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Review article

Actinobacillus succinogenes: Advances on succinic acid production and prospects for development of integrated biorefineries

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ABSTRACT

Actinobacillus succinogenes is a wild-type bacterial strain, isolated from bovine rumen, known as one of the most efficient natural producers of succinic acid. Herein, the factors contributing to the fermentative production of succinic acid by *A. succinogenes* are reviewed with particular focus on raw materials, culture conditions, significance of carbon dioxide availability and downstream separation and purification. The metabolic potential of this strain is evaluated through discussion of the pathways involved in succinic acid production, genome analysis as well as the development of *A. succinogenes* mutants. The review also addresses the importance of by-product formation during fermentation that constitutes an important aspect regulating succinic acid production by *A. succinogenes*. The prospect of integrating succinic acid production in future biorefineries is assessed.

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1. Introduction

Succinic acid ($C_4H_6O_4$) is nowadays established as a key platform chemical for the bio-economy era according to several reports and increasing industrial interest for commercialisation [1,2]. Its significance as a platform intermediate is based on the reactivity of the two functional carboxylic groups leading to versatile end-products, the high fermentation efficiency, the utilization of numerous carbon sources and inexpensive renewable resources as feedstocks, and the cost-competitiveness of biotechnological production over petrochemical synthesis.

Various chemical technologies have been developed for the production of succinic acid (SA), including paraffin oxidation [3] and catalytic hydrogenation or electrolytic reduction of either maleic acid or maleic anhydride [4,5]. The paraffin oxidation technology employs a calcium or manganese catalyst to obtain a mixture of dicarboxylic acids. Subsequently, distillation, crystallization and drying are used to purify the succinic acid, which is produced in relatively low yield and purity through this process. The catalytic hydrogenation technology is a mature industrial process that could be carried out in homogeneous or heterogeneous catalyst systems. Although succinic acid can be obtained in high yield and purity, the operation of the hydrogenation technology is expensive and may cause serious environmental problems.

Conventional industrial applications for succinic acid include the production of polybutylene succinate and polybutylene succinate-terephthalate (9%), polyester polyols (6.2%), the food industry as acidulant, flavorant and sweetener (12.6%), the pharmaceutical industry (15.1%), and the production of resins, coatings and pigments (19.3%) [6]. In the bio-economy era, succinic acid will evolve into a platform intermediate, as a replacement for maleic anhydride, for the production of various bulk/intermediate chemicals such as 1,4-butanediol, γ-butyrolactone, tetrahydrofuran, *N*-methyl-2-pyrrolidone, 2-pyrrolidone, succinimide, succinic esters, maleic acid/maleic anhydride among others [7]. Succinate and its derivatives (e.g. adipic acid and 1,4-butanediol) could be applied for the manufacture of biodegradable polymers (e.g. polyamides and polyesters). For instance, its market is expected to grow in the production of polybutylene succinate and polyurethanes such as polyethylene succinate.

The utilization of succinic acid as platform chemical necessitates its production at a cost around $1 per kg succinic acid as it is required for the production of commodity products by the chemical industry. Microbial bioconversion could lead to cost-competitive production of succinic acid due to certain advantages including high carbon source to succinic acid theoretical conversion yield, significant reduction of greenhouse gas emissions and non-renewable energy consumption [4,8], and high potential for CO_2 sequestration due to CO_2 fixation involved in the reductive TCA cycle leading to succinic acid production. The market potential and the advantages provided by bioprocessing have led to investment by several companies (Table 1) in the construction of industrial facilities for fermentative production of succinic acid with varying capacities [2,9,10]. The current industrial activity for succinic acid production is currently positioned at a Technology Readiness Level of 8 with manufacturing facilities constructed in Europe and North America [10]. The market price of bio-based succinic acid is around 2.94 $/kg, while the respective market price of both bio- and fossil-based succinic acid is around 2.5 $/kg [10]. The annual production capacity of bio-based succinic acid in the period 2013–2014 was around 38,000 t that constitutes 49% of the total market [10]. The bio-based succinic acid market is expected to reach 600,000 t by 2020 with a projected market size of $\$539 \times 10^6$, but this is regarded as an optimistic scenario because a production cost of 1 $/kg has been considered and the current production cost is much higher [10].

Bioprocessing costs and environmental impact are highly dependent on the selection of the raw material, the upstream pre-treatment stages required to produce a nutrient-complete fermentation medium, the fermentation stage and the downstream separation and purification of succinic acid (especially in the case of high purity grade required for biopolymer formulation). All these stages are highly dependent on the microorganism and the fermentation conditions employed. Producing bulk bio-based platform chemicals will require the construction of industrial plants including several bioreactors with capacities in the scale of hundred cubic meters per bioreactor. Therefore, the use of facultative anaerobic microorganisms, as in the case of *Actinobacillus succinogenes*, will reduce bioreactor costs due to the absence of aeration that increases significantly capital and operating costs. Therefore, the selection of the microorganism depends on both bioprocessing and physiological aspects (e.g. oxygen and specific nutrient requirements, flexibility in raw material utilization, optimum fermentation parameters).

The carbon sources used in current industrial fermentations are mainly purified sugars or glucose syrups from corn. The utilization of agricultural residues and industrial side streams is necessary in order to create a sustainable bio-based succinic acid production. Although succinic acid producing bacteria can directly assimilate some industrial by-product streams (e.g. cheese whey and cane molasses), other raw materials (e.g. lignocellulosic residues and starch-rich waste streams) cannot be readily consumed. In the latter case, optimum pre-treatment schemes should be developed increasing also the complexity of downstream separation stages due to the remaining nutrients in the fermentation broth. Therefore, the utilization of crude renewable resources will eventually lead to sustainable production of bio-based succinic acid only through refining of the original resource in an analogous manner that refining has been applied to corn and petroleum [11]. In this way, the production of value-added co-products will provide the profitability margin required for the development of sustainable bioprocesses.

Several reviews have been published focusing on various aspects of succinic acid production including both general reviews [7,12–14] as well as more specific ones focusing on the prospect of *Escherichia coli* [15], comparison of natural versus genetically engineered strains [16], the utilization of lignocellulosic biomass or various renewable carbon sources [14,17], the development of wheat-based biorefining [18], downstream separation schemes [19], recent commercialization attempts [2] and metabolic engineering approaches [20]. This review focuses on the utlisation of *A. succinogenes* for succinic acid production covering its metabolic potential, bioprocess development and integrated biorefinery options leading to the co-production of various end-products using side streams from current industrial processes (e.g. food supply chain waste, biofuel industry, pulp and paper industry).

2. Succinic acid producers

Table 2 presents information regarding fermentation efficiency and conditions reported for various succinic acid producing strains. Fermentative succinic acid production has been accomplished by both wild-type and genetically engineered strains. *Actinobacillus succinogenes*, *Basfia succiniciproducens* and *Mannheimia succiniciproducens* are the most promising wild-type bacterial strains as they are able to consume numerous carbon sources, are facultative anaerobes, can achieve high fermentation efficiency and are classified as biosafety level 1 microorganisms by DSMZ and ATCC. These strains have been isolated from the rumen. The highest succinate concentration (105.8 g/L) has been produced by *A. succinogenes* FZ53 mutant using glucose with a yield and

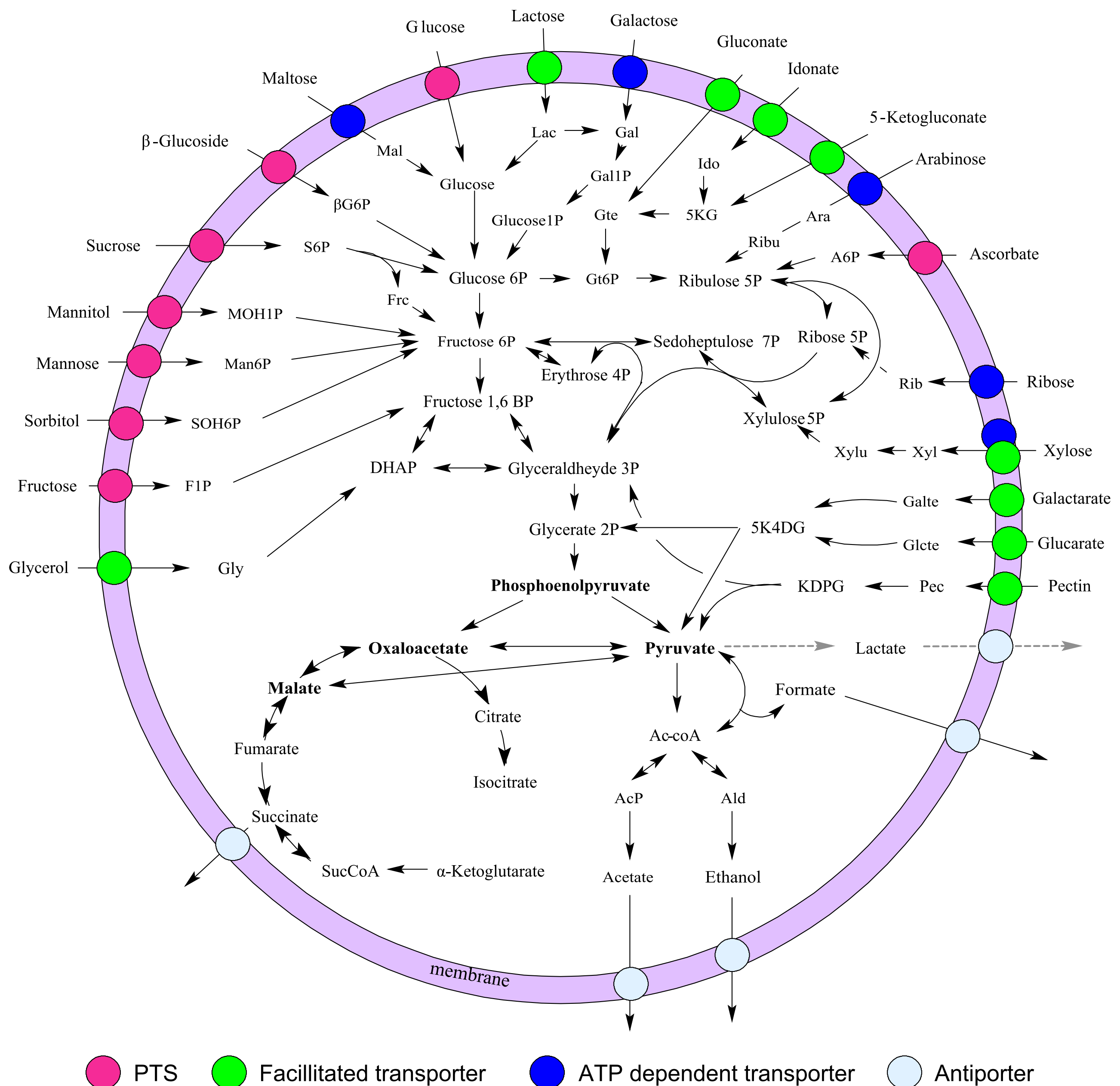


Fig. 1. *A. succinogenes* metabolic pathways and possible sugar transporters. 5K4DG: 5-dehydro-4-deoxy-D-glucarate; 5KG: 5-ketogluconate; A6P: Ascorbate-6-phosphate; AcCoA: Acetyl-CoA; AcP: acetyl-phosphate; Ald: Aldehyde; Ara: Arabinose; DHAP: dihydroxyacetone phosphate; E4P: Erythrose-4-phosphate; F1,6P: Fructose-1,6-biphosphate; F1P: Fructose-1-phospshate; F6P: Fructose-6-phosphate; G1P: Glucose-1-phosphate; Gal: Galactose; Gal1P: Galactose-1-phosphate; Galte: Galactarate; Glc: Glucose; Glcte: Glucarate; Gly: Glycerol; Gt6P: Gluconate-6-phosphate; Gte: Gluconate; Ido:Idonate; KDPG: 2-keto-3-deoxy-6-phosphogluconate; Lac: Lactose; Mal: Maltose; Man6P: Mannose-6-phosphate; MOH1P: Mannitol-1-phosphate; Pec: Pectin; R5P: Ribose-5-phosphate; Rib: Ribose; Ribu: Ribulose; Ru5P: Ribulose-5-phosphate; S6P: Sucrose-6-phosphate; S7P: Sedoheptulose-7-phosphate; SOH6P: Sorbitol-6-phosphate; SucCoA: Succinyl-CoA; Xu5P: Xylulose-5-phosphate; Xyl: Xylose; Xylu: Xylulose; βG6P: β-Glucoside-6-phosphate. Lactic acid production occurs in the case of other succinic acid producing strains (e.g. *Basfia succiniciproducens*).

productivity of 0.82 $g_{SA}/g_{glucose}$ and 1.36 g/L/h, respectively [21]. Contrary to other bacterial strains, *B. succiniciproducens* has not been studied to a great extent. Its metabolic fluxes have been investigated and two mutant strains have been developed [23].

