Review

State of the Art on the Microbial Production of Industrially Relevant Organic Acids

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Abstract: The industrial relevance of organic acids is high; because of their chemical properties, they can be used as building blocks as well as single-molecule agents with a huge annual market. Organic acid chemical platforms can derive from fossil sources by petrochemical refining processes, but most of them also represent natural metabolites produced by many cells. They are the products, by-products or co-products of many primary metabolic processes of microbial cells. Thanks to the potential of microbial cell factories and to the development of industrial biotechnology, from the last decades of the previous century, the microbial-based production of these molecules has started to approach the market. This was possible because of a joint effort of microbial biotechnologists and biochemical and process engineers that boosted natural production up to the titer, yield and productivity needed to be industrially competitive. More recently, the possibility to utilize renewable residual biomasses as feedstock not only for biofuels, but also for organic acids production is further augmenting the sustainability of their production, in a logic of circular bioeconomy. In this review, we briefly present the latest updates regarding the production of some industrially relevant organic acids (citric fumaric, itaconic, lactic and succinic acid), discussing the challenges and possible future developments of successful production.

Keywords: industrial biotechnology; microbial cell factories; organic acids; bioeconomy

1. Introduction

The current industrial system relying on fossil resources is putting at serious risk our living conditions on the planet. Globally, industry is responsible for over 30% of all greenhouse gas emissions, mainly represented by CO_2, methane, nitrous oxide and fluorinated gasses (Sources of Greenhouse Gas Emissions—EPA). Most of these gasses arise from the production and use of bulk materials such as cement, metals, chemicals and petrochemical products, as well as from other human activities such as intensive farming. This description exemplifies the consequences of our model of growth, mainly based on a linear economy, which we thought to be sustainable when it was responding to the needs of a few billion people. Changing the paradigm from a linear to a circular economy and from the depletion of resources to exploitation of renewable biomasses is a promising alternative to reconnect human activities with biogeochemical cycles [1].

About 20 years ago, anticipating the current scenario, the U.S. Department of Energy (DOE) had identified twelve building-block chemicals [2]. Some years later, lactic acid was included in the list of the top-ten chemicals (Table 1) [3]. About 70% of the listed compounds are organic acids, which are good examples of building blocks. They find applications in the food & beverage industry, pharmaceutical and cosmetic segments, nutrition, and animal feed. In particular, chemical industries use organic acids as basic compounds for a

wide variety of polymer and solvent production processes. In addition, they can be used as preservatives, as they can avoid the accumulation of reactive species, which are detrimental for cellular metabolism, if not for life [4,5]. It is not surprising, therefore, that the global annual organic acids market is projected to grow to about EUR 35 billion in 2027 [6].

Table 1. The 2004 and 2010 "revisited" US Department of Energy's top Sugar-derived Building Block Chemicals [2,3].

2004	2010
1,4-Diacids (Succinic, fumaric and malic acids)	3-Hydroxypropionic acid/aldehyde
2,5-Furan dicarboxylic	Bio-hydrocarbons
3-Hydroxybutyrolactone	Ethanol
3-Hydroxy propionic acid	Furans
Aspartic acid	Glycerol and derivatives
Glucaric acid	Lactic acid
Aylitol/Arabitol	Levulinic acid
Glutamic acid	Succinic acid
Itaconic acid	Sorbitol
Levulinic acid	Xylitol
Glycerol	
Sorbitol	

Interestingly, organic acids can be produced by microbial metabolism, in some cases naturally, in some others after redirecting the carbon flux towards the desired target(s). Nonetheless, in terms of the production process, this implies non-trivial assumptions; the biocatalyst has to be able to transform a given biomass into a specific product, with maximized titer, yield and productivity. Furthermore, when commodities are produced, these parameters have to match with a final selling price of about or ideally below EUR 2 per kg [7]. Translating this concept into a feasible industrial process implies that the biocatalyst is forced to produce a non-natural product or, even if natural, to accumulate it up to non-physiological titer in a non-natural environment, possibly starting from a non-preferred substrate. In general, the engineered cell factories are proposed to overcome the main issues occurring during the determination of fermentation performances and, above all, during the scale-up process. Indeed, one of the main challenges remains the possibility to obtain strain(s) able to maintain the same performance of the lab-scale productions. The recent review by Wehrs et al. [8], provides a comprehensive analysis toward strains implementation. In particular, they underline the profound differences of the environment characterizing commercial-scale bioreactors if compared with laboratory-scale cultivation systems. The fluctuation and heterogeneity of chemical-physical parameters in large bioreactors and the genotypic instability or drift observed during long cultivations are prominent examples of issues that are often neglected or difficult to reproduce at lab-scale. This gap makes it extremely difficult to predict strains' performances and consequently, optimal engineering design.

Despite the fact that these descriptions could lead to the conclusion of an unattainable goal, thanks to strain and process engineering, an increasing number of microbial processes are matching scale-up requirements and are emerging as real alternatives to chemical synthesis at industrial scale. Among the many organic acids that can be obtained by microorganisms, some have a prominent space in industry, both for tradition and in terms of competitiveness with traditional production: microbial citric, succinic, lactic, itaconic, lactobionic, gluconic, fumaric, propionic and acetic acid have approached the market, with different degrees of penetrance and success. From a chronological point of view, citric acid was the first organic acid industrially produced by a microbial cell factory, followed by lactic, fumaric, succinic and itaconic acid (Scheme 1).

In this review we present a state of the art on microbial processes for these organic acids which successfully reached the industrial, commercial, or piloting scale of production. Companies currently involved in this important challenge for our future will be listed

and, when publicly available, information about the microbial strain currently in use will be given.
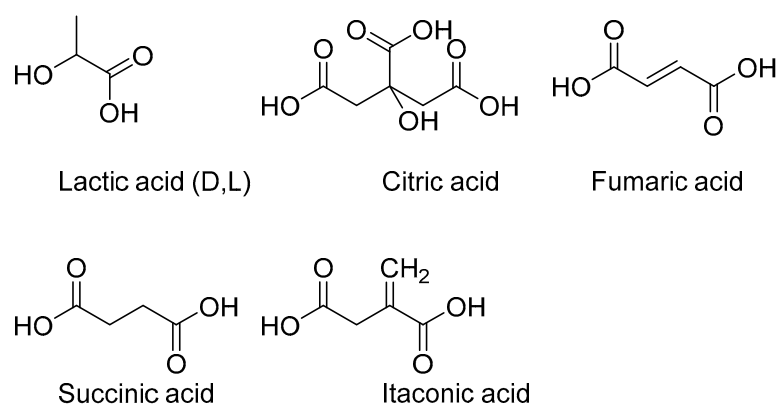
Scheme 1. Structures of organic acids produced at pilot scale by microbial processes and discussed in this review.

2. From First Evidence and Studies to Current Pilot or Commercial-Scale Production

2.1. Citric Acid ($C_6H_8O_7$, 192.124 $g \cdot mol^{-1}$, pKa$_1$ 3.13, pKa$_2$ 4.76, pKa$_3$ 6.39)

Citric acid (CA, 2-hydroxy-propane-1,2,3-tricarboxylic acid) is an intermediate of the TCA cycle, and therefore present in virtually all living organisms.

