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REVIEW

Microbial production of specialty C4 dicarboxylic acids from maleic anhydride

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Summary
Maleic acid and its anhydride have been, historically, the main substrate for the production of four-carbon dicarboxylic acids (C4 diacids). Malic, fumaric and tartaric acids are widely used as food accidulants, which improve the flavour profile of new kinds of beverages and foods. In the last decade, the range of their applications greatly expanded in particular in pharmaceutical, chemical and building industries. The compounds can be prepared from maleic acid by chemical or biotechnological processes, or by a combination of these two ways. In this article, the chemical properties, new application trends and the various strategies for the production of all isomers of specialty C4 diacids are reviewed.

Keywords
dicarboxylic acids; maleic acid; fumaric acid; tartaric acid; epoxysuccinic acid; malic acid

Organic acids with one or more carboxylic groups occur widely in nature and may come from plant, animal and microbial sources. They can be present in various forms such as esters, amides or peptides, and therefore their presence is not always obvious. At the present time, industrial production is oriented towards the biotechnological production of organic acids using microorganisms or enzymes. Currently, more than 130 organic acids or their derivatives can be prepared in this way [1].

Maleic anhydride and maleic acid (*cis*-2-butenedioic acid; *cis*-1,2-ethenedicarboxylic acid, $C_4H_4O_4$) are multifunctional chemicals that have been a focus of attention from the commercial point of view for many years. Maleic acid forms white crystals that are easily soluble in water, alcohol or acetone. It is an unsaturated organic dibasic acid whose carboxylic acid groups are next to each other in the *cis* form. Its *trans* form isomer is fumaric acid. Maleic anhydride is prepared commercially by oxidation of benzene or by the reaction of butane with oxygen in the presence of a vanadium catalyst. The physical properties of maleic acid and fumaric acid are very different, the *cis* isomer being less stable. Maleic acid is used for preparation of fumaric acid by catalytic isomerization. So far, maleic acid and its anhydride are important starting materials especially in non-food industries such as the manufacture of alkyds and unsaturated polyester resins, surface coatings, lubricant additives, plasticisers, copolymers and various agricultural chemicals. With the development of biotechnology, there are increasing efforts to use non-conventional and readily available starting materials for the biosynthesis of food and pharmaceutically important substances. Recently, maleic anhydride has been considered as a good starting material for the chemical and biotechnological preparation of several organic acids with expanding applications in the food and pharmaceutical industries. The current possibilities of organic acid synthesis from maleic anhydride, and the respective biosyntheses of these substances, are shown schematically in Fig. 1. Some acids (fumaric acid, D-malic acid, L-malic acid, succinic acid or itaconic acid) can be prepared directly from maleic acid using microbial processes. Others may be prepared only by a combination of chemical and microbial synthesis (all isomers of tartaric acid), or only by chemical synthesis (*cis*- and *trans*-epoxysuccinic acid).

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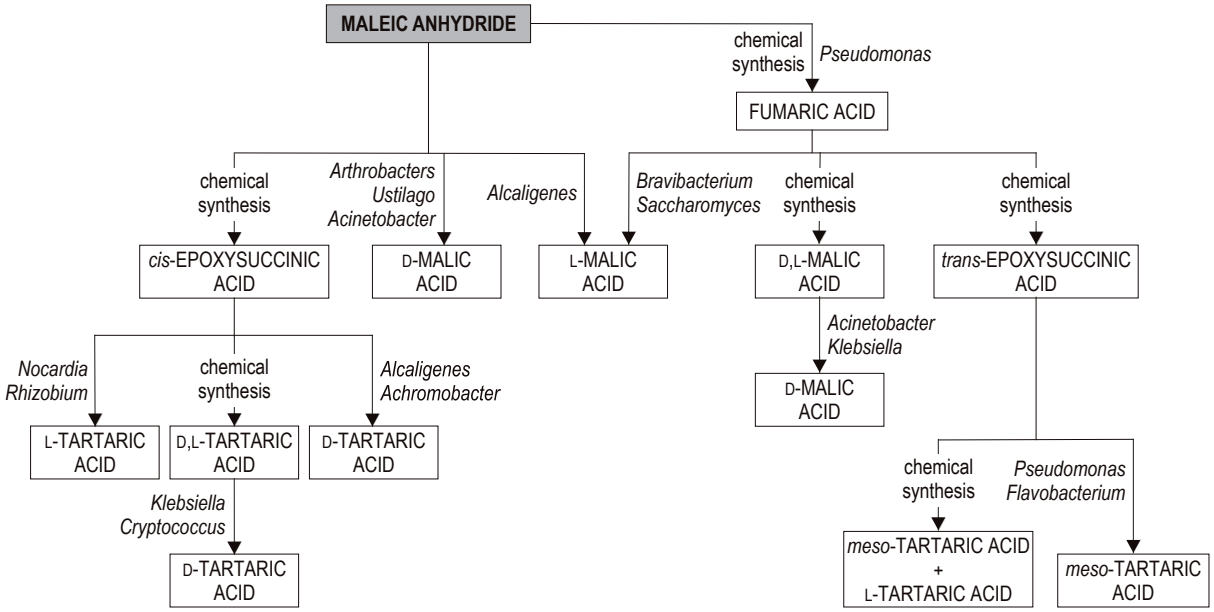


Fig.1. C_4 dicarboxylic acids that can be synthesized from maleic anhydride.