Highly efficient genetically engineered *E. coli* strains have been constructed for succinic acid production, such as *E. coli* strain AFP111/pTrc99A–pyc that produced 99.2 g/L of succinic acid concentration in dual phase fermentations with the highest reported yield of 1.1 $g_{SA}/g_{glucose}$ and a productivity of 1.3 g/L/h [24]. *Corynebacterium glutamicum* ΔldhA-pCRA717 could be a promising microorganism for succinic acid production due to the high productivity (3.17 g/L/h), final concentration (146 g/L) and yield (0.92 $g_{SA}/g_{glucose}$) achieved [26]. Recent research focuses on the development of genetically engineered yeast strains that can produce succinic acid at low pH in order to reduce the unit operations in downstream separation and purification of succinic acid [2,27].

The main advantages of *A. succinogenes* exploitation for succinic acid production are the utilization of numerous carbon sources, adequate tolerance to inhibitors and sufficient fermentation efficiency even with crude renewable resources. The main disadvantages lie on the fastidious nature of *A. succinogenes* for nitrogen sources (e.g. yeast extract) and vitamins (e.g. biotin), the near neutral optimum pH required and the limited genetic engineering tools for its genetic manipulation. The implementation of succinic acid production by *A. succinogenes* in integrated biorefineries using complex renewable resources would provide

Table 1
Industrial facilities for bio-based succinic acid production.

Company	Capacity	Start-up	Raw material	Fermentation/ Microorganism	Downstream recovery critical stage	Investment made in	Reference
BioAmber (DNP/ard)	3000 t/y demo plant	2010	Wheat glucose	*E. coli*	Electrodialysis	Europe, Pomacle, France	[9,10]
BioAmber, Mitsui	30,000–50,000 t/y	Under construction	Corn glucose	Low pH culture is targeted using *Candida krusei*	Direct succinic acid separation when low pH conditions are used	Sarnia, Ontario, Canada	[2,10]
BioAmber, Mitsui	70,000–200,000 t/y	Two plants to be constructed	–	–	–	North America	[10]
Reverdia (joint venture between Roquette & DSM)	10,000 t/y	2012	Starch/sugars	Low pH culture is targeted by *S. cerevisiae*	Direct separation of the succinic acid	Cassano Spinola, Italy	[2,10]
Myriant, ThyssenKrupp	1000 t/y	2013	Glucose	*E. coli*	Ammonia precipitation	Leuna, Germany	[10]
Myriant	14,000 t/y	2013	Corn glucose	*E. coli*	Ammonia precipitation	Lake Providence, Louisiana, USA	[2,9,10]
Succinity (joint venture between BASF & Corbion-Purac)	10,000 t/y	2013	Glycerol/sugars	*B. succiniciproducens*	Magnesium hydroxide as neutralizer followed by recycling	Montmelo, Spain	[2,10]

Table 2
Fermentation efficiency and conditions for different succinic acid producing strains.

Fermentation parameters	*A. succinogenes* FZ53	*M. succiniciproducens* LPK7	*B. succiniciproducens* *JF4016*	*E. coli* AFP 111/pTrc99A-pyc	*E. coli* KJ060	*C. glutamicum* ΔldhA-pCRA717	*S. cerevisiae* SUC-297	*Y. lipolytica* Y-3314
Carbon source	Glucose	Glucose	Glucose	Glucose	Glucose	Glucose	Glucose	Glycerol
Nutrient sources (g/L)	Yeast extract, (5–15), corn steep liquor (10–15, vitamins	Yeast extract (5)	Yeast extract (5), peptone (5), vitamins	Yeast extract, (10), Tryptone (20), biotin, thiamine	$(NH_4)_2HPO_4$, $NH_4H_2PO_4$, thiamine, betaine	Urea (2), yeast extract (2), casamino acid (7), $(NH_4)_2SO_4$ (7), biotin, thiamine	$(NH_4)_2SO_4$, vitamins	Yeast extract (10), peptone (10), uracil, leucine
Gas supply	CO_2 (0.05–0.1 vvm	CO_2 (0.25 vvm)	CO_2 atmosphere	Dual–phase cultures	Anaerobic environment	Oxygen deprivation	Air and CO_2	Aerobic shake flasks
pH	7.2–6	6.5	nk[a]	7	7	nk[a]	5	6.8–5.8
Neutralisers	$MgCO_3$, $Mg(OH)_2$	Ammonia solution	$MgCO_3$	NaOH, HCl	$NaHCO_3$, KOH, K_2CO_3	NaOH, bicarbonate	KOH	$CaCO_3$
Yield ($g/g_{glucose}$)	0.82	0.76	0.49	1.1	0.92	0.92	nk	0.36
Productivity (g/L/h)	1.36	1.8	0.53	1.3	0.9	3.17	0.45	0.27
Succinic acid (g/L)	105.8	52.4	20	99.2	86.6	146	43	45.5
By-products[b]	Ace: Prop: Pyr:	Mal: Pyr	For: Ace: Lac: EtOH	Ace: EtOH	Mal: Ace: Lac	Ace: Lac: Mal Pyr	EtOH: Gly: Mal	nk[a]
Reference	[21]	[22]	[23]	[24]	[25]	[26]	[27]	[28]

[a] nk: not known.
[b] Ace: acetic acid, For: formic acid, Pyr: pyruvic acid, Pro: propionic acid, Lac: lactic acid, Mal: malic acid, EtOH: ethanol, Cly: glycerol.

nutrient-complete fermentation media at lower cost than commercial nutrient sources. The use of neutralizers and the neutral pH of the fermentation broth resulting from *A. succinogenes* cultures affect both fermentation and downstream separation costs. Lower pH values of the fermentation broth reduce the downstream separation cost as the pH value affects the dissociation level of succinic acid ($pKa_1 = 4.16$ and $pKa_2 = 5.6$) [2].

3. The metabolic potential of *A. succinogenes*

A. succinogenes is a Gram-negative, facultative anaerobic, non-motile, non-spore forming, capnophilic, pleomorphic rod, which was isolated from bovine rumen [29] and taxonomically was placed in the *Pasteurellaceae* family, based on 16S rRNA amplification. Its taxonomical order has been formed as follows: Bacteria; Proteobacteria; Gammaproteobacteria; Pasteurellales; *Pasteurellaceae*; *Actinobacillus succinogenes*. *A. succinogenes* is mesophilic and grows well at 37–39 °C in chemically defined media. The microorganism is capable of consuming a wide range of C5 and C6 sugars as well as various disaccharides and other carbon sources, such as glucose, xylose, arabinose, mannose, galactose, fructose, sucrose, lactose, cellobiose, mannitol, maltose and glycerol [30–35]. The use of more reduced carbon sources than glucose, such as sorbitol, glycerol and mannitol, results in higher succinic acid yields. However, the utilization of C5 sugars, such as xylose and arabinose, results in lower succinate yields [36].

3.1. Metabolic pathway towards succinic acid production

Glucose is transported into the cell with the action of a permease. Phosphorylation of glucose occurs not only through the phosphoenolpyruvate dependent phosphotransferase system [37], but also through a hexokinase activity [21]. Furthermore, glucose-6-phosphate is catabolized to phosphoenolpyruvate (PEP) through the glycolytic pathway, while the oxidative pentose phosphate pathway (OPPP) has limited contribution in the catabolism of glucose. Thus, NADPH is formed from NADH through transhydrogenase and/or by the combination of NADH-oxidizing malate dehydrogenase and NADP-reducing malic enzyme activity [37], while NADH is produced through the activity of pyruvate dehydrogenase and formate dehydrogenase. Moreover, NADPH requirements affected the flux distribution between C3 and C4 pathways, mainly due to pyruvate dehydrogenase and formate dehydrogenase activities. The glyoxylate and Entner-Doudoroff pathways are not present in the fermentative metabolism of *A. succinogenes*. The four most important nodes on flux distribution

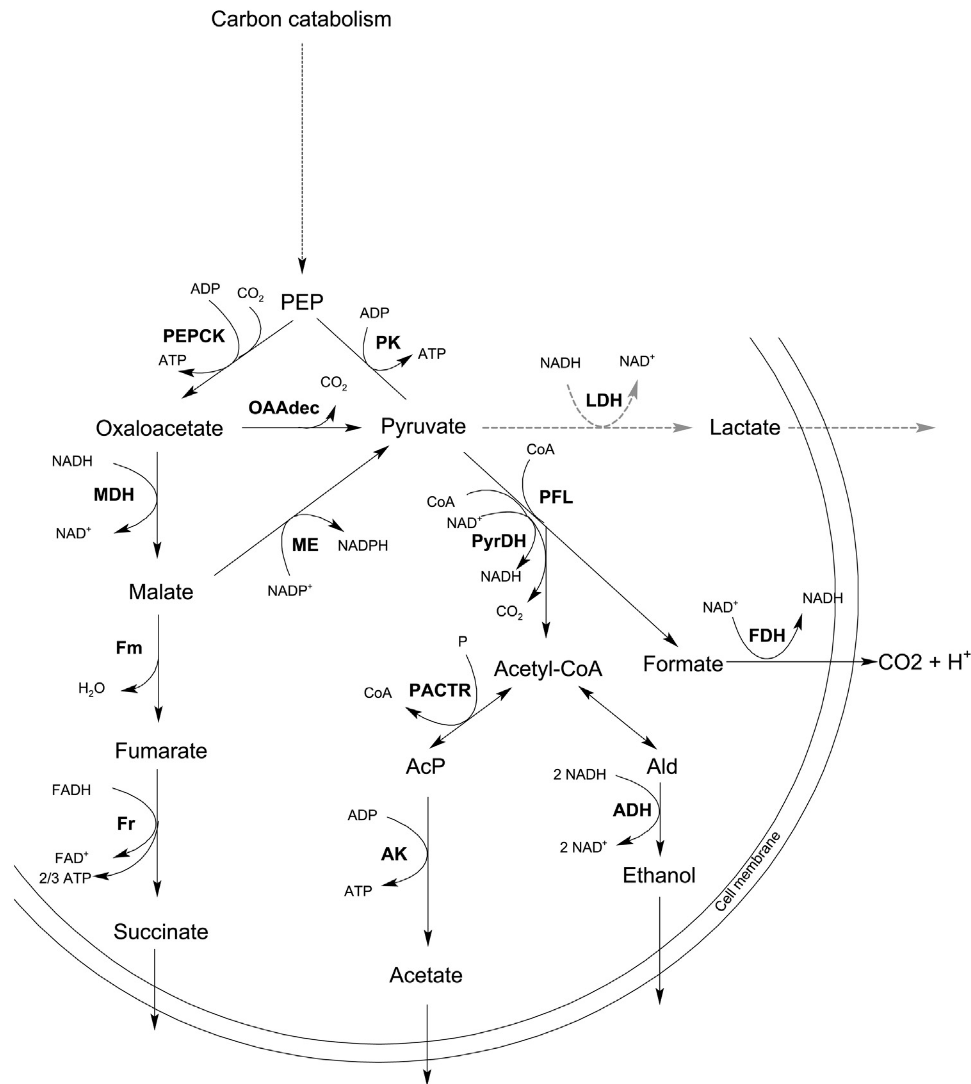
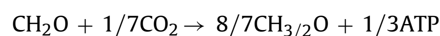


Fig. 2. Co-factors and enzymes that participate in the C3 and C4 metabolic pathways in *A. succinogenes*. Lactic acid production occurs in the case of other succinic acid producing strains (e.g. *Basfia succiniciproducens*).

for succinate production in *A. succinogenes* are phospoenolpyruvate (PEP), oxaloacetate (OAA), malate and pyruvate (Figs. 1 and 2) [38].

Metabolic flux analysis has established that the major route to succinic acid flows from phosphoenolpyruvate to oxaloacetate, malate, fumarate and finally to succinate catalysed by PEP carboxykinase, malate dehydrogenase, fumarase and fumarate reductase [37]. PEP could be converted into formate, acetate and ethanol via the C3 pathway and into succinate via the C4 pathway, with malic enzyme and OAA decarboxylase holding the potential role for the conversion into pyruvate, hence forming reversible shunts between C3 and C4 pathways [39]. PEP carboxykinase (EC 4.1.1.49) is considered to be the key enzyme for the production of succinic acid with subsequent ATP formation, since it is capable of catalyzing the conversion of PEP into OAA (ΔG: 5.6 kJ mol^{-1}) through the consumption of CO_2 [37]. Furthermore, malate dehydrogenase (EC 1.1.1.299) catalyzes the conversion of oxaloacetate into malate with NADH as a co-factor (ΔG: −32.1 kJ mol^{-1}), while fumarate is generated from malate (ΔG: 3.6 kJ mol^{-1}) with the use of the enzyme fumarase (EC 4.2.1.2). Fumarate reductase (EC 1.3.1.6) is a NADH dependent enzyme which catalyzes the conversion of fumarate into succinate, with generation of 2/3 of ATP (ΔG: −71.4 kJ mol^{-1}). Pyruvate kinase (EC 2.7.1.40) occurs in the first reaction of the C3 pathway, involved in the conversion of PEP into pyruvate (ΔG: −30.6 kJ mol^{-1}), producing 1 molecule of ATP [37]. Pyruvate could be converted into formate, acetate, ethanol and biomass. Pyruvate-formate lyase (EC 2.3.1.54) uses coA as a cofactor to convert pyruvate into acetyl-coA and formate (ΔG: −11.2 kJ mol^{-1}). The values of ΔG presented above have been calculated for pH 7 and standard conditions (http://equilibrator.weizmann.ac.il/).