CA was crystallized for the first time in 1784 by the Swedish chemist Karl Scheele; the procedure relied on treatment of the lemon juice with calcium hydroxide to obtain calcium citrate, subsequently treated with sulfuric acid to give citric acid [9].

Commercial production of citric acid started in England in 1826 from unripe Italian lemons and the Italian monopoly lasted until the late nineteenth century [10]. During World War I, export of lemons from Italy stopped and chemical production from glycerol (invented in 1880 by Grimoux and Adams [11]) took over. However, due to the increased importance of citric acid, fermentation became competitive enough and quickly replaced chemical synthesis. The first evidence of microbial production of CA dates back to 1893, when Wehmer observed that *Penicillium glaucum* could produce citric acid from sugars, and two years later, two strains of *Penicillium*, at that time named *Citromyces* spp., were isolated [12]. Unfortunately, production with *Citromyces* was never industrially significant because of the occurrence of contaminations and the long fermentation time. Later, Currie in 1917 found that, in various strains of *Aspergillus niger*, the production and secretion of CA was stimulated by growing them in a nutrient medium with an initial pH of 2.5–3.5 [13]. Afterwards, in 1919, the pharmaceutical company Pfizer, in cooperation with Currie, started the first pilot plant for CA production in New York using the fermentation process developed by the chemist and named SUCIAC "Sugar Under Conversion to Citric Acid". Already in mid-1920 the fermentative production of CA surpassed the extractive production from lemons [14,15].

Citric acid is a colorless, odorless, and highly water-soluble compound. It is safe for human health and the environment and is considered almost indispensable and hard to substitute in light of the number of applications in very different sectors, such as food and beverages, the pharmaceutical, nutraceutical and cosmetics industries. The great majority of the produced citric acid (>65%) is addressed to the food and beverage (F&B) sector where it is employed both as a preservative, thanks to its antioxidant properties, and as flavor enhancer, used to balance the sweetness of juices, soft drinks and jams [16,17]. CA is certified as safe (Generally Regarded As Safe) by the Food and Drug Administration in the US and, together with its salts, is EU-approved as a food additive, being indicated as E330 (citric acid), E331 (sodium citrate), E332 (potassium citrate) and E333 (calcium citrate).

The second main user is the pharmaceutical sector, where CA is used in the preparation of effervescent tablets, as an anticoagulant in blood transfusions, as a crosslinking agent
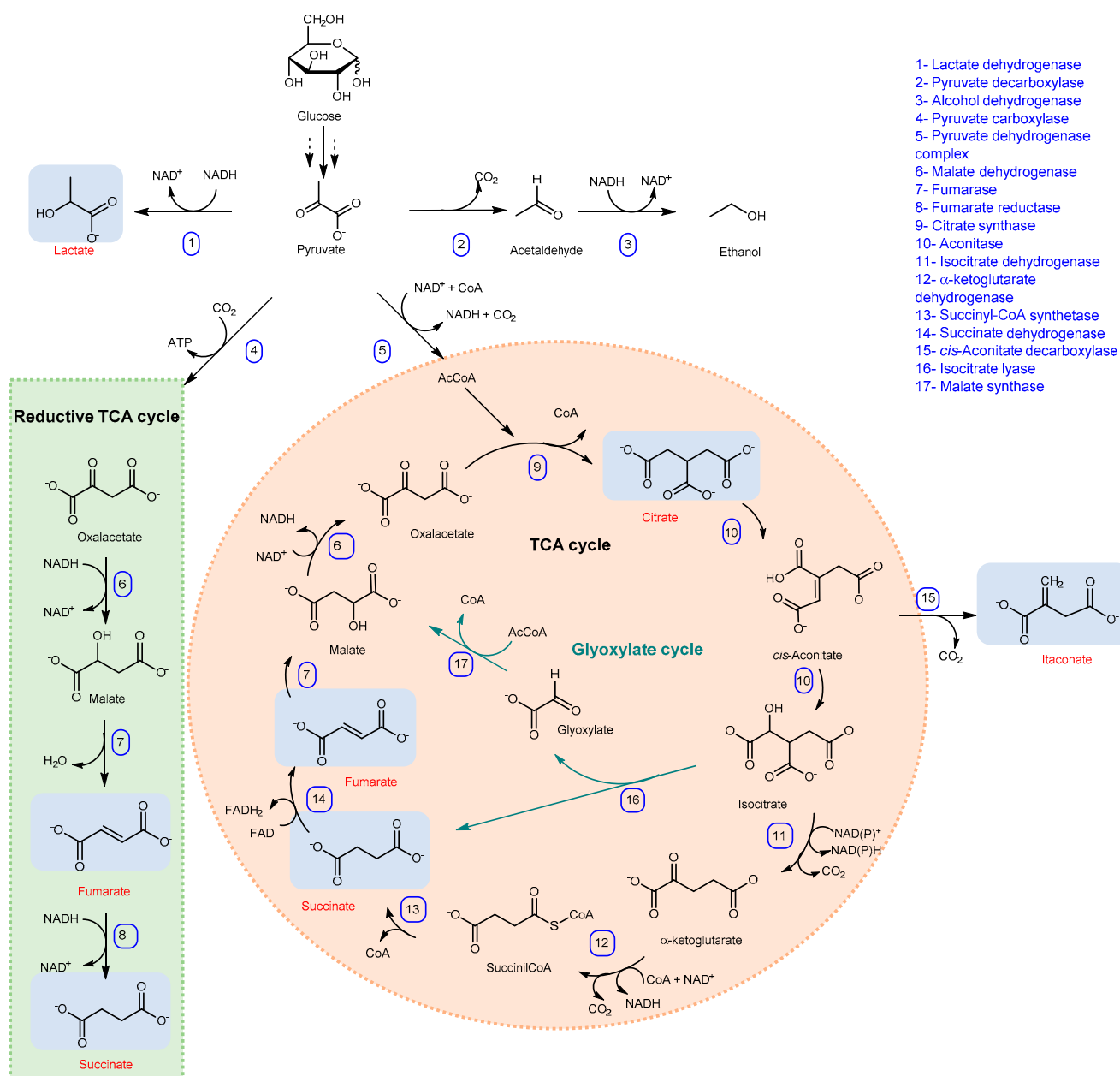
in films for controlled drug release, and as a palatability enhancer of unpleasant drugs. Moreover, many APIs are administered in the form of citrate salts [17]. Other uses deal with the detergent, cleaning and polymer industries and environmental remediation, and have been extensively reviewed by Ciriminna at al. [17]. More recently, the use of CA as one of the components of deep eutectic solvents, promising in the valorization of residual lignocellulosic biomasses, was proposed [18].

Many microorganisms have been found to produce and accumulate CA. For instance, some bacteria (such as *Bacillus* sp., *Arthrobacter* sp., *Corynebacterium* sp., *Brevibacterium* sp.), yeasts (such as *Candida tropicalis*, *C. oleophila*, *Yarrowia lipolytica, Hansenula anomala)* and filamentous fungi, mostly belonging to the *Aspergillus* genus, have been reported as CA producers [19,20]. Despite this wide portfolio, most of these microorganisms are not able to afford commercially exploitable amounts of CA, and thus, *A. niger* is still the preferred workhorse for productive purposes thanks to its robustness in industrial fermentation conditions. In fact, more than 80% of the world's citric acid production is derived from *A. niger*, although some processes with the yeast *Y. lipolytica* have been reported [21]. The yeast-based process is, however, plagued by the significant production of by-products, such as isocitric acid, that inevitably lower the final product yield.

