome these challenges. For example, use of renewable substrates such as first and second-generation feedstocks (2G) to lower the cost of the substrate, optimizing various process parameters, configuration of fermenters, different operational techniques and genetic modifications to host organisms to improve the yield were studied (Amulya and Mohan, 2022; Mancini et al., 2020). With respect to downstream processing, various techniques such as crystallization techniques, reactive extraction, membrane technologies, electrodialysis and electrochemical extraction have been investigated (Mancini et al., 2020).

In this review, research progress with respect to the use of different second-generation feedstocks (2G) and different bioconversion strategies for SA production have been discussed. Biosynthesis of SA along with a detailed overview on the genetic and process engineering

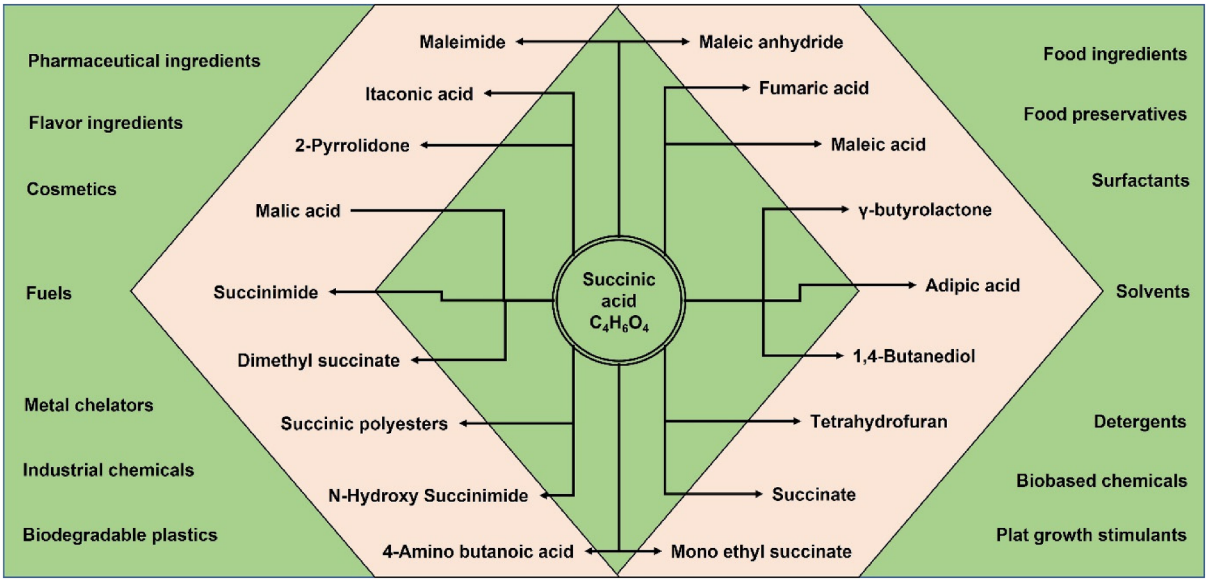

Fig. 1. Applications of succinic acid and its chemical derivatives.

approaches for improved production of SA from 2G feedstocks is elaborated. Furthermore, bottlenecks for separation and purification of SA from fermented broth and applications of SA in industries are also discussed.

2. Second generation feedstocks as the carbon source

Although first generation feedstocks are the cleaner source of feedstocks due to their starch and sugar contents, their use as a carbon source for the production of bio-based SA would be debatable and a serious issue. This is due to the need to address food security for the growing propulation (Fig. 2). Therefore, an alternative approach is to find the most suitable, non-edible, and renewable feedstock such as second-generation feedstocks to produce bio-based SA.

2.1. Agricultural residues or lignocellulosic biomass

Due to rapid growth in global population and demand for food, agricultural practices are increasing, resulting in huge quantities of post-harvest residues (Vivek et al., 2019), particularly in Asian countries, where maize, wheat, rice, and sugarcane are the major agricultural crops. Accounting the whole cultivation area, significant amounts of crop residues will be available as agricultural wastes. The traditional methods of disposing these wastes are landfilling, incineration or composting. The impact of landfilling and incineration is against the green policies, since these are potential causes for air pollution and emission of harmful GHGs. Although composting has several advantages and generates biogas and biofertilizers, the process economics and the competition with chemical fertilizers limit its market value (Chen et al., 2016).

Agricultural wastes are composed of organic polymeric carbon in the form of cellulose and hemicellulose. These agricultural residues are termed lignocellulosic biomass (LCB) due to their composition i.e. 25 – 30% w/w cellulose, 40 – 50% w/w hemicellulose, 15 – 25% w/w lignin, and 5 – 10% w/w ash (Batista Meneses et al., 2020; Peinemann and Pleissner, 2020). The cellulose and hemicellulose upon depolymerization generate a glucose and xylose rich mixture of hexoses and pentoses, respectively. Although LCB is composed of fermentable sugars, access to those sugars is hindered due to recalcitrance of plant cell walls. The cellulose constitutes of an inner skeleton, surrounded by hemicellulose, and together encrusted by a lignin matrix. Pretreatment (Canilha et al., 2013) using acid (HCl, H_2SO_4, H_3PO_4) and alkali (NaOH, Na_2SO_3, NH_4OH) removes the lignin, enhancing the depolymerisation of the cellulose and hemicellulose. In the initial pretreatments the ionized H^+ ions generated at high temperatures (160 – 220 °C) attack the ether bonds and depolymerize the lignin and hemicellulosic fraction of LCB, providing an access to the enzyme for saccharification (Chen et al., 2021). Further, the enzyme accessible polymers (cellulose and hemicellulose) can be saccharified by cellulases and xylanases, respectively. LCB such as oil palm fonds, rice straw, wheat straw, corn stalk, corn husk, and corn cobs are rich in cellulosic content, with an estimation of 1.5 trillion tons/year cellulose production from these residues (Akhtar et al., 2014). The sugars obtained after the enzymatic hydrolysis are supplemented for production of value-added chemicals like organic acids, biopolymers, diols, amino acids, and nutraceuticals. Bioconversion of LCB into SA involves the following steps: (i) initial pretreatment of LCB residues for soluble lignin removal, (ii) enzymatic hydrolysis of polysaccharides like cellulose and hemicellulose into their respective components i.e. hexoses and pentoses, and (iii) fermentation of these sugars into SA by a suitable host. These processes for SA production using LCB are discussed in the following sections.

2.1.1. Separate hydrolysis and fermentation (SHF)

SHF is a two-step process in which LCB residues are saccharified by hydrolytic enzymes and then the fermentable sugars obtained are converted into target metabolites by chassis microbial strains. The process can be either performed as single or dual unit operations. In single unit operation, the initial saccharification and later fermentation can be carried out in a single bioreactor. Whereas in a dual unit operation, the saccharification is carried out in a hydrolysis reactor and the sugars and residual solids are separated using membrane filtration. The fermentable sugars are later fermented in fermentor. In both scenarios, higher efficiencies can be reached because both the saccharification and fermentation can be carried out under different optimum temperatures. However, these processes can differ with respect to cost of equipment and process economics.

Several strains of bacteria and yeast were reported to utilize the saccharified sugars from LCB residues to produce SA. For example, in an anaerobic cultivation performed in a 3L bioreactor, *A. succinogenes* supplemented with enzymatic hydrolysate of chemically (3% H_2O_2) pretreated hemp resulted in maximum SA titers of 21.9 g/L with 0.83 g/g sugar yield (Gunnarsson et al., 2015). Along with the feedstocks, process engineering could provide increased product titers.

