Production of Multifunctional Organic Acids from Renewable Resources

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Recently, the microbial production of multifunctional organic acids has received interest due to their increased use in the food industry and their potential as raw materials for the manufacture of biodegradable polymers. Certain species of microorganisms produce significant quantities of organic acids in high yields under specific cultivation conditions from biomass-derived carbohydrates. The accumulation of some acids, such as fumaric, malic and succinic acid, are believed to involve CO₂ fixation which gives high yields of products. The application of special fermentation techniques and the methods for downstream processing of products are described. Techniques such as simultaneous fermentation and product recovery and downstream processing are likely to occupy an important role in the reduction of production costs. Finally, some aspects of process design and current industrial production processes are discussed.

Keywords. L-Aspartic acid, Citric acid, Fumaric acid, Itaconic acid, Lactic acid, L-Malic acid, Succinic acid, Aspergillus niger, Aspergillus terreus, Lactobacillus, Rhizopus oryzae, Rhizopus arrhizus, Rhizopus oryzae

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1 Introduction

Many naturally occurring organic acids are multifunctional with the potential to be used as raw materials for the manufacture of biodegradable plastics in addition to their traditional uses in foods and other applications. In order for biologically produced organic acids to be competitive in the market place, the production of these chemicals must be inexpensive. One way to achieve this goal is to utilize inexpensive substrates for organic acid production. Another way is to improve the fermentation process to lower the cost. This paper reviews some recent research developments concerning the aspects of the biological production of some of the wider used organic acids.

2 Lactic Acid

Lactic acid (2-hydroxypropionic acid) is a naturally occurring multifunctional organic acid that is found in many food products, particularly in those which involve natural or processed fermented food preparations. Currently, more than 70% of lactic acid is used as acidulents, food preservatives, and feedstock for the manufacture of calcium stearoyl-2-lactylates in the baking industry. The consumption of lactic acid is estimated to be around 30 million lb in the US with an estimated increase of 6% per year. Therefore, lactic acid is an intermediate-volume specialty chemical used chiefly for food processing.

Recently, lactic acid has been considered to be an essential component for the production of many nonfood products including polylactic acid (PLA). Due to its chemical properties, lactic acid has the potential to become a very large-volume, commodity-chemical intermediate. It can be produced biologically from carbohydrates to serve as the feedstock for slow release carriers, biodegradable polymers, oxygenated chemicals, environmentally friendly solvents, and other intermediates. Recently, lactic acid production has received more attention because of the development of PLA plastics, which are 100% biodegradable [1] and have been approved for use by the Food and Drug Administration. PLA plastics have many characteristics similar to the thermoplastics now used in packaging consumer goods and may become the feedstock for environmentally benign polymers [2].

For the reasons mentioned, lactic acid has the potential to become a very large-volume, commodity-chemical “green” product that can be produced biologically from carbohydrates to serve as the feedstock for polylactate. This potential demand is estimated at 5.5 to 5.7 billion lb/year (or 2.5 to 3.4 million tons). Due to this potential, many large corporations have been involved in product and process development of lactic acid and polylactate production (Chemical Market Reporter, October 28, 1996).

Lactic acid is produced on the industrial scale by chemical and biological means. The most commonly used synthetic method is based on the hydrolysis of acrylonitrile derived from acetaldehyde and hydrogen cyanide. On the other hand, biological production that accounts for about 50% of the current total
acid capacity is primarily carried out by bacterial fermentation of simple sugars. Lactic acid is the smallest molecule that exists in two isomeric forms that also occurs in nature. Chemical methods can only produce a racemic mixture of the stereoisomers. In contrast, biologically produced lactic acid can be obtained in either form of the isomer or as a mixture of the two in different proportions [3].

2.1
Bacterial Fermentation

2.1.1
Fermentation Pathway

Lactic acid is a metabolic product of simple carbohydrates produced by many species of bacteria, yeasts, and mycelial fungi mainly through the fermentative metabolic pathway. The stoichiometry for homofermentative production of lactic acid from hexose can be expressed as:

\[ \text{C}_6\text{H}_{12}\text{O}_6 \rightarrow 2 \text{C}_3\text{H}_6\text{O}_3 \]

Therefore, this bioconversion does not lose any atoms of carbohydrate or produce any carbon dioxide. For the conversion of pentoses, the stoichiometry can be expressed as:

\[ \text{C}_5\text{H}_{10}\text{O}_5 \rightarrow \text{C}_3\text{H}_6\text{O}_3 + \text{C}_2\text{H}_4\text{O}_2 \]

The bioconversion products are one lactic acid and one acetic acid. Again, there is no loss of material from pentoses [4]. Therefore, lactic acid fermentation has an advantage over other bioconversion processes due to its high product yield and environmental friendliness.

2.1.2
Industrial Fermentation

Lactic acid was the first organic acid to be manufactured industrially by fermentation and L. delbrueckii is the preferred organism. It facilitates the homo-lactic fermentation with a temperature optimum of 50°C and pH of 5.5–6.5. Substrates may be simple sugars or starch. Starch is commonly hydrolyzed either by acid or amylases prior to fermentation. Typically, the medium is buffered with excess calcium carbonate and is kept suspended by agitation. The incubation temperature is maintained at about 50°C until the sugar is metabolized, usually in 48 to 96 h. Carbon dioxide is evolved from added CaCO3 during the lactic acid production stage. This helps to maintain the fermentation under the anaerobic conditions that are required for optimal productivity. Concentrated sweet whey can also be used as the substrate with lactose fermenting L. bulgaricus as the biocatalyst due to its ability to ferment lactose [5,6]. Other less expensive unconventional substrates such as food processing wastes and cellulosic materials have also been tested for lactic acid production.
2.1.3 Process Consideration

Biological production of lactic acid is complicated primarily due to economical considerations arising from product inhibition and the required downstream processing of dilute aqueous product streams. The standard method of biological lactic acid production is the anaerobic fermentation by *Lactobacillus* in a batch reactor [7]. The conventional process requires the base to be added to the reactor to control the pH and the use of calcium carbonate to precipitate the lactate. This process produces a lactate salt that must be acidified (usually by sulfuric acid) to recover the lactic acid, with calcium sulfate as an undesirable by-product.

2.1.4 Process Improvement

To increase the volumetric productivity and to reduce the costs in lactic acid fermentation, high cell density fermentation has been studied through the growth of bacterial cells on activated charcoal, a cell-recycle reactor [8], or a membrane reactor [9]. Recovery of the final product has been examined using liquid extraction [10] and solid adsorbents. This is done either in a product-stripping side stream or by adding directly to the CSTR reactor. Alternatively, in situ product removal during the fermentation offers the advantage of minimizing process waste streams by eliminating the need for reactor pH control and lactic acid recovery.

Recent examples of process improvement have been reported by Davison and Thomson [11] and Kaufman et al. [12]. They studied the simultaneous fermentation and recovery of lactic acid in a biparticle fluidized-bed reactor using *L. delbrueckii* as the biocatalyst. The immobilized bacterial cells (on calcium alginate beads of 0.7–0.8 mm diameter) were fluidized in the liquid media in a column reactor (see Fig. 1). During fermentation, solid particles of lactic acid adsorbent (polyvinylpyridine resin) are added batchwise to the top of the reactor, and fall countercurrently through the biocatalyst. After the adsorbents have fallen through the reactor, they are recovered and the adsorbed lactic acid is recovered. The adsorbents not only remove acid produced but also effectively maintain the broth pH at optimal levels. The increase in lactic acid production is significant. The reported volumetric productivity of 4.6 g/l/h was a 12-fold increase over the reactor without the adsorbents.