Citric acid is produced during the TCA cycle through the condensation of oxaloacetate and acetyl-CoA, resulting in 1 mol of CA from each mol of glucose with a maximum theoretical yield of 1.067 *g/g* glucose (Scheme 2). Although the enzymatic activities responsible for CA production have been identified and investigated, much less is known about the biochemical and physiological aspects of CA accumulation [22]. In fact, the exact mechanism underlying CA accumulation in *A. niger* has not been completely understood; several hypotheses have been proposed and the debate is still open. It is clear that CA overflow is the result of the synergistic effect of different imbalanced conditions, such as low pH, low iron concentration, limited availability of nitrogen and phosphate, and excess oxygen and carbon sources [10].

A. niger can use several substrates for its growth (fructose, glucose, galactose, mannose, glucose); however, glucose and sucrose, with a slight preference for the latter, are the preferred carbon sources for CA production [10]. Sucrose was demonstrated early on to be the best carbon source, and CA production still mainly relies on sucrose-based feedstocks. The use of molasses as residual carbon source substrate was reported already in 1948 at an industrial scale [23]. However, due to the impellent need to make production of CA more sustainable and less dependent on sucrose and sucrose-based feedstocks, the use of various carbon-rich residual biomasses has been evaluated. The exploitation of residual biomasses with different polymeric compositions is possible thanks to the presence in *A. niger* of a pool of extracellular hydrolyzing enzymes, such as invertase, glucoamylase, α-glucosidase and α-amylase. Several examples, both in scientific literature and patents, describe the use of corn, fruits, lignocellulosic and starchy materials [19,24–28] (Table 2).

The availability of high-producing strains is essential from an industrial point of view in order to develop highly efficient processes. Until recently, the main strategy for strain improvement was random chemical or physical mutagenesis followed by screening. These protocols, although time consuming, successfully allowed the improvement of CA yields. For instance, a combination of UV exposure, ethyl methane sulfonate (EMS) and acridine orange treatment to *A. niger* UMIP 2564 resulted in a 3.2-fold increase in product yield [29]. Adeoye et al. reported a 45.97-fold increase in citric acid production by *A. niger,* FUO 2 strain, subjected to UV radiation and cultivated on cassava peel substrate [30].

1- Lactate dehydrogenase
2- Pyruvate decarboxylase
3- Alcohol dehydrogenase
4- Pyruvate carboxylase
5- Pyruvate dehydrogenase complex
6- Malate dehydrogenase
7- Fumarase
8- Fumarate reductase
9- Citrate synthase
10- Aconitase
11- Isocitrate dehydrogenase
12- α-ketoglutarate dehydrogenase
13- Succinyl-CoA synthetase
14- Succinate dehydrogenase
15- *cis*-Aconitate decarboxylase
16- Isocitrate lyase
17- Malate synthase

Scheme 2. Representation of the main metabolic pathways involved in the production of citric, fumaric, itaconic, lactic and succinic acid.

The release in 2007 of the first *A. niger* genome data (strain CBS 513.88) [31] paved the way to more targeted modifications such as those aimed at modifying carbon source utilization and uptake [32,33], enhancing product secretion [34], and modifying the mycelial morphology of the fungus, which is known to be critical for CA production in submerged fermentations [35]. Other attempts were made by improving the efficiency of the mitochondrial electron transport chain [36] or by inserting genes involved in the reductive branch of the tricarboxylic acid (rTCA) cycle [37].

Table 2. Citric acid production with different substrates, microorganisms, and fermentation strategies.

Microorganism	Substrate	Citric Acid (g/L)	Fermentation Mode	Reference
A. niger MH 15-15	Glucose	31	Shake flask	[38]
A. niger MH 15-15	Sucrose	53	Shake flask	[38]
A. niger	Corn stover	136.3	Simultaneous saccharification and fermentation	[39]
A. niger GMCC 5751	Liquefied corn	151.7	Batch	[40]
Adapted *A. niger*	Corn powder and citric acid effluent	157.3	Batch	[41]
Mutant *A. niger* MO-25	Beet molasses and chicken feather peptone	68.8	Shake flask	[28]
Mutant *A. niger* UMIP 2564	Sucrose	96.3	Shake flask	[29]
Mutant *A. niger* UMIP 2564	Beet molasses	98.3	Shake flask	[29]
Mutant *A. niger* UMIP 2564	Cane molasses	91.2	Shake flask	[29]
A. niger 831f	Liquefied cassava	162.7	Repeat-fed batch	[42]
Y. lipolytica NRRL YB-423	Glycerol	21.6	Shake flask	[43]

Production of CA with *A. niger* can be achieved by three different fermentation strategies: submerged, surface and solid-state fermentation, with each method having advantages and disadvantages.

Liquid surface fermentation (SF) was the original technique used for the first industrial manufacture since 1919, and until recently, some small- and medium-scale production plants adopted this method because of the low installation and operation costs. Typically, fermentation is carried out in trays with a capacity of 50–100 L, mounted one over another in aseptic chambers with controlled humidity and temperature. After spore germination, which is usually completed within the first 2 days, the fungus grows as mycelial mat. Finally, after 8–12 days of fermentation, the liquor can be separated from the mycelia and further processed for CA recovery [44].

Solid-state fermentation (SSF), also known as the Koji process, employs a solid insoluble material that acts both as substrate and support in a low-water activity environment. Different reactor configurations can be employed, such as flasks, trays, horizontal drums and glass columns [45]. SSF for CA production has several advantages over submerged and surface fermentation, since it allows the exploitation of agro-food waste materials [25] and is intrinsically correlated with lower energy and water consumption. Conversely, during SSF it is difficult to have full control of the process parameters; therefore, it is difficult to scale up and to standardize, requires longer process time and higher overall costs for product recovery compared to other strategies. For these reasons, the SSF production mode is still far from being industrially applicable [44].

Currently, most citric acid globally is produced by submerged fermentation (SmF) with either stirred tank or bubble-column reactors. Compared to surface fermentation, submerged fermentation requires more sophisticated equipment but, in turn, is characterized by higher yields, lower fermentation times, ease of process standardization and automation, and less risk of contamination [16,44].

A crucial step in CA production is represented by the downstream process, which has been estimated to account for 30–40% of the selling price [46]. It must be considered that the complexity of the procedure can be greatly influenced by the type of fermentation (SSF, SF or SmF) and by the fermentation substrate; for instance, the use of agro-food wastes may require additional steps to obtain a high-purity product. At the end of the fermentation, microbial biomass is removed by filtration, after which the broth can be subjected to different treatments to obtain the purified citric acid. The classical method for

CA recovery, which remains the most used, relies on tri-calcium citrate salt precipitation caused by the addition of calcium oxide at 90 °C and pH 7, followed by treatment with sulfuric acid that results in soluble citric acid and calcium sulfate [16]. Calcium sulfate is filtered off, whereas citric acid undergoes further purification steps. An alternative method for CA recovery is based on liquid–liquid extraction with various solvents (such as aliphatic alcohols, ketones and tertiary amines) [47,48]. More recently, other methods have been proposed, such as supercritical CO_2 [49], adsorption on cationic resins and electrodialysis [50,51].