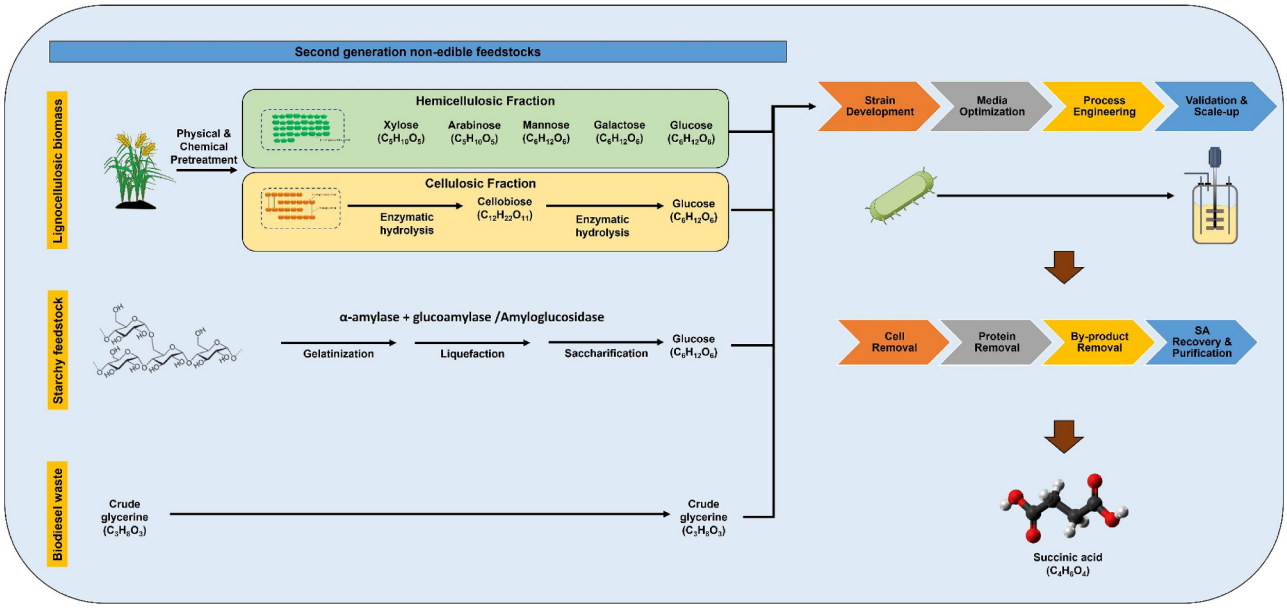

Fig. 2. Types of 2G feedstocks and their monomeric components for succinic acid biosynthesis.

A. succinogenes 130Z strain, which is known to accumulate high titers of SA, was immobilized on a custom-made polypropylene impellor with perforated tubes acting as the support for the formation of a biofilm. The cultivation was carried out in chemostat mode with various dilution rates, using non-detoxified xylose-rich corn stover hydrolysate. It was observed that the strain could accumulate 39.6 g/L SA, with 0.78 g/g yield and 1.77 g/L/h productivity (Bradfield et al., 2015). As the strain performed well in non-detoxified hydrolysate, *A. succinogenes* might have resistance to fermentation inhibitors produced during the chemical and thermal pretreatment process. Further evaluation on strain efficacy could improve the knowledge for providing commercial status for SA production. Xylose-rich corn stover hydrolysate as the feedstock was supplemented to facultative anaerobic, non-pathogenic gram-negative, capnophilic *Basfia succiniciproducens*, resulting in 30 g/L SA, 0.69 g/g yield, and 0.43 g/L.h productivity (Salvachúa et al., 2016b). *Arundo donax*, a perennial herbaceous crop, was pre-treated with steam explosion and further enzymatic hydrolysis was carried out using 140 units of commercial cocktail Novozymes NS22201. The *A. donax* hydrolysate, a mixture of hexoses and pentoses, was supplemented as a substrate for newly isolated *B. succiniciproducens* BPP7. The strain could accumulate 17 g/L SA, with 0.75 mol/mol yield (Cimini et al., 2016). It was observed that *B. succiniciproducens* can simultaneously assimilate both glucose and xylose for SA biosynthesis, suggesting that further focus on metabolic and process modifications could improve the final titers and yield. Cimini et al., 2019 further developed the fed-batch process by implementing simulations through global mass balance calculations, and material flow analysis, resulting in 37 g/L SA with 0.8 g/L/h productivity. In a repeated batch fermentation strategy, using an enzymatic hydrolysate derived from *Agave tequilana* bagasse, *A. succinogenes* was able to accumulate 33.6 g/L SA with 0.38 g/g yield, and 1.32 g/L h productivity (Corona-González et al., 2016).

In UK and most of the European countries, sugar beet is the most important commodity crop providing almost 40% of the world's sugar production with annual trade of 270 million tons. Sugar beet pulp (SBP) is the major by-product from the sugar beet refinery. For example, Wessington Plant, in UK, produces 400 K tonnes of sugar by processing sugar beets, with 350 K tonnes of SBP as the by-product, which can be either provided as the animal feed or incinerated for energy purposes. With the biorefinery concept, supplementing with SBP hydrolysate, the *A. succinogenes* 130Z strain was observed to accumulate 30 g/L SA, with 0.8 g/g yield, and 0.62 g/L.h productivity. In terms of dry weight, 268 g of SA, 20% protein, 303 g of pectin, 78.6 g of phenolic rich content could be obtained from 1 kg sugar beet pulp (Alexandri et al., 2019a). In a novel biorefinery approach, semi simultaneous saccharification and co-fermentation (SSSCF) strategy, *A. succinogenes* supplemented with SCB resulted in 41 g/L, 0.32 g/g and 0.3 g/L.h SA titers, yield, and productivity, respectively. Initially in this approach, the pretreated solids were sterilized (121 °C, 15 min) and enzymatically hydrolysed for 24 h. Later without impeding the enzymatic (cellulase) reaction, the nutrients, and nitrogen sources required for fermentation were added into the reactor was flushed with CO_2 for maintaining anaerobic conditions, followed by inoculation (Chen et al., 2021). In a similar strategy, semi simultaneous saccharification and fermentation (SSSF), using *Landoltia punctata* (duckweed) hydrolysate supplemented to a *A. succinogenes* GXAS137 strain, resulted in 65.31 g/L SA, which is comparatively higher than in SSF (52.41 g/L) and SHF (62.12 g/L) processes. Later when the SSSF strategy was reproduced in 2L bioreactor, final concentrations of 75.46 g/L SA, with 0.82 g/g yield, and 1.35 g/L.h productivity was achieved (Shen et al., 2018).

In a simultaneous saccharification and fermentation strategy, oil palm empty fruit brunches were enzymatically hydrolysed using optimized process conditions (39.5 FPU/g enzyme loading, 36 °C, and pH – 5). *A. succinogenes* strain was able to accumulate 42.9 g/L SA with 0.61 g/g yield (Akhtar et al., 2020). In a study to understand the behaviour of *A. succinogenes* DSM 22257 in representative fermentable sugars of LCB using the mixed sugars like glucose, mannose, arabinose and xylose in their respective (ratio of 5:1:2:4) concentrations, 27 g/L SA was achieved in comparison with 26.5 g/L SA attained with glucose alone as carbon source (Ferone et al., 2017). The in-silico simulation of the fermentation conditions depicted that the expected metabolite concentrations are 25.5 g/L SA, 16 g/L acetic acid (AA) and 11.8 g/L formic acid (FA), but the experimental results turned out to be 27 g/L SA, 5.8 g/L AA, and 2.9 g/L FA. It was also observed that the strain was able to assimilate hexoses and pentoses simultaneously with the carbon flux more towards SA, compared to AA and FA. The synergistic effect of these sugars in SA production can be further evaluated using LCB hydrolysates (Ferone et al., 2017).

An interesting study on impact of organic acids used for initial pretreatment of the LCB, on final titers of SA by *A. succinogenes* 130Z strain was carried out. It was observed that citric acid pretreated LCB hydrolysates displayed a higher total (94%) and individual (92% glucose and 96% xylose) sugar consumption, which is significantly higher than the sulfuric acid pretreated hydrolysates that contained total (77.5%) and individual (83.4% glucose and 69.7% xylose) sugars. It was obvious that the fermentative inhibitors (g/100 g), AA (1.97 vs 5.67), furfural (0.0000 vs 0.0002), and hydroxymethyl furfural (HMF) (0.0004 vs 0.0017), were higher in the case of sulfuric acid pretreatment than the citric acid (Bukhari et al., 2020). However, the performance of citric acid in terms of sugar release and enzyme digestibility was far lower than sulfuric acid, but further studies are required on optimization of process parameters to improvise the citric acid mediated LCB pretreatment. The strain was also evaluated using dilute acid pretreated xylose-rich corn stover hydrolysate resulting in 42.8 g/L SA, with 0.74 g/g and 1.27 g/L. h yield and productivity (Salvachúa et al., 2016a). In another approach, alkali pretreated oil palm frond (OPF) was subjected to enzymatic hydrolysis producing 0.5 g reducing sugar/g biomass. The hydrolysate obtained was supplemented to *A. succinogenes* 130Z strain resulting in 36.6 g/L SA with 0.71 g/g and 0.61 g/L.h, yield and productivity (Indera Luthfi et al., 2016). With these varied feedstocks, and simultaneous sugar consumption efficiency *A. succinogenes* can be of significant commercial potential. LCB residues after the initial pretreatment usually result in xylose-rich pretreated liquors. Later after enzymatic hydrolysis, saccharified glucose is released from cellulose and valorisation of both fermentable sugars is of utmost importance to improve the economics of the bioprocesses.