2.1.5 Selection of Adsorbents

The criteria for selecting the proper adsorbent are: capacity, specificity, ease of regeneration, and the ability to withstand repetitive regeneration. Although a resin exhibiting all of the desired properties has yet to be found, one specific resin, polyvinylpyridine (PVP) Reillex 425, appears satisfactory and has been tested for the recovery of lactic acid [13].
2.1.6 Product Recovery

Several methods have been explored for the economical recovery of lactic acid from fermentation broth including extraction with solvent, electrodialysis, ion-exchange adsorption (see [14] for review), and reverse osmosis [15]. Wang et al. [16] studied nondispersive extraction for the recovery of lactic acid from broth using a hydrophobic membrane. This method performed favorably when compared to the ion-exchange or the electrodialysis method.

2.1.7 Fermentation of Starchy Materials

The conventional method of lactic acid production from starchy materials such as barley, corn, potato or rice requires pretreatment by gelatinization and liquefaction. This is usually carried out at an elevated temperature of about 90 - 130°C for at least 15 min, followed by saccharification of the starch by amy-
lases to glucose and subsequent conversion of glucose to lactic acid by fermentation. However, this method involves many reaction steps that require additional reactors. Alternatively, fermentation can be conducted simultaneously with the presence of amylases and biocatalyst known as "simultaneous saccharification and fermentation (SSF)". This method eliminates the need for complete hydrolysis of starch to glucose prior to fermentation. In SSF, the liquefied starch is used in the presence of glucoamylase to continuously hydrolyze starch to produce lactic acid. Linke and Javananen [17] demonstrated the advantages of SSF by carrying out barley starch fermentation with L. casei. Lactic acid concentration as high as 137 g/l was obtained from barley starch within 48 h. The reported lactic acid yield was 98% from an initial starch concentration of 130 g/l. The highest lactic acid accumulation in this report was 162 g/l under high substrate concentration.

2.1.8 Direct Fermentation of Starchy Materials

Some starch degrading Lactobacilli such as L. amylophilus [18] and L. amylo- virans [19, 20] are able to produce lactic acid from liquefied yet unhydrolyzed cassava, potato and corn starch. However, the final lactic acid concentration is low (<20 g/l) with low acid yield (<70%).

2.1.9 Municipal Solid Waste (MSW)

L. pentosus was used by McCasky et al. [21] for the production of lactic acid under static conditions from acid-hydrolyzed MSW. The substrate had the following composition: glucose, 16.4 g/l; xylose, 6.5 g/l; mannose, 14 g/l; and galactose, 8.4 g/l. After 3 d at 32°C in the presence of CaCO3, 65 g/l lactic acid were produced from 100 g/l total carbohydrate with acid yields ranging from 70 to 85% based on the total sugars consumed. In a subsequent study [22], lactic acid concentration was increased to 78 g/l with 91% lactic acid weight yield when the nitrogen source and phosphate concentration were optimized.

2.1.10 Cellulosic Biomass

Cellulose powder and milled newspaper were used by Abe and Takagi [23] as the substrate for lactic acid production by L. delbrueckii in the presence of cellulase derived from Trichoderma reesei. The highest conversion rate was at pH 5. After 120 h of fermentation, the amounts of lactic acid produced from cellulose (100 g/l) and newspaper (50 g/l) were 52 and 23 g/l, respectively.

Chen and Lee [24] studied lactic acid production from dilute acid pretreated α-cellulose and switchgrass by L. delbrueckii NRRL-B445 in the presence of a fungal cellulase in a fermentor extractor employing a microporous hollow fiber membrane (MHF). This reactor system was operated in a fed-batch mode with continuous removal of lactic acid by in situ extraction. A tertiary amine (alamine
336) was used as the extractant for lactic acid. The extraction capacity was greatly enhanced by the addition of a long-chain alcohol. A solvent mixture of 20% aminon 336, 40% oleyl alcohol, and 40% kerosene was most effective at pH 5.

2.2 L-Lactic Acid Production by Rhizopus

In addition to lactic acid producing bacteria, a few mycelial molds belonging to Rhizopus are good lactic acid producers. The ability of Rhizopus to produce only L- (+)-lactic acid aerobically under nitrogen-limited environments has been studied [25–28]. Compared to bacterial fermentation, Rhizopus requires only inorganic salts. In addition, Rhizopus cultures are more tolerant to a low pH environment. Consequently, pH maintenance is not as stringent as bacterial culture during lactic acid fermentation. Furthermore, Rhizopus molds are amylolytic that can produce lactic acid from starch materials directly. For example, R. oryzae NRRL 385 was used to ferment starch derived from barley, cassava, corn, oat, and rice to L-lactic acid [25].

Previous work on Rhizopus fermentation was carried out using free cells in stirred-tank fermentors. Difficulties were encountered in poor oxygen supply and low fermentation efficiency because of the increase in the fermentation broth viscosity due to the formation of large and soft mycelial aggregates. Therefore, a good oxygen transfer rate and good mixing of Rhizopus mycelia in the fermentor are two important considerations for lactic acid accumulation.

![Fig. 2. Schematic diagram of the reactor setup for the simultaneous production and recovery of L-lactic acid (26)](image-url)
Yang et al. [26] used xylose as the carbon source for Rhizopus to grow into mycelial pellets (about 1 mm in diameter). With these restricted mycelial pellets, lactic acid was produced readily and the pellets could be used repeatedly over a 22-d period with good acid yield. When PVP was incorporated into the fermentation system, lactic acid was also produced in the absence of the neutralizing agent. With this fermentor/adsorber system, fermentation can be performed as effectively as when CaCO₃ is used (see Fig. 2 for schematic diagram of the fermentation system).

Du et al. [29] studied lactic acid fermentation by R. oryzae ATCC 52311 in a bubble column to enhance gas and mass transfer. This column fermentor (3.5-L working capacity) was constructed with a polycarbonate pipe and equipped with a perforated plate located at the bottom of the column to serve as the air sparger. The reactor does not require an agitator and is able to distribute air bubbles evenly. With this reactor, Rhizopus produces a higher yield of acid than in a stirred-tank fermentor. To initiate fermentation, the germinated spores are prepared and inoculated into the bubble column. The physical forms (mycelial filamentous or mycelial pellets) of fungal growth can be manipulated by adjusting the pH of the fermentation broth. A final lactic acid concentration of 62 g/l was obtained from 78 g/l glucose (ca. 0.8 g lactic acid/g glucose consumed) with an average specific productivity of 1.46 g/l/h.

2.2.1 Lactic Acid from Xylose

R. oryzae NRRL 395 is able to utilize xylose to produce lactic acid, but the production rate is much slower than with glucose as the substrate. Figure 3 shows the kinetics of lactic acid production from glucose and xylose as reported by Yang et al. [26].

2.2.2 Production of Lactic Acid with Immobilized Cells

L-Lactic acid fermentation using R. oryzae has also been studied using immobilized cells. Dong et al. [30] employed polyurethane foam cubes (macropores larger than hundreds of microns with the pore volume fraction greater than 0.9) as the carriers for Rhizopus immobilization. The immobilized cells can reach 450 g fresh cell (67 g dry)/l cube. The amount of immobilized cells was 30% higher than those immobilized with calcium alginate as the carrier [31]. With this immobilized cell system, lactic acid production reached 37 g/l after 8 h with acid yields approaching 78% based on glucose consumed. Furthermore, the immobilized cells can be used in a repetitive manner for more than 10 batches without lose of activity. Likewise, Tamada et al. [32] immobilized Rhizopus cells in a polymer prepared from the γ-ray-induced polymer of polyethylene glycol dimetharylate. The specific rates of lactic acid production with the immobilized cells were 1.8 times greater than free cells. Lin et al. [33] immobilized Rhizopus mycelium on the plastic surface of a rotary biofilm reactor (RBC) to carry out lactic acid production from glucose.
2.2.3 Solid-State Fermentation

Soccol et al. [34] used R. oryzae NRRL 395 to produce lactic acid from glucose-impregnated sugar cane bagasse. In the presence of CaCO₃, 137 g/l L-lactic acid was produced from 180 g/l glucose with a productivity of 1.43 g/l/h.