A recent techno-economic and environmental analysis on CA production comparing recovery by precipitation, ion exchange and solvent extraction indicated that the ion exchange method is the most environmentally friendly, whereas solvent extraction, followed by precipitation, are the most economically convenient. Other factors having a large impact on the final selling price were electricity cost, by-product credit, feedstock cost, fermenter cost and wastewater treatment cost [52].

Global citric acid production rose from 0.5 million tons in 1992 [53] to more than 2.55 million tons in 2020, and a moderate growth is expected in 2021–2026 [54].

The fermentative process used nowadays for citric acid production is mainly via submerged fermentations of *A. niger* grown on molasses and/or other carbohydrates. The main producers (in alphabetic order) are: Archer Daniels Midland-ADM (Chicago, IL, USA), Cargill (Minneapolis, MN, USA), COFCO Biochemicals (Beijing, China), FoodChem (Shanghai, China), Jungbunzlauer (Basel, Switzerland) and Tate and Lyle (London, UK).

In the last two decades, production was mostly located in China [16,17], which is now acting as the major player in citric acid production, as can be easily inferred by patent analysis over the last two decades. In fact, China has >80% of the total applied patents regarding citric acid production and downstream [16].

2.2. Fumaric Acid ($C_4H_4O_4$, 116.07 g·mol^{-1}, pKa$_1$ 3.03, pKa$_2$ 4.44)

Fumaric acid (FA) is a *trans* four-carbon dicarboxylic acid, intermediate in the TCA cycle, isolated for the first time from the plant *Fumaria officinalis*.

Fumaric acid is a specialty chemical with a number of applications in very diverse fields. It is used as an additive and acidulant in food and beverages; being 1.5-fold more acidic than citric acid, lower amounts are required (0.91 g of fumaric acid *vs* 1.36 g of citric acid are required to achieve the same taste [55]). Moreover, it is supplemented into ruminal feed to decrease methanogenesis [56] and in quail's feed has shown a number of benefits including improved growth, immune response and intestinal health [57]. Importantly, fumaric acid has two carboxylic groups and one double bond, characteristics that make it suitable for esterification and polymerization to produce, to cite some, paper resins, unsaturated polyester resins (UPR), biodegradable polymers, plasticizers and carboxylating agents for rubber [58]. Recently, its esters (FAEs) have shown important pharmaceutical applications in the treatment of sclerosis and psoriasis [59,60].

FA is mainly produced starting from petroleum-derived chemicals (mainly benzene, *n*-butane, *n*-butene) by conventional chemical processes based on the isomerization of maleic acid, obtained, in turn, from the hydrolysis of its anhydride [58]. Alternatively, enzymatic processes based on the activity of maleate isomerases (from *Bacillus*, *Pseudomonas* or *Arthrobacter*) on maleic acid have been developed [61,62]. However, this route had poor success as confirmed by the few available reports.

The first industrial production of FA was started by Pfizer using *Rhizopus arrhizus* in the 1940s, with a production of 4000 tons/year [58]. Later, the fermentative route was discontinued and replaced by the more economically convenient classical chemical route. However, recently, as for several other platform chemicals, the shortage of fossil resources and the need for green and sustainable processes gave a new boost to microbial production.

The most relevant species from a productive point of view are those belonging to the genus *Rhizopus* (*arrhizus*, *oryzae*, *nigricans* and *formosa*), which can accumulate and secrete large amounts of fumaric acid through the cytosolic reductive branch of the TCA

(rTCA) pathway under aerobic conditions and limited nitrogen availability [58] (Scheme 2). However, not all *R. oryzae* strains are able to produce FA. In fact, some studies revealed that those strains able to produce FA did not belong to *R. oryzae* and should be reclassified as *R. delemar* [63], however, to avoid confusion, they are still named as *R. oryzae*. The rTCA cycle takes place in the cytosol starting from pyruvate and involves three reactions: (i) condensation, in presence of ATP, of pyruvate and carbon dioxide catalyzed by pyruvate carboxylase to produce oxaloacetic acid; (ii) conversion of oxaloacetic acid into malate by malate dehydrogenase; (iii) conversion of malate to fumarate catalyzed by fumarase. However, the rTCA cycle does not lead to the production of ATP, which must be produced by the oxidative branch of the TCA cycle in mitochondria or by ethanol formation. When nitrogen becomes limiting and cell growth stops, glucose metabolism and CO_2 fixation continue, leading to the accumulation of fumarate [58]. The simultaneous occurrence of both TCA cycles was demonstrated in *R. arrhizus* also by carbon labeling studies [64]. The rTCA cycle has a theoretical maximum yield in non-growth conditions of 2 mol of FA per mol of glucose (1.3 *g/g*); however, experimental yields are much lower. This is mainly ascribed to the co-existence of the TCA cycle that fulfils the cell energy requirements and to the formation of fermentation by-products such as ethanol and lactic acid [65].

Although high concentrations of fumaric acid have been obtained using recombinant strains of *R. oryzae* [66,67], *Escherichia coli* [68,69], *Saccharomyces cerevisiae* [70,71], and *Torulopsis glabrata* [72], the major titer of fumaric acid is ascribable to the native fumaric acid-producing *Rhizopus* species [73], which are at present the main producers at industrial scale, and most of the research on fumaric acid is based on these microorganisms. So far, the highest titer of FA (>130 g/L) was obtained by Du Pont with *R. arrhizus* NRRL 1526 using glucose as the carbon source as claimed in the US patent US4877731A [74].

Glucose is indeed the most used carbon source for processes with *Rhizopus* species. However, to improve the sustainability of the process, several efforts were addressed to assess the use of alternative substrates and, in particular, of residual biomasses. Sebastian et al. reported the production of FA starting from perennial grasses hydrolysates using *R. oryzae* strains [75] with 0.3–0.5 g of FA per g of sugar. Production of FA was also achieved with *R. oryzae* via fed-batch SSF and SHF processes employing alkali-pretreated corncob [76]. Other examples deal with the use of food wastes [77,78], starchy materials [79–81], lignocellulosic materials [82,83], crude glycerol [84] and brewery wastewater [85] (Table 3). Two of the most relevant key factors for process optimization of FA production are the control of fungi morphology and of the pH of the production medium. Morphology of the fungi is considered one of the major technical challenges during fumaric acid production in submerged fermentations, and it is one of the most important variables that may hamper industrial scale production. In fact, during their growth, filamentous fungi can exhibit three different morphologies: pellets, filaments, and clumps. Filamentous mycelium is the most productive morphology; however, its formation causes operational problems due to the high viscosity of the broth. Clumps are the less productive form, because their inner part suffers from nutrient and oxygen limitations, leading to the production of ethanol and other fermentation by-products. Pellet morphology is preferred over the others since it allows efficient mass transfer while avoiding the increase in medium viscosity. Interestingly, it was observed that a lower pellet diameter corresponds to a more efficient mass transfer of both nutrients and oxygen, and thus increased yield [86]. Several reports showed how fungi morphology can be controlled by acting on different parameters, such as nitrogen source, spore concentration in the inoculum, pH, temperature and agitation rate [87–90]. Moreover, immobilization of cells onto a solid support was also proposed as a method to overcome morphology problems. Different metallic nano- and micro-particles were evaluated for their ability to influence *R. oryzae* 1526 morphology and pellet size. Microparticles of Fe_3O_4 at a concentration of 200 µg/mL were found to be the most effective providing the highest FA titer (42 g/L) [91]. Unusual materials were also exploited as immobilization supports; for instance, *R. arrhizus* RH 7-13-9 immobilized on loofah fibers allowed to achieve 30 g/L of fumaric acid when cultivated in a stirred tank reactor [92]. Another

example by Naude et al. reports the use of *R. oryzae* cells attached to a polypropylene tube for continuous fermentation [93].