2.1.2. Consolidated bioprocessing (CBP)

Consolidated bioprocessing (CBP) combines production of hydrolytic enzymes, hydrolysis of LCB residues and microbial fermentation in a single reactor using a single microorganism or a microbial consortium. Enzyme production, hydrolysis and fermentation occurs at different optimum operating conditions, hence developing a microbial consortium that can produce enzymes, and later ferment the saccharified sugars to end products would be an efficient approach (Vivek et al., 2019). For example, *E. coli* Suc260, a genetically engineered strain for SA production was able to accumulate 60.76 g/L SA, with a yield of 0.8 g/g glucose, when the competitive by-product pathways were eliminated. Further, the strain was modified to accumulate SA from cellobiose, a disaccharide available in the LCB hydrolysate. To complement this characteristic feature in *E. coli* Suc260, the β-glucosidase (*BglA*) gene from *Paenibacillus* sp. M1, was expressed. The mutant strain *E. coli* Suc260 (pTbglA) was evaluated on pure cellobiose, and pretreated SB hydrolysate consisting of 25.30 g/L cellobiose, 9.70 g/L glucose, 5.9 g/L arabinose, and 7.1 g/L xylose, resulting in 26.5 and 24.3 g/L SA with 0.88 and 0.89 g/g sugar yield, respectively (Dong et al., 2017). Whereas a consortium of hemicellulase (hemicellulose (xylan) → xylose) producing *Thermoanaerobacterium thermosaccharolyticum* M5 and *A. succinogenes* 130Z was able to produce 32.5 g/L SA with a yield of 0.39 g/g using xylan as the substrate. Later when untreated corncobs were provided as feedstock, 12.51 g/L SA was accumulated (Lu et al., 2020). However, there are serious challenges such as low hydrolytic efficiency, low SA yields and productivity that need to be addressed for

successful demonstration of CBP.

2.2. Biodiesel industry derived crude glycerol

Crude glycerol (CG) is a low value by-product produced during the transesterification process of fats (triglycerides) for biodiesel production. For every 100 kg of biodiesel produced, approximately 10 kg of CG is obtained as waste (50 – 70% purity) containing fatty acid methyl esters, fatty acids, ash, methanol, and other contaminants (Vivek et al., 2017). Glycerol is more reduced in nature than glucose, and bioconversion of 1 mol of glycerol to pyruvate generates 2 mol of NADH, which is advantageous favouring the production of reduced compounds like SA and 1,3-propanediol (Li et al., 2016). Most of the studies related to bioconversion of glycerol to SA are using genetically modified *E. coli* or through the oleaginous yeast *Y. lipolytica*, but there are two recent studies where bacterial isolates were able to accumulate high amounts of SA using crude glycerol. A wild type isolate AKR177 was able to utilize both pure and crude glycerol efficiently producing 117 g/L and 86.9 g/L SA with conversion yields of 1.3 and 0.9 g/g, respectively (Kuenz et al., 2020). However, the strain was not yet identified, but observed to be having a significant potential in accumulating high amounts of SA. Table 1 compiles the usage of different 2G feedstocks as substrates for fermentation of SA. *Y. lipolytica*, an unconventional strictly aerobic yeast, depends on the TCA cycle and electron transport chain for its growth and development. It was observed that *Y. lipolytica* can accumulate various organic acids like citric acid, isocitric acid, and α-ketoglutaric acid. Using developed genetic tools and available whole genome information, an engineered *Y. lipolytica* strain was developed for

Table 1
Succinic acid production using 2G feedstocks.

Microorganism	Feedstock	Pretreatment conditions	Enzymatic hydrolysis	Saccharification efficiency (%)	Mode of Fermentation	SA Titer (g/L)	SA Yield (g/g)	SA Productivity (g/L.h)	Reference
Actinobacillus succinogenes TISTR 1994T	Sugarcane Trash (SCT)	Organosolv (50% v/v ethanol), incubated at 140 °C, 60 mins, and 20% w/v NaOH	Cellulase (Cellic Ctec2) Incubation: 50 °C, 150 rpm for 96 hrs	96.99	Batch	41.39		0.86	Pakchamni et al., 2022
A. succinogenes ATCC 55618	Sugarcane bagasse (SCB)	Alkaline hydrogen peroxide; 5.74% v/v H_2O_2, Incubated at 65.6 °C, 5 h.	Cellulase (6 FPU/g), Hemicellulase (100 U/g), Whey protein (20 mg/g), and Sphorolipid (30 mg/g)	70% Glucose 69% Xylose	Fed-batch	42.3	0.64	0.7	Zhang et al., 2022
A. succinogenes 130Z	Corn fiber	Liquid hot water treatment, incubated at 180 °C, 10 mins	Cellulase (Cellic Ctec2) Incubation at 50 °C, 200 rpm for 72 h.	93.3% Glucose 39.6% Xylose	Batch	27.8	0.61	0.58	Vallecilla-Yepez et al., 2021
A. succinogenes DSM 22257	SCB	Thermochemical pre-treatment	N/A	N/A	Fed-batch	28.7	0.27	0.40	Oreoluwa Jokodola et al., 2022
A. succinogenes DSM 22257	Olive pits	Dilute acid pre-treatment (2% v/v H2SO4; 121 °C, 30 mins)	N/A	N/A	Fed-batch	33.6	0.27	0.46	Oreoluwa Jokodola et al., 2022
A. succinogenes ATCC 55618	Oil Palm Empty Fruit Bunches	Sequential inorganic salt pre-treatment (15% w/v Na_3PO_4, 121 °C, 30 mins); (5% w/v $ZnCl_2$, 121 °C, 30 mins)	Cellulase (40 FPU/g) (Cellic Ctec2) Incubation at 37 °C, 200 rpm	–	Simultaneous Saccharification and Fermentation (SSF)	65.2	0.65*	1.09	Khairil Anwar et al., 2021
A. succinogenes ATCC 55618	Wheat flour	Gelatinization at 75 °C for 10 mins; autoclaving at 121 °C, 120 mins	Glucoamylase from fungal fermentation (*Aspergillus awamori*)	–	Batch with flour hydrolysate + Complex medium	27.2	0.65	1.01	Du et al., 2007
					Batch with flour hydrolysate + Complex medium - Vitamins	35.6	0.82	0.56	
					Batch with flour hydrolysate + fungal hydrolysate + Minerals	23.2	0.54	0.33	
					Batch with flour hydrolysate + Fungal hydrolysate	15.9	0.47	0.31	
A. succinogenes ATCC 55618	Wheat flour	N/A	Enzyme cocktail from *A. awamori* and *A. oryzae*, and proteases (55 °C, 500 rpm, 24 h)	–	Batch	64.2	0.81	1.19	Du et al., 2008
A. succinogenes ATCC 55618	Glycerol	N/A	N/A	N/A	Batch	24.39	0.95	2.13	Margarida et al., 2014
					Fed-batch	49.6	0.92	0.62	

*Yield calculated based on the grams of biomass used.

accumulating SA by deleting the gene or replacing the promoter of succinate dehydrogenase (Succinate + FAD → Fumarate + $FADH_2$), resulting in a mutant strain that can accumulate 40.5 g/L SA, with 0.36 g/g yield (Yuzbashev et al., 2010). A similar approach was conducted in another strain of *Y. lipolytica*, Po1f by knocking out the SDH5 subunit of succinate dehydrogenase. The resultant mutant PGC01003, produced high titers of SA, but high concentrations of AA were found as by-product, affecting the cell growth and metabolism (Gao et al., 2016). Later the research group focussed on eliminating AA by deleting the CoA-transferase gene (*Ylach*). Further heterologous overexpression of phosphoenol pyruvate (PEP) carboxykinase (Oxaloacetate + GTP → PEP + GDP + CO_2) from *S. cerevisiae* and endogenous succinyl-CoA synthase (Succinyl-CoA + ADP + PO_4^- → Succinate + ATP + CoASH), 110.7 g/L SA with 0.53 g/g without any pH control was achieved (Cui et al., 2017). To improve the glycerol uptake rate, and to increase the SA titers, the glycerol kinase (Glycerol + ATP → Glycerol – 3 – Phosphate + ADP) (GUT1) gene was overexpressed in PGC01003 strain, the resulting mutant strain RIY420 produced 178 g/L SA, with 0.46 g/g yield and 0.44 g/L/h productivity in a fed-batch mode of fermentation (Ong et al., 2020). The strategy tends to be industrially viable, as there are no by-products, and the SA is produced in acidic form rather than in salt form, which makes downstream processing much easier.