Maleic acid is a petrochemical product and, therefore, its price is highly dependent on crude oil prices, which are very volatile and often fluctuate over short intervals. Currently, the prices of petrochemicals are declining because of the shale gas revolution, which has helped to ease the pressure on petrochemical supply and prices. The market volume of maleic acid was approximately 1 900 000 t in 2011 [2].

This review provides information on the biotechnological production of fumaric, epoxysuccinic, tartaric and malic acids from maleic anhydride, which provides an alternative to conventional chemical ways of production. Basic characteristics and new applications of the selected diacids are also briefly summarized.

FUMARIC ACID

Fumaric acid (*trans*-1,2-ethylenedicarboxylic acid, $C_4H_4O_4$) occurs naturally in many microorganisms, plants and animals. It is named after the plant *Fumarate officinalis* from which it was first isolated. Together with malic acid, fumaric acid is a component of the tricarboxylic acid cycle and also of the glyoxal acids cycle. From the chemical point of view, fumaric acid is a weak unsaturated dicarboxylic acid with a molecular weight of 116.1 g·mol^{-1} and a melting point of 287–300 °C. Its water solubility is 7 mg·ml^{-1} at 25 °C and 98 mg·ml^{-1} at 100 °C. It is slightly soluble in methanol and completely insoluble in chloroform and benzene [3].

Fumaric acid contains a double bond and easily participates in addition reactions or other reactions typical of dicarboxylic compounds. Industrially, the most common application of fumaric acid is in esterification and polymerization of polyvinyl acetate or styrene monomers. In particular, it is used in the production of polyester and alkyd resins, paints and furniture colourings. In the food industry, fumaric acid is used as a powdered beverage ingredient and is added to gelatin desserts, puddings and cakes. In the meat industry, it is added to canned meat and poultry products to stabilize colour. In addition, fumaric acid is the starting material for the industrial production of other organic acids and amino acids [4].

Commercially, fumaric acid is being prepared since 1932 by catalytic isomerization of maleic acid, which is produced from butane:

$$C_4H_{10} + 3{,}5\ H_2O \rightarrow C_4H_2O_3 + 4H_2O$$

In the 1940s, fumaric acid was manufactured by Pfizer (New York, New York, USA) through fermentation using filamentous fungi *Rhizopus arrhizus* (about 4 000 tons per year). However, the economic advantages of chemical synthesis gradually forced the company to stop production by fermentation [4]. The annual production of maleic anhydride reached 1.807 million tonnes in 2007, of which approximately 3 % was used for the preparation of fumaric acid, corresponding to 90 000 tons

per year [5]. Although the yield of fumaric acid from maleic anhydride is 112% (w/w), the price of the starting material is gradually increasing (associated with the growth of fossil fuel prices) and thus the production of fumaric acid is becoming more expensive. For this reason, fermentation processes related to fumaric acid production have been receiving increasing attention. Filamentous fungi of the genus *Rhizopus* were identified as the best fumaric acid-producing strains among different microorganisms tested. The traditional carbon source in fermentation was glucose, but xylose, saccharose and other saccharides were also described as substrates for fumaric acid production [1].

Interest in fungal fermentation has also increased as it has been found that microorganisms are capable of using starchy and cellulosic feedstocks as starting materials for fermentation [1]. Because of the low fungal glucoamylase activity, improvement in the simultaneous saccharification and fermentation of cheap starchy materials was recently proposed for the production of fumaric acid using mutant strains of *R. oryzae* [6]. A fumaric acid titer of 32.18 $g \cdot l^{-1}$ with a productivity of 0.44 $g \cdot l^{-1} \cdot h^{-1}$ was obtained using raw maize powder (100 $g \cdot l^{-1}$ total sugar) as a substrate of saccharification and fermentation.

The last interesting strategy is food waste fermentation technology, resulting in fumaric acid production. Food waste is a promising potential feedstock, rich in proteins and carbohydrates. When used as a fermentation medium, the yield of fumaric acid was found to reach 32.68 $g \cdot l^{-1}$ with a productivity of 0.34 $g \cdot l^{-1} \cdot h^{-1}$. The results indicated that food waste could be a promising production medium for providing a high yield of fumaric acid in the future [7].

Immobilization techniques for *Rhizopus* spp. were studied to open up the possibility of a continuous mode of fumaric acid production. Several suitable inert and cheap materials (e.g. expanded polystyrene, wood shavings or polyurethane sponge) were tested as carriers for immobilization of fungal spores, mostly on a laboratory scale [4]. The latest immobilization device using net and wire for the fungal fermentation of glucose was found to be a feasible and promising method for the commercial scaling up of fumaric acid production. Immobilization fermentation was found to yield identical fumaric acid production compared to the free-cell mode (32.03 $g \cdot l^{-1}$ vs 31.23 $g \cdot l^{-1}$), with higher volumetric productivity (1.335 $g \cdot l^{-1} \cdot h^{-1}$ vs 0.217 $g \cdot l^{-1} \cdot h^{-1}$) and a shortened fermentation time (from 144 h to 24 h) [8].