Table 3. Fumaric acid production with different substrates, microorganisms, and fermentation strategies.

Microorganism	Substrate	Fumaric Acid (g/L)	Fermentation Mode	Reference
R. arrhizus NRRL 1526	Glucose	130	Batch	[74]
R. arrhizus NRRL 1526	Perennial grasses	8.9	Shake flask	[75]
R. oryzae ATCC20344	Lignocellulosic Syrup	34.2	Shake flask	[82]
R. arrhizus RH7-13	Food Waste	32.7	Shake flask	[78]
R. oryzae NRRL 1526	Brewery wastewater	31.3	Shake flask	[85]
R. arrhizus RH-07-13	Glucose and glycerol	22.8	Shake flask	[84]
R. arrhizus RH-7−13-9	Glucose	17.5	Simultaneous fermentation and separation	[94]
Mutant *E. coli* E2	Glycerol	41.5	Fed-batch	[69]
Mutant *S. cerevisiae*	Glucose	33.1	Shake flask	[71]

Fumaric acid production is strongly affected by the fermentation pH which, if not properly controlled through the addition of a neutralizing agent, quickly drops from 5.0 to 2.0 within the first 20 h of fermentation. The most frequently used neutralizing agent is $CaCO_3$; however, its use is correlated with several issues. First, calcium fumarate is poorly soluble at 30 °C, and is consequently present as a precipitate that aggregates with cells, resulting in a high viscous suspension. Secondly, the conversion of the carboxylate salt to the free carboxylic acid requires the addition of H_2SO_4 which produces $CaSO_4$ (gypsum), which is associated with several environmental concerns [65]. Unfortunately, the use of other neutralizing agents (i.e., $Ca(OH)_2$, $NaHCO_3$) is associated with lower yields and productivity [95]. In particular, sodium fumarate is highly soluble in water and high fumarate concentration may cause product inhibition [96]. Moreover, Na^+ ions can in some cases negatively affect the cell metabolism [95].

An efficient strategy for pH control during fermentation is represented by the simultaneous fermentation-separation process (SFS), that, in addition, allows the alleviation of product inhibition phenomena, which cause reduced yields and productivity [94].

FA is produced in about 90,000 tons/y; MBI international (Lansing, MI, USA) uses the fungus *R. oryzae* for production [97], while for Myriant corporation (Quincy, MA, USA), there are indications about the development of different engineered strains of *E. coli* and *S. cerevisiae* for organic acids production, including fumaric acid, from specific carbon sources [98,99].

2.3. Itaconic Acid ($C_5H_6O_4$, 130.10 g·mol^{-1}, pKa$_1$ 3.85, pKa$_2$ 5.45)

Itaconic acid (IA) is an unsaturated dicarboxylic acid, also known as methylene succinic acid. It was firstly discovered in 1836 by Baup during the distillation of citric acid. However, the name itaconic acid its due to Crasso, who synthesized it through decarboxylation of *cis*-aconitic acid [100].

Itaconic acid can be considered mainly as a chemical building block, as it is exclusively used in non-food applications. It is used in styrene-butadiene rubber, synthetic latex, methyl methacrylate (MMA), unsaturated polyester resins (UPR), and as a chelant dispersant [101,102].

The production of IA by microbial fermentation is preferred to chemical synthesis, which is long, scarcely efficient and expensive because it is carried out starting from a substrate, citric acid, with the same cost of itaconic acid, and it requires high temperature, implying high energy consumption [103,104]. Itaconic acid is produced in about 41.400 tons/y. The first industry plant was founded by Pfizer Co. Inc (Brooklyn, 1955), which also developed a patented aerobic fermentation process to produce itaconic acid [101]. While in the past decades the majority of the itaconic acid production was ascribable to

companies located in different parts of the world, such as Cargill, Iwata Chemicals and Melle, currently it is completely in the hands of Chinese industries [105,106].

The fungus *Aspergillus terreus* is known to be the best IA producer (about 160 g/L) [107], also at commercial level, for its extraordinary tolerance to low pH, high yield and titer of product, as described by [103,108]. In the last ten years, the basidiomycete *Ustilago maydis*, also known as a natural producer, due to its similarity to yeasts, gained attention as a candidate for large scale production, even if the IA yield on glucose is lower than that of *A. terreus* (0.2 *g/g versus* 0.48 *g/g* on glucose, respectively) [109–111] (Table 4).

Moreover, some other yeasts and fungi have been exploited as natural producers of itaconic acid, but have never reached the commercial level (i.e., *Candida* sp. and *Pseudozyma antarctica*) [112,113].

From a metabolic point of view, IA production from glucose in *A. terreus* mainly involves glycolysis and TCA (tricarboxylic acid cycle), where pyruvate produced by glycolysis is transported in the mitochondrial compartment to become acetil-CoA, which together with oxaloacetate, catalyzes the reaction to obtain citrate, and further into *cis*-aconitate, a substrate of *cis*-aconitate decarboxylase (CAD) responsible for the decarboxylation in itaconic acid (Scheme 2) [114]. IA is then transported outside the cell by a facilitator superfamily protein (mfsA gene) [115]. Differently, for the filamentous fungus *Ustilago maydis*, due to the lack of the CAD gene, the decarboxylation of *cis*-aconitate into itaconic acid occurs by the action of *trans*-aconitate decarboxylase (Tad1), preceded by isomerization through the cytosolic enzyme aconitate-*D*-isomerase (ADI). Afterwards, the membrane transport protein ITP1 allows the excretion of itaconic acid through the cell wall [116]. It is important to highlight that, since decarboxylation takes place in the cytosol, while *cis*-aconitate is produced in the mitochondrion, the action of a mitochondrial tricarboxylate transporter protein (Mtt) is necessary as well [102]. During the years, engineering strategy and/or mutagenesis techniques were developed to increase itaconic acid production [104]. The native producer of itaconic acid *U. maydis* was engineered by overexpressing the genes involved in itaconic acid production in *A. terreus* (rai1 and mttA) and deleting genes involved in the synthesis of by-products (cyp3, dgat, MEL and UA). The resulting strain, grown on glucose with a continuous feeding strategy, showed an itaconic acid titer and yield of 74.9 g/L and 0.54 g/g, respectively [117]. In addition, non-native producers of itaconic acid, due to some interesting characteristics for the fermentation (e.g., organic acid tolerance and production of a considerable amount of TCA intermediates), have also been considered for genetic modifications. The low pH-tolerant *Pichia kudriavzevii*, already engineered to produce some organic acids, has undergone multiple genetic modifications (introduction of CAD gene from *A. terreus*, overexpression of a native mitochondrial tricarboxylate transporter (mttA), and deletion of the gene encoding isocitrate dehydrogenase (ICD)), to establish itaconic acid production [118].