2.3. Food and bakery wastes

Food waste is a serious global issue, initially when FGFs were used for production of value-added chemicals and fuels, it lead to huge debate on food vs feed, but there is an unaccountable food loss across the supply chain including harvesting, transport, storage, processing, packaging, distribution, marketing and household usage. Every year around 1.5 billion tonnes of food is wasted worldwide, accounting for one-third of global food production (Rex et al., 2017). Food waste disposal in landfills provides a possible hazardous challenge to ecosystems, the environment, and society due to its high nutritional composition (Rex et al., 2017). Food wastes contain up to 30 – 60% carbohydrate, 10 – 40% lipids and around 5 – 10% protein, thus making them a good substrate for SA production. For example, mixed food hydrolysate consisting of 31.9 g/L glucose and 280 mg/L free amino nitrogen was supplemented to genetically engineered an *E. coli* strain resulting in 29.9 g/L SA with a yield of 0.22 g/g food waste (Sun et al., 2014b). Similarly, an enzyme cocktail consisting of 2% glucoamylase, 1% cellulase, 2% hemicellulase, and 0.25% pectinase was used to hydrolyse mixed fruit and vegetable waste at pH 5.0 and 55 °C, the resultant hydrolysate has 56.7 g/L glucose. Using the food waste hydrolysate and corn steep liquor, a genetically engineered *Y. lipolytica* PSA02004 (Δ*SDH* and evolved to grow on glucose) strain was able to accumulate 140.6 g/L SA with 0.69 g/L/h productivity (Li et al., 2018b).

3. Genetic engineering strategies to improve SA titers

3.1. Biochemistry and physiology of succinic acid production

Various native strains including *Actinobacillus succinogenes, Mannheimia succiniciproducens, Anaerobiospirillum succiniciproducens, Corynebacterium crenatum, Bacteroides amylophilus, Clostridium thermosuccinogenes, Escherichia coli, Klebsiella pneumoniae, Paecilomyces varioti, Ruminococcus flavefaciens and Succinivibrio dextrinosolvens* can accumulate SA as an end product. In addition, many microbial species have an ability to synthesize SA as an end product or as an intermediate, which is further metabolized. Among these microorganisms, the capnophilic ruminal facultative anaerobic, non-pathogenic, gram-negative bacterium *A. succinogenes* is considered as an industrially potent microbial strain for SA production (Dessie et al., 2018; Nghiem et al., 2017). Most of the SA producing strains were isolated from the rumen, because SA acts as an important metabolic precursor for propionate biosynthesis, which is absorbed by the rumen colon wall and is further oxidized to meet the energy and other metabolic demands of the animals. SA is derived as an intermediate product from hexose and pentose through three different microbial assimilation routes, reductive branch (Equation (1)) of TCA cycle, which is active under anaerobic conditions, oxidative branch of tricarboxylic acid (TCA) (Equation (2)) pathway primarily active under aerobic conditions, or glyoxylate (Equation (3)) pathway which is active under aerobic conditions when the cells are adapted to grow on carbon source containing two carbon atoms such as acetate (Dessie et al., 2018). However, in most of the cases, in the oxidative TCA and glyoxylate pathways, SA is an intermediate, which is further converted into fumarate or other metabolites. Hence, to realize succinate accumulation through oxidative or glyoxylate pathways, the succinate dehydrogenase gene should be blocked preventing further oxidation to fumarate.

$$C_6H_{12}O_6 + 0.86CO_2 \rightarrow 1.71C_4H_6O_4 + 0.86H_2O \quad (1)$$

$$C_6H_{12}O_6 \rightarrow C_4H_6O_4 + 2CO_2 + NADH \quad (2)$$

$$2Acetyl - CoA + 2H_2O + NAD^+ \rightarrow C_4H_6O_4 + 2CoASH + NADH + 2H^+ \quad (3)$$

In anaerobic conditions, the reductive pathway predominates and succinate acts as the terminal electron acceptor. Phosphoenolpyruvate (PEP) is converted to SA through various intermediates of the TCA cycle such as oxaloacetate (OAA), malate and fumarate with expense of 4 electrons or 2 mol of NADH and 1 mol of CO_2 (Equation (4)).

$$PEP + CO_2 + 2NADH \rightarrow C_4H_6O_4 + 2NAD^+ \quad (4)$$

In the glucose assimilation, 2 NADH molecules are generated through glycolysis, and SA biosynthesis requires 2 NADH molecules per SA, assuming that the whole carbon flux is directed towards SA. In this case the maximum molar theoretical yield from glucose would be limited to 1 $mol_{SA}/mol_{Glucose}$. The genes involved in the SA biosynthesis are regulated in an orderly fashion, and the major enzymes involved are: (i) PEP carboxykinase (*PEPCK*) [EC 4.1.1.38] or PEP carboxylase (*PEPC*) [EC 4.1.1.31], (ii) malate dehydrogenase (*mdh*) [EC 1.1.1.37], (iii) fumarate reductase (*frd*) [EC 1.3.1.6], and (iv) fumarase (*fr*) [EC 4.2.1.2]. Enzyme PEPC (*E. coli*) or PEPCK (*A. succinogenes*) replenishes the OAA in the TCA cycle, by fixing CO_2 along with PEP. Then the OAA produced is converted to malate in the presence of *mdh*, and further metabolized to fumarate, catalysed by fumarase. The fumaric acid produced is later reduced to SA in the presence of fumarate reductase, the key enzyme in anaerobic SA biosynthesis. A sequence similarity exists between *frd* and succinate dehydrogenase (*sdh*) of oxidative TCA cycle, and both catalyse the interconversion of the fumarate to succinate. The enzyme characterization also revealed that the functional characteristics, substrate specificity and enzyme kinetics were similar between *frd* and *sdh* enzymes.

In *A. succinogenes,* SA biosynthesis is regulated by the amount of CO_2 levels, hence theoretically, to produce 1 mol of SA, 1 mol of CO_2 is required. At increased CO_2 levels, the carbon flux is through carboxylation of PEP to OAA, rather than to pyruvate, making a positive impact on SA accumulation, whereas reduced CO_2 concentration diverts the flux towards pyruvate, resulting in mixed acid fermentation with by-products like acetate, lactate and ethanol. In *E. coli*, SA can be formed either in aerobic or anaerobic conditions. In aerobic conditions, acetyl-CoA produced from pyruvate enters the TCA cycle, and in subsequent biochemical reactions, SA is produced by succinyl-CoA synthetase and is further oxidized to fumarate by succinate dehydrogenase (*sdh*). Hence, a wild type *E. coli* strain cannot accumulate SA in aerobic conditions but blocking the oxidation step either by inactivation or deletion of the *sdhA* gene results in accumulation of SA. In contrast, in anaerobic conditions *E. coli* undergoes mixed acid fermentation, with acetate, formate, lactate and ethanol as the major products, and succinate in lower concentrations. Another potential route for SA accumulation in *E. coli* is the glyoxylate pathway, that converts 2 mol of acetyl-CoA and 1 mol of OAA to 1 mol SA and 1 mol malate. Malate formed can be later converted to

SA at the expense of 1 mol NADH. Usually, the expression of the glyoxylate pathway is induced when the microbial cell starts feeding on acetate accumulated during mixed acid fermentation, the pathway is regulated by *IclR* transcriptional repressor, encoded by *iclR* gene. Theoretically, during excess CO_2 availability, one mole of glucose can be converted into 2 mol of SA. However, each mole of SA requires 2 NADH molecules, so for the generation of 2 mol of SA, 4 NADH molecules are required. However, 1 mol of glucose produces only 2 NADH molecules, thus mandating the need for 2 extra NADH molecules causing a bottleneck for higher SA accumulation. The fixation of CO_2 during bio-based SA production is deemed important since it can mitigate 4.5–5 tons of CO_2 per ton of SA produced compared to petrochemical-based SA.