Theoretically, 1 mol of succinic acid can be produced from the fixation of 1 mol of CO_2 and 0.5 mol of glucose, 0.6 mol of xylose or 1 mol of glycerol. In parallel, 2 moles of NADH are oxidised through the reductive pathway of the TCA cycle during the conversion of OAA to malate and fumarate to succinate. This means that the additional reducing capacity (i.e. NADH) required in the C4 pathway should be supplied by other parts of the metabolism (e.g. glycolysis, C3 pathway). The maximum glucose to succinic acid conversion yield is 1.12 g/g and is given by the following stoichiometric equation



$$CH_2O + 1/7CO_2 \rightarrow 8/7CH_{3/2}O + 1/3ATP$$

The theoretical yield cannot be achieved due to biomass and by-product formation. The latter is essential for the regeneration of NAD(P)H and ATP. The production of 1 mol of acetic acid is

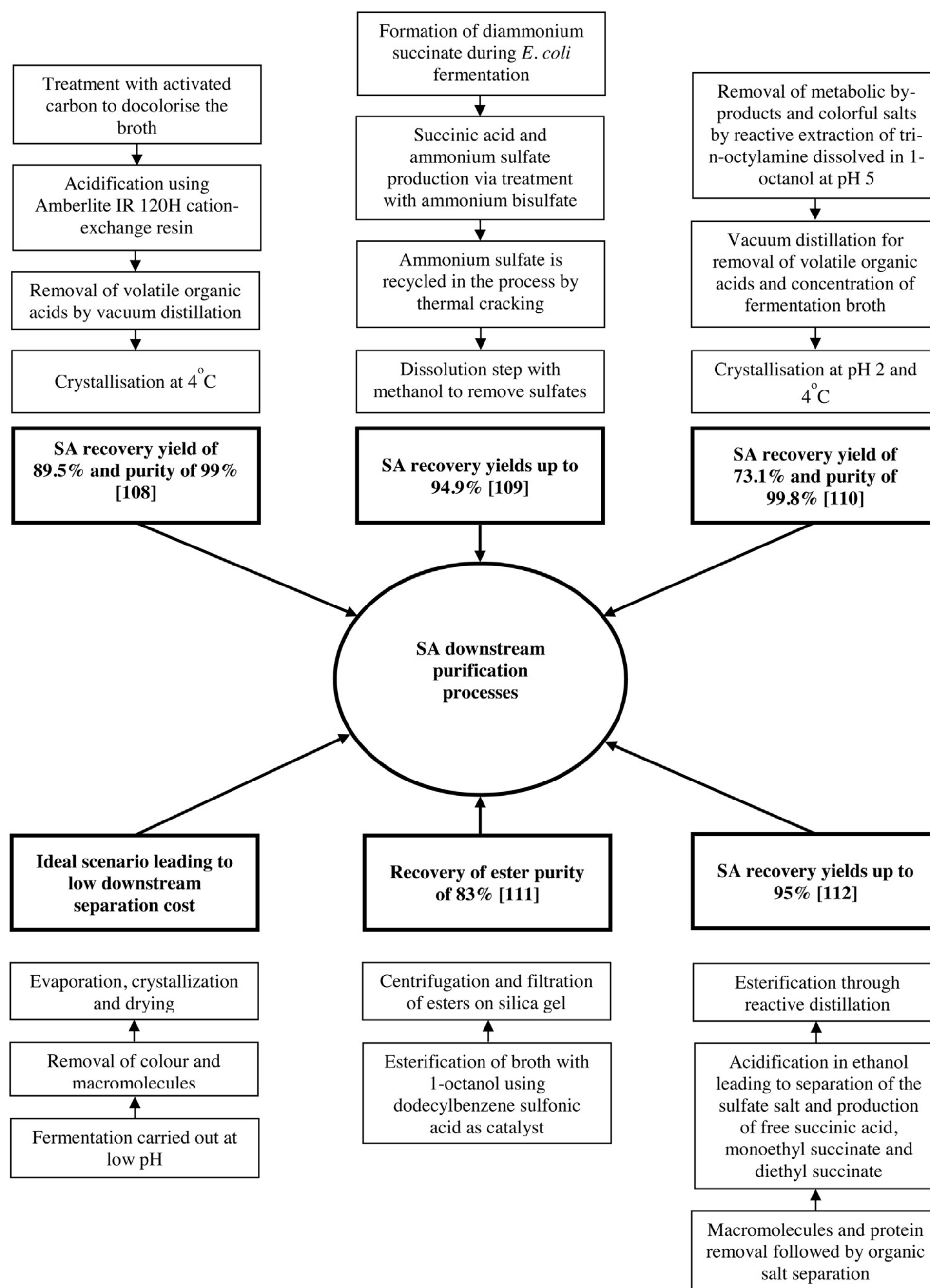


Fig. 3. Representative downstream separation processes for succinic acid purification.

accompanied with the generation of 1 mol of ATP. One mole of formate (through pyruvate-formate lyase) is produced along with 1 mol of acetyl-CoA, a precursor of structural molecules and finally biomass. Subsequently formate can be converted into CO_2 and H^+, catalysed by formate dehydrogenase, which can serve as electron donor for the production of succinic acid. The C3 pathway results in the production of NADH that is necessary for the production of succinic acid by the C4 pathway resulting in different glucose to succinic acid theoretical conversion yields depending on the metabolic pathway employed.

The key factor to achieve higher carbon source to succinic acid conversion yields is to reduce by-product formation. Homo-fermentative succinic acid production in *A. succinogenes* fermentations can be achieved by genetic manipulation or evolutionary adaptation to create improved strains, such as *A. succinogenes* FZ6 and FZ53 mutants [21]. Furthermore, the development of continuous fermentations with cell immobilisation could lead to less by-product formation and higher productivities than batch and fed-batch cultures [40,41]. *A. succinogenes* cells form biofilms during continuous cultures [40] resulting in reduced acetyl-CoA requirements for biomass formation and therefore low acetic acid and formic acid concentrations in the effluent.

3.2. *Influence of substrate and product inhibition on* A. succinogenes *bioprocesses*

Succinic acid production via fermentation is hampered by substrate and product inhibition. Inhibition on growth by metabolic product formation could be a result of two mechanisms: (1) regulation of homeostasis and (2) membrane breakdown due to osmotic stress. The lipophile, undissociated form of weak acids can enter the cell at neutral pH values. In elevated acid concentrations, pH

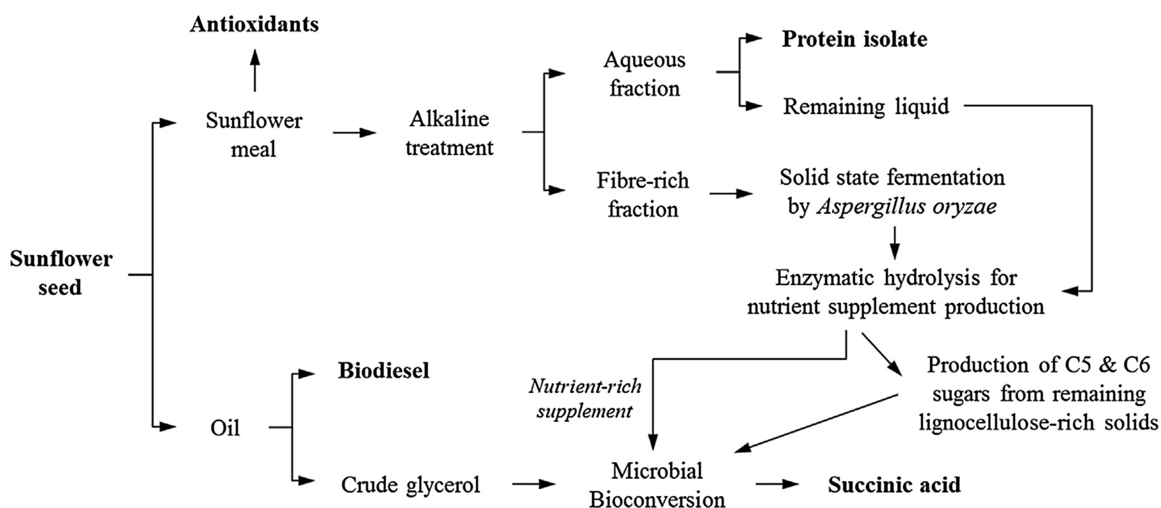


Fig. 4. Integrated biorefinery based on the utilization of biodiesel industry by-products.

homeostasis is maintained through the synthesis of excessive ATP and the regulation of H^+-ATPase, which expels protons from the cytoplasm (proton motive force) against the concentration gradient to maintain ΔpH in low levels (between the inner and outer surface of the cell). High osmotic stress occurs when the medium contains high concentrations of sugars and/or minerals, causing cell shrinkage, subsequent breakdown of the membrane and cell death.

Table 3 presents indicative substrate and product inhibition for *A. succinogenes* and *E. coli* strains cultivated mainly on glucose. Substrate inhibition was tested in the case of *A. succinogenes* CGMCC1593 growth on glucose [42] and sugar cane molasses [43] demonstrating that inhibition started at 50–60 g/L of glucose concentration and 65 g/L of total sugar concentration, respectively. The growth of the strain *A. succinogenes* 130Z was completely inhibited at initial glucose concentrations higher than 158 g/L [44], while the glucose to succinic acid conversion yield was decreased and the lag phase was increased at glucose concentrations higher than 100 g/L. Formate imposed the most inhibitory effect on succinic acid production, while the inhibitory concentrations of acetate, ethanol, formate, pyruvate and succinate were 46, 42, 16, 74 and 104 g/L, respectively [44]. The product inhibition of *A. succinogenes* 130Z was compared to 3 mutant strains of *E. coli* [47] showing that *E. coli* NZN111 is more tolerant than *A. succinogenes* 130Z in the case of succinic, formic and acetic acids.

3.3. Oxido-reduction potential of A. succinogenes

Succinic acid is excreted either by diffusion or through a transport protein on the cell membrane [48]. The process involves the oxidation of a substrate and the transfer of electrons to a final electron acceptor. Fumarate is used as an electron acceptor in fumarate respiration, which is converted into succinic acid [29] and excreted through an antiporter [48]. Pyruvate is oxidized to release reducing power (i.e. NADH) that can be used in the C4 pathway. Thus, the provision of more reduced carbon sources as well as the supply of reducing power from external sources (e.g. electrically reduced neutral red in an electrochemical bioreactor system) could enhance the production of succinate and ATP under controlled conditions [49]. When H_2 or electricity is not used as additional reducing power, pyruvate should be oxidized in order to produce an electron donor for fumarate reduction resulting in decreased succinate yield and ATP synthesis via electron transport-mediated phosphorylation [49].

The redox potential or oxido-reduction potential is a measure of the tendency towards reduction or oxidation. Li et al. [50] investigated the influence of the redox potential on the fermentation profile of *A. succinogenes* NJ113 by addition of potassium ferricyanide as the oxidant agent and dithiotreitol as the reducing agent. At a redox potential level of −350 mV, succinic acid production, yield and productivity increased and by-product formation decreased compared to the rest of the redox potential levels tested (no redox potential regulation, −100, −300, −350, −400, −450 mV). This occurred due to a significantly higher NADH/NAD^+ ratio observed at −350 mV [50].

Xi et al. [51] reported that biotin concentrations in the range of 2–10 mg/L result in enhanced succinic acid production when a chemically defined medium is used. Considering that succinic acid is formed from fumaric acid, the role of biotin in the pathway is to provide cytochrome b and electron transfer exits [51]. Furthermore, when 5-aminolevulinic acid (a biosynthetic precursor of heme) or heme (an electron carrier that can increase the reduction potential) replaced biotin in the chemically defined medium, similar fermentation results were obtained indicating that biotin biosynthesis could be achieved from 5-aminolevulinic acid.