Microbial production of fumaric acid from maleic acid can also be achieved using bacteria of the genera *Arthrobacter*, *Pseudomonas* and *Alcaligenes*. *Arthrobacter* sp. TPU 5446 was used for the isolation and characterization of maleate *cis-trans* isomerase. This enzyme catalyses isomerization of maleate to fumarate. The enzyme is very unstable and, during its purification, a large amount of activity is lost [9]. In 1997, a screening of bacteria with maleic acid as a substrate was performed using 600 soil samples collected at different locations in Japan [10]. Twenty strains with the ability to convert maleic acid into other organic acids were found, four strains showing an increased maleate *cis-trans* isomerase activity. The highest enzyme activity was found in *Pseudomonas alcaligenes* XD-1, in which the molar yield of fumaric acid was about 70% after 6 h of incubation. On the other hand, approximately 18.4% of L-malic acid by-product was produced at the same time. Further study of this strain showed that the fumarase activity could be inhibited by heat treatment of the cells (70 °C, 1 h). Moreover, the addition of calcium ions to the reaction mixture during the conversion increased the thermostability of maleate *cis-trans* isomerase. Using all of these modifications, a maximum yield of 95% of fumaric acid was achieved [11].

Recently, the model microorganisms *Escherichia coli* and *Saccharomyces cerevisiae* were genetically and metabolically engineered to increase their fumaric acid production [12, 13].

EPOXYSUCCINIC ACID

Epoxysuccinic acid (*cis*-2,3-oxiranedicarboxylic, $C_4H_4O_5$) was studied from time to time for potential use in industry, particularly in connection with production of tartaric acid. In the 80s and 90s, preparation of various derivatives of this acid and their use in the pharmaceutical industry were described. The advantage of these compounds is their extremely low toxicity. In 1983, several derivatives of *trans*-epoxysuccinic acid were synthesized, which proved to be good inhibitors of thiol protease activity [14]. A beneficial effect was confirmed in the treatment of muscle diseases. *trans*-Epoxysuccinic acid is easily transformed to β-hydroxy-L-aspartic acid, which has excellent antibacterial properties and can be used as a good starting material for the synthesis of optically specific single β-lactam antibiotics [15].

Epoxysuccinic acid exists in two forms, namely, *cis* and *trans* stereoisomers. Both can be prepared from maleic anhydride. *cis*-Epoxysuccinic acid is prepared via oxidation of maleic acid in

the presence of a catalyst. Soluble salts of palladium and vanadium were the first catalysts used. The oxidizing agent can be oxygen, copper salts, nitric acid or alkali metal nitrates. This procedure is used in the production of D,L-tartaric acid and *cis*-epoxysuccinic acid is formed as an intermediate. By modification of the reaction conditions, it is possible to obtain highly pure *cis*-epoxysuccinic acid. Maleic anhydride is mixed in distilled water with NaOH, hydrogen peroxide and catalyst – sodium tungstate or sodium molybdate. Sodium maleate is subsequently converted to sodium *cis*-epoxysuccinate at 60–70 °C and pH 4–6 within 4 h. The yield of the reaction is 96%. Sodium *cis*-epoxysuccinate can be used for the microbial production of tartaric acid. Examples of derivatives of *cis*-epoxysuccinic acid are sodium, ammonium, potassium and calcium salts. The production process of high purity salts of *cis*-epoxysuccinic acid from maleic anhydride was later modified and epoxidation was carried out in aqueous-alcoholic solution (30–90% aqueous solution of alcohol, such as methanol, ethanol or propanol) [16].

cis-Epoxysuccinic acid is prepared only by chemical synthesis, microorganisms are not known to be able to synthesize this acid or its salts. *trans*-Epoxysuccinic acid can be prepared in two ways, either by epoxidation of fumaric acid (D- or L-forms) or via a microbial pathway (L-form; Fig. 1). The course of epoxidation is similar to the preparation of the *cis*-form of the acid, the difference being only in the amount of the added catalyst. In order to achieve 80–86% conversion, a five-fold higher concentration of tungstate or molybdate salts is required. The temperature is kept between 65 °C and 75 °C [17].

MARTIN and FOSTER [18] described the production of *trans*-epoxysuccinic acid by fermentation from glucose or ethanol using the filamentous fungi *Paecilomyces varioti, Penicillium vineferum,* and *Aspergillus fumigatus*. The product was formed either during fermentation or, alternatively, mycelium after fermentation can be separated and used for biotransformation of pure carbohydrate solutions. Studies of the mechanism of biosynthesis showed that *trans*-epoxysuccinic acid is synthesized from C2 compounds via the glyoxylic acid cycle. The carbon skeleton of fumarate is transformed to *trans*-epoxysuccinate. The "epoxy oxygen" of the *trans*-epoxysuccinate solution comes directly from the molecular oxygen [19]. Production of *trans*-L-epoxysuccinic acid by fermentation was also studied by the YAMAGUCHI group in 1991. The authors used new strains of filamentous fungi *Aspergillus clavatus, Aspergillus fumigatus, Neosartorya fischeri, Paecilomyces elegans, Talaromyces wortamannii,* and *Byssochlamys nivea* [15].