Genetic modification of the cellulolytic strain for production of organic acids, or vice versa, could increase the metabolic burden, when the genes responsible for either of the pathways are expressed in one single strain. Various chassis strains of *E. coli, Corynebacterium glutamicum, A. succinogenes, Y. lipolytica* and *S. cerevisiae* were constructed either to increase the substrate consumption, or to re-route the carbon flux towards SA accumulation. Table 2 summarizes the strains genetically modified to utilize various second-generation feedstocks for SA production.

3.1.1. *Escherichia coli*

Aerobic or anaerobic culture conditions are not favourable for SA accumulation for a wild-type strain of *E. coli*. However, due to its fastidious growth, and ease of genetic engineering with available genome information and tools, different strategies of random mutagenesis, pathway engineering, and evolutionary engineering were carried out to develop an engineered *E. coli* strain for SA production. In *E. coli* under anaerobic conditions, the carbon flux is more towards acetate, lactate and ethanol compared to SA. Hence, to reduce the by-products, pyruvate formate lyase (*pfl*) and lactate dehydrogenase (*ldhA*) genes responsible for formate and LA production were deleted resulting in a double knockout mutant *E. coli* NZN111. Unfortunately, the strain lost its characteristic growth on glucose under anaerobic conditions, as the inactivation of NADH dependent lactate dehydrogenase, decoupled the NAD^+ regeneration efficiency of the strain. Later overexpression of malate dehydrogenase (*mdh*), that performs the similar function, resulted in 31.9 g/L SA with a yield of 1.19 mol/mol glucose (Wang et al., 2009). The strain was further subjected to spontaneous chromosomal mutation for glucose phosphotransferase (*ptsG*) for improved substrate consumption, and heterologous overexpression of pyruvate carboxylase (*Rhizobium etli pyc* gene) which assists in conversion of pyruvate to oxaloacetate, resulting in the strain *E. coli* AFP111. The mutant strain cultivated in a dual fermentation strategy, initial aerobic cultivation for growth followed by anaerobic phase for SA production, was able to accumulate 99.2 g/L SA, with 1.74 mol/mol yield and 1.3 g/L h productivity (Chatterjee et al., 2001; Vemuri et al., 2002).

In bacterial fermentations, the maintenance of pH is of utmost importance as acidic conditions do not favor the growth and metabolite production, but bio-SA production at low pH favors the operational and investment costs as well as simplifies the downstream process. To implement the strategy of bacterial fermentation in acidic conditions, the AFP111 strain was modified by overexpressing the glutamate decarboxylase (*gadBC* operon) system. The gadBC system regulates the intracellular H^+ accumulation under acidic conditions by performing the proton consuming decarboxylation reaction and performs export of γ-aminobutyrate (GABA) for glutamate through a putative antiporter (gadC). The resulting strain BA201 showed a 1.2-fold increase in SA production at pH 5.6, than its parent strain AFP111 (Wu et al., 2017). Chen and associates performed simultaneous saccharification and fermentation using an engineered NZN111 strain that could accumulate 127.13 and 106.17 g/L SA using hydrolysed cassava starch and cassava powder, respectively (Chen et al., 2014).

Sugarcane molasses is considered as the most abundantly available first-generation feedstock, with approximately 50% w/w sugars (sucrose, glucose, and fructose), with sucrose as the major fraction, but *E. coli* cannot utilize sucrose. Hence a sucrose utilizing operon consisting of an invertase (*CscA*), and sucrose permease (*CscBK*) system was expressed in *E. coli* KJ122, resulting in 56 g/L SA in 10 L bioreactors with 0.96 g/g yield and 0.77 g/L.h productivity (Chan et al., 2012). In a similar approach, CscA with outer membrane *OmpC* anchoring motif and CscBK genes are expressed in another strain *E. coli* AFP111, the resulting strain accumulated 79 g/L SA in the dual phase fermentation strategy as described above with 1.19 mol/mol hexose yield (Ma et al., 2014).

SA is a higher energy metabolite compared to acetate, lactate, and ethanol, i.e. SA is produced in expense of energy rich molecules like ATP, NADH, NADPH and H_2 that drive the CO_2 fixation towards reductive SA accumulation. It was observed that 0.8 mmol of H_2 in the presence of excess CO_2 increases the carbon flux towards SA (Ahn et al., 2016; Tan et al., 2014). As an energy intensive process, the SA production was carried out through a bio-electrochemical approach. *E. coli* is an electrically inactive model, hence the genes involved in biological electron transfer, such as a c-type outer membrane cytochrome (*MtrC*), a periplasmic *c*-type cytochrome (*MtrA*), a non-heme outer membrane β-barrel protein (*MtrB*), and an inner-membrane associated quinol oxidase (CymA) was heterologously expressed from *Shewanella oneidensis,* an electroactive microbe, using formate as an external electron donor, and neutral red as electron carrier. The mutant *E. coli* T110 could produce SA with 1.10 mol/mol yield (Wu et al., 2019).

Another strategy for SA accumulation in *E. coli* is through induction of glyoxylate pathway genes under aerobic conditions. This can be possible by engineering the global transcription factor or knocking down the catabolite repressor. In a study by Zhu et al., (2016), the catabolite activator/repressor (*Cra*) was inactivated through error prone PCR, after high through put screening, a mutant strain was able to accumulate 79.8 g/L SA, that is 22.8% higher than the parent strain (Zhu et al., 2016). Myriant employed a genetically modified *E. coli* strain for SA biosynthesis in a 15000-ton capacity plant since 2013.

3.1.2. *Corynebacterium glutamicum*

Corynebacterium glutamicum is a heterotrophic, facultative anaerobic bacterium with industrial potential in production of various amino acids. During the incubation, the phase transition from aerobic to anaerobic conditions resulted in cessation of growth and accumulation of LA, AA, and SA. As the strain has potential to accumulate SA, the competing lactate biosynthesis pathway was inactivated by the deletion of lactate dehydrogenase (*ldh*) gene, and the native pyruvate carboxylase gene was overexpressed. The resulting mutant strain *C. glutamicum* Δ*ldh*-pCRA717 accumulated 146 g/L SA with 1.4 mol/mol yield through high cell density fermentation. Often in the biological production, product mediated inhibition is observed, where the end-product intervenes the central metabolic pathways, ABC transporter system, substrate consumption rate, transcriptional regulation, DNA repair system or biosynthesis of essential metabolites for growth and development. Similar behaviour was observed when the *C. glutamicum* cells were exposed to 0.25 M (~30 g/L) SA. To overcome these inhibitions Chung et al., 2017 overexpressed a global transcriptional regulator gene *NCgl0275*, which was observed to be downregulated during exposure of *C. glutamicum* cells to 0.25 M SA. The mutant strain displayed an increase in glucose uptake rate and 37.7% increase in SA production. Further on re-routing the carbon flux towards OAA, the strain could accumulate 152.2 g/L SA with a yield of 1.1 g/g glucose. The *NCgl0275* gene which showed this effect was characterized to be the *whi-B* regulatory gene, involved in cell division, differentiation, starvation survival and stress response (Chung et al., 2017). In another approach to increase the glucose consumption rate and NADH supply, the H^+-ATPase activity of the *C. glutamicum* NC-3–1 strain was reduced by point mutations. The mutant strain displayed a 39% (113 Vs 81 g/L) increase in SA production, and 29% increase in the yield (0.94 Vs 0.73 g/g). The generation of

Table 2
Summary of genetic alterations in native and non-native microorganisms for improved utilization of 2G feedstocks and SA production.