3.4. Genome analysis of A. succinogenes

The genome of *A. succinogenes* was sequenced by McKinlay et al. [52]. Although this is the largest genome of the family *Pasteurellaceae* characterized so far, a genome size of 2.3 Mb (GenBank accession number CP000746) is generally considered as relatively small. The genome contains 2079 protein coding genes out of 2199 genes. Compared to other *Pasteurellaceae*, *A. succinogenes* and *M. succiniciproducens* are more closely related. These strains have 78% of the open reading frames (ORFs) in common (1735), while 488 are found only in *A. succinogenes* and 442 only in *M. succiniciproducens*. Furthermore, 60% (1252) of 2081 automated KEGG comparisons were mostly similar to *M. succiniciproducens*, while the GC content was determined by HPLC to be 45.1 mol% [53]. *A. succinogenes* contains a 39,489 bp prophage genome, which is useful for phage based genetic engineering but it might be risky for industrial fermentations due to possible lysis of the cells. A USS1 repeat (9 nt sequence) has been shown to be present in *A. succinogenes* (1690 copies and density of 0.73 USS/bp), which functions for natural competence. Although various *Pasteurellaceae* strains are pathogens, *A. succinogenes* and *M. succiniciproducens* lack pathogenicity, since they do not encode sequences of virulence traits or for the production of leukotoxin, cytolethal toxin and hemolysin. Sequences that function for the production of sialic acid, incorporation of choline into

Table 3
Inhibitory product and glucose concentrations on succinic acid production (initial inhibitory concentrations are enclosed in parenthesis). Missing data are either not mentioned or not investigated in the literature-cited references.

Microorganism	Glucose (g/L)	Succinic acid g/L)	Pyruvic acid (g/L)	Lactic acid (g/L)	Formic acid (g/L)	Acetic acid (g/L)	Ethanol (g/L)	Total products (g/L)	Reference
A. succinogenes CGMCC1593	>100 (50–60)	–	–	–	–	–	–	–	[42]
A. succinogenes CGMCC1593	>100 (65)[a]	–	–	–	–	–	–	–	[43]
A. succinogenes 130Z^T (ATCC 55618)	158 (100)	59.4	59.2	–	10.8 (1.8)	33.7	42	–	[44]
A. succinogenes 130Z^T (ATCC 55618)	>100 (57.4)	–	–	–	–	–	–	–	[45]
A. succinogenes 130Z^T (ATCC 55618)	–	40 (9.8)	–	–	–	–	–	–(20)	[46]
A. succinogenes 130Z^T (ATCC 55618)	–	50 (<40)	–	18 (<9)	35.2 (<8.8)	20 (<10)	–	–	[47]
E. coli NZN111	–	80 (<20)	–	18 (<9)	52.8 (<8.8)	60 (<20)	–	–	
E. coli AFP111	–	80 (<20)	–	18 (<9)	35.2 (<8.8)	40 (<20)	–	–	
E. coli BL21	–	>80 (<40)	–	18 (<9)	35.2 (<8.8)	60 (<20)	–	–	

[a] Total sugars from pretreated cane molasses.

lipopolysaccharide as well as iron uptake, through hemoglobin and transferrin utilization, have not been identified.

Various genes involved in the transport of a wide range of sugars have been characterized (Fig. 1). The transport is achieved either through PTS, an ATP-dependent transporter or with the application of a facilitated transporter, while an assumption is often used about the specific function of each sugar transporter. Gene Asuc_0496 encodes a sugar transport protein that possibly functions as glucose facilitator, while Asuc_1504, Asuc_0131 and Asuc_0084 encode possible sugar kinases. Genes that encode glyoxylate pathway enzymes and Entner-Doudoroff enzyme phosphogluconate dehydratase were not present in the genome of *A. succinogenes*, whereas genes that encode for the glycolytic cycle as well as the pentose phosphate pathway are also present. However, although Asuc_0152, Asuc_0374 and Asuc_1471 could be part of operons that encode for 2-keto-3-deoxy-6-phosphogluconate (KDPG) aldolases, possibly functioning in glucuronate or galacturonate degradation pathways, the growth substrates triggering activation of these genes still remain unknown. The most important enzymes composing the incomplete TCA cycle are encoded by the following genes: Asuc_0221 (PEP carboxikinase), Asuc_1612 (malate dehydrogenase), Asuc_0956 (fumarase), Asuc_1813-6 (fumarate reductase) and Asuc_1564-5 (succinyl-CoA synthetase). Asuc_1199 encodes a carbonic anhydrase which is most likely the key enzyme for making CO_2 available, since it catalyses the reaction that converts CO_2 to HCO_3^- and protons. However, genes that encode for PEP carboxylase are absent. The C3 pathway enzymes identified include Asuc_0942-4 encoding for pyruvate dehydrogenase, Asuc_0207 for pyruvate formate-lyase, Asuc_0005 for lactate dehydrogenase and Asuc_1261-6 for the different subunits of formate dehydrogenase. Additionally, energy for anabolic processes can be gained from reverse fluxes between C4 and C3 pathways, which are particularly affected when CO_2 or H_2 are present. Specifically, malic enzyme and OAA are the nodes where the reverse fluxes between C4 and C3 pathways occur, while the genes encoding for these enzymes are Asuc_0669 and Asuc_0301-3, respectively [52].

Investigation of the relationship between genome, biochemistry and cell physiology is crucial for understanding the regulatory effects of genes and enzymes, which can lead to the reconstruction of the metabolic network and *in silico* computation of its integrated functions. Although a limited number of genomic and proteomic studies have been performed up to date [52,54], it is evident from the relevant literature that a deeper understanding of the biological control mechanisms of *A. succinogenes* growth is required for the development of sustainable succinic acid production. Therefore, research should focus on application of high-throughput experimental technologies combined with mathematical models to identify gene targets for modification and to develop bioprocess engineering strategies for control and optimization purposes [55].

3.5. Genetically modified strains

A wide variety of tools is available to induce genetic diversity and strain development, which can be achieved by targeted genetic manipulation (gene introduction, deletion and overexpression) or through exposure to extreme conditions (e.g. mutagenic chemical agents, UV exposure and radiation). So far, random mutagenesis and directed evolution have been applied in *A. succinogenes* to enhance the production of succinic acid and/or to reduce the formation of by-products. Although suitable shuttle vectors have been constructed and were successful in homologous gene expression in *A. succinogenes* [56], there are no focused metabolic engineering studies targeting the improvement of succinic acid production by *A. succinogenes*. In contrast, several genetically modified *E. coli* strains have been developed to produce succinic acid from various carbon sources (including glucose and xylose) as well as to achieve yields and productivities similar to (or even higher than) natural succinic acid producing bacteria [24,25,57,58].

Two mutant strains *A. succinogenes* FZ6 and FZ21 (Table 4) with formate lyase and formate dehydrogenase deletions have been reported [21]. Specifically, the mutant FZ6 was used for the production of succinic acid by fumarate alone that was accomplished only in the presence of an external electron donor (e.g. reduced neutral red or H_2) [59]. *A. succinogenes* NJ113 was cultivated in high concentration of ammonium ions (253 mmol/L) resulting in various adapted strains (Table 4) [60]. Batch fermentation was also used for adaptation in osmotic stress [61] using the strain *A. succinogenes* mutant YZ0819, which was cultivated in the presence of 0.7 M NaCl. The mutant strain *A. succinogenes* CH050 formed was capable of producing 66 g/L of succinic acid from 95 g/L of glucose, while mutant YZ0819 produced 48 g/L of succinic acid with a yield of 0.69 g/g. Furthermore, Zheng et al. [62] reported a new mutation technique based on nitrosoguanidine and UV exposure followed by protoplast fusion. *A. succinogenes* CGMCC1593 was the parental strain and the best succinic acid producing mutant constructed was F3-II-3-F that resulted from the third protoplast fusion [62].

In an attempt to overcome the limitation caused by the lack of suitable shuttle vectors for the development of engineered *A. succinogenes*, an expression vector was constructed (pLGZ901) holding the capacity to carry a foreign gene under the control of a pckA promoter. The vector was succesfully introduced into *A. succinogenes* by electroporation [63], while the transformation was tested

Table 4
Mutants and engineered strains of *A. succinogenes*.

Genetic modification strategy	Parental strain	Mutant	Glucose (g/L)	Succinic acid (g/L)	Yield SA (g/g)	Productivity SA (g/L/h)	Formic acid (g/L)	Acetic acid (g/L)	Reference
Adaptation in sodium monofluoroacetate (0.01–0.08 mol/L)	*A. succinogenes* 130Z		97	79.8	0.82	nk	7.5	21.4	[21]
		FZ6	112	92.1	0.82	nk	0	5.2	
		FZ21	110	101	0.92	nk	0	16.4	
Adaptation in ammonium ions (NH_4^+) (0.253 mol/L)	*A. succinogenes* NJ113		45	0	0	0	nk	nk	[60]
		YZ0819	45	33.01	0.73	nk	nk	nk	
		YZ0206	45	31.71	0.71	nk	nk	nk	
		YZ05	45	29.24	0.65	nk	nk	nk	
		YZ03	45	28.11	0.63	nk	nk	nk	
Adaptation in NaCl (0.7 mol/L)	*A. succinogenes* mutant YZ0819		95	48	0.69	0.69	7.74	11.4	[61]
		Mutant CH050	95	66	0.73	0.74	8.8	12.45	
Genome shuffling by protoplast fusion on mutants derived from nitrosoguanidine (NTG) and UV treatment	*A. succinogenes* CGMCC1593		77.1	55.2	0.72	1.25	0	5.8	[62]
		VI-10-C	106.7	77.9	0.73	1.62	0	7.1	
		F1-IV-9-D	106	84.2	0.79	1.75	0	7.1	
		F2-III-6-D	110.7	87.3	0.79	1.82	0	6.1	
		F3-II-3-F	121.1	95.6	0.79	1.99	0	6.2	

using Tn3 AmpR, Tn10 TetR and Tn9CmR as selection markers that provide resistance to ampiciclin, tetraciclin and chloramphenicol, respectively. Furthermore, pGZRS-1 was identified as a stable replicon. In a different study, two stable *E. coli-M.* succiniciproducens/*A. succinogenes* shuttle vectors were constructed (pMEx and pMVSCS1) and introduced through optimised electoporation [56]. The copy numbers of pMEx and pMVSCS1 were 9.9 and 1.7 in *M. succiniciproducens*, while in *A. succinogenes* the copy numbers were 9.9 and 2.5, respectively. Although homologous (*fumC*) gene expression was succesfully tested in both *M. succiniciproducens* and *A. succinogenes*, heterologous (fluorescent proteins) genes were only expressed in *M. succiniciproducens*.

All genetic alterations of *A. succinogenes* that led to higher succinic acid production have been achieved so far by adaptation and metabolic evolution [21,60–62]. Lee and Kim [64] proposed ten strategies in order to achieve industrial production of a metabolic product. According to the strategies proposed by Lee and Kim [64], in the case of *A. succinogenes* the following strategies could be elaborated in future studies: (1) Increasing tolerance to product, (2) Removing negative regulatory circuits limiting overproduction, (3) Rerouting fluxes to optimize cofactor and/or precursor availability, and (4) Diagnosing and optimizing metabolic fluxes towards product formation. Although genetic engineering strategies to enhance succinic acid production in *A. succinogenes* have not yet been reported, shuttle vectors have been successfully constructed and tested for their functionality. Lee et al. [65] suggested genetic engineering strategies to improve product yield, titer and productivity. One possible solution for *A. succinogenes* would be the enhancement of succinic acid titer and yield by gene amplification of the pathways that lead to the desired product or deletion of genes that control pathways leading to the formation of other products. Genes that take part in the reductive pathway of the TCA cycle (*PEPCK*, *MDH, Fm* and *Fr*) could be amplified or genes responsible for by-product formation (*AK*, *PFL*) could be knocked out in order to favor the fluxes to the C4 pathway (Fig. 2).

4. Bioprocessing aspects of succinic acid production by *A. succinogenes*

4.1. Utilisation of renewable resources as nutrient-complete fermentation media

Various industrial waste and by-product streams (e.g. sugar cane molasses, cheese whey, crude glycerol from biodiesel production, wheat milling by-products, sake lees) and agricultural residues (e.g. corn fiber and corncob, sugarcane bagasse, bio-waste cotton) have been evaluated for the production of succinic acid mainly by *A. succinogenes* (Table 5). Production of succinic acid requires significant quantities of complex nitrogen sources such as yeast extract. The reduction of succinic acid production cost necessitates the utilization of low-cost nitrogen sources supplied either by separate renewable resources, such as corn steep liquor (CSL), or by the same renewable resource that also provides the carbon source (e.g. wheat milling by-products, waste bread). For instance, the use of whey as carbon source achieved succinic acid yield of 0.72 $g_{SA}/g_{lactose}$ in the presence of yeast extract, which was only slightly reduced (0.71 $g_{SA}/g_{lactose}$) in the presence of CSL [85]. The utilization of agri-industrial waste and by-product streams may also supply other nutrients, such as minerals and vitamins.