Both forms of *trans*-epoxysuccinic acid (D- and L-forms) can now be used for the chemical or microbial production of *meso*-tartaric acid.

TARTARIC ACID

Tartaric acid (2,3-dihydroxybutanedioic, $C_4H_6O_6$) is the most important acid of musts and wine. In the pure state it occurs as colourless, transparent crystals which are odourless, having an acid taste. Tartaric acid is soluble in water, alcohol and ether. It is sold in technical, crystalline, powder and granular forms. Structurally tartaric acid contains two hydroxyl and two carboxyl groups attached to an ethane hydrocarbon system and possesses two centres of symmetry. It, therefore, exists as four different forms namely: D(–)-tartaric acid, L(+)-tartaric acid (they are optically active); racemic (D,L)-tartaric acid and *meso*-tartaric acid, which are optically inactive. The most important physico-chemical properties of all tartaric acid forms are shown in Fig. 2. The specific rotation

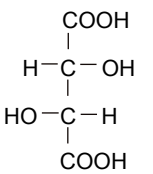
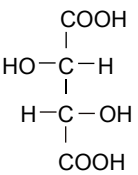
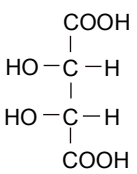
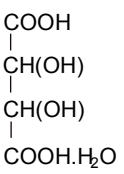
Structure				
Form	L-form	D-form	*mezo*-form	racemic
Melting point [°C]	167–170	167–170	159–160	203–204
Density at 20 °C [g·ml^{-1}]	1.760	1.760	1.737	1.697
Solubility at 20 °C [kg·l^{-1}]	1.39	1.39	1.25	0.206

Fig. 2. Physico-chemical properties of tartaric acid.

at 20 °C ($[\alpha]^{20}$) of 20% aqueous solution of L(+)-tartaric acid is 11.9° ± 1.28°. MIURA et al. [20] published a value of +12.84° for a commercially available L(+)-tartaric acid and +12.81° for L(+)-tartaric acid obtained from bioconversion using bacteria of the genus *Nocardia*.

Tartaric acid exists in nature, occurring in L(+)-form in fruits. In plants, it is partly free and partly present as K, Ca or Mg salts. It is present in a variety of fruits, in particular in all parts of grapes and grape leaves. One kilogram of leaves contains 13–16 g of tartaric acid. During the growth of grapes, the amount of tartaric acid gradually increases and a portion of the free tartaric acid binds to potassium to form wine stone. Tartaric acid forms salts, specifically bitartrate and tartrate.

Uses

Wine, food and beverages accounted for approximately 68 % of world consumption of tartaric acid in 2009. Global consumption in these applications was forecasted to grow at an average annual rate of 3.4 % during 2009–2014. Tartaric acid has distinct and common uses in the wine sector. It is added to wine to balance naturally present malic and tartaric acids. It was used traditionally in wine grape musts, but low-cost citric acid has now forced it out completely. Another important market segment is the beverages industry, including the instant beverages sector. In baking, tartaric acid is used in the form of diacetyl tartaric acid ester of mono- and diglycerides (DATEM) to improve the quality of flours with low gluten content and to improve the overall flavour of bread. It is also added to pectin and starch jellies, puddings and drops. Tartaric acid can be used in many other fields including teas, snacks, textile, fertilisers, tannery and building materials, namely, cement and gypsum, as an antisolidifying agent in the production of plaster and cement, and as an anticaking agent in gypsum processing. In pharmacy, it has new applications, where it serves as an important intermediate in antibiotic, effervescent antacid and chiral compound production. Tartaric acid derivatives are also explored as potential therapeutic agents for the treatment of HIV [21].

Tartaric acid is produced worldwide. Large producers are found in countries with a large wine industry and having chemical industry groups using organic synthesis, polymer chemistry and biotechnology as their core technologies. Major players in the marketplace include wine producers such as Caviro (Faenza, Italia), Legre-Mante (Marseille, France), Industrias Vinicas (Santiago, Chile), Tarac Technologies (Nuriootpa, Australia) and chemical companies such as Thirumalai Chemicals (Mumbai, India), Toray Industries (Tokyo, Japan), Changmao Biochemical Engineering (Changzhou, China), Hangzhou BioKing Biochemical (Hangzhou, China) and Ninghai Organic Chemical Factory (Ningbo, China). Global demand for tartaric acid was 60 600 t in 2013 and is expected to reach 87 200 t by 2020 [22].

Technology and production process

There are various feedstocks and producers used for manufacturing tartaric acid. The following processes based on different feedstocks are used:

- from tamarind leaf,
- from grape waste and wine stone,
- from maleic acid/maleic anhydride/fumaric acid,
- from glucose by microbial fermentation,
- from epoxysuccinic acid by biotransformation.