Microorganism	Genetic modification	Feedstock	Pre-treatment conditions	Enzymatic hydrolysis	Saccharification efficiency (%)	Mode of Fermentation	SA Titer (g/L)	SA Yield (g/g)	SA Productivity (g/L.h)	Reference
Escherichia coli AFP184	Deletion of *pflB*, spontaneous mutations in *ldhA* and *ptsG*	Sweet sorghum syrup	Dilute acid hydrolysis using 0.36 M HCl, Incubation at 75 °C, 10 mins.	–	100	Batch	27	0.34	–	Klasson et al., 2022
E. coli SD121	Overexpression of the *ppc* gene	Xylose mother liquor from Corncob or Sugarcane bagasse	N/A	N/A	N/A	Batch	52.1	0.63	0.62	Wang et al., 2014a
E. coli BA204	Deletion of *pflB, ldhA, ppc* gene, and overexpression of *pck* gene increasing the demand for ATP during the anaerobic phase of fermentation	Corn stalk	Dilute acid hydrolysis (2% v/v H_2SO_4, 121 °C, 2.5 h)	N/A	N/A	Batch	11.13	1.03*	0.7	Liu et al., 2012
Corynebacterium glutamicum	Expression of heterologous xylose utilization pathway (*xylA and xylB*) and deletion of *ldhA, pta, ackA*	Corn cobs	Dilute acid pretreatment (2% v/v H_2SO_4, 105 °C, 2 h)	N/A	N/A	Two stage Batch (Aerobic and Anaerobic)	40.8	0.69	0.85	Wang et al., 2014b
A. succinogenes 130Z	NTG Chemical mutagenesis	Napier grass	Alkaline pre-treatment 10% NaOH: Incubation at 90 °C, 1 h.	Cellulase (Cellic Ctec2) Incubation: 50 °C, 150 rpm for 96 hrs	–	Batch Fed-batch: hydrolysate and glycerol (10:1) Fed-batch: hydrolysate and glycerol (1:1)	– – –	0.58 0.65 0.88		Lee et al., 2022
Saccharomyces cerevisiae	Overexpression of heterologous glycerol dehydrogenase (*Opgdh*), homologous (*DAK1*), endogenous (*PYC2*)	Crude glycerol & CO_2	–	–	–	Batch	20	0.35	0.27	Malubhoy et al., 2022
Yarrowia lipolytica PSA02004	Glucose metabolism was restored by Adaptive evolution	Organic fraction of municipal solid waste hydrolysate	–	–	–	Fed-batch	48.7	0.37	0.49	Stylianou et al., 2021
Y. lipolytica PSA2004PP	Overexpression of XDH, XR and XK	SCB	Thermochemical pretreatment	N/A	N/A	Batch	5.6	0.13	0.09	Prabhu et al., 2020
Y. lipolytica PGC01003	Deletion of YLSDH5	Crude glycerol	N/A	N/A	N/A	Fed-batch	160.2	0.4	0.4	Gao et al., 2016

Yield calculated based on total reducing sugars; pflB*: Pyruvate Formate Lyase; *ldhA*: D-lactate dehydrogenase; *ptsG*: Phosphotransferase system; ppc: Phosphoenol pyruvate carboxylase; pck; Phosphoenol pyruvate carboxykinase; xylA: Xylose isomerase; xylB: Xylulose kinase; pta: Phosphate acetyltransferase; DAK1: Dihydroxyacetone kinase; PYC2: Pyruvate carboxylase; XDH – Xylitol Dehydrogenase; XK – Xylulose Kinase; XR – Xylose Reductase; YLSDH5: Succinate dehydrogenase.

energy packets (ATP) is through oxidative and substrate level phosphorylation, and H^+-ATPase has a significant role in the oxidative phosphorylation. Hence, by reducing the H^+-ATPase, ATP generation is downregulated, and to meet the demand, substrate level phosphorylation (SLP) should be accelerated. For that, glycolysis and glucose consumption should be overexpressed, such that surplus NADH molecules for SLP will be generated (Xu et al., 2016). An engineered *C. glutamicum* strain was commercially used by Ajinomoto and Mitsubishi Chemicals jointly since 2006. Along with *A. succinogenes*, *C. glutamicum* can also assimilate glucose to SA, unlike *A. succinogenes,* the wild type *C. glutamicum* lacks the xylose assimilatory pathway. In a study, the xylose assimilatory genes xylose isomerase (*XylA*) (D-xylose → D-Xylulose) and xylulokinase (*XylB*) (D-Xylulose + ATP → Xylulose – 5 – phosphate + ADP) were heterologously expressed in *C. glutamicum* (Wang et al., 2014b). The mutant strain with the xylose assimilatory pathway produced 40.8 g/L SA, with 0.69 g/g yield, and 0.85 g/L.h productivity using xylose rich corn cob hydrolysate (Wang et al., 2014).

3.1.3. Actinobacillus succinogenes

Actinobacillus succinogenes was evaluated and considered as one of the potent SA producers that can grow on a wide range of carbon sources like glucose, arabinose, fructose, sucrose, glycerol, and lactose. The native *A. succinogenes* strain was known to accumulate 50 – 65 g/L SA under anaerobic conditions with acetate as the major by-product. To increase the carbon flux towards SA and decrease the by-product accumulation, 11 strains from a mutant library of *A. succinogenes* CGMCC 1593 strain were subjected to genome shuffling. After three rounds of genome shuffling, the resulting mutant strain F3-11–3-F could produce 95.6 g/L SA, which is a 73% increase in comparison to the parent strain. Further genomic analysis revealed that the genes involved in glycolysis glucokinase, frutcose-1,6-bisphosate aldolase, PEP carboxykinase and fumarase had elevated activity, and genes responsible for by-product accumulation like pyruvate kinase, pyruvate formate lyase, and acetate kinase were downregulated (Zheng et al., 2013). As explained in the case of *C. glutamicum*, the bacteria are in-efficient at low-pH fermentations compared to yeast strains. To increase the tolerance of *A. succinogenes* to low pH levels, the strain BC-4 was mutated through adaptive laboratory evolution, the resultant strain had a 2.95- and 3.25-fold increase in titers and productivity at pH 5.8 compared to the parent strain. The adaptive evolution improved the pH homeostasis by increasing the ratio of medium chain fatty acid to long chain fatty acids, that lowers the permeability of H^+ into cytoplasm, and also the enzymes involved in ATP generation were accelerated (Zhang et al., 2020). Combining these two characteristics increased SA production and low pH tolerance, the strain AS-F32 was developed through genome shuffling, which produced 31.2 g/L SA at pH 4.8 (Hu et al., 2019).

Understanding the microbial genetic make-up and construction of a chassis strain with carbon flux towards the product of interest is very much important before optimizing the process and operating conditions for maximizing the titers. In a study conducted by Guarnieri et al., (2017), metabolic engineering capabilities of *A. succinogenes* were explained, deletion of the competing pathways lactate, and formate, and overexpression of malate dehydrogenase resulted in increased accumulation of SA, a similar observation through prediction tools and in-silico optimization was explained. The deletion of phosphate acetyltransferase (acetyl-CoA + phosphate → CoA + acetyl phosphate), acetyl kinase (ATP + acetate → ADP + acetyl phosphate), and phosphoenol pyruvate carboxykinase (ATP + oxaloacetate → ADP + phosphoenolpyruvate + CO_2) could be effective in SA production (Nag et al., 2018). Compared to *E. coli* and *C. glutamicum*, relatively less metabolic engineering of *A. succinogenes* was reported.

3.1.4. Yarrowia lipolytica

Yarrowia lipolytica is an oleaginous, aerobic yeast belonging to the family Dipodascaceae. In eukaryotic organisms, the cellular mechanism is well developed to maintain the intracellular pH changes more efficiently than prokaryotes. As SA production requires higher energy requirements, and NADH generation, and increased performance in acidic conditions, these yeasts are highly preferable. *Y. lipolytica,* is an unconventional yeast, generally regarded as safe (GRAS), was evaluated as a potent strain for SA production. The *Y. lipolytica* strain PSA3.0 was adapted in a fibrous bed reactor to accumulate SA at low pH 3.0 using a glucose based medium. The adapted strain was able to accumulate 76.8 g/L SA with 0.23 g/L. h productivity (Li et al., 2018a). During the SA production through *Y. lipolytica*, acetate was observed to be a major by-product and reason for drastic changes in the extracellular pH. The carbon flux towards acetate is also reducing the SA titers, hence the acetyl-CoA hydrolase gene responsible for carbon flux towards acetate was deleted. In the resulted strain, PEPCK (PEP + CO_2 → OAA) from *S. cerevisiae* and endogenous succinyl-CoA synthase (succinyl-CoA → Succinate) was overexpressed in a fed-batch mode the mutant strain accumulated 53.6 g/L SA with 0.61 g/g glucose yield without any pH control in the bioreactor (Yu et al., 2018). With a similar modification, and further deletion of the succinate dehydrogenase (sdh5) gene in the strain PGC01003 in a glycerol-based fermentations 198.2 g/L SA was accumulated in a fed-batch fermentation (Li et al., 2017).