Sugar cane molasses has been employed, after pretreatment with sulfuric acid, in fed-batch cultures for succinic acid production by *A. succinogenes* CGMCC1593 leading to the production of 55.2 g/L at a productivity of 1.15 g/L/h [43]. Shen et al. [73] identified the optimum concentrations of total sugars of cane molasses (85 g/L), yeast extract (8.8 g/L), and $MgCO_3$ (63.1 g/L) that led to the production of 64.3 g/L of succinic acid concentration at 60 h fed-batch fermentation. As a comparison, the succinic acid concentration (37.3 g/L and 55.8 g/L) and productivity (1.04 g/L/h and 0.77 g/L/h) achieved by genetically engineered *E. coli* strains were approximately in the same range [86,87].

Crude glycerol is a highly promising industrial by-product stream for succinic acid production because glycerol is a more reduced carbon source than C5 and C6 sugars. Vlysidis et al. [66] reported the production of 26.7 g/L of succinate concentration at a yield and productivity of 0.96 g/g and 0.23 g/L/h, respectively. Limited glycerol consumption during cell growth by *A. succinogenes* could be improved by the supplementation of external electron acceptors such as dimethylsulfoxide that led to the production of 49.6 g/L of succinic acid concentration with a productivity of 0.96 g/L/h and a yield of 0.64 $g_{SA}/g_{glycerol}$ in fed-batch cultivation [30].

Wheat milling by-products have been utilised for the production of succinic acid employing a two-stage bioprocess [70]. Initially, amylolytic and proteolytic enzymes were produced via solid state fermentations on bran-rich wheat milling streams using the fungal strains *Aspergillus awamori* and *Aspergillus oryzae*. Crude fermented solids were subsequently used to hydrolyse the starch and protein contained in wheat milling by-products. The hydrolysates were used as the sole fermentation feedstock for the production of 50.6 g/L succinic acid using the strain *A. succinogenes*. The

Table 5
Bio-based succinic acid production in fermentations utilizing different raw materials and microbial strains.

Carbon source	Strain	Nitrogen–nutrient source (g/L)	Type of fermentation, working volume	SA concen. (g/L)	SA productivity (g/L/h)	Yield ($g_{SA}/g_{totalsugars}$)	SA:LA:FA:AA (mol/mol)[a]	Ref.
Representative succinic acid production from pure carbon sources by *A. succinogenes*								
Glucose	*A. succinogenes* 130Z	YE (6)/CSL (10)	CO_2 sparging, continuous, 0.158 L	48.5	nk	0.84	1:0:0:0.38	[40]
Glucose	*A. succinogenes* CGMCC 1593	YE (10)/CSL (6)/Vit	CO_2 sparging, fed-batch, bioreactor, 3 L	60.2	1.3	0.75	1:0:0.13:0.31	[42]
Glycerol	*A. succinogenes* 130Z	YE (5–10)/Vit	CO_2 sparging, batch, bottle reactors, 0.07 L	26.7	0.23	0.96	1:0:0.15:0.14	[66]
Glycerol	*A. succinogenes* 130 Z	YE (10)	CO_2 sparging, fed-batch, bioreactor, 1.5 L	49.6	0.62	0.92	1:0:0.39:0.16	[30]
Sucrose	*A. succinogenes* NJ113	YE (10)/CSL (5)	CO_2 sparging, fed-batch, bioreactor, 1.5 L	60.4	2.16	0.72	1:0:0.55:0.29	[35]
Cellobiose	*A. succinogenes* NJ113	YE (10)/CSL (5)	CO_2 sparging, batch, bottles, 0.03 L	38.9	1.08	0.66	1:0:0:0.69	[34]
Representative succinic acid production from crude renewable resources by *A. succinogenes*								
Corn fiber	*A. succinogenes* FZ6 (mutant)	YE (10), Biotin (10 μg)	CO_2 sparging, batch, vials, 0.01 L	70.6	0.70	0.88	1:0:0.01:0.08:f	[21]
	A. succinogenes NJ113	YE (10)/CSL (5)	CO_2 sparging, batch, bioreactor, 4.5 L	35.4	0.98	0.72	nk	[67]
Corncob	*A. succinogenes* CICC 11014	YE (11)	CO_2 sparging, batch anaerobic bottles, 0.025 L	23.6	0.49	0.58	nk	[68]
Corn stover	*A. succinogenes* CGMCC 1593	CSL (20)	CO_2 sparging, batch SSF[b], bioreactor, 2 L	47.4	0.99	0.72[c]	1:0.06:0.06:0.44	[69]
Corn straw	*A. succinogenes* CGMCC1593	YE (15)	CO_2 sparging, fed-batch, bioreactor, nk	53.2	1.21	0.82	1:0:0:0.22	[32]
Corn stalk	*A. succinogenes* CGMCC 2650 or BE-1	YE (30)/Urea (2)	CO_2 sparging, batch, nk	17.8	0.56	0.66	nk	[33]
Wheat milling by-products	*A. succinogenes* 130Z	YE (2.5)	CO_2 sparging, batch, bioreactor, 0.5 L	62.1	0.91	1.02	nk	[70]
Waste bread	*A. succinogenes* 130Z	Bread hydrolysate (200 mg/L free amino nitrogen)	CO_2 sparging, batch, bioreactor, nk	47.3	1.12	nk	nk	[71]
Cotton stalk	*A. succinogenes* 130Z	YE (30)/Urea (2)	CO_2 sparging, batch SSF[b], flasks, nk	63	1.17	0.64	nk	[72]
Cane molasses	*A. succinogenes* CGMCC 1593	YE (10)	CO_2 sparging, fed-batch, bioreactor, nk	55.2	1.15	nk	1:0:0.16:0.32	[43]
Cane molasses	*A. succinogenes* GXAS137	YE (8.8)	CO_2 sparging, fed-batch, bioreactor, 0.8 L	64.3	1.07	0.76	1:0:0:0.39	[73]
Sugarcane bagasse cellulose	*A. succinogenes* NJ113	YE (10)/CSL (5)	CO_2 sparging, batch bioreactor, 1.5 L	20	0.61	0.65	1:0:0:1.28	[34]
Sugar cane bagasse	*A. succinogenes* NJ113	YE (10)/CSL (5)	CO_2 sparging, batch, bioreactor, 1.5 L	23.7	0.99	0.79	1:0:0:0.37	[74]
Sugarcane bagasse	*A. succinogenes* CIP 106512	YE (2)	CO_2 sparging, batch, bioreactor, 1.5 L	22.5	1.01	0.43	nk	[75]
Macroalgal hydrolysate	*A. succinogenes* 130Z	YE (16.7)	CO_2 sparging, batch bioreactor, 1.5 L	33.0	1.27	0.75	1:0.18:0.28:0.54:g	[76]
Rapeseed meal	*A. succinogenes* 130 Z	YE (15)	CO_2 sparging, fed-batch SSF[b], bioreactor, 1.2 L	23.4	0.33	0.115[d]	1:0:0:0.71	[77]
Whey	*A. succinogenes* 130Z	YE (5)/Pep (10)	CO_2 sparging, batch, bioreactor, 1.2 L	21.3	0.43[c]	0.44	1:0.02:0.68:0.78:h	[31]
Sake lees hydrolysate	*A. succinogenes* 130Z	SLH/YE/biotin	CO_2 sparging, batch bioreactor, 1.5L	52.3	1.74	0.85	1:0:0:0.30	[78]
Representative succinic acid production from pure carbon sources by various strains								
Glucose	*A. succiniciproducens* ATCC 53488	YE (5)/Pep (10)/$(NH_4)_2SO_4$ (5)	CO_2 sparging, batch, bioreactor, nk	32.2	1.19	0.90	1:0:0:0.52	[79]
Galactose	*A. succiniciproducens* ATCC 29305	YE (2.5)/Pep (2.5)/$(NH_4)_2SO_4$ (5)	CO_2 sparging, batch bioreactor, 1 L	15.3	1.46	0.90	1:0:0:0.60	[80]
Glucose	*E. coli* AFP184	CSL (33)/$(NH_4)_2SO_4$ (3)	Dual phase, batch,	45.4	2.84	0.92	1:0:0:0.24	[81]
Xylose			bioreactor, 8 L	29.2	1.79	0.69	1:0:0:0.45	
Fructose				27.7	1.54	0.46	1:0:0:0.34	
Glucose	*E. coli* AFP111	(NH_4)2HPO$_4$ (8)/NH_4Cl (0.2)/$(NH_4)_2SO_4$ (0.8)/Vit	Dual phase, fed-batch, bioreactor, 3 L	101	1.18	0.78	1:0:0:0.07	[82]
Representative succinic acid production from crude renewable resources by various strains								
Corn stalk	*E. coli* SD121	YE (10)/Tryp (20)/$(NH_4)_2SO_4$·7H2O (3)	Dual phase, batch, bioreactor, 1L	57.8	0.96	0.87	1:0:0:0.29:i	[83]
Whey	*A. succiniciproducens* ATCC 29305	CSL (20)/Tryptophane (0.02)	CO_2 sparging, fed-batch, bioreactor, nk	34.7	1.02	0.91	nk	[84]
	A. succiniciproducens ATCC 29305	CSL (20)/Tryptophane (0.02)	CO_2 sparging, continuous, bioreactor, nk	19.8	3	0.64	nk	
	M. succiniciproducens MBEL55E	CSL (7.5)	CO_2 sparging, batch, bioreactor, 1L	13.4	1.18	0.71	1:0.06:1.10:0.73	[85]
	M. succiniciproducens MBEL55E	YE (2.5)	CO_2 sparging, batch, bioreactor, 1L	13.5	1.21	0.72	1:0.05:1.11:0.74	
	M. succiniciproducens MBEL55E	CSL (5)	CO_2 sparging, continuous, bioreactor, 0.5L	10[e]	3.9[e]	0.69[e]	1:0:0.80:0.79	
Cane molasses	*E. coli* AFP111/pTrcC-cscA	$(NH_4)_2HPO_4$ (8)/NH_4Cl (0.2)/$(NH_4)_2SO_4$ (0.7)/Vit	Dual phase, fed-batch, bioreactor, 1.5L	37.3	1.04	0.79	1:0:0:0.17:j	[86]
Cane molasses	*E. coli* KJ122-pKJSUC-24T	$(NH_4)_2HPO_4$ (19.9)/$NH_4H_2PO_4$ (7.5)/Vit	CO_2 sparging, batch, bioreactor, 7.5 L	55.8	0.77	0.96	1:0:0:0.18	[87]
Softwood hydrolysate	*E. coli* AFP184	YE (15)/CSL (15)/$(NH_4)_2SO_4$ (3.3)	Dual phase, batch, bioreactor, 0.7 L	42.2	1.00	0.72	nk	[88]
Pre-treated wood hydrolysate	*M. succiniciproducens* MBEL55E	YE (5)	CO_2 sparging, batch, bioreactor, 1 L	11.73	1.17	0.56	1:0.23:0.45:0.59	[89]
Pre-treated wood hydrolysate	*M. succiniciproducens* MBEL55E	YE (5)	CO_2 sparging, continuous, bioreactor, 0.5 L	7.98	3.19	0.55	nk	

Nitrogen Source: YE: Yeast extract, CSL: Corn steep liquor, Tryp: Tryptone, Pep: Peptone, Vit: Vitamin supplementation.
nk: not known.
[a] mol/mol ratio of fermentation by-products SA: Succinic acid, LA: Lactic acid, FA: Formic acid, AA: Acetic acid.
[b] Simultaneous saccharification and fermentation.
[c] Yield: $g_{succinic\ acid}/g_{substrate}$.
[d] Yield: $g_{succinic\ acid}/g_{dry\ matter}$.
[e] Maximum value observed during continuous fermentation at different dilution rates.
[f] Also propionic acid (3 g/L).
[g] Also ethanol: (2.5 g/L).
[h] Also ethanol (3 g/L).
[i] Also ethanol (1.62 g/L).
[j] Also pyruvic acid (1.2 g/L).