One of the first procedures used for the preparation of tartaric acid was isolation from pulp residues that are formed as a waste in the production of grapefruit juice. The pulp was boiled with water and the present alcohol was distilled off. The hot mixture was then cooled and crystals of potassium bitartrate were formed. The crystallization solution contained about 90 % of this salt. Another natural source of tartaric acid is tamarind, available mainly in the south of USA, India, south-east Asia and in the Carribean. The leaves of this plant contain 8–15 % L-tartaric acid (w/w, dry matter). The process consists of digestion of leaves under suitable conditions and precipitation of the acid as calcium tartrate. The crystals are broken down by charcoal and re-crystallized. However, there is no report of the commercial exploitation of this process, maybe because of its low yields of tartaric acid (about 7 %).

The traditional and the most widely used method of tartaric acid production is isolation from wine fermentation waste products, namely, wine stone that is potassium hydrogen tartrate. Therefore, the major producers of tartaric acid are in the countries with the largest wine industries (Italy, France, Portugal). A salt is prepared from the wine stone, namely, calcium tartrate tetrahydrate. Sulphuric acid is then applied to the salt and the tartaric acid formed comes out of solution after thickening in the crystalline form. The disadvantage of commercial production of tartaric acid in this way is the limited availability and seasonality of the starting material. Therefore, new processes (chemical and microbiological) for economical production of tartaric acid are of interest. One chemical method is epoxidation of maleic

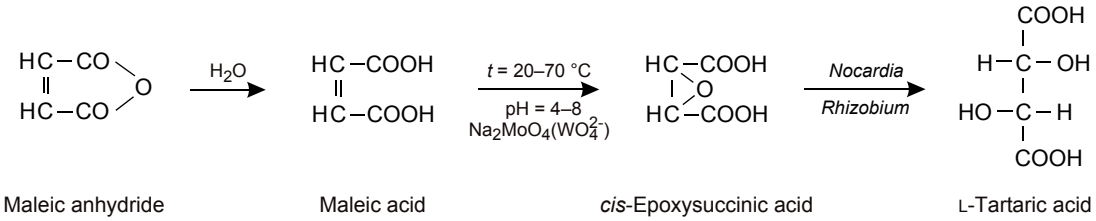


Fig. 3. Chemoenzymatic synthesis of L-tartaric acid from maleic anhydride.

acid or fumaric acid, yielding DL-tartaric acid (or *meso*-tartaric acid) by introducing to the reaction hydrogen peroxide, $CaCO_3$ (or NaOH, depending on the prepared salt) and a catalyst, of which the most common are tungstate or molybdate. The reaction is carried out at 60–75 °C and at pH 4.5–5.5. The yield of DL-tartaric acid is approximately 95–97% [23]. The racemic form of tartaric acid is much less soluble than its D- or L-form, therefore limiting its commercial application. L(+)-tartaric acid is very important in the food and pharmaceutical industry since the D-form is considered as harmful to human health in food [24]. Natural resources of L-tartaric acid are limited, therefore a simple and environment-friendly manufacturing process is desirable. Today, there are two known biotechnological processes for L-tartaric acid preparation, namely, fermentation of saccharidic substrates and biotransformation of epoxysuccinic acid.

The former technology, not yet applied on an industrial scale, is the fermentation of saccharidic substrates or 5-keto-gluconic acid by bacteria of the genus *Gluconobacter***.** These bacteria were successfully used for the industrial production of many food, pharmaceutical and cosmetics products, e.g. 6-amino-L-sorbose, 2-keto-L-gulonic acid, dihydroxyacetone and gluconates. One important characteristic of *Gluconobacter* strains is their ability to convert glucose to 5-keto-D-gluconic acid, which can be easily converted into L-tartaric acid [25]. Strains of *Gluconobacter suboxydans* and *Gl. oxydans* are know to catalyse the oxidation of glucose to gluconic acid and, subsequently, to 2-keto-D-gluconic acid and 5-keto-D-gluconic acid by gluconate dehydrogenases. The reaction can be accelerated by the addition of a catalyst, vanadate [25] or noble metals [26]. The disadvantages of this procedure are the small yields of tartaric acid and the formation of by-products, mainly 2-keto-D-gluconic acid and glycolic acid. In the 1980s, mutant strains of *Gl. suboxydans* that produced twice the amount of tartaric acid in comparison with the original strain were prepared [27]. In recent years, to improve the yields of 5-keto-D-gluconic acid, a precursor of the industrially important L-tartaric acid, *Gluconobacter* strains were genetically engineered. KLASEN et al. [28] cloned the gene encoding the gluconate-5-dehydrogenase into an expression vector and expressed it in *E.coli* and *Gl. oxydans.* HERRMANN et al. [29] reported an increase of 20% in 5-keto-D-gluconic acid production by a new genetically modified strain *Gl. oxydans* DSM 2343.