Table 4. Itaconic acid production with different substrates, microorganisms, and fermentation strategies.

Microorganism	Substrate	Itaconic Acid (g/L)	Fermentation Mode	Reference
A. terreus DSM 23081	Glucose	160	Fed-batch	[107]
A. terreus NRRL 1960	D-Xylose	53.97	Batch	[119]
A. terreus NRRL 1960	Bleached eucalyptus pulp	37.5	Shake flask	[120]
U. maydis MB215	Glucose	44.5	Batch	[109]
Mutant *U. maydis* MB215	Glucose	80	Fed-batch	[121]
Mutant *U. maydis* MB215	Glucose	74.9	Continuous feeding	[117]
U. vetiveriae TZ1	Glycerol	34.7	Batch	[122]
A. terreus CICC40205	Wheat bran hydrolysate	34.2	Shake flask	[123]
A. terreus M69	Corn stover hydrolysate	33.6	Shake flask	[124]

Furthermore, the best citric acid producer *A. niger* was engineered by the introduction of *cadA* and *acoA* genes, two known key enzymes for itaconic acid production, which allowed IA production by an extension of the citric acid production pathway [125].

From an economical point of view, since the maximum yield of IA is reached by glucose fermentation of *A. terreus*, alternative carbon sources should be used to make the process competitive with petrochemical-derived products. Unfortunately, low-cost substrates (including potato starch, plant-derived hydrolysate, lignocellulosic biomasses) are related to nutrient deficiency and toxicity problems, which lead to poorly competitive yields compared to the ones obtained with pure sugars [126]. In addition, most residual biomasses require pre-treatment steps in order to be metabolized by the cells. A recent review about itaconic acid [101] reported different IA producer strains (native or not) modified in order to exploit some residual biomasses as alternative carbon sources.

Moreover, it is important to highlight that to replace the petroleum-based itaconic acid efficiently with the biotechnological one, a price reduction should occur (at least of USD 0.5/kg from the current cost of USD 1.5–2.0/kg) [126,127].

2.4. Lactic Acid ($C_3H_6O_3$, 90.07948 $g \cdot mol^{-1}$, pKa$_1$ 3.78)

Lactic acid (LA) was first isolated from sour milk by the Swedish chemist, Karl Wilhelm Scheele, in 1780, but only in 1857 did the chemist and microbiologist Louis Pasteur attribute its production to certain microorganisms [128]. LA appears colorless to yellow at 15 °C and at a pressure of 1 atm [129]. It is known to occur in two isomeric forms, (L(+)- or D(−)), due to the chiral carbon that confers optical activity to the monomer, as well as the racemic form, DL-Lactic acid [130].

The production of LA occurs by chemical synthesis or fermentation. Microbial fermentation is very attractive for lactic acid production since it allows to obtain pure isomers (L(+)- or D(−)-LA) instead of racemic mixture (DL-LA), with the latter being typical of the chemical synthesis [131]. Furthermore, the chemical synthesis involves the use of polluting substances (e.g., lactonitrile and the highly toxic hydrogen cyanide), which have high costs of production too [129]. These days, more than 90% of lactic acid is produced by fermentation processes [132]. Currently, many companies are involved in LA production at the commercial level: ADM (Chicago, IL, USA), Aldon Corporation (Avon, NY, USA), Anhui COFCO Biochemical and GALACTIC (Bengbu, China), Cargill (Minneapolis, MN, USA), ChonggingBofei Biochemical Products (Chongging, China), CorbionPurac (Amsterdam, The Netherlands), Galactic (New Orleans, LA, USA), Henan Jindan Lactic Acid Technology (Dancheng Henan, China), Musashino Chemical (Tokyo, Japan), Nature Works LLC (Minnetonka, MN, USA), Symbra Technology BV (Etten-Leur, The Netherlands), Tedia Company Inc (Fairfield, OH, USA), Yangtze Labre (Yangtze, China) [133]; among these, the biggest producers at industrial scale are Corbion and Cargill (producing about 240,000 and 180,000 tons, respectively) [134]. LA is mostly used as monomer of the biobased and biodegradable polylactic acid (PLA) for biodegradable plastics. PLA is today considered among the most promising polymers for bioplastics. PLA finds applications in packaging, agriculture, transport, electronics, textiles, and others. At present, the main suppliers of PLA are Cargill (Minneapolis, MN, USA) Galactic (Escanaffles, Belgium), Purac, (Amsterdam, The Netherlands), FKuR (Willich, Germany), and Shimadzu (Kyoto, Japan) [135,136].

The microorganisms used for microbial fermentation are different, depending on the isomer to be produced. Several microorganisms can produce pure L-LA, including bacteria, fungi, cyanobacteria, algae and yeasts [137,138]. Moreover, other strains are known for D-LA production, for instance *Lactobacillus delbrueckii*, *Sporolactobacillus*, *Escherichia coli*, and *Bacillus coagulans* [139] (Table 5).

Although other microorganisms have been identified as lactic acid producers, as described above, nowadays lactic acid bacteria (LABs) still represent the best producers at industrial scale [136]. They produce LA by glycolysis (Scheme 2) in anaerobic conditions, although they are micro-aerophilic or aerotolerant as well, at a temperature in the range of 25–45 °C (depending on the microorganism), and a pH between 5.5 and 6.5 [133,140]. It is possible to distinguish homofermentative LABs, belonging to the genera *Streptococcus*, *Lactococcus*, *Enterococcus*, *Pediococcus* and *Lactobacillus*, which produce lactic acid as main product, and heterofermentative LAB (as *L. brevis*, *L. fermentum* and *L. reuteri*) in which,

together with lactic acid, there are other by-products such as ethanol, acetic acid and carbon dioxide. At the metabolic level, homofermentative LABs produce two molecules of LA per mole of glucose with a theoretical yield of 1 *g/g* through the Embden-Meyerhof-Parnas pathway, whereas the yield for heterofermentative LABs is 0.5 *g/g* or 0.6 *g/g*, depending on the pathway, phosphogluconate and phosphoketolase, respectively [136,141].

Generally, homofermentative strains use hexose sugars such as glucose to produce lactic acid as the only product, whereas pentose sugars such as xylose can enter in the phosphoketolase pathway to obtain lactic acid and other by-products, which leads to yield reduction and increase in the recovery cost [133]. Since homofermentative LABs can reach yield values very close to the maximum theoretical one, they were considered for industrial production.

In order to optimize lactic acid production, some aspects that can affect the fermentation process should be considered, including temperature, sugar concentration, effect of nutrients, mixed sugars, and by-product formation [133].

A critical role is held by the pH of the fermentation: the preferred form of the product is the undissociated one, which means that the final pH of the medium should be well below the pK_a of LA, corresponding to 3.78. However, at this pH, most LABs show irreversible damage to their metabolic functions [142]. Therefore, several neutralizing agents are added to the fermentation broth to maintain the pH and protect the microbial cells. Unfortunately, the use of $Ca(OH)_2$ or $CaCO_3$ causes the formation of calcium lactate that must be removed through acid treatment (H_2SO_4), generating, as discussed above for other organic acids, calcium sulfate (gypsum), a waste product with serious disposal concerns [143,144].