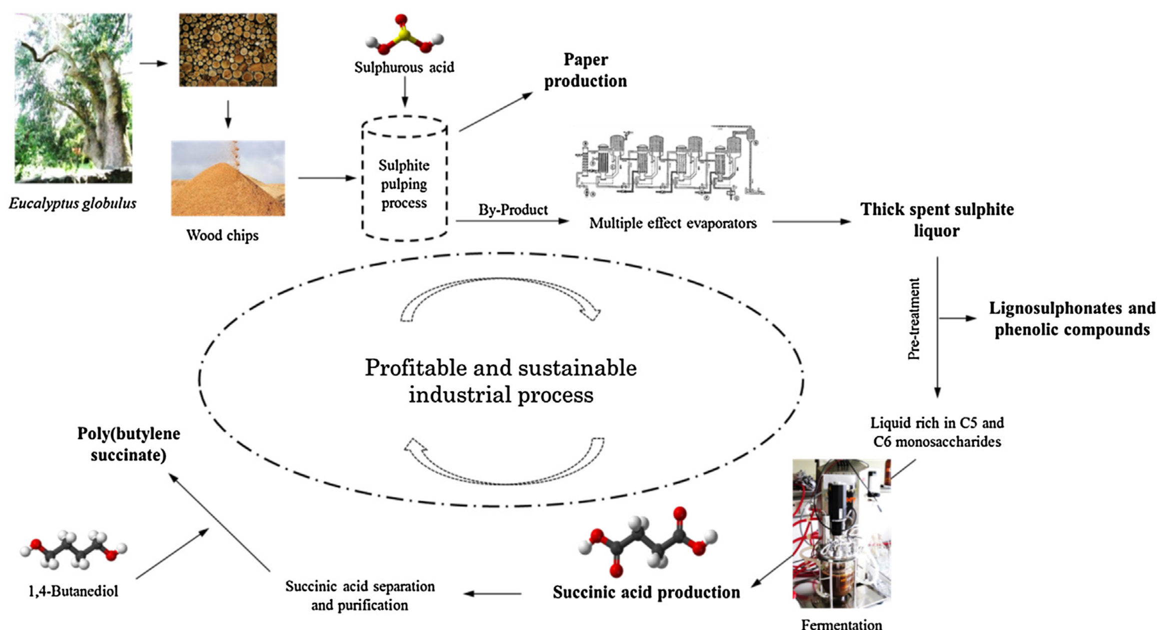


Fig. 5. Integrated biorefinery based on the utilization of spent sulphite liquor.

utilization of 20% (v/v) inoculum at the beginning of fermentation led to the production of 62.1 g/L succinic acid.

Enzymatic hydrolysates of sake lees (pretreated with 0.5% sulfuric acid) supplemented with 2.5 g/L of yeast extract and 0.2 mg/L biotin led to the production of 36.3 g/L succinic acid with a productivity of 1.21 g/L/h and a yield of 0.59 $g_{SA}/g_{glucose}$ [78].

Lignocellulosic biomass has also been used for succinic acid production. Pretreatment of lignocellulosic biomass should be carried out via combined thermo-chemical and enzymatic treatment in order to produce C5 and C6 sugars. Chen et al. [67] utilized corn fiber (containing 31.6% hemicellulose, 21.7% cellulose and 15.4% starch) hydrolysates produced via sulfuric acid pretreatment followed by $CaCO_3$ neutralization and activated carbon absorption (targeting the removal of furfural) for the production of 35.4 g/L succinic acid with a yield of 0.72 g/g_{sugars} and a productivity of 0.98 g/L/h using the strain *A. succinogenes* NJ113. Hydrolysates from waste corncob produced via dilute acid pretreatment were used as xylose and arabinose rich media (constituting around 90% of total sugars) supplemented with yeast extract (11 g/L) and $MgCO_3$ (38 g/L) as neutrilisation agent for the production of 23.6 g/L succinic acid with a yield of 0.58 g_{SA} per g consumed sugars and a productivity of 0.49 g/L/h [68].

Cereal straws are abundant renewable resources with 35–45% cellulose, 20–30% hemicelluloses and 8–15% lignin [32]. Corn straw hydrolysate has been demonstrated to be more efficient for the production of succinic acid compared to hydrolysates derived from rice and wheat straw [32]. Fed-batch fermentation with the strain *A. succinogenes* CGMCC1593 cultivated on corn straw hydrolysates, rich mainly in glucose and xylose, produced by combined alkali pretreatment followed by enzymatic hydrolysis led to the production of 53.2 g/L of succinic acid concentration with a yield of 0.82 g/g and a productivity of 1.21 g/L/h [32]. Cotton stalks pretreated by steam explosion followed by $NaOH/H_2O_2$ treatment were employed in simultaneous saccharification and fermentation at 40 °C for the production of succinic acid (63 g/L) by *A. succinogenes* 130Z with a productivity of 1.17 g/L/h and a conversion yield of 0.64 g/g [72].

Sugarcane bagasse hydrolysates containing glucose (8 g/L), arabinose (5 g/L), xylose (4 g/L) and cellobiose (25.7 g/L) led to complete consumption of all sugars and production of 20 g/L of succinic acid concentration with a yield of 0.65 g/g and a productivity of 0.61 g/L/h using the strain *A. succinogenes* NJ113 [34]. Ultrasonic pretreatment of sugarcane bagasse followed by hydrolysis with dilute acid led to the production of 23.7 g/L of succinic acid concentration with a yield and productivity of 0.79 g/g and 0.99 g/L/h, respectively [74].

Chen et al. [77] demonstrated that rapeseed meal could be employed as a renewable resource for succinic acid production providing both carbon and nitrogen sources. The rapeseed meal hydrolysate was produced via pre-treatment with dilute sulfuric acid followed by hydrolysis by a commercial pectinase preparation. Fed-batch fermentations with *A. succinogenes* ATCC 55618 were carried out with simultaneous saccharification using a pectinase formulation leading to the production of a succinic acid concentration of 23.4 g/L with a yield of 0.115 $g_{SA}/g_{dr\ matter}$ and a productivity of 0.33 g/L/h [77].

The pulp and paper industry produces significant quantities of spent liquors that contain high concentrations of sugars derived mainly from hemicellulose degradation. Alexandri et al. [90] reported the production of succinic acid by *A. succinogenes* and *B. succiniciproducens* in batch cultures using crude and pretreated spent sulphite liquor produced by the sulphite pulping process. The spent sulphite liquor contains predominantly xylose with lower quantities of galactose, glucose, mannose and arabinose. Besides *A. succinogenes*, *E. coli* AFP 184 can also consume xylose [91]. When this strain was cultivated in diluted spent sulphite liquor in dual phase fermentations, where aeration was required for cell growth in the first phase and CO_2 supply was required for the production of succinic acid in the second phase, a succinic acid concentration of 5.2 g/L was produced from 13.9 g/L total sugars (unpublished data).

Waste and by-product streams from the food-industry could be employed for the production of succinic acid. Around 47.3 g/L of succinic acid with productivity of 1.12 g/L/h were produced by *A. succinogenes* cultivated on waste bread hydrolysates produced via hydrolysis of starch and protein contained in waste bread by crude enzymes produced via solid state fermentation [71]. Spent yeast from breweries and wineries could be employed for nitrogen and other nutrient supplementation after autolysis or enzymatic hydrolysis in order to release the intracellular nutrients. Jiang et al. [92] reported that spent brewer's yeast hydrolysate supplemented with vitamins could successfully replace the addition of 15 g/L of

yeast extract, resulting in the production of 46.8 g/L of succinic acid concentration with a yield of 0.69 $g_{SA}/g_{glucose}$. Besides spent yeast, the corn steep liquor derived from corn refining could be employed as nutrient-rich supplement [93].

The high carbohydrate content of macroalgae (up to 60% dry matter) could be used for succinic acid production. Fermentation of algal hydrolysates [76], containing around 45 g/L and 7.5 g/L of glucose and mannitol respectively, with *A. succinogenes* 130Z resulted in 33.78 g/L of succinic acid with a yield of 0.63 $g_{SA}/g_{consume\ sugars}$ and a productivity of 1.5 g/L/h.

4.2. Effect of pH regulators and osmotic stress

Cellular maintenance and regulation of intracellular enzymatic activities are processes known to be highly pH dependent. Therefore, the pH level is often a key parameter in fermentation processes with most bacterial cultures requiring near neutral pH values for optimal performance. Succinic acid production via fermentation results also in the production of other organic acids (e.g. formic, acetic acids) necessitating the use of neutralising agents to prevent acidification of the medium. At near neutral pH values, these organic acids are present in their dissociated forms resulting in the production of the respective salts via neutralization during fermentation. Wang et al. [94] reported an optimum pH value of 7.5 that led to maximum succinic acid production via fermentation of *A. succinogenes* ATCC 55618, while the utilization of NaOH or KOH as individual neutralizing agents led to decreased production of succinic acid due to severe cell flocculation. Inhibition of growth was observed when NH_4OH or $Ca(OH)_2$ were used as neutralising agents [94]. A combination of 5 M NaOH and 40 g/L of $MgCO_3$ prevented cell flocculation and resulted in 27.9% higher succinic acid concentration (59.2 g/L) than the fermentation where NaOH was used as the sole neutralizing agent.

Liu et al. [42] reported stable succinic acid production at a pH range of 6–7.2, whereas $MgCO_3$ was the best neutralizing agent when compared to $CaCO_3$, Na_2CO_3, NaOH and NH_4OH in fermentations carried out with *A. succinogenes* CGMCC1593 using glucose as carbon source. Cell flocculation was observed at 12 h when NaOH or Na_2CO_3 were used, while the utilisation of $MgCO_3$ did not cause any cell flocculation. The use of $CaCO_3$ decreased the production of succinic acid, while NH_4OH resulted in total inhibition of growth and acid formation. Chloride, sulphate and phosphate ions were found not to inhibit the microbial growth. Chloride salts were used to determine the cation caused the inhibitory effect. Although sodium and calcium ions are known to be essential for cell growth, initial concentrations higher than 0.2 mol/L NaCl and 0.1 mol/L $CaCl_2$ are inhibitory to microbial growth and succinic acid production. When $MgCl_2$ was used in a concentration range of 0–0.3 mol/L, growth and succinic acid production remained stable.

Li et al. [95] demonstrated that $MgCO_3$ is the most efficient neutraliser among Na_2CO_3, $NaHCO_3$, $Mg(OH)_2$, $Ca(OH)_2$, $CaCO_3$, NaOH and $NH_3{\cdot}H_2O$ regarding *A. succinogenes* NJ113 cell growth, glucose utilization, succinic acid production and glucose to succinic acid conversion yield. The neutralisers $Ca(OH)_2$, $CaCO_3$ and $NH_3{\cdot}H_2O$ suppressed cell growth resulting in low succinic acid production. Li et al. [95] reported that addition of mixed neutralisers of $Mg(OH)_2$ and NaOH at a ratio of 1:1 could result in similar succinic acid production efficiency as in the case that only $MgCO_3$ is used as neutraliser. This could be attributed to the strong alkalinity and solubility of NaOH and the necessity of the cofactor Mg^{2+} for PEP carboxykinase.

McKinlay et al. [38] reported that a $NaHCO_3$ concentration of 25 mM leads to 1.3–1.4 times higher *A. succinogenes* growth rate than any other $NaHCO_3$ concentration used when a defined medium (i.e. phosphate-buffered medium containing glutamate, cysteine and methionine as the required amino acids, NH_4Cl as the main nitrogen source, as well as vitamins and minerals) was used for succinic acid production.

In the studies presented above [94,42,95], the most effective pH regulator was $MgCO_3$ providing CO_2 and Mg^{2+} ions that serve as cofactors for PEP carboxykinase, the first enzyme in the reductive branch of the TCA cycle.

The fluidity and permeability of the cell membrane requires the presence of Ca^{+2} for energy and transfer regulation [95,96]. Elevated concentrations of Ca^{+2} potentially interfere with cell membrane regulation and acid accumulation, while K^+ are crucial for maintenance of intracellular osmotic pressure as well as exchange of acids and bases. Furthermore, Na^+ is involved in intracellular pH regulation [48] and nutrient uptake, whereas high concentrations may result in hyperosmotic stress. Apart from the influence of $MgCO_3$ on succinic acid production, Mg^{+2} do not interrupt the stability of the membrane and cell flocculation is not observed. Lee et al. [97] reported that the maximum cell concentration of *Anaerobiospirillum succiniciproducens* decreased when NaCl concentration was higher than 4 g/L. Moreover, Fang et al. [98] investigated the effect of three osmoprotectants in *A. succinogenes* NJ113 fermentations performed with high NaCl concentrations. *A. succinogenes* NJ113 was strongly inhibited in NaCl concentrations higher than 0.3 mol/L. However, proline was demonstrated as an excellent osmoprotectant and it was more effective compared to trehalose and glycine betaine.

4.3. Supply of CO_2

Dissolved CO_2 is present in the broth in equilibrium with bicarbonate (HCO_3^-) and carbonate (CO_3^{2-}). When gaseous CO_2 is supplied during fermentation, the concentration of dissolved CO_2 is dependent on the components of the fermentation broth, the temperature, the agitation, the flow rate of CO_2 and the CO_2 partial pressure. High dissolved CO_2 concentrations can be achieved when carbonate (e.g. $MgCO_3$) or bicarbonate salts are supplied during *A. succinogenes* fermentation regardless gaseous CO_2 supply [99]. The consumption of CO_2 during fermentation, leads to the conversion of carbonates and bicarbonates into CO_2 in order to maintain the reaction equilibrium. The diffusion of carbonates and bicarbonates through cell membranes requires ATP consumption [100]. However, supplying high concentration of insoluble carbonates is not practical from an industrial point of view. Samuelov et al. [101] reported that the affinity for CO_2 of the enzymes responsible for its fixation is low. This means that high CO_2 partial pressures are required to divert the metabolic flux towards succinic acid production.