The most effective method of production of L-tartaric acid is biotransformation of the inexpensive *cis*-epoxysuccinic acid by an enzyme, *cis*-epoxysuccinate hydrolase (CES hydrolase). CES hydrolase, a member of the epoxide hydrolases group (EC 3.3.2.3), hydrolyses the epoxy ring of *cis*-epoxysuccinic acid to form D-tartaric acid or L-tartaric acid. The enzyme is found in cells of some bacterial strains from the following genera: *Achromobacter, Alcaligenes, Rhizobium, Corynebacterium, Pseudomonas, Rhodococcus* and *Nocardia* [30]. In these microorganisms, the activity of CES hydrolase is stimulated by the presence of *cis*-epoxysuccinic acid in the culture medium. KAMATANI et al. [31] first described the process by which *cis*-epoxysuccinate is converted into a natural form of tartaric acid using the bacterial strain *Rhizobium validum*. *cis*-Epoxysuccinic acid or its derivatives used in the tartaric acid production can be easily obtained by epoxidizing maleic acid with hydrogen peroxide in the presence of tungstate or molybdate salts as catalysts (see the section on epoxysuccinic acid). The catalysts have no effect on CES hydrolase activity and can be separated from the reaction mixture in the last step, after biotransformation of *cis*-epoxysuccinic acid to L-tartaric acid (Fig. 3) [32].

The CES hydrolase enzyme activity in the microorganisms can be increased by the addition of detergents and various ions during the bioconversion [33] or by protein and genetic engineering methods [34]. The biotransformation can be carried out using microorganisms in the form of living cells, dried cells, permeabilized cells, cell extracts

or purified enzymes. Although many papers deal with the isolation and purification of epoxide hydrolases [35, 36] and the cloning and sequencing of epoxide hydrolase genes from microorganisms, microbial production of L-tartaric acid is still more used than the application of a purified enzyme because of problems with enzyme instability in its pure form and its associated high cost [37–39].

Further improvement in the efficiency of the production process can be achieved by whole-cell immobilization. This is a method for repeated use of the biocatalyst and increasing the productivity of the biotransformation process. Gelatine beads [40], pectate gel beads [33, 41], sodium alginate-cellulose sulphate-poly(methylene-*co*-guanidine) capsules [42], and κ-carrageenan [43, 44] were investigated as matrices for immobilization.

A recent discovery is the isolation and identification of a novel strain of *Labrys* sp. BK-8 that produces *cis*-epoxysuccinate hydrolase (CESH) [44]. Free and immobilized cells of the strain showed a high conversion rate (> 99%), enantioselectivity (> 99.5%) and storage stability (> 90 days). A conversion rate of 97% was maintained after ten repeats of application. The bacterial strain provides a new alternative, with good stability, for the industrial biosynthesis of L(+)-tartaric acid.

Recent studies focused on improving the selectivity and stability of epoxide hydrolases by protein and genetic engineering [34, 45]. The relevant enzymes were successfully cloned and expressed in *E.coli*. Overexpression of epoxide hydrolases could improve understanding of the functional role of the enzyme and could result in the production of large amounts of efficient biocatalysts for the industrial production of tartaric acid.

MALIC ACID

Malic acid (2-hydroxybutanedioic acid, $C_4H_6O_5$) was first isolated in 1785 from unripe apples and the name "malic" comes from the Latin word for apple "malum". Malic acid is found in fruits, in particular apples, pears, grapes, melons, cherries, grapefruit, plums and other fruits and vegetables. Although the grapes contain tartaric acid, their main ingredient is malic acid, which is rapidly converted into lactic acid during wine ageing. In addition, malic acid is also found in other higher plants, animals and microorganisms (bacteria, yeasts, filamentous fungi), as it is an important intermediate in cellular metabolism and a member of the tricarboxylic acid cycle. Malic acid is a dicarboxylic acid, with one asymmetric carbon atom, occurring in three forms. The D(+)- and L(–)-isomers of malic acid have optical activity, the racemic mixture of (D,L)-malic acid is optically inactive. Malic acid is soluble in water, alcohol and ether. In the pure state, it forms odourless white crystals.

Applications

L-Malic acid has better organoleptic properties than citric acid and it is widely used in the food industry as an acidificant for soft-drinks and natural juices. There are no limits for the acceptable daily intake of L-malic acid, while the limit for the D-isomer is 100 mg per kilogram of body weight per day in human food. Excepting therapeutic purposes, Food and Drug Administration (Silver Spring, Maryland, USA) and the World Health Organization (Geneva, Switzerland) do not allow the use of D,L-malic acids in baby foods [46]. L-Malic acid is added to a variety of foods, such as candied fruit, gelatin, chewing gum, yoghurts, puddings, and salad dressings. Its growing consumption is related to the increased use of high-intensity sweeteners in new kinds of beverages, and in foods such as "sports" and "energy bars", as well as in "energy" and "protein" drinks. Malic acid prolongs sourness in food products compared with other acidulants, such as citric acid, supresses aftertastes caused by the addition of sweeteners, nutraceuticals, vitamins, fibres or antioxidants and, thereby, improves the flavour profile of "diet" beverages and foods. Because of its complexing properties it is used as a chelating agent for the removal of metal impurities in edible oils and fats. In medicine, it is used to treat liver dysfunction and hyperammonemia. Malic acid has also a good position in the cosmetics industry. Malic acid, together with α-hydroxy acids (AHA-products), is added to creams to reduce wrinkles and it is also used in the producion of „heavy sweet" perfumes.