3 Citric Acid

Citric acid (2-hydroxy-1,2,3-propanetricarboxylic acid) is one of the most widely used naturally occurring organic acids with an estimated worldwide production of over 400,000 tons per year. It is used mostly as an ingredient in food, confectionery, and beverages. Citric acid can form a wide range of metallic salts that are useful as sequestering agents in industrial processes, as anticoagulant blood preservatives, and as antioxidants. Other potential uses include applica-
tion in the cosmetic industry and environmental remediation in which citric acid can be used as a scrubber to remove sulfur dioxide from flue gases. It then reacts with H2S to give elemental sulfur and the regenerated citrate.

Citric acid can be produced in high productivity and high yield by fermentation of simple sugars mainly by the mycelial fungus, Aspergillus niger, although some processes use the yeast Yarrowia lipolytica (Candida lipolytica or Saccharomyces lipolytica) with n-alkane as the substrate.

3.1 Biochemical Pathways

Because of its economic significance, a great deal of work has been done on the biochemistry of citric acid production. There are four major metabolic sequences involved in citric acid accumulation by A. niger: the conversion of carbohydrates via the glycolytic pathway to yield pyruvate; the anaplerotic formation of oxaloacetate from pyruvate; the accumulation of citric acid within the cell membrane; and the secretion of citric acid to outside cells. Step 2 has been shown to be of great importance in the metabolic control of the entire citric acid biosynthesis process. Figure 4 shows the generalized scheme of carbon flow to citrate in A. niger [35]. There is considerable information available on the properties of the enzymes involved and the steady state concentration of metabolites during citric acid accumulation. In spite of this, many questions still remain unsettled as to the exact mechanism determining the high yield and high rate of citric acid accumulation. The biochemistry and physiology of citric acid production by A. niger and, to a lesser extent, by the yeast Yarrowia lipolytica mainly with n-alkane as the substrate has been reviewed [35–38].

3.2 Factors Affecting Citric Acid Production

The kinetics of citric acid fermentation by A. niger from a sucrose/mineral salt medium has been detailed in a review by Berry et al. [36]. Five successive phases are involved in citric acid accumulation (see Fig. 5):

![Diagram of citric acid fermentation process]

Fig. 4. Generalized scheme of carbon flow from glucose to citrate in A. niger [35]
Fig. 5. Relationship between the biomass proliferation, the specific growth rate, and the specific rate of citric acid production ([36]).
(1) Initiation of spore germination followed by the exponential growth phase, 
(2) Period of growth disturbance caused by the exhaustion of nitrogen (e.g. am- 
monia) and phosphate in the medium, 
(3) Citric acid production phase, 
(4) Citric acid accumulation phase, and 
(5) Declining phase.

3.2.1 Morphology of A. niger

Similar to most mycelial fungi, A. niger can grow into a wide variety of physical 
forms depending on the strain, the initial cell density, the nutrient conditions, 
the growth environment, and the type of fermentor. For efficient production of 
citric acid in a stirred-tank fermentor, it is generally agreed that the physical 
form of mycelial growth is important. The nutritional factors affecting mycelial 
growth and physical appearance have been studied extensively (see review ar-
ticles by Mathey [38]).

Nutritional factors affecting the morphology of the developing mycelia 
greatly influence the performance of the biocatalyst. The development and the 
morphology of the germinated spores, in turn, determine the high yield and 
high productivity of citric acid. Many reports indicate that the shape of hyphae 
growth and their aggregation are crucial to the ultimate yield. Ideally, the hy-
phae of the newly germinated spores should be abnormally short, bulbous, and 
should have many short branches [39-42]. It is known that this state of mor-
phological development is brought about by the concentration of Mn²⁺ [39]. 
Further development of the germinated spores should also form small more or 
less spherically shaped pellets with a diameter of less than 0.5 mm with a 
smooth, hard surface [42]. Such pellets are formed when a number of factors are 
controlled. These are pH, Mn²⁺, Fe³⁺, aeration, agitation, and the amount of 
initial spore inoculum. Based on the experience in the authors' laboratory, de-
velopments of this type of morphology and the maintenance of this type of 
morphology are probably the requisites for successful submerged citric acid ac-
cumulation [43].

3.2.2 Effect of Nitrogen

It is generally concluded that nitrogen limitation is required for citric acid ac-
cumulation. Normally in defined media, nitrogen is supplied as ammonium sul-
face or nitrate. Within 24 h after the inoculation of spores, ammonium ions are 
rapidly consumed with the excretion of stoichiometric amounts of protons. As 
a result, the vegetative growth of germinated spores slows down due to the 
decrease in pH [44] and citric acid accumulation ensues. The optimum nitro-
gen concentration has been determined to be between 1 and 4 g/l depending on 
the nature of the substrate, the fungal strain, and the method of fermentation. 
For an extended period of fed-batch fermentation, the addition of an extra 
nitrogen source is beneficial to maintain the activity of the biocatalyst [45].
3.2.3
Effect of Phosphate

The effect of phosphate levels on citric acid fermentation is somewhat uncertain. Kubicek and Kohr [45] have shown that citric acid accumulation occurs when phosphate is limited. The effect of phosphate on balanced growth during citric acid fermentation was studied by Shu and Johnson [46]. Phosphate may be related to the regulation of enzyme systems and the overall energetics. More recent research, however, indicates that the phosphate effect is not very pronounced if the balance of trace elements is maintained [43]. The optimum phosphate concentration was determined to be between 1 and 4 g/l.

3.2.4
Effect of pH

The pH of the medium is vital for a good yield of citric acid. Ideally, the pH should fall below 2 within a few hours after the initiation of the spore inoculation. At high pH, A. niger tends to accumulate gluconic acid. This is due to the activation of mycelial-bound glucose oxidase at high pH while at low pH (<2), this enzyme is inactive [47]. The pH of the medium will also affect the ionization of citric acid. At pH values of about 2, most of the citrate will be present as either citrate<sup>3-</sup> or citrate<sup>2-</sup>, whereas at an internal pH of about 7, the citrate will be present mainly as citrate<sup>1-</sup>. It has been suggested that only citrate<sup>2-</sup> ions can be transported out of the mycelium easily [48].

Therefore, the optimal pH for citric acid accumulation is between 2 and 3. In this pH range, mycelial vegetative growth is not excessive. It also minimizes the formation of other acids such as gluconic and oxalic acid. The optimal starting pH is usually at about 2–2.5 but varies depending on the nature of the substrate.

3.2.5
Temperature

Temperatures between 28–30°C are the normal range for obtaining high rates of accumulation and high yields of citric acid. At higher temperatures the fermentation rates are very rapid and abundant mycelial growth occurs causing a low yield of acid with higher levels of by-products. Conversely, at lower temperatures, acid yields are higher with the reduced rates of fermentation. Ideally, the temperature should be high during the initial stage of fermentation and low during the latter stage of acid accumulation.

3.2.6
Aeration

Citric acid fermentation is an aerobic process; an increase in the oxygen supply results in an increase in citric acid yields during submerged fermentation. An interruption in the aeration, even briefly, during fermentation has been known
to have an irreversible impact on the ability of the mycelial pellets to produce citric acid even though the growth and the viability of the biocatalyst remains unaffected. The influence of dissolved oxygen on citric acid accumulation has been examined [49].