The strain *Y. lipolytica* PGC01003 initially after deletion of the *sdh*5 gene involved in the oxidative TCA cycle, exhibited impaired the growth on glucose, but after adaptive evolution for 21 days (14 generations), the strain regained the glucose consumption rate (0.3 g/L.h). The adapted strain accumulated 65.7 g/L SA using yeast extract, peptone and glucose (Yang et al., 2017). When the *sdh* gene was deleted, the strain impaired the growth on glucose, instead the promoter of *sdh* gene was truncated reducing 77% of its activity, following to that gene in the glyoxylate pathway, oxidative TCA cycle and heterologous expression of PEPCK from *A. succinogenes* was carried out. The mutant strain was also adapted on glucose to reduce the length of the lag phase. The resulting strain could accumulate 35.3 g/L SA at pH 5.0, with 0.26 g/g glucose yield (Babaei et al., 2019). Although the *Y. lipolytica* strain was known to utilize diverse carbon sources, the strains have a cryptic xylose utilization pathway, i.e. strains cannot grow on xylose as sole carbon source in a glucose or glycerol – xylose co-fermentation, the strain can accumulate SA and xylitol. Hence, it was understood that the yeast has transporters for xylose, but the downstream enzymes responsible for the xylose flux into central carbon metabolism was inactive. In a recent study, the xylose assimilatory pathway was overexpressed by chromosomal integration and the resulting strain *Y. lipolytica* PSA02004PP could accumulate 22.3 g/L SA (Prabhu et al., 2020).

3.1.5. Saccharomyces cerevisiae

Saccharomyces cerevisiae is a well characterised industrially developed eukaryotic strain with widely available genetic information and metabolic tools for rewiring the carbon flux towards the desired product. Like *Y. lipolytica,* these conventional yeasts offer an advantage in growing at lower pH range of 3 – 6. *S. cerevisiae* wild type strain cannot accumulate SA, and unlike other SA producers, the reductive TCA pathway genes are thermodynamically under unfavourable conditions for SA accumulation (Ahn et al., 2016). Hence, any genetic modification to enhance the SA titers should be carried out in the oxidative TCA pathway. In a complex metabolic engineering strategy, the subunits of succinate dehydrogenase (*sdh1* and *sdh2*), and isocitrate dehydrogenase (*Idh1*) were deleted and the mutant could accumulate SA with 0.11 mol/mol glucose (Raab et al., 2010). Further, the GPD1 gene that encodes glycerol-3-phosophate dehydrogenase (DHAP + NADH → G-3-P + NAD^+) and fumarase (Fumarate → Malate) was deleted and during the operation the biotin and urea levels in the media composition were optimized to produce 12.97 g/L SA with 0.21 mol/mol glucose at pH 3.8 (Yan et al., 2014). Although titers are lower than other SA producers, *S. cerevisiae* has various advantages compared to the host strain. Further in-silico assessments and in-vitro pathway modifications could result in increased SA production. Reverdia is employing the genetically modified *S. cerevisiae* for production of SA in their 10,000-ton capacity plant

since 2011.

4. Separation and purification of SA from fermented broth

Biological production of SA is economically not viable and competitive compared to the petrochemical route because of the complicated recovery from the fermented broth. In general, recovery and purification processes account for 50–60% of processing expenses. As a result, economical and efficient recovery and purification technologies for industrial production of SA are desired. Precipitation, salting-out, reactive extraction, electrodialysis, and direct crystallization are some of the separation and purifications methods proposed for bio-SA recovery (Sun et al., 2018).

4.1. Precipitation

Precipitation is a classical industrial method for recovery of organic acids from an aqueous fermentation broth. The method involves the precipitation of organic acid with calcium hydroxide or calcium oxide. After the precipitation of the calcium salt of SA in the fermented broth, the precipitate is treated with concentrated sulfuric acid to produce SA and $CaSO_4$ (gypsum). Gypsum is an environmentally unfriendly by-product produced in equimolar amounts to SA, with a 15% loss of acid as well (Datta et al., 1992). The technique presents many disadvantages such as high quantity of by-product formation and high operation cost, which prevents its application as a feasible process from commercial production. Furthermore, the process is reported to be very slow and is less energy efficient. Even precipitation using ammonia was reported for SA recovery, where ammonium ions are used to regulate the pH during the fermentation resulting in diammonium salt of SA. The precipitate upon treatment with sulfate ions at low pH aid in the recovery of SA and ammonium sulfate. This process can be more preferred than calcium precipitation, as the by-product ammonium sulphate has applications in both upstream and downstream processes of biological production. With this approach approximately 93% recovery yield was reported (Yedur et al., 2001). Lee and associates utilized Napier grass hydrolysate as the carbon source for SA production, resulting in 0.58 g/g yield, further the fermented broth was subjected to sequential processes like ultrafiltration, single stage electrodialysis for concentration of organic acids, decolourization through activated carbon treatment, and finally precipitation resulted in 74.7% SA recovery with purity of 99.4% (Lee et al., 2022). In this process waste formation is reduced and the reagents can be recycled. However, the disadvantage of the process is high energy use and corrosion of equipment due to low pH.

4.2. Salting-out extraction

Salting-out extraction (SOE) systems use an organic solvent as the extractant and an inorganic salt as the salting-out reagent to recover hydrophilic metabolites from the fermentation broth while rejecting the majority of soluble proteins, cells, and other soluble and insoluble components, either in the aqueous phase or as an intermediate layer separating the upper light and lower heavy phases. This can omit the implementation of centrifugation and filtration steps. The technique was implemented for SA recovery from the fermented broth using various organic solvents (acetone, ethanol, butanol, and methanol), and salts (sodium carbonate, dipotassium hydrogen phosphate, and ammonium sulfate). The partition behaviour of SA between the aqueous phase and the organic phase determines the type of solvent to be used for the separation. The partition co-efficient (K_D) is defined as, at equilibrium, the ratio of the SA concentration in the organic phase to its concentration in the same form in the aqueous phase, calculated using equation (5):

$$K_D = \frac{[SA]_{organic}}{[SA]_{aqueous}} \quad (5)$$

A study by Sun et al. (2014a) investigated the partition behaviour of SA in different organic solvents and two salt systems. The results showed that the extraction yield of SA was higher in the acetone phase (72.31%), when compared to other solvents using ammonium sulphate [$(NH_4)_2SO_4$]. It was also observed that SA was better distributed in an SOE system consisting of acidic salts than that of basic salts.

A similar study by Alexandri et al. (2019b) on the recovery of SA from fermentation broth using an acetone - $(NH_4)_2SO_4$ system yielded in 50% recovery. The low yield was attributed to the presence of xylose in the fermentation broth. A recommendation of salting-out extraction combined with crystallization would give a higher yield (Alexandri et al., 2019b). The partitioning behaviour of SA was also studied by increasing acetone and ammonium sulfate concentrations, with 15% (w/w) ammonium sulfate and 30% (w/w) acetone as the optimal concentrations and 84.9% recovery yield was achieved (Gu et al., 2014).

4.3. Reactive extraction

Reactive extraction is separation and purification of organic acids using high molecular weight amines (reactants) and is a well-known approach. It is regarded as an efficient and cost-effective method in an industrial scale for extracting of SA, because it is operated at room temperature and pressure (Alexandri et al., 2019b). Long-chain aliphatic primary, secondary, and tertiary amines have been proposed for the reactive extraction of SA from aqueous solutions in downstream processing. Reactive extraction of SA with dioctyl amine in 1-octanol at pH 2 recovered 73% of SA whereas only 34.2% at pH 5 (Alexandri et al., 2019b). With pKa values 4.21 and 5.63, lower the pH of the fermentation broth, more the SA is found in acidic form and the higher the complex formation with amine following the extraction. Kurzrock and Weuster-Botz discovered that reactive extraction using trihexylamine in 1-octanol or dihexylamine and diisooctylamine in 1-octanol and 1-hexanol, as an extractants recovered >95% SA from an aqueous solution at pH 2.0 (Kurzrock and Weuster-Botz, 2010). The secondary amines had a higher extraction efficiency using polar solvents as diluents compared to primary and tertiary amines. In another study SA was recovered using tri-n-octyl-amine in 1-octanol, where the pH of the broth was maintained lower than 4.2 resulting in reduced extraction of SA (Jun et al., 2007). In the solutions with lower pH the majority of the acid molecules are in undissociated form, along with SA and other impurities in the fermentation broth, which compete for the H^+ ions resulting in lower efficiencies (Jun et al., 2007).

4.4. Direct crystallization

In separation of solid–liquid mixtures, crystallization has high efficiency in terms of recovery yield and purity. During the process of crystallization, initially seed crystals are added to the aqueous solution, to initiate the process, and it is one of the parameters (seed loading) during the optimization of crystallinity and purity. In a study where fermented broth with 151.44 g/L SA, produced by *A. succinogenes* ATCC55618 using cassava root hydrolysate, was subjected to crystallization resulted in 99.35% purity (Thuy et al., 2017).