More recently, malic acid has been used in the production of a polymer, also known as polymalic acid (PMA). PMA is being evaluated as a biodegradable, bioresorbable component in drug delivery systems, particularly as a non-toxic, slow release carrier for covalently bound drugs [47]. A further non-food growing application of malic acid is in the chemical synthesis of 1,4-butanediol as a building block compound that can be further converted to resins, polymers or plastics.

The issue of the preparation of malic acid is still current, because there is still a deficit between production and demand. The global market demand for malic acid exceeded 70 000 t in 2015 and is expected to reach 102 000 t by 2020, with an annual growth rate of 4.2 % [48].

Methods of production

Malic acid has conventionally been produced by chemical synthesis through the hydration of fumaric acid or maleic acid at high pressure and high temperature. This process leads to the formation of a racemic mixture of D- and L-isomers of malic acid, which is much less soluble than the D- or L-form, therefore limiting its commercial application [49]. The traditional methods of malic acid production through the extraction of fruits or eggshells provide the natural L-form of this acid but have many limitations, such as the low content of malic acid in the natural sources, low efficiency of the extraction processes, high energy consumption and high environmental pollution [50, 51]. An alternative route of L-malic acid production is via microbiological processes. An industrial realization has been attained through biotransformation of fumaric acid to L-malic acid using a key enzyme, fumarase, or using cells containing fumarase. The other process is a one-step fermentation leading from glucose to L-malic acid. The last technology uses microorganisms producing polymalic acid, which is subsequently hydrolysed by a strong acid.

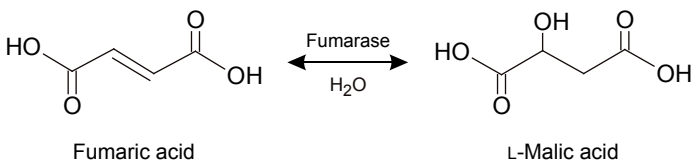
Enantiomerically, pure L-malic acid is desirable for food, pharmaceutical and polymerization applications. The biotechnological method is economically more viable than the chemical synthesis of the L-isomer. While bioconversion of fumarate to malate is an established industrial process, no commercial process is available for the production of L-malic acid from saccharidic substrate at present.

Biotransformation of fumaric acid to L-malic acid

The most efficient process for the preparation of natural L-malic acid is bioconversion from fumaric acid (Fig. 4). The globally dominant production of L-malic acid through the conversion of fumaric acid is carried out by bacterial producers immobilized in κ-carrageenan [5]. The production of L-malic acid in this way began in 1974 using *Brevibacterium ammoniages* bacteria. In 1977, this species was replaced by a better producer, namely, *Brevibacterium flavum*. The yield of malic acid was approximately 70 % of the theoretical yield and the unused fumarate was recycled. The enzymatic reaction was conducted at neutral pH and the products were the salts of malic acid. One of the disadvantages was the production of succinic acid as a by-product. Production of this unwanted acid can be inhibited through the incubation of cells in a solution of fumarate containing bile extract before immobilization [52]. The use of this incubation step not only leads to inhibition of succinate dehydrogenase, but also to multifold increase in fumarase activity. Bile extract, cholic acid, deoxycholic acid and detergents can also be used as inhibitors of succinic acid production. Bioconversion was carried out with 80–86 % yields and unreacted fumarate was recycled. Other studies in this area focused on the genus *Brevibacterium*, increasing the fumarase activity or immobilizing the enzyme or cells in a suitable matrix [53, 54]. The latest improvement was a "one-pot" coupling conversion system in which immobilized *Brevibacterium* sp. cells transform fumaric acid to malic acid and the residual fumarate is converted to L-aspartic acid by immobilized *E. coli* [55]. The production of L-malic acid was also described for members of the genera *Lactobacillus* [56], *Corynebacterium*, *Escherichia* [57] and *Nocardia* sp. [58].

The bioconversion of fumaric acid to L-malic acid was also studied in several yeast strains. Those most studied are strains of *S. cerevisiae* and *S. bayanus* [59–61]. The production of succinic acid was found to be inhibited by incubation of cells in a solution with malonic acid. Increase in fumarase activity is possible to be achieved through the use of detergents or through the use of genetically engineered microorganisms. Sodium dodecyl sulphate was used to increase the membrane permeability of *S. cerevisiae* and, due to this, the activity of fumarase was found to increase 60-fold compared to its original activity [59]. Permeabilized *S. cerevisiae* cells covalently immobilized on a microchannel surface was also used for malic acid production [62].

In the 1990s, extensive genetic studies related to malic acid production in *S. cerevisiae* were performed. Recombinant strains of *S. cerevisiae* were prepared and immobilized in an agarose gel, producing L-malic acid at yields of 80–90 % [63]. Excellent results were obtained with the use of



Fumaric acid L-Malic acid

Fig. 4. Bioconversion of fumaric acid to L-malic acid catalysed by fumarase.

a recombinant strain of *S. cerevisiae* immobilized in alginate-silica beads. Malic acid was produced in a special bioreactor and the conversion from fumaric acid to malic acid was nearly 100 % [64]. A genetically modified *S. cerevisiae* strain immobilized in a polyacrylamide gel was used for malic acid production without giving rise to by-products [65].