3.2.7 Trace Elements

Trace elements are considered to be the main factor influencing the success of submerged citric acid production. They affect the biocatalyst in a two-stage response: inhibit growth at a suboptimal level and stimulate growth at a supra-optimal level. It is well known that some trace elements are more important than others in regulating the proper development of the germinating spores and the subsequent hyphae development. In general, the concentration of trace elements that promote growth is detrimental to acid accumulation. Many variations in the requirements of trace elements for optimal citric acid fermentation have been reported in the literature and have been reviewed [35–38]. The optimum concentration of trace elements varies depending on the substrate, the fungal strain and the mode of fermentation.

3.2.7.1 Fe²⁺/Zn²⁺/Cu²⁺

A small quantity of Fe²⁺ accompanied by a limited quantity of Zn²⁺ is essential for obtaining a high citric acid yield. At low Zn²⁺ concentration (below 1 uM) growth becomes limited and citric acid production ensues. If additional Zn²⁺ is added during the production phase (up to 2 uM) then growth can be resumed. Cu²⁺ has been used as an antagonist to Fe²⁺. Too much Cu²⁺ can affect the uptake of Mn²⁺.

3.2.7.2 Mn²⁺

Mn²⁺ has a special effect on the morphological development of the germinated spores, the subsequent mycelial growth, and citric acid production. According to Clark et al. [40], as little as 1 ppb of Mn²⁺ addition will cause a reduction in the citric acid yield by as much as 10%. The detrimental effect of Mn²⁺ on the continuous accumulation of citric acid has been demonstrated by Kubicek et al. [49]. Figure 6 shows the effect of Mn²⁺ addition during citric acid fermentation. A similar effect was also observed with Fe²⁺ addition.

3.3 Industrial Processes

The citric acid production process can be divided into five phases: the preparation of substrates; the preparation of inoculum; the preparation of medium nutrients; the fermentation parameter control; and the product recovery.
Fig. 6. Effect of addition of 1 ppm of manganese on citric acid production [40]

Three processes are currently in practice for the commercial production of citric acid: the Koji, the shallow-pan, and the submerged process. The Koji process is utilized when a solid substrate is the feedstock. The shallow-pan process, that was used during the early stages of industrial production of citric acid, has more or less been replaced by submerged fermentation due to higher productivity and labor saving.

3.3.1 Submerged Fermentation

The submerged process has become the method of choice because it requires less labor to operate, uses less space, is easier to automate, gives a higher production rate, and generates a higher product yield. Several reactor designs such as traditional stirred-tank, bubble fermentor, air-lift fermentor, or air-lift loop fermentor have been used but stirred tanks are the most commonly practiced reactors. Fermentors of all types must be constructed of high-grade stainless steel. This is to avoid the contamination from trace elements, particularly Mn²⁺, that can be released into the medium under the low pH environment and by the continuous accumulation of citric acid.

Aeration is a significant cost factor in the industrial production of citric acid. The industrial practice uses relatively low aeration rates, initially at 0.01 vvm and rises to 0.5 to 1 vvm as fermentation proceeds. For the bubble column,
enough air has to be supplied to maintain a high dissolved oxygen level and to maintain a suitable rheology of the broth. The reactor may be held above atmospheric pressure to increase oxygen dissolution.

The submerged process takes 3 to 10 d to complete, depending on the method used. Although very high yields are possible, the productivity is a more important consideration on an industrial basis. It is rare that the process is allowed to continue to the maximum yield. The typical kinetics of citric fermentation in a bubble column is shown in Fig. 7 and the kinetics of high yield (up to 360 g/l) in a fed-batch bubble column fermentation is shown in Fig. 8.

3.3.1.1 Substrate Preparation

Traditional submerged fermentation typically uses cane and beet molasses as the source of carbohydrate for citric acid production. All molasses and other crude carbon sources may need pretreatment to regulate the proper amount of heavy metal ions. More recently, starch hydrolysates (corn syrups) have been
used more frequently than molasses. When crude substrates are to be used it is often necessary to reduce/remove the level of trace elements (e.g., Mn²⁺ and Fe²⁺). In molasses, the control of metal levels is achieved by treatment with either sodium or potassium ferrocyanide [42] either before or after inoculation of spores. Ferrocyanide not only removes trace metals by chelation but also results in the restriction of fungal growth. A similar effect can be achieved by the addition of excess Cu²⁺. The amounts of ferrocyanide are in the range of 0.5 to 3 g/l of fermentation medium, normally in the 1.5 to 2 g/l range. Whereas relatively pure substrates such as glucose syrup or sucrose solution are used, ion-exchange resins can be used to remove the trace elements.

3.3.1.2 Medium Formulation

Nitrogen is usually supplied as an ammonium salt and/or in combination with other nitrogen sources such as urea. The concentration range varies from 1 to
3 g/l. Phosphates are usually added to give a final concentration of between 1 and 2.5 g/l. Trace elements are supplied according to the original levels in the substrate. Again, careful control of trace element levels is important to limit fungal growth and to achieve maximum citric acid accumulation.

3.3.1.3 Product Recovery

In the final stage of the fermentation, the mycelia are filtered off and citric acid precipitated out of the solution as calcium citrate by the addition of Ca(OH)\textsubscript{2}. Citric acid is regenerated using sulfuric acid then cleaned up before being finally purified by crystallization. In a typical batch fermentation process, the final acid weight yield is about 80 - 85% and the recovery efficiency is around 90%. Recently, Annadurai et al. [51] examined the recovery of citric acid from fermentation broth by calcium carbonate precipitation and subsequent acid hydrolysis. They proposed empirical equations to predict the recovery of citric acid by the precipitation method.

3.3.2 The Surface Process

Shallow stainless steel containers are filled with already inoculated medium. Humid air is blown over the surface of the solution for 5 to 6 d, after which dry air is used. As the fermentation progresses, thick mats of mycelium (a few cm in thickness) form on the surface. Citric acid is formed mainly from the bottom layer of cells that have direct contact with the medium. Normally it takes 8 to 10 d to reduce the sugar concentration to 10 to 30 g/l from an original concentration of 200 g/l. The pH of the solution is lowered from the 5-6 range to 1.5-2.0 due to the continuous accumulation of citric acid. The final yield of citric acid is in the range of 80 - 85% of the sugar utilized [35].

3.3.3 The Koji Process

This method (solid-state fermentation) has been employed for the fermentation of starchy substrates, cellulosic materials, agricultural residues, and food processing wastes. The method utilizes the ability of fungus to produce cellulase and amylases. Since the crude materials normally contain excess trace elements such as Mn\textsuperscript{2+} and Fe\textsuperscript{2+} that interfere with citric acid accumulation, strains that are insensitive to trace elements are often used to carry out this type of fermentation. Typically, solid substrate (pH 5.5) with ca. 70% moisture is inoculated with fungal spores and incubated at 30°C for about 5 to 10 days. With starchy materials, amylases can be supplemented to increase initial sugar concentrations. Likewise, cellulase can be supplemented to enhance cellulose utilization of cellulosic substrates. Often during solid-state fermentation, reactor blockage occurs because of the compacting of solid substrate and fungal growth which causes the channeling of the air supply and temperature and gas
3.4 Production of Citric Acid with Immobilized Cells

In recent years citric acid has been produced on the laboratory scale using A. niger immobilized on calcium alginate gel, polyacrylamide gel, polyurethane foam, and disks of rotating biological contactors (RBC). (see [53,54] for related references). For economic reasons, it is impractical to produce citric acid on an industrial scale using the immobilized cell system at the present time.

4 Fumaric Acid

Fumaric acid is a naturally occurring organic acid that is commonly used as a food acidulant and beverage ingredient. With a double bond and two carboxylic groups, fumaric acid is a good source for chemical syntheses. It is useful for making polyesters and other types of synthetic polymers and has many potential industrial applications, such as in the manufacture of synthetic resins, biodegradable polymers and as the intermediate for chemical and biological syntheses. Essentially all of the fumaric acid produced commercially is derived from petroleum-based maleic acid by a catalytic isomerization process.