For the successful production of LA, different modification technologies were considered. More recently, Tian et al. described the application of mutagenesis, adaptive evolution and metabolic engineering on lactic acid bacteria [145].

Along with LAB, other microorganisms have been evaluated as potential lactic acid producers. Some of the most interesting are the *Bacillus* strains which are able to grow at high temperatures (up to 55 °C), avoiding further sterilization of the culture media, and allow the exploitation of second-generation biomasses, since they may tolerate some by-products generated by pre-treatments [146–148].

In addition to bacteria, fungi, for example those belonging to the genus *Rhizopus* [141], and yeasts have attracted attention for their intrinsic ability to tolerate low pHs.

In respect to yeasts, while the initial preference was for the budding yeast *Saccharomyces cerevisiae*, which was the first engineered for lactic acid production [149], other yeasts have been exploited.

The natural fermentation occurring in yeasts leads, in most cases, to ethanol and CO_2 formation, therefore there is a need to re-route pyruvate to the desired product. The engineering strategy most often used is the overexpression of a heterologous lactate dehydrogenase gene (*LDH*), coupled, in some cases, to the partial or complete deletion/inactivation of pyruvate decarboxylase (*PDC*) genes [150,151]. These modifications were often combined with further engineering or evolution to overcome poor growth on glucose related to the deficiency in 2C intermediates, usually deriving from acetaldehyde (as reviewed in [152]), and with the ability to tolerate low pH. Recently, Park at al. described the high titer of D-lactic acid (154 g/L) reached by the genetically modified yeast *Pichia kudriavzevii* (also known as *Issatchenkia orientalis*) [153], but the pH of the medium of 4.7 cannot assure the advantage of having the product in undissociated form.

Lactic acid production can be also improved by co-culture of strains with similar growth characteristics, allowing the cultivation of homofermentative strains which can take the advantage from heterofermentative ones, which normally produce low amounts of lactic acid due to the accumulation of other products [154]. Co-cultures are an interesting perspective also for the simultaneous saccharification and fermentation (SSF) strategy, and for the use of mixed sugars; both are typical conditions when raw materials are considered as fermentation substrate [133].

The increasing demand for lactic acid is due to the spread of end-products industries which include food & beverages, cosmetics, pharmaceuticals, polymers [133]. It is estimated that, by 2028, the market for lactic acid will reach USD 5.02 billion, and is expected to grow by about 8.0% from 2021 to 2028 [155].

For a commodity such as LA, it is crucial to consider all the factors which contribute to the final cost, and at the same time, it is relevant to take sustainability into account, as large production is expected. The use of cheap raw materials, which do not release dangerous by-products after pre-treatments, could be the ideal condition for economic lactic acid production [156]. Among the second-generation biomasses, the lignocellulosic ones are the most interesting for LA production due to their abundance [129]. However, they need pretreatments and enzymatic hydrolysis to release the sugars, toxic compounds can be released along with these steps, and the final purification of LA is more complex. All these elements need to be accurately considered. Recently, in order to analyze all the variables related to lignocellulosic biomasses pre-treatment and use for LA fermentation, Li et al. exploited the BioSTEAM platform to conclude that the optimization of separation process and fermentation is essential for a successful production process [157].

Indeed, the downstream affects the final cost of lactic acid, of which 50% is ascribable to recovery and purification [158]. As already discussed above, due to the decrease in fermentation pH as result of LA accumulation, neutralizing agents are used for LAB-based production [133]. This method is well known as precipitation and is still the conventional one for LA separation today [144]. Alternatively, MgO can substitute $Ca(OH)_2$ or $CaCO_3$, with the advantage that it can be reused after the lactic acid extraction. Li et al. recently described the pros and cons of the separation and purification techniques known today for LA [159]. Certainly, the recovery process is extremely complex considering the composition of the fermentation broth (cells, sugars, inorganic salts, and other organic acids) [160].

Table 5. Lactic acid production with different substrates, microorganisms and fermentation strategies.

Microorganism	Substrate	Lactic Acid (g/L)	Fermentation Mode	Reference
Mutant *P. kudriavzevii*	Glucose	154 (D-Lactic acid)	Fed-batch	[153]
Evolved *L. delbrueckii* S-NL31	Soybean meal	112.3 (D-Lactic acid)	Fed-batch	[161]
Evolved and mutated *S. cerevisiae*	Glucose	82.6 (D-Lactic acid)	Fed-batch	[162]
Mutant *S. cerevisiae*	Glucose	40 (D-Lactic acid)	Fed-batch	[163]
Mutant *E. coli*	Glycerol	115 (D-Lactic acid)	Fed-batch	[164]
L. delbrueckii ssp. bulgaricus	Orange peel waste	45 (D-Lactic acid)	Separate hydrolysis and fermentation	[165]
L. delbrueckii	Molasses and corn steep liquor	162 (D-Lactic acid)	Fed-batch	[166]
B. coagulans A107	Defatted rice bran (DRB)	75.9 (L-Lactic acid)	Batch	[137]
L. brevis ATCC 367 and *L. plantarum* ATCC 21028	Corn stover	24 (L-Lactic acid) 31.2 (L-Lactic acid)	Simultaneous sequential	[167]
L. casei	Sugarcane molasses	120.23 (NS)	Batch	[168]
Mutant *S. cerevisiae*	Glucose	142 (L-Lactic acid)	Fed-batch	[169]
R. oryzae LA-UN-1	Glucose	162 (L-Lactic acid)	Fed-batch	[170]
L. rhamnosus DUT1908	Starchy biomass	108 (L-Lactic acid)	One step liquefaction, saccharification and fermentation	[171]
Mutated and evolved *L. paracasei* NCBIO01	Glucose	202 (L-Lactic acid) 9.06 (D-Lactic acid)	Open fermentation	[172]
B. coagulans and *L. rhamnosus*	Cassava bagasse	113 (L-Lactic acid)	Simultaneous saccharification and co-fermentation	[173]
Mutant *L. plantarum* NCIMB 8826	Raw corn starch	50 (L-Lactic acid)	Batch	[174]
L. paracasei subsp. paracasei2	Food waste	34 (L-Lactic acid)	Batch	[175]

NS: not specified.

2.5. Succinic Acid ($C_4H_6O_4$, 118.09 $g \cdot mol^{-1}$, pKa₁ 4.2, pKa₂ 5.6)

Succinic acid (SA) is a dicarboxylic acid usually produced by hydrogenation of fossil-derived maleic anhydride, but due to the drawbacks associated with petroleum-chemical production (e.g., greenhouse gas emission and non-renewable energy consumption), nowa-

days the fermentative route is preferred for its production. Several studies have shown that the production of succinic acid by fermentation can also lead to a reduction in costs [176].

It is defined as "bio-succinic" acid, derived from plants, animals and microorganisms, although it is principally produced by anaerobic fermentation. In 1546 the German mineralist and doctor Georgius Agricola purified succinic acid for the first time from amber; afterwards, it was produced by microbial fermentation [177].