Zou et al. [99] investigated the effect of dissolved CO_2 concentration, gaseous CO_2 partial pressure as well as the supplementation of $MgCO_3$ on the production of succinic acid by *A. succinogenes* ATCC 55618. When only gaseous CO_2 was supplied, the dissolved CO_2 concentration was not sufficient in order to maximize succinic acid production. The supplementation of 40 g/L of $MgCO_3$ and 101.33 kPa of CO_2 partial pressure (achieved by supplying gas with 100% CO_2 content) resulted in the highest succinic acid concentration (60.4 g/L), productivity (0.84 g/L/h) and yield (0.58 $g_{SA}/g_{glucose}$). Xi et al. [100] reported that, when *A. succinogenes* NJ113 was cultivated at a stirring speed of 200 rpm, a CO_2 partial pressure of 0.1 MPa, pH of 6.8, temperature of 37 °C and 150 mM $NaHCO_3$, the optimum CO_2 fixation rate of 0.57 g/L/h could be achieved leading to a succinic acid concentration of 51.6 g/L with a yield of 0.76 g/g.

Anthropogenic energy-related CO_2 emissions could be used for succinic acid production targeting carbon sequestration and recycling of CO_2 rather than simple carbon capture and storage [102]. The availability of CO_2 may increase due to its market price of around 60–450 $/t and an annual estimated carbon capture and storage potential of 3.6 Gt [102]. The cost associated with the

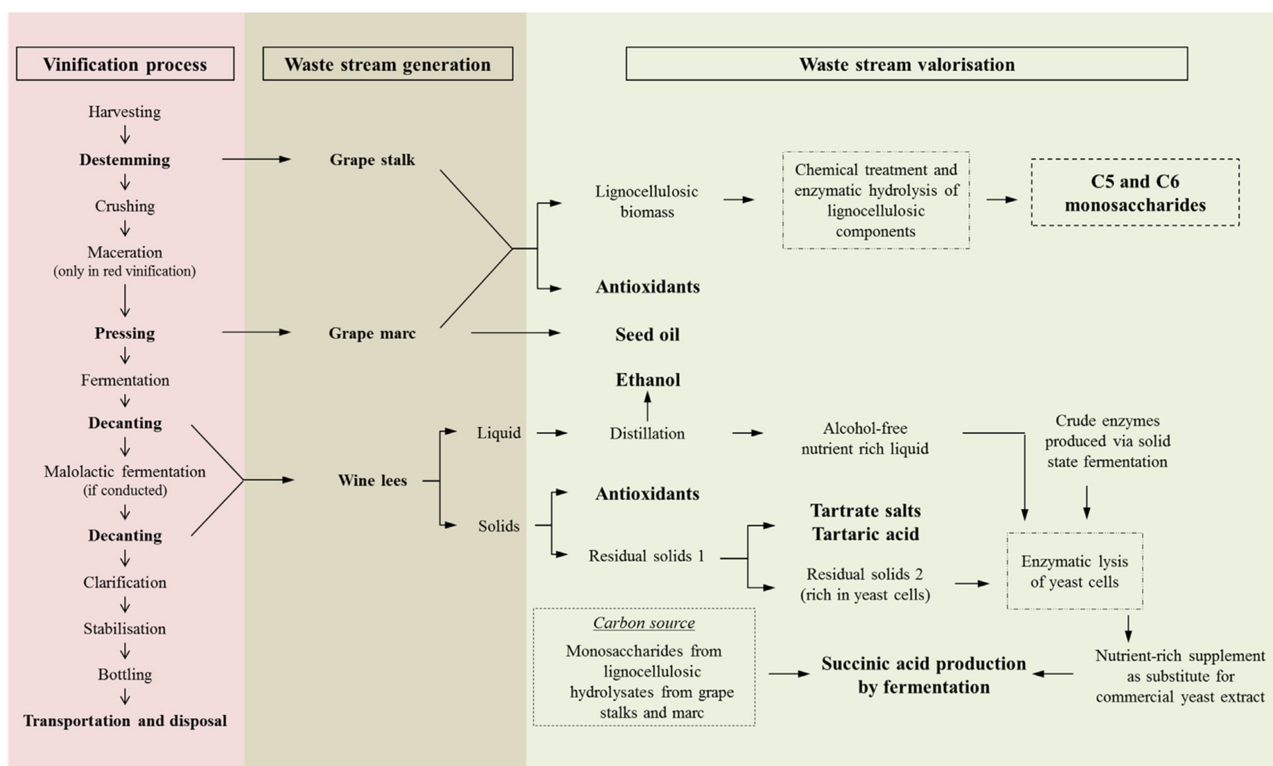


Fig. 6. Integrated biorefinery based on the utilization of winery by-products.

transportation of CO_2 should be minimized as it could be a significant contribution to the total cost when the capture site is far from the recycling/storage site. An alternative solution could be the precipitation of CO_2 with a Mg^{2+} solution for the production of $MgCO_3$ that could be used in fermentative succinic acid production [102].

4.4. Type of bioreactor and operation mode

A. succinogenes was used for the production of succinic acid in different types of bioreactors and operation modes, including utilization of immobilized biocatalysts, integrated fermentation and separation systems and batch, fed-batch and continuous modes of operation. Fed-batch operation with maintenance of a glucose concentration at 10–15 g/L during feeding could enhance succinic acid concentration and productivity [42]. Continuous systems have been also employed for the production of succinic acid. Bradfield et al. [103] investigated continuous succinic acid production with biofilm formation using perlite packing in an external recycle bioreactor to achieve high productivity (6.35 g/L/h at a dilution rate of 0.56 h^{-1}) and constant succinic acid yield of 0.69 g per g of consumed glucose that was independent of fermentation conditions. In a subsequent publication, Bradfield and Nicol [40] reported the production of 48.5 g/L of succinic acid concentration with a yield of 0.91 g/g during continuous fermentation of *A. succinogenes* using glucose and CO_2 feeding into a biofilm reactor operated with external recycling. It was observed that the succinic acid to acetic acid ratio (2.4–5.7 g/g) was increased with increasing glucose consumption (10–50 g/L), while the formic acid to acetic acid ratio was gradually decreased to almost zero with increasing glucose consumption. Urbance et al. [104] reported the production of 10.4 g/L and 40 g/L of succinic acid concentrations in continuous and repeated batch biofilm fermentations employing a plastic composite support.

Yan et al. [105] studied the production of succinic acid by immobilised *A. succinogenes* cells in spiral cotton fiber packed in a modified bioreactor. The fibrous-bed bioreactor was used in 4 cycles of fed-batch fermentations leading to the production of 98.7 g/L of succinic acid concentration with a yield of 0.89 $g_{SA}/g_{glucose}$ and a productivity 2.77 g/L/h. Li et al. [106] employed fed-batch fermentation integrated with *in situ* product removal by an expanded-bed adsorption system leading to increased succinic acid concentration (up to 145.2 g/L) with average yields and productivities of 0.52 $g_{SA}/g_{glucose}$ and 1.3 g/L/h, respectively.

4.5. Downstream processing of succinic acid rich fermentation broths

Downstream purification of succinic acid mainly focuses on the separation of pure crystals from various impurities (e.g. proteins, colour) and by-products generated during fermentation. The separation of metabolic by-products (mainly acetate and formate in the case of *A. succinogenes*) is crucial in order to produce succinic acid crystals of high purity, even in the range of 99.5% [107]. Several methods have been evaluated for the purification of succinic acid (Fig. 3).

Precipitation of organic acids with calcium hydroxide is the conventional separation process employed by the traditional fermentation industry. Following treatment with calcium hydroxide, calcium succinate is recovered by filtration and converted into succinate with the addition of sulfuric acid. The resulting solution is treated with activated carbon and succinic acid is separated using ion exchange resins [7]. The crystals of succinic acid are purified via evaporation to concentrate the solution followed by crystallisation [7]. However, the purification of succinic acid by precipitation with calcium hydroxide presents significant disadvantages, such as the equimolar production of calcium sulfate which is considered a waste stream as well as the high demand for calcium hydroxide and sulfuric acid that cannot be recovered and recycled.

Table 6
Potential production of succinic acid based on by-product streams produced by various industrial plants.

Industrial plant	Availability of waste stream (million t)	Practical carbon source to succinic acid conversion yield (taken from Table 5) (g/g)	Potential succinic acid production from by-product steam (million t)[a]	Potential added-value co-products
Biodiesel	3 for crude glycerine	0.92	0.14	–
Biodiesel or edible oil production processes	35.8 for rapeseed meal 14.9 for sunflower meal	0.43–0.88 (only the carbohydrate content is considered)	0.16–0.33 0.12–0.25	Phenolic-rich extract with antioxidant properties and protein isolates
Pulp and paper	Around 210 × 10^6 m^3/y of thick liquor containing an average sugar concentration of 145 g/L	0.43–0.88	0.65–1.34	Phenolic-rich extract with antioxidant properties and lignin derivatives (e.g. lignosulphonates)
Wheat straw	74	0.43–0.88	1–2.1[b]	Waxes and bioenergy/chemicals from lignin
Wineries	18.7 considering prunings, stalks and pomace	0.43–0.88	0.04–0.09[c]	Bioactive compounds, pectins, tannins, ethanol and tartrate salts
Wheat and rye losses during processing and consumption in EU27	23.95	1.02 (based on wheat milling by-products)	0.5[d]	Lipids for biodiesel or oleochemical production
DDGS production from corn ethanol	31.6	0.43–0.88	0.4–0.8[e]	Lipids and proteins

[a] Assuming 5% raw material utilization.
[b] A moderate polysaccharide content of 60% has been assumed.
[c] A 10% cellulose/hemicellulose content has been assumed.
[d] Assuming 40% starch content.
[e] Assuming 53% carbohydrate content.

An alternative process employing diammonium succinate produced via neutralisation with an ammonia-based solution during fermentation of *E. coli* resulted in a succinic acid recovery yield of up to 94.9% [109]. Diammonium succinate is converted into succinic acid and ammonium sulfate via treatment with ammonium bisulfate and/or sulfuric acid. The ammonium sulfate could be thermally cracked into ammonia and ammonium bisulfate, which could be reused in the succinic acid production process. The purity of succinic acid crystals could be increased by removing sulfates, which may co-crystallize with succinic acid, via methanol purification. However, ammonia or ammonium based neutralisers do not lead to efficient succinate production by *A. succinogenes* [42].

The relatively high pH values (6.2–7.2) employed during fermentation results in the conversion of succinic acid, as well as other organic acids, into ionized-succinate salts. Other compounds present in the fermentation broth, including carbohydrates, proteins and amino acids, mainly remain non-ionized [7]. Electrodialysis is a technology where separation between ionized and non-ionized compounds is achieved through ion exchange membranes. Bipolar membrane electrodialysis has been employed for the separation of succinic acid from fermentation broths [113]. The divalent cations (e.g. Mg^{2+}) in the succinate salts produced during *A. succinogenes* fermentation should be replaced by sodium ions in order to minimize operational limitations of bipolar membrane electrodialysis. The sodium hydroxide produced by this process, besides succinic acid separation, could be recycled in the fermentation. However, the high capital and operating costs, the complexity of the fermentation broth, the effect of other acids on purity and the operation efficiency of this process need further optimissation in order to ensure industrial implementation [113].

Reactive extraction is a promising process for the separation of carboxylic acids from fermentation broths [114]. The efficiency of reactive extraction is dependent on the amine and solvent used and the stoichiometry of the amine–acid complexes formed. The pH value of the fermentation broth and the dissociation constants (pK_a) of the acids present in the broth are crucial in the extraction efficiency achieved during reactive extraction. An advantage of reactive extraction is the ambient temperature and pressure conditions usually employed. Kurzrock and Weuster-Botz [115] compared 448 different amine–solvent mixtures for the extraction of succinic acid from aqueous succinic acid solutions resulting in separation yields of more than 95%, which was higher than the 78–85% extraction yield achieved when *E. coli* fermentation broths were used. Huh et al. [110] employed a reactive extraction system comprising of tri-*n*-octylamine combined with 1-octanol followed by evaporation, crystallization and drying to achieve succinic acid separation with purity of 99.8% (w/w) and yield of 73.1% (w/w). In this case, the reactive extraction targeted the selective removal of by-products (e.g. acetic acid, pyruvic acid, maleic acid) and salts from diluted *M. succiniciproducens* fermentation broth [110].