As the intermediate of the metabolic TCA cycle, fumaric acid is often found as a metabolic product produced by microorganisms. Many species of mycelial fungi produce small amounts of citric, fumaric, malic, succinic, and other organic acids as metabolic by-products during oxidative metabolism. Some mycelial fungi, particularly those belonging to Rhizopus, are known to produce significant quantities of fumaric acid from glucose under special cultural conditions [55–57].

Although the production of fumaric acid from either glucose, sucrose, starch, or molasses by fermentation using Rhizopus was in commercial operation during the 1940s, it was discontinued due to low productivity and the cheap source of petroleum-derived feedstock.

4.1 Pathway Leading to Fumaric Acid Accumulation

Fumaric acid is an intermediate of the TCA cycle. This oxidative pathway will generate one mole of fumarate per mole of glucose consumed. During active cell growth, however, this pathway cannot lead to a significant accumulation of fumarate. Fumarate generated in the TCA cycle is mainly utilized for the biosynthesis of cell constituents. Early experiments on the production of fumaric acid indicated the possible involvement of the reductive branch of the TCA cycle in fumaric acid accumulation. The carbon dioxide fixing reductive branch is
capable of producing two moles of fumarate per mole of glucose consumed as
given in the follow reaction:

\[ C_6H_12O_6 + 2 \text{CO}_2 \rightarrow 2 \text{HOOCCCH} = \text{CHOOH} + 2 \text{H}_2\text{O} \]

The enzyme responsible for CO₂-fixation and fumarate accumulation in
*Rhizopus* is pyruvate carboxylase (EC 6.4.1.1) which is mainly localized in cyto-
plasm [58-60]. This enzyme catalyzes the ATP-dependent condensation of py-
ruvate and CO₂ to form oxaloacetic acid. The ability of *Rhizopus* to incorporate
carbon dioxide to produce fumarate has been studied employing \(^{14}\text{CO}_2\) and
\(\text{NaH}^+\text{CO}_3\) [61, 62]. Other enzymes involved in fumaric acid accumulation in-
clude fumarase and malate dehydrogenase. Both enzymes are also found in cytosol [63].
Kenealy et al. [63] studied the effect of inhibitors (chlorampheni-
col and cycloheximide) on fumarate accumulation indicating that mitochond-
dria are probably not involved in fumarate accumulation. The accumulation of
fumarate from glucose by *Rhizopus* is believed to operate entirely through the
cytosolic pathway. The cytosolic pathway is induced in the mycelial fungi under
conditions of nitrogen limitation, high carbon to nitrogen ratios, pH of 5.5 or
higher and in the presence of CaCO₃ [60, 63].

By limiting the nitrogen source, cell growth can be kept to a minimum.
During this growth-restricted stage, fumaric acid can be accumulated with a
theoretical maximal yield of 2 moles per mole of glucose consumed or 1.29 g
fumarate/g glucose consumed on a weight basis [63]. In reality, the cytosolic
pathway requires the supply of NADH from the TCA cycle. Therefore, the maxi-
mum obtainable yield is about 0.93 g fumarate/g glucose consumed or close to
1.45 moles of fumarate from each mole of glucose [63, 64].

4.2 Nutritional Factors

*Rhizopus* has very little nutritional demands. Normally, *R. oryzae* and related
species require only substrates and inorganic salts to give a satisfactory
fermentation performance. The addition of some minor elements may en-
hance mycelial growth or fumaric acid accumulation. The principal stimula-
tory metals were found to be Zn⁺² and Mg⁺², which give the maximum stimu-
lation of fumaric acid fermentation at a concentration of 10 and 30 ppm,
respectively. Phosphorus, present as the phosphate ion, is required at 200 ppm
for optimal production of fumaric acid [64]. Cu⁺² may inhibit fumarate
production at 1 ppm or higher. The effect of Zn⁺² and other metal ions has
been studied [61, 64, 65]. Similar to citric acid fermentation by *A. niger*, the
amount and type of trace elements required to achieve maximum fumaric acid
yield vary depending on the fungal strain, the physical state of fermentation,
the presence of other trace elements, and the nature of the substrate. Trace
metals alone with pH of the growth medium also influence the morphology of
mycelial growth. A recent study by Chou et al. [65] shows that very small,
distinctive mycelial pellets can be obtained by special medium formulation
(see Fig. 9).
4.3 Submerged Stirred-Tank Fermentation

The production of fumaric acid by fermentation using *Rhizopus* in submerged cultures has been the most commonly employed method. Rhodes et al. [64] studied fumaric acid production by *R. arrhizus* strain NRRL 2582 in a 20-l baffled stainless steel fermentor (10-l working volume) and established the standard for the conditions in stirred-tank fermentation. In their study, the best results were obtained when the continuous addition of CaCO₃ as the neutralizing agent was used. Other neutralizing agents such as NaOH and KOH are not as satisfactory due to the effect of the ionic strength from the accumulation of soluble fumarate salts when the concentrations in the broth reach 35–40 g/l. Conditions established by Rhodes et al. to obtain the best yield (0.65 g fumaric acid/g sugar consumed) of fumaric acid include a simple salt solution, 100–160 g/l sugar concentration, 0.5 vvm aeration, 300 rpm agitation rate, 33°C incubation temperature, and pH of 5.5–6.0. In most cases, fumaric acid produced accounted for about 80% of the total organic acids accumulated.

To initiate the fermentation, *Rhizopus* spores are inoculated directly into the fermentor. The production of calcium fumarate normally follows a regular pattern. Initially, there is slow utilization of sugars accompanied by the development of mycelial growth and the initiation of acid production. This is followed by the rapid consumption of sugars and the accumulation of fumarate. However, the solubility of calcium fumarate in the broth is limited. During fermentation, growing mycelia form interlocking hyphae often mingled with solid calcium carbonate and calcium fumarate crystals. This results in the formation of a very viscous solution which leads to an increase in diffusion and oxygen transfer restrictions, an operational problem that slows down the fermentation rate.

In fumaric acid fermentation, the most critical control factor is the amount of nitrogen present. Nitrogen is the main factor in the regulation of mold vegetative growth or fumaric acid production. On the other hand, hydrodynamical forces influence the morphology of fungi and the bioparticle sizes. Therefore,
minimizing the branch extension of the hyphae to obtain small mycelia particles is important [65].

4.4 Problems Encountered During Fumaric Acid Fermentation

The fumaric acid fermentation by Rhizopus is an aerobic process that requires oxygen. Usually, an agitated-sparged stirred-tank fermentor is employed to achieve a good oxygen transfer rate. There are several problems encountered during submerged stirred-tank fermentation. In a typical fumaric acid fermentation, the presence of a neutralizing agent, particularly CaCO₃, is required to maintain the proper pH and to supply CO₂ as the co-substrate. The solubility of calcium fumarate and fumaric acid are only 21 and 7 g/l, respectively. Due to its low solubility, fumarate crystallizes easily and forms conglomerates with fungal mycelia that result in complication during fumarate recovery. Gangl et al. [66] used Na₂CO₃ to neutralize the fumaric acid produced in order to avoid the complicated product recovery process. Federici et al. [67] used KOH/K₂CO₃ as the neutralizing agent and CO₂ source for the conversion of starch hydrolysate (glucose syrup) to fumaric acid. In general, fumaric acid productivity (g/l/h) is lower when Na₂CO₃ is the neutralizing agent.

Another difficulty encountered is the tendency of Rhizopus sporangiospores to grow into mycelial mats or mycelial clumps. The growing mycelia will anchor onto the inside elements of the reactor such as the bafflers, propellers, and heat exchanger (see Fig. 10, unpublished observation). This results in the inter-

![Fig. 10. Picture of Rhizopus mycelia attached to a fermentor (unpublished observation)](image-url)
ference of oxygen and mass transfers and therefore enhanced ethanol production at the expense of acid accumulation. One way to avoid this problem is to grow the cells in a specially formulated growth medium to obtain small compact mycelial pellets (0.5–1 mm, see Fig. 9) before subjecting the cells to the non-growth fermentation medium. Other methods include the use of an air-lift loop fermentor or rotary biofilm contactor (RBC) to carry out the fumaric acid fermentation.

4.5 Air-Lift Loop Fermentation

Du et al. [68] employed a laboratory air-lift loop fermentor for fumaric acid fermentation. This fermentor (10-L working capacity) has a concentric draught tube inside the reactor that is used as the riser section to form an inner gas-liquid loop (see Fig. 11 for configuration). This reactor does not require an agitator. It is also designed to provide an even distribution of air bubbles and carbon dioxide in the broth that is necessary to provide a high yield of acid. With *R. oryzae* ATCC 20344, a fumaric acid production rate of 0.81 g/l/h was obtained with a weight yield of 0.75 g/g glucose consumed. In another study, Du et al. [69] conducted fumaric acid fermentation in an air-lift loop fermentor with two different sizes of mycelial pellets. Results show a high yield of fumaric acid (0.865 g fumaric acid/g glucose consumed) is obtained with smaller pellets.

![Fig. 11. Air-lift loop fermentor with product recovery system [69]](image-url)
4.6 Fumaric Acid Production with a Rotary Biofilm Contactor (RBC)

A rotary biofilm contactor (RBC) is a vessel that contains plastic plates that act as the supports for fungal mycelial growth. The plates are mounted onto a horizontal shaft and placed in the vessel containing the medium (see Fig. 12 for reactor configuration). During the growth stage, Rhizopus mycelia are grown onto the plastic surface of the plates and form the “biofilm” and are immobilized on the surface of the plates (Fig. 13). During the fermentation stage, the shaft rotates slowly exposing the biofilm periodically to the gas space on the upper portion of the RBC for good oxygen absorption, and to the lower portion with the liquid medium for substrate uptake and excretion of fumaric acid. Similar types of reactors have been used for industrial wastewater treatment [71].

Cao et al. [70] operated a 2-L RBC fermentor with a 0.9-L working liquid volume for fumaric acid production with CaCO₃ as the neutralizing agent. With R. oryzae ATCC 20344, a fumaric acid production rate of 3.78 g/L/h was obtained with a weight yield of 0.75 g/g glucose consumed. A final calcium fumarate concentration of 75.5 g/L was obtained after 24 h from an initial glucose concentration of 98.7 g/L. The volumetric productivity achieved using the RBC is almost four times higher than that using a stirred-tank system (0.94 g/L/h) with CaCO₃ as the neutralizing agent.

\[ \text{Fig. 12. Rotary biofilm contactor with product recovery system [72]} \]
In another experiment, an adsorption column (with anion-exchange resin or polyvinylpyridine) was coupled to the RBC to carry out simultaneous production and recovery of fumaric acid without the addition of CaCO₃ (for configuration, see Fig. 12). With this system, Cao et al. [72] were able to obtain a fumaric acid yield of over 90% of the theoretical maximal yield (0.85 g fumaric acid/g glucose consumed). The specific productivity of 4 g/lh obtained with this system is higher than RBC fermentation with CaCO₃ as neutralizing agent. The adsorbed acid can be recovered as pure product after elution with NaCl followed by the acidification of the recovered sodium fumarate. The same pre-grown biofilm in the RBC can be used repeatedly provided the immobilized fungal mycelial are activated periodically with nitrogen-rich solution.

4.7 Production of Fumaric Acid from Xylose

Fumaric acid can also be produced from xylose. The rate of xylose fermentation is much slower than with glucose with a specific productivity of only about 0.075 g fumaric acid/hg biomass. Kautola and Linko [73] used immobilized R. arrhizus with polyurethane foam to ferment xylose. A specific productivity of 0.087 g/lh was obtained when the initial xylose concentration was 100 g/l and the resident time was 10.25 days.

4.8 Production of Fumaric Acid with immobilized Rhizopus

Fumaric acid production using immobilized Rhizopus cells has also been studied. Petruccioli et al. [74] immobilized R. arrhizus NRRL 1526 on polyurethane sponge to carry out repeated batch fumaric acid production from glucose syrup with KOH/KCO₃ as the neutralization agent and CO2 source. Although the yield (12.3 g/l) is low, it provides the possibility of using immobilized Rhizopus for the continuous production of fumaric acid.
4.9 Fumaric Acid from Starch Hydrolysates

Fumaric acid production from starch hydrolysate by \textit{R. arrhizus} NRRL 1526 was studied by Federici et al. [75] in a 3-L stirred-tank fermentor with CaCO$_3$ and KOH/KCO$_3$, as the neutralizing agent and CO$_2$ source. The fermentation conditions for fumaric acid production by this fungus from potato flour has been optimized by Moresi et al. [76].

5 L-Malic Acid

Similar to fumaric acid, L-malic acid is also a naturally occurring four-carbon dicarboxylic acid and an intermediate in the TCA cycle. It has been used in many food products, primarily as an acidulant. L-Malic acid is compatible with all sugars with low hygroscopicity and good solubility. In addition, it has therapeutic value for the treatment of hyperammonemia and liver dysfunction and as a component for amino acid infusion. L-Malic acid has been the subject of interest because of its increased application in the food industry as a citric acid replacement and its potential use as a raw material for the manufacture of biodegradable polymers.

Traditionally, L-malic acid has been obtained from its natural source, apple juice. However, this is impractical due to the small quantities in which it occurs. Large-scale production of malic acid can be achieved by chemical methods through the hydration of either maleic or fumaric acid. It can also be obtained by biological hydration of chemically derived fumaric acid to L-malic acid mediated by fumarase (fumarate hydratase, EC 4.2.1.2) or by the fungal fermentation of simple sugars. The synthetic method generates a racemic mixture of the stereoisomers, D- and L-malic acid. In contrast, the biological method produces only L-malic acid. For human consumption, L-malic acid is preferred. Likewise, the single isomer is preferred as the feedstock for biodegradable polymers. Large-scale production of L-malic acid from simple sugar or cheap cellulosic biomass is possible. However, it is expensive because of the relatively low concentration of acid produced and the high product recovery cost. The most common method for the biological production of L-malic acid is by enzymatic hydration. It is mediated by fumarase of chemically derived fumaric acid using \textit{Brevibacterium} strains [77–79].

5.1 Malate from Fumarate

Fumarase is an enzyme component of the TCA cycle that catalyzes the reversible reaction of fumarate to L-malate with equilibrium favoring malate production. It is a soluble enzyme with high turnover number. In one report, fumarate content in some organisms can be as high as 1000 mg/kg of wet cells [80]. Theoretically, a malate weight yield of 115% can be obtained from fumarate. However, in reality, a weight yield of 90–95% is often obtained.
Since the 1970s, malate has been produced from fumarate using immobilized *Brevibacterium* as the biocatalyst in a continuous process on a large scale [77]. Because fumarase is one of the essential enzymes involved in the basic oxidative metabolism, it is likely that fumarase is active in most living organisms. However, high fumarase activity has been found in only a few bacterial species [81]. Eukaryotic microorganisms such as mycelial molds or yeasts are not known to have very active fumarase activity for in vivo conversion of fumarate to malate. There are few reports using immobilized baker's yeast, *Saccharomyces cerevisiae*, for malate production from fumarate. However, the specific activity of yeast conversion is only about one-third compared with those by bacterial systems [82]. Neufeld et al. [83] studied L-malate formation by immobilized *S. cerevisiae* that was amplified for fumarase in the presence of a surfactant. The highest specific activity achieved was reported to be three times higher than the bacterial system. Likewise, Wang et al. [84] reported that a genetically modified yeast strain, *S. cerevisiae* SHT2, possesses high in vivo fumarase activity.