Lin et al., (2010) have reported the high purity (95%) of SA and recovery (89%) yield from direct crystallization. A resin-based vacuum distillation-crystallization method was used to recover SA crystals from fermented broth using *A. succinogenes*. The cation exchange resin, Amberlite IR 120H, was used to convert succinate, formate, and acetate into free acid form from the salt form, and through vacuum distillation at 60 °C, other organic acids were distilled from the fermented broth, then SA was selectively separated by crystallization (Lin et al., 2010). In comparison, the study conducted by direct crystallization at 4 °C using *A. succinogenes* fermented broth maintained at pH 2, resulted in a SA yield and purity of 28% and 45%, respectively (Luque et al., 2009). At 4 °C and pH 2.0, SA is only 3% water-miscible, while the other acid by-products, such as lactic acid, AA and FA are highly water-miscible,

which might be the reason for lower extraction. Omwene and associates presented two different processes consisting of (i) ion-exchange chromatography followed by direct crystallization, and (ii) sequential cationic exchange chromatography, activated carbon treatment, membrane filtration, vacuum distillation, and crystallization. The final SA recovery yields from process I and II, were 78 and 65%, with 98.5 and 96.7% crystal purity, respectively (Omwene et al., 2021).

Although high purity and yields are reported for few of the recovery techniques, there are still challenges to overcome the limitations of the downstream processing techniques (Table 3).

5. Techno economic and lifecycle assessment of bio-based SA production

To successfully commercialize bio-based SA production, it is important that the economic, environmental and social aspects of the process need to be assessed. Lifecycle and techno-economic analysis studies have been carried out to understand the commercial implementation of SA production processes. The environmental performance of a SA biorefinery process employing bread waste was evaluated using a cradle-to-factory-gate life cycle assessment approach (Gadkari et al., 2021). GHG emissions and non-renewable energy use (NREU) was assessed. In comparison to the fossil-based system, waste bread fermentation depicted better environmental profile. Nevertheless, in comparison to other biomass feedstocks such as corn wet mill or sorghum grains, 50 % higher GHG emissions were observed. NREU was significantly lower (46%) than fossil-based systems. Steam and heating oil used in the process contributed the highest to NREU and GHG emissions.

A detailed techno-economic analysis was also carried out by Lam and co-workers to estimate the process economics. The process had a return on investment of 12.8 % and a payback period of 7.2 years (Lam et al., 2014). SA production through three different routes such as fermentation at low pH using yeast, anaerobic fermentation at neutral pH and use of ammonium sulfate in the downstream processing was evaluated using a cradle-to-gate LCA model (Cok et al., 2014). These processes were compared with the conventional maleic anhydride and adipic acid production processes. The results depicted that fermentation at low pH using yeast with direct crystallization had the lowest environmental impact in comparison to other bioprocesses and petrochemical routes. Data from a commercial bio-based SA production plant, the Myriant corporation facility was assessed using a cradle-to-gate LCA. Non-renewable fossil cumulative energy demand and GHG emissions were lower in comparison to the petrochemical alternative (Moussa et al., 2016). However, when apple pomace was used as a feedstock for SA production, the global warming potential (GWP) per kg SA produced, was found to be significantly higher than that of other bio-based processes (like those using corn, sorghum grains or sugar cane as feedstock) as well as that of fossil-based processes. LCA of SA production from food waste as well as indirect land-use changes and conventional and societal life cycle costing of the process were studied (Albizzati et al., 2021). It was reported that the GWP for bio-based SA production using food waste as feedstock was 2.2 0.03 kg CO_2-eq./kg SA. The study also reported that use of burden-free steam, recirculation of oil and NaCl, decrease in the use of potassium chloride, and increase in product yield could further reduce the GHG emissions. Nevertheless, it was reported that overall economic and societal costs will only reduce with an increase in the plant capacity by 43%. It is suggested that emphasis should be laid on the pre-treatment costs when LCB is used as a feedstock. The consumption of chemicals and energy and generation of inhibitory compounds are the major concerns associated with pre-treatment. Although various pre-treatments have been investigated, a common pre-treatment still cannot be used for a wide range of LCBs. Although enzymatic pre-treatment is effective, reducing the cost associated with it should be explored. Process simulation methodologies should be first implemented to get a preliminary idea about the potential of a LCB biorefinery process.

6. Limitations and future perspectives

SA has been considered as an important value-added bulk chemical due to its diverse applications. However, economic, environmental, and sustainability concerns, expected the processes to be developed using crude renewable feedstocks rather than pure sugars from edible sources. Decades of research has been progressing on consolidated bioprocessing (CBP), where a strain or consortium can be developed to effectively utilize lignocellulosic biomass for production of value-added products like SA. BioAmber, Reverdia, Myriant and Succinity are international players involved in biomanufacturing of SA. Despite so many advantages associated with the fermentative route, these companies are witnessing a decline in bio-based SA due to higher cost of production in comparison to fossil route. To address this issue, the major drivers or limitations to be addressed and where the future research needs to be concentrated are as follows:

(i) Pretreatment: The pretreatment is considered the most expensive process step in a 2G biorefinery making use of crude renewable sources and can contribute up to 30% of the total cost. It has a pervasive impact on the cost of all biological processing operations downstream, therefore, developing cost-effective pre-treatment methods for extraction of fermentable carbon from waste streams is a must.

(ii) Substrate range: With the advanced genetic tools and techniques, an efficient strain that can utilize multiple substrates like hexoses, and pentoses, without carbon catabolite repression must be developed.

(iii) Strains: Current native and non-native SA producers resulted in high titers and yields of SA either in anaerobic cultivation or in the presence of CO_2 or $CaCO_3$ as the co-substrate. However, replicating the

Table 3
Advantages and disadvantages of product recovery steps for SA manufactured via fermentative route.

Technique	Calcium precipitation	Salting -out extraction	Reactive extraction	Direct crystallization
Advantages	-Adaptable to existing mature equipment, technology, and infrastructure. -Viable process for commercial bio succinate production with very low technological barriers and risks	-Low cost, -Low interfacial tension -Good resolution -High yield -Simplicity of scaling up the system	-Cost-efficient -High output and low energy consumption	-Few unit operations -Easy operation -No reagents addition
Dis-advantages	-Slow -Requires high energy consumption -Production of calcium sulfate -Calcium sulphate cannot be sold directly due to odour and colour impurities		-Conventional extraction agents have low performance -Quite complicated -Extraction agent and diluent are expensive	-Low yield and purity -Desalination and deproteinization is required -Recrystallization is often required
Yield (%)	73	84.9	73	91.86
Purity (%)	97.2	86	97.2	99.3

same in a commercial scale would be difficult, as maintaining anaerobic conditions or sparging CO_2 would add additional costs to the process.

(iv) Successful scale up: One of the barriers for commercial viability of bio-based products is successful scale up of lab-based results at commercial level. The future work should focus on designing of process engineering approaches for translating the results at large scale to meet industrial standards/benchmarks.

(v) Separation and purification: Although various microbial strains have been constructed to produce SA in higher titers, there is no specific downstream process that could effectively separate and purify SA from the fermented broth. The main limitation of the bioprocess is operation at neutral pH, which results in SA salt formation, complicating the downstream process.

Hence, with the current advancements and combination of inter-disciplinary research the barriers can be overcome in the foreseeable future resulting in development of comprehensive and effective bio-process for the production of SA.

7. Conclusions

SA is a significant commodity chemical and precursor for various speciality chemicals and other additives. With myriad of applications, and potential to reduce the carbon footprint, the renewable, sustainable, and economical production of SA is of large interests. With current titers and yields, bio-based production processes are competitive with petroleum refinery. However, further advancements in upstream and downstream processes could provide profits in terms of economics and reduction of GHG emissions. Development of biorefinery processes using lignocellulosic and starchy wastes achieving good carbon conversion efficiency and techno-economic feasibility to achieve minimal environment footprints is of great interest.

CRediT authorship contribution statement

Vivek Narisetty: Conceptualization, Methodology, Software, Writing – review & editing. **Maureen Chiebonam Okibe:** Writing – review & editing. **K. Amulya:** Writing – review & editing. **Esther Oreoluwa Jokodola:** Writing – review & editing. **Frederic Coulon:** Writing – review & editing. **Vinay Kumar Tyagi:** Writing – review & editing. **Piet N.L. Lens:** Writing – review & editing. **Binod Parameswaran:** Writing – review & editing. **Vinod Kumar:** Supervision, Conceptualization, Data curation, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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