A downstream process based on treatment with activated carbon, acidification, vacuum distillation, crystallization and drying for the purification of succinic acid crystals has been evaluated leading to relatively high purity (90–97%) and yield (61–75%) in the case of simulated broths and lower purity (45%) and yield (28%) in the case of actual fermentation broths [116]. Lin et al. [108] reported high purity (99%) and separation yield (89.5%) of succinic acid crystals via transformation of the salt forms of succinate and other by-products into the free acids via treatment with a commercial cation-exchange resin Amberlite IR 120H, evaporation of the resulting broth to concentrate the succinic acid and remove the other acids, and crystallization of succinic acid.

Orjuela et al. [112] employed acidification and esterification in ethanol as a novel approach for the recovery of succinic acid. The fermentation broth was initially treated for cell, macromolecules and protein removal followed by separation of the salts of organic acids that were subsequently placed in ethanol along with a slight stoichiometric excess of sulfuric acid. This process led to separation of the sulfate salt and the succinate was recovered as free succinic acid, monoethyl succinate and diethyl succinate in ethanol, a mixture that was subsequently esterified via reactive distillation [112]. The recovery of succinate obtained with this process was higher than 95% for both simulated succinate salt solutions and actual fermentation broth mixtures.

Succinic acid could be ideally converted directly in the fermentation broth into less miscible products. Budarin et al. [117,118] demonstrated that the water insoluble diethyl succinate could be generated by aqueous phase esterification of succinic acid with ethanol that separates easily from the aqueous fraction as a second layer in good purity. The catalyst dodecylbenzene sulfonic acid could esterify succinic acid from fermentation broths with 1-octanol in two-phase systems at a final ester purity of 83% and conversion yield of 85% [111]

Commercial implementation of succinic acid production requires low number of unit operations for succinic acid purification that could be achieved through implementation of fermentations carried out at low pH, where the organic acids will be present in their undissociated forms. Bioamber is targeting the production of bio-based succinic acid at low pH using recombinant yeast strains [119].

4.6. Techno-economic analysis for succinic acid production

The cost-competitiveness of succinic acid production has been evaluated when different carbon sources were used including pure sugars, such as sucrose [120] and glucose [121], and crude renewable resources, such as crude glycerine from biodiesel production processes [122,123]. Efe et al. [120] carried out a conceptual design of a succinic acid production process with plant capacity of 30,000 t using hypothetical low pH fermentation and cane sugar as carbon source. The downstream separation process was based on cell removal via centrifugation, succinic acid adsorption using ZSM-5 zeolite followed by desorption using hot water, a flash drum, evaporation, crystallization and drying resulting in succinic acid crystals with 99.5% purity. The minimum selling price, considering a zero net present value at the end of the life cycle of the plant (10 years), was estimated at 2.26 $/kg at a discount rate of 10%. The contribution of the downstream process was estimated at 0.36 $/kg succinic acid. The total capital investment was estimated at more than $146.6 million with around 11% attributed to the downstream separation process. The bioreactors are the major cost contributor to the total capital investment. Efe et al. [120] suggested that heat integration and process improvement could significantly decrease the downstream separation cost.

Posada et al. [122] carried out techno-economic evaluation of succinic acid production using pure glycerol (98% w/w) and a downstream separation stage based on reactive extraction with tri-*n*-octylamine and 1-octanol as extraction and diluent agents, respectively. The unitary production cost for succinic acid varied in the range of 2.01-2.95 $/kg considering a plant production capacity of around 460 kg/h [122]. Vlysidis et al. [123] evaluated the design of a small scale succinic acid production plant integrated in an existing biodiesel plant with an annual capacity of 430 t of succinic acid production. The unitary product cost and the minimum selling price were estimated at 2.3 and 3.64, respectively, for an interest rate of 7% and 20 years of plant lifetime [123].

Orjuela et al. [124] carried out process design and techno-economic evaluation for the downstream separation of succinic acid based on dissolution and acidification of succinate salt in ethanol, followed by reactive distillation to produce succinate esters. A capital investment of $75 million and a net processing cost of $1.85 per kg succinic acid were estimated at 54,900 t per year plant capacity and 100 g/L succinic acid concentration in the fermentation stage.

The raw material used, the fermentation stage and the downstream separation process contributes the major cost to succinic acid production. The fermentation stage contributes the highest expenditure to the fixed capital investment. Process integration in existing industrial facilities could reduce logistics related costs. The utilization of waste and by-product stream could reduce the raw material costs depending on the pretreatment cost of the raw material. Succinic acid production usually requires expensive nutrient supplement, such as yeast extract. The production of succinic acid crystals with purity higher than 99% is essential in order to reach high yields in the subsequent chemical conversion of succinic acid to various products. For instance, the polymerization of succinic acid to PBS requires a succinic acid purity of at least 98%, and hence, efficient purification processes need to be developed to reach this target [125].

The cost of utilities is a critical factor in all cost estimates as significant quantities of electricity and steam are required for the sterilization process, the agitation of the bioreactors and the downstream separation process. However, utilities requirements can be reduced by implementing heat integration techniques. Kastritis et al. [126] developed an optimum design for a heat exchange network in a biodiesel plant co-producing succinic acid so as to minimise the annual operational cost of the plant. The total annual cost was reduced by 17.2% and the operational cost by 46.4% by implementing heat integration methodologies [126].

5. Biorefinery concepts including succinic acid production

The fermentation efficiency is the most important parameter that decides the cost of manufacture of metabolic products. The higher the final succinic acid concentration and volumetric productivity achieved, the lower the capital investment. The higher the carbon source to succinic acid conversion yield achieved, the lower the raw material requirements. Furthermore, by-product formation and neutral pH conditions during fermentation lead to more downstream stages for succinic acid purification. Besides fermentation efficiency, refining of renewable resources will lead to the production of versatile end-products with diversified market outlets improving the profitability margin of succinic acid production. Table 6 presents potential biorefinery concepts for simultaneous production of succinic acid and various value-added co-products.

In 2021, the worldwide production of crude glycerine is projected at around 3×10^6 t/y based on the expected biodiesel production from edible vegetable oils of 30×10^6 t/y [127]. If it is assumed that 5% of crude glycerine is used for succinic acid production then around 0.14×10^6 t/y could be produced at a glycerol to succinic acid conversion yield of 0.92 g/g (taken from Table 5). The production of succinic acid could be also integrated in biorefineries based on the valorization of oilseed meals (Fig. 4). This concept involves the utilization of crude glycerine and the carbohydrate content of oilseed meals as carbon sources for fermentative succinic acid production combined with the extraction of value-added co-products from oilseed meals. A fraction of the protein content in the oilseed meal could be used as substitute for yeast extract. Kachrimanidou et al. [128] have demonstrated the production of antioxidant-rich fractions, protein isolate and poly(3-hydroxybutyrate) from sunflower meal. The protein content in rapeseed and sunflower meals is around 35–40% and 20–40%, respectively [11]. Protein isolates could be used as higher value animal feed, whereas their hydrolysates could be used as food additives. Furthermore, rapeseed and sunflower meals contain around 19% and 35% of carbohydrates, respectively [11]. Considering the conventional use of rapeseed and sunflower meals as animal feed, if 5% of the worldwide production in 2012/2013 of rapeseed meal (35.8×10^6 t/y) and sunflower meal (14.9×10^6 t/y) are used for succinic acid production [129], it is estimated that around $0.16–0.33 \times 10^6$ and $0.12–0.25 \times 10^6$ t of succinic acid per year could be produced from the cellulose and hemicelluloses contents of these two meals, respectively, allowing for a mixed sugar to succinic acid conversion yield of 0.43–0.88 g/g (taken from Table 5).

By-product streams from the current pulp and paper industry are mainly composed of lignin derivatives, phenolic compounds, C5 and C6 sugars, small amounts of cellobiose and various minerals, impurities and derivatives of lignocellulose degradation (e.g. acetic acid, furfural). Phenolic compounds and higher molecular weight lignin derivatives (e.g. lignosulphonates produced by the sulphite pulping process) could be separated by liquid–liquid extraction and membrane filtration to avoid the degradation of sugars (Fig. 5). Alexandri et al. [90] has demonstrated the extraction of phenolic-rich fractions with strong antioxidant activity from spent sulphite liquor produced by the sulphite pulping process of hardwood *Eucalyptus globulus*. Phenolic-rich extracts and lignosulphonates could be used in various applications including utilization of antioxidants in polymer formulation and utilization of lignosulphonates in conventional cement production or novel polymer production. If 5% of the remaining sugars in thick liquors are used for succinic acid production then around 0.65×10^6–1.34×10^6 t/y of succinic acid could be produced taking into consideration that the average sugar concentration in thick spent sulphite liquor or black liquor is around 145 g/L and the quantity of thick liquor produced is around 210×10^6 m^3/y [11].

Wheat straw production in the EU will be approximately 74×10^6 t by 2020 [130]. Only 17.5% of total straw production can be used as feedstock in the bio-economy sector, after allowing straw usage for soil improvement (75%), power generation (2.5%) and animal husbandry (5%) [130]. If it is assumed that wheat straw contains a moderate polysaccharide content of 60% [131], then it could be estimated that 1–2.1×10^6 t/y of succinic acid could be produced from only 5% of total wheat straw utilisation. Wheat straw could be processed for the extraction of value-added components [132]. For instance, Deswarte et al. [133] have developed a process based on supercritical CO_2 extraction of waxes from straw that could be used as soaps, detergents, lubricating grease, wax coatings, surfactants, cosmetics and metal chelators among others, allowing in the same time for minimal change in the nature of the lignocellulosic material. Lignin could be also used either for energy generation or material and chemical production.

Wine production generates many side streams, such as prunings from vineyards as well as stalks, pomace and lees from wineries. Prunings, stalks and pomace/marc contain cellulose and hemicellulose together with free sugars from pomace/marc depending on the vinification process. These streams also contain bioactive compounds, pectins and tannins that could be extracted as value-added co-products. The wine lees are rich sources of yeast cells, tartrate salts, ethanol and antioxidants. Dimou et al. [134] developed a process leading to the extraction of antioxidants, ethanol and tartrate salts as value-added co-products followed by the conversion of yeast cells into a nutrient rich hydrolysate via enzymatic hydrolysis that could be used as nutrient supplement in various fermentation processes. The annual production of wine grape in EU is around 24×10^6 t leading to the production of 14×10^6 t of prunings, 1.2×10^6 t of stalks, 3.5×10^6 t of pomace/marc and 10^6 t of wine lees. In the case of holistic utilization of winery side streams, at least 70% should be utilized for soil improvement. If it is assumed a conservative 10% cellulose/hemicellulose content and only 5% usage potential of prunings, stalks and pomace/marc, the production of 0.04–0.09×10^6 t/y of succinic acid can be estimated. If it is assumed that 5% of the total wine lees is yeast cells, around 50,000 t of yeast extract would be available for succinic acid production. It should be also stressed that technologies could be developed for the utilization of CO_2 produced during wine production as supplement in fermentative succinic acid production. Fig. 6 presents a potential biorefinery concept targeting the production of succinic acid and various value-added co-products from winery by-products.

Bakery and confectionery industries generate by-products that are rich in starch, sucrose, lipids and proteins. Hydrolysis of starch to glucose and proteins to small peptides or aminoacids could be used as a nutrient-rich fermentation medium for the production of succinic acid. The losses and wastes of wheat and rye in Europe generated at the industrial bread baking and the consumption stages of the whole value chain are 7.45×10^6 and 16.5×10^6 t/y [135]. If it is assumed that these waste streams contain 40% starch, it is estimated that 0.5×10^6 t/y of succinic acid could be produced from only 5% of wheat and rye losses and wastes, assuming a glucose to succinic acid conversion yield of 1.02 g/g (taken from Table 5). If it is assumed a 10% protein content in the aforementioned waste streams then around 2.4×10^6 t/y of protein would be available as nitrogen source in fermentation processes.

Bioethanol production is currently mainly produced from corn and wheat in the USA and Europe, respectively. The dry milling of cereal grains generates distillers dried grains with soluble (DDGS) as by-product stream that contains carbohydrates, lipids, protein, minerals and vitamins [136]. The main conventional use of DDGS is animal feed. The production of DDGS in the USA amounted to 31.6×10^6 t in 2012 [137]. The average total carbohydrate content in DDGS is around 53%. If only 5% of total DDGS production is used as fermentation feedstock, around 0.4–0.8×10^6 t/y of succinic acid could be produced. DDGS could be also used for the extraction of lipids and proteins.

6. Conclusion

Succinic acid is currently established as an industrially important platform chemical that could be used as precursor for the development of a sustainable chemical industry. *A. succinogenes* can be considered as one of the most promising natural succinic acid producers, while its ability to utilize a wide range of carbon sources can facilitate the utilization of different raw materials. Although several downstream separation methods have been suggested so far, the efficient purification of succinic acid is still considered as a critical parameter for the application of the process at large scale. The development of genetically engineered *A. succinogenes* strains is essential in order to facilitate industrial implementation. Finally, the integration of bio-based succinic acid production into existing industrial facilities and the development of integrated biorefineries will lead to significant economic and environmental benefits.

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