5.2 Fermentative Production of L-Malic Acid

L-Malic acid can also be produced from simple sugars in significant quantities by a number of *Aspergillus* species [85] and by the wood mushroom, *Schizophyllum commune* [86]. Similar to fumarate fermentation by *Rhizopus*, special cultural conditions are required for the production of L-malic acid by *Aspergilli* from glucose. *A. flavus* produced a high weight yield of L-malic acid from glucose in a stirred-tank fermentor with CaCO₃ as the neutralizing agent. The high yield of malic acid is believed to be due to the ability of the fungus to incorporate CO₂ into the product during the L-malic acid producing phase [85]. The activity of pyruvate carboxylase and its location in the cytoplasm of the cells are believed to be the key factors in the high L-malic acid yield.

5.3 Production of L-Malic Acid by *A. flavus*

Battat et al. [87] used *A. flavus* ATCC 13697 as the biocatalyst for the production of malic acid from glucose in a 16-l stirred-tank fermentor. The optimal fermentation conditions are as follows: agitation rate, 350 rpm; Fe²⁺, 12 mg/l; nitrogen (as ammonium sulfate), 271 mg/l; phosphate, 1.5 mM. Total amount of CaCO₃ added was 90 g/l. Fermentation was carried out at 32°C for up to 200 h. Under the aforementioned conditions, 113 g/l of L-malic acid were produced from 120 g/l glucose utilized with an overall productivity of 0.59 g/l/h. Based on the molar yield, it was 128% for malic acid and 135% for total acid (malic, fumaric and succinic acid). The increase in acid accumulation during the course of incubation coincides with the increase in the activities of NAD⁺-malate dehydrogenase, fumarase and citrate synthase.
5.4 Production of L-Malic Acid by Schizopyllum commune

Kawagoe et al. [88] used S. commune as the biocatalyst for the production of malic acid from glucose (50 g/l) in an 8-l air-lift column fermenter (7.2-1 working volume). The optimal fermentation conditions are as follows: superficial air velocity, 1.7 cm/s; temperature, 27°C; nitrogen (as ammonium nitrate), 10 g/l; CaCO₃, 50 g/l. After 110 h of fermentation, 43 g/l of L-malic acid were produced from 50 g/l glucose.

5.5 Mixed Culture Fermentation

L-Malic acid can also be produced from glucose using a combination of a fumaric acid producer (Rhizopus arrhizus) and an organism with a high fumarase activity in the same fermentor [89,90].

5.6 Biochemical Aspects of L-Malic Acid Production

The cytosolic location of pyruvate carboxylase responsible for the accumulation of high concentrations of malic acid has been demonstrated in A. flavus [87]. This enzyme and other related enzymes (e.g. malate dehydrogenase) are induced in the mycelial fungi under conditions of nitrogen limitation, high carbon-to-nitrogen ratios, a pH of 5.5 or higher, and in the presence of CaCO₃. The protein synthesis inhibitor, cycloheximide, inhibits both malate accumulation and malate dehydrogenase activity without affecting fumarase activity [91]. Similarly, inhibitors of pyruvate carboxylase, such as avidin, inhibit the formation of malic acid. These results suggest that both malate dehydrogenase and pyruvate decarboxylase are required for malic acid accumulation.

Recently, S. cerevisiae has been studied in regard to the biochemical regulation of malic acid production [92,93]. This common yeast has been shown to produce small amounts of fumaric and malic acid (less than 10 g/l) from glucose under environmentally stressed conditions. Similar to the malic acid production pathway of Aspergillus, the cytosolic reductive pathway of acid synthesis and accumulation has also been shown in S. cerevisiae. A 13C NMR study following the label from glucose to malic acid indicates the following reactions lead to malic acid accumulation: pyruvate → oxaloacetate → fumarate → malate. The involvement of cytosolic fumarase in the conversion of fumaric acid to malic acid has been collaboratively recently. Wang et al. [94] have shown the ability of a cytoplasmic respiratory deficient mutant of S. cerevisiae to convert fumarate to malate without the participation of mitochondria fumarase.

6 L-Aspartic Acid

Aspartic acid is an amino diacid that is commonly used as an ingredient in the synthesis of the artificial sweetener, Aspartame. Aspartic acid is also a useful
monomer for the manufacture of polyesters, polyamides, and other chemical
derivatives. The polymeric form of aspartic acid, polyaspartic acid, is an ingredi-
ent in cleaning compounds that can be used as a replacement for EDTA and
other widely used chemicals. The potential market for polyaspartate can be as
high as US$ 450 million per year. The key to a wider use of polyaspartate is a
cheap source of L-aspartic acid (Chemical Market Reporter, 18 October 1996).

L-Aspartic acid can be produced by direct fermentation of sugars using bac-
terial strains. However, commercially it has been produced by the amination of
fumaric acid by immobilized biocatalysts that have high aspartase (EC 4.3.1.1) ac-
tivity in a fixed-bed reactor. Suitable microbes for the industrial bioconversion
of fumaric acid to L-aspartic acid include strains of Brevibacterium, Coryn-
ебacterium, E. coli, and Pseudomonas. A weight yield of 110% can be obtained
in the conversion of fumaric acid to asparagic acid as shown in the following:

\[
\text{fumaric acid} + \text{NH}_3 \rightarrow \text{L-aspartic acid}
\]

The first commercial production of L-aspartic acid was started in 1973 by the
Tanaba Seiyaku Company, Japan. The process uses aspartase contained in whole
microorganisms and involves the immobilization of E. coli on polyacrylamide
gel or carrageenan. The immobilized cells are then subjected to treatment in
order to increase cell permeability. The substrate, fumaric acid, is dissolved in a
25% ammonia solution and the resulting ammonium fumarate is then passed
through the reactor containing the immobilized E. coli. The reaction is exother-
omic and the reactor has to be designed to remove the heat produced. The con-
version of fumaric acid to aspartic acid is more economical than the direct fer-
mentation of sugars. The key to economical production of L-aspartic acid for
expanded use is a cheaper and more abundant source of fumaric acid.

7 Succinic Acid

Succinic acid is a flavor-enhancing organic acid used in dairy products and fer-
mented beverages. This acid and its derivatives are widely used as specialty
chemicals with applications in polymers, foods, pharmaceuticals, and cosme-
tics. Succinic acid is a valuable four-carbon intermediate that can be converted
by catalytic processes into 1,4-butanediol, tetrahydrofuran, and γ-butyrolac-
tone. It can also be easily esterified to dimethyl succinate, which is marketed as
an environmentally friendly solvent [95].

Succinic acid is currently manufactured by the hydrogenation of maleic an-
hydride to succinic anhydride, followed by hydration to succinic acid. A fer-
mentation process for succinic acid production is desirable because in such
processes, renewable resources such as starchy crops and other agricultural
products can be used as feedstock for the biological production of succinic acid.
It addition, a high purity product, which is required as raw material for polymer
manufacture, can be obtained.

Succinic acid is one of many organic acids (e.g. acetic, butyric, caproic,
formic, propionic and succinic acid) produced from glucose by many rumen
anaerobic bacteria. Only a very few accumulate succinate as the anaerobic end-

product in significant concentration. One of such rumen anaerobes, *Fibrobacter succinogenes*, is capable of utilizing cellulose to produce succinic acid. With pulped shredded office paper, *F. succinogenes* produced 3.2 g/l succinate after 90 h from 10 g/l substrate with acetate (0.28 g/l) and formate (0.07 g/l) as the co-products [96]. By far the best succinate producer is *Anaerobiospirillum succiniciproducens*, a non-ruminal anaerobic bacterium. According to Nghiem et al. [97], a maximum of 32 g/l of succinic acid was produced by this anaerobe in 27 h at pH 6.0 with a CO₂ sparge rate of 0.08 l/h/min with a yield of 0.99 g succinate/g glucose consumed.

The degree of succinic acid accumulation by *A. succiniciproducens* is influenced strongly by the culture pH and by the availability of CO₂. Samuelov et al. [98] studied the effect of CO₂ and pH on growth, fermentation kinetics, carbon balance, and the profile of the product formed by this organism. They also measured the changes in the levels of key catabolic enzymes with respect to pH and available CO₂. They arrived at the following conclusions (see Fig. 14):

1. Glucose is metabolized via the Embden-Meyerhof-Parnas (EMP) route to generate phosphoenolpyruvate,
2. Oxaloacetate is formed from PEP with CO₂ as the co-substrate,
3. Reduction of oxaloacetate results in malate formation,
4. Under excess CO₂/HCO₃ growth conditions, phosphoenolpyruvate carboxy-kinase levels increase while lactate dehydrogenase and alcohol dehydrogenase levels decrease,
5. The accumulation of succinic acid is a growth-associated event, resulting from the regulation of electron sink metabolism.

In contrast, Millard et al. [99] showed that the over expression of phosphoenolpyruvate carboxylase in *E. coli* is responsible for succinic acid accumulation and not phosphoenolpyruvate carboxykinase.

![Fig. 14. Proposed catabolic pathway for glucose fermentation in A. succiniciproducens](98)
Table 1. Summary of fermentation results obtained at 32 h [104]

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Succinic acid concentration (g/l)</td>
<td>47.5</td>
</tr>
<tr>
<td>Succinic acid yield (g/g fumaric acid consumed)</td>
<td>93%</td>
</tr>
<tr>
<td>Specific productivity of succinic acid (g/g biomass/h)</td>
<td>0.183</td>
</tr>
<tr>
<td>Volumetric productivity of succinic acid (g/l/h)</td>
<td>1.46</td>
</tr>
<tr>
<td>Succinic acid/acetic acid (g/g)</td>
<td>1.14</td>
</tr>
</tbody>
</table>

Succinic acid can also be generated from fumarate [100] or citrate [101] in the presence of a readily metabolizable carbon source to serve as the hydrogen donor. When citrate is the hydrogen acceptor, it is split into oxaloacetate and acetate by citrate lyase. Oxaloacetate is in turn converted into succinate [102]. The rate of conversion and yield of succinate from fumarate can be enhanced by the amplification of genes that synthesize fumarate reductase [103, 104]. Table 1 shows the fermentation results reported by Wang et al. [104]. In addition, succinic acid can be generated from glucose with mixed culture fermentation, in which fumarate produced by a Rhizopus culture is converted into succinate by a bacterial culture [105].

8 Itaconic Acid

Itaconic acid (methylene succinic acid) is an unsaturated five-carbon dicarboxylic acid. It is a by-product of the destructive distillation of citric acid. The reaction leading to itaconic acid from citric acid is as follows:

citric acid + H₂O → aconitic acid + CO₂ → itaconic acid + citraconic acid

Aconitic acid is also present in sugar cane juice and is believed to interfere with sucrose crystallization. Removal of calcium aconitate by sugar refiners, and its subsequent conversion to itaconic acid by heating the solution, was the principal source of itaconic acid until the fermentation process was developed.

Itaconic acid is a useful feedstock for the synthesis of polymers. This is due to the ability of the methylene group of this acid to engage in polymerization reactions. The resulting polymers are used in carpet backing and paper coating. Another significant reaction product of itaconic acid is the formation of N-substituted pyrrolidinones with amines. The products are used in detergents, shampoos, and other products in which their surface activity is useful [106].

In 1929 Kinoshita [107] identified itaconic acid as the major metabolic product of A. itaconicus. Later research showed that A. terreus is a better biocatalyst for itaconic acid accumulation. A number of yeast strains belonging to Candida and Rhodotorula [108] can also accumulate a limited amount of itaconic acid. Patents on the industrial production of itaconic acid using Aspergillus as the biocatalyst from molasses were issued in 1961. The currently preferred industrial process uses improved strains of A. terreus as the biocatalyst. The most often studied itaconic acid producers are A. terreus NRRL 265 and A. terreus NRRL 1960.

The biochemistry of the itaconic acid biosynthesis is very similar to that of citric acid. The general pathway from hexose to pyruvate (via the EMP pathway)
to the TCA intermediate is the same. The metabolic flux limitation appears to occur at the same place as in the citric acid biosynthesis [109], although the precise route of the conversion of pyruvate to itaconate is still uncertain.

In 1985, Bonnarre et al. [110] used the analytical techniques that combine isotopic tracing, nuclear magnetic resonance spectroscopy, and mass spectroscopy to compare the enzyme systems of intact cells of high- and low-producing strains of A. terreus. Results show that itaconate formation requires de novo protein synthesis. During acid formation, TCA cycle intermediates increase in both strains. Furthermore, data showed that both the EMP pathway and the TCA cycle are involved in itaconate biosynthesis. Based on the biosynthetic pathway (Fig. 15), one itaconate molecule is produced from one hexose molecule with a theoretical weight yield of 72.2%. The actual yield should be lower due to the loss of carbon to biomass accumulation and cell maintenance.

Similar to citric acid and other organic acid fermentations by mycelial fungi, dissolved oxygen, trace metal elements, low pH environment, nitrogen and carbon sources, and phosphate have profound impact on itaconic acid accumulation. Figure 16 shows typical kinetics of itaconic acid fermentation [111]. Itaconic acid fermentation is an aerobic process; an increase in the oxygen supply results in an increase in acid yields during submerged fermentation. An interruption in the aeration for 5 min leads to a complete cessation of itaconic acid production, which can only slowly restore 24 h after the resumption of aeration. The effect of aeration interruption is due to cessation of protein synthesis and the loss of ATP supply. Continuous generation of ATP is believed to be required for the transport of produced itaconic acid outside of the cell membrane [112]. The effect of dissolved oxygen concentration and impeller speed on itaconic acid production by A. terreus was reported by Park et al. [113].

The production rate and yield of itaconate are influenced by the presence of divalent cations (Fe²⁺, Zn²⁺, and Ca²⁺), physical forms of fungal growth, aeration, and the presence of neutralization agents (e.g., CaCO₃). Using A. terreus NRRL 1960 as the biocatalyst, Gyamerah [114] indicated that the frayed and

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**Fig. 15.** Suggested pathway of itaconic acid biosynthesis from glucose in A. terreus [110]
loose growth form with a size of 0.1 to 0.5 mm in diameter gave the best itaconate yield with the optimal divalent cation concentration shown as follows: Fe\(^{2+}\), 0.06 mg/l; Zn\(^{2+}\), 0.1 mg/l; and Ca\(^{2+}\), 0.23 g/l. The best results showed an itaconic yield of 0.6 g/g sucrose utilized.

Yahiro et al. [115] conducted itaconic acid production from glucose using stirred-tank and air-lift reactors. Results indicate that the air-lift reactor has a much higher productivity (0.64 g/l/h) than the stirred-tank reactor (0.48 g/l/h). Final itaconic acid concentration reached 65 g/l after 96 h of fermentation. Likewise, Okabe et al. [116] used an air-lift bioreactor using a modified draft tube for itaconic acid production and obtained an enhanced itaconic acid yield.

With the objective of producing itaconic acid directly from starchy materials, Kizirina