The production of L-malic acid was also studied in *Dipodascus* yeast strains**.** The fumarase activity of intact and disintegrated cells of *D. magnusii* CCM 8235 was 8–10 fold higher than in *S. cerevisiae* and the formation of succinic acid, as a by-product, was not detected. The cells were permeabilized using detergents octylphenol ethoxylate (chemical name Triton X-305, non-ionic surfactant) and sodium dodecyl sulphate (anionic surfactant) [66]. Other yeasts were also used, including *Pichia* sp. and *Candida rugosa*, immobilized in a polyvinylalcohol gel, as well as *Zygosaccharomyces rouxii* [67].

The enzymatic bioconversion of fumarate to L-malate described in this chapter requires petroleum-derived maleic anhydride or fumaric acid as substrates. Recent trends have focused on the use of renewable or waste materials for the efficient and cost-effective bio-based production of organic acids. In recent years, diverse one-step fermentation processes have been developed as sustainable alternatives to petroleum-based malate. The latest advances in the production of L-malic acid by fermentation are briefly reviewed below.

One-step direct microbial fermentation

The production of L-malic acid can be carried out, in fermentation processes, by biosynthesis from glucose via four pathways that occur in microorganisms [68]. This production process is characterized by simplicity and good yields with the use of saccharidic substrates (glucose, saccharose). The optimal metabolic pathway for L-malic acid preparation begins from pyruvate, which is the product of glycolysis. Pyruvate is transformed by pyruvate carboxylase to oxaloacetate and, subsequently, reduced to malate by the enzyme malate dehydrogenase. This pathway is also called the reductive pathway of citric acid (rTCA) and occurs naturally in the cytosol of various microorganisms, including the filamentous fungus genus *Aspergillus* (species *A. niger*, *A. nidulans*, *A. oryzae*, *A. flavus* and *A. parasiticus*) [69, 70]. In general, *Aspergillus* strains were recognized as very important organisms for the production of various organic acids (citric acid, succinic acid and gluconic acid). Many research groups showed that production of L-malic acid by *Aspergillus* from saccharidic substrates is possible and most of them also demonstrated very good results with engineered strains [51, 69].

More recently, L-malic acid-producing yeasts *S. cerevisiae* were engineered [71]. Through overexpression of plasmid-borne genes encoding pyruvate carboxylase, cytosolic malate dehydrogenase and a heterologous malate transporter, production of 59 $g \cdot l^{-1}$ malate, with a malate yield of 0.42 $mol \cdot mol^{-1}$ of glucose, was achieved. However, a disadvantage of the engineered strains is the production of high amounts of pyruvate. Therefore, these methods need to be improved further for effective malate production [71].

Engineered strains of *E. coli* were also used as malate producers, producing higher L-malic acid concentrations and achieving higher productivities than those by engineered strains of *S. cerevisiae* [51].

Acid hydrolysis of poly-malic acid

A novel approach to L-malic acid production is acid hydrolysis from poly-malic acid [72, 73]. The production of poly-malic acid by *Aureobasidium pullulans* was tested in a fermentation process in a stirred-tank bioreactor. The main carbon source was glucose or glucose-containing materials. Pure malic acid was produced from poly-malic acid through hydrolysis with 2 $mol \cdot l^{-1}$ sulphuric acid at 85 °C. The yield of malic acid from batch fermentation was 47.3 $g \cdot l^{-1}$ and the yield of malic acid from fed-batch fermentation was 87.6 $g \cdot l^{-1}$. Fed-batch fermentation with cells immobilized in a fibrous-bed bioreactor led to the highest concentration of the product at 144.2 $g \cdot l^{-1}$. This method for poly-malic acid and malic acid production could be effective and economically viable for industrial application [73].

D-Malic acid production

Maleic acid is not a natural compound, but some organisms can metabolize it through the activity of “malease” (maleate hydratase, EC 4.2.1.31). The product, D-malic acid, can be used as a chemical intermediate in organic syntheses. In the 1990s, screening for microorganisms capable of converting maleic acid to D-malic acid at a high optical purity was performed. The ability to produce D-malic acid was found in bacteria of the genus *Arthrobacter*, with a yield of D-malic acid of 70–72 % and an optical purity of 100 % [74]. Later, eukaryotic microorganisms of the genera *Ustilago*, *Saccharomycopsis* and *Rhodotorula* were successfully used for D-malic acid production [75]. A second possibility for D-malic acid production is the utilization of L-malic acid from a racemic

mixture of D- and L-malic acid using the bacteria of the genera *Klebsiella*, *Acinetobacter*, *Serratia* or *Pseudomonas* [76, 77].

CONCLUSIONS

The demand for bulk and speciality organic acids is increasing due to the growing production of organic acid-containing foods and materials. New applications of these organic acids are also increasing the demand. While bulk carboxylic acids, such as citric or lactic acids, still have a strong position on the market, industrial producers have recently focused on speciality high-value organic acid production. Fumaric, malic and tartaric acids belong to this group of organic acids. All of them can be produced by chemical or biotechnological processes from maleic anhydride. Currently, traditional industrial methods for their preparation are prevalent. The increasing environmental concerns and limited natural sources suggest that the production of organic acids from renewable sources will probably become the only alternative in the future.

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