The first microorganisms recognized as succinic acid producers were bacteria isolated from rumen, such as *Actinobacillus succinogenes*, *Anaerobiospirillum succiniciproducens*, *Mannheimia succiniciproducens*, *Basfia succiniciproducens*, *Bacillus fragilis* and also some fungi such as *Fusarium*, *Aspergillus* and *Penicillium* species.

Among them, the most promising for industrial production is *A. succinogenes*; several fermentation aspects including carbon and nitrogen sources, as well as pH regulator, should be considered for a successful production process [178].

Unfortunately, the production of succinic acid by microbial fermentation is not competitive compared to petro-chemical products, as often happens for different organic acid production. The cost of the product per kg is USD 2.94 and USD 2.5, for bio-succinic acid and the combined bio- and fossil-based succinic acid, respectively [179]. Until now, the pivotal companies which produce succinic acid, according to the biorefinery concept, at commercial scale are Myriant (Quincy, MA, USA), Reverdia (Cassano Spinola, Italy) and Succinity (Montmelò, Spain) [180]. Succinic acid represents the key precursor for a wide range of bulk chemicals including 1,4-butanediol, gamma-butyrolactone, tetrahydrofuran, adipic acid, n-methylpyrrolidone, linear aliphatic esters, or biodegradable polymers (butylene succinic acid) (PBS) [181,182].

In most cases, succinic acid is produced by exploiting the reductive TCA cycle (Scheme 2) through anaerobic or micro-aerobic fermentation, principally by rumen bacteria [183]. In addition, there are two other pathways, the glyoxylate pathway and the oxidative TCA cycle, for succinate production [184].

In the last few years, several engineering strategies have been developed for the production of succinic acid by microbial fermentation. For this purpose, microorganisms such as *S. cerevisiae*, *Y. lipolytica*, *E. coli*, *P. kudriavsevii*, *B. succiniciproducens* have been considered, especially for their tolerance to low pH, essential to exceed the downstream costs, and for the many engineering tools available [176] (Table 6). The joint venture of DSM and Roquette, Reverdia, developed the modified strain of *S. cerevisiae*, SUC-297, for succinic acid production through the dual-phase fed-batch fermentation of glucose [185]. Likewise, other companies have worked to obtain mutated strains to increase their plant capacity, as reported by Ahn et al. [186].

At the same time, as for the other commodities before, low-cost feedstocks must be considered to obtain a sustainable bio-product. A two-stage pH fermentation, to promote the undissociated form of the acid, was proposed by Stylianou et al. [187] using as carbon sources the organic fraction of municipal solid waste (OFMSW) hydrolysates. In addition, other second-generation biomasses (e.g., mixed food, agricultural and textile waste as well as crude glycerol) have been successfully used as described by Li et al. [176].

The yield and efficiency of "bio-succinic" acid depends on the metabolic pathway involved. For example, the production of succinic acid by the reductive TCA cycle in anaerobic conditions can lead to defective cellular growth; Vemuri et al. proposed a dual-phase fermentation, which consists of an aerobic growth phase and an anaerobic production phase, combining glyoxylate and reductive TCA cycle, obtaining 99.2 g/L of succinic acid [188]. More recently, a mixed culture of two microorganisms, *S. cerevisiae* and *A. succinogenes*, was also exploited to create an efficient co-culture system for succinic acid production [189].

Table 6. Succinic acid production with different substrates, microorganisms, and fermentation strategies.

Microorganism	Substrate	Succinic Acid (g/L)	Fermentation Mode	Reference
Isolate AKR177	Pure glycerol	117	Fed-batch	[190]
Isolate AKR177	Crude glycerol	86.9	Fed-batch	[190]
A. succinogenes CCTCC M2012036	Sugarcane bagasse	39.9	Batch	[191]
		70.81	Fed-batch	
Mutant *E. coli* MH28	Glycerol	84.3	Batch	[98]
Mutant *S. cerevisiae* SUC-297	Glucose	43	Fed-batch	[185]
Mutant *B. succiniciproducens* LU15224	Glycerol and maltose	69.8	Batch	[192]
Mutant *Y. lipolytica*	Crude Glycerol	209.7	Fed-batch	[193]
Mutant *E. coli*	Glucose	99.2	Dual-phase	[188]
Mutant *Y. lipolytica*	Glucose	101.4	Fed-batch	[194]
Mutant *Y. lipolytica*	Glycerol	110.7	Fed-batch	[195]
Evolved *Y. lipolytica*	Mixed food waste	71.6	Fed-batch	[196]

3. Discussion and Conclusions

The examples reported are the best way of demonstrating that microbial-based production can at least compete, if still not replace, the fossil-based production of chemical platforms. Nonetheless, it was mentioned that many other commodity chemical platforms have been produced by microbial fermentation but have not reached the market yet.

Robustness of cell factories and scaling-up gaps are the prominent limitations. In respect to ameliorating the performances of cell factories, several aspects, often interconnected, need to be taken into account. The stoichiometry and thermodynamics of the native or heterologous pathways have to be balanced and considered, respectively, especially when the product of interest is implied to close or reroute other pathways as well. This also results in the possibility of calculating the theoretical yield and compensate for redox imbalance or availability of (micro)nutrients, to avoid by-product formation or/and energy overflow. Another relevant aspect is to evaluate the formation of oxidized products or free radicals, as a consequence of an energy imbalance or the fermentation settings [197].

In respect to the issue of scaling up, a promising support to fill the gap is offered by innovation in the field of scaling-down models, supported by the miniaturization of fermentation units, still fully equipped for online measurements. The very crucial point is to ask the system how to better simulate what happens in large-scale fermenters and how to prioritize the selected parameters, which can be decided only by sharing data and information and by having the whole process, including the final downstream, in mind [8]. In this respect, systems and synthetic biology can give a tremendous boost to our descriptive, predictive, and engineering capability, for example by including fluctuating cultivation conditions in quantitative descriptions and implementing cells with biosensors or molecular switchers tailored to compensate for stresses. These perspectives are particularly relevant if we consider, as recently reviewed, that the cost of bringing a process of commodity production to industrial scale can cost from USD 100 million to 1 billion [198].

As a final consideration, while it can be securely assessed that all the mentioned products are biobased, exactly as for biofuel production, the next question will be if they are really sustainable, as for most of them the feedstock is constituted by first-generation sugars. In order to exploit renewable substrates for the microbial production of organic acid, it is important to consider the difficulties related to the assimilation and metabolization by the cells. Recently, the genetic manipulation of membrane transporters involved in the import of second-generation biomasses has been summarized [199]. Nevertheless, there are different examples of improvements in the use of this kind of feedstock for organic acid production [200]. In parallel, great attention is more and more devoted to the utilization of CO_2 as a carbon source, also for organic acid production [201,202], and not only in dependence on photosynthesis.

To conclude, thanks to experience in microbial biotechnology that has matured over the last decades, it is now very clear that obtaining the proof of concept for a process of

production is no longer the limiting step. The main bottlenecks towards the development of profitable and competitive microbial and biobased processes are the scale-up parameters, which are very often difficult to replicate at laboratory-scale. The recent advances in technologies and in quantitative analysis, together with the tremendous advances in synthetic biology, will be crucial to boost research as well as the technology transfer of microbial organic acid production in the next few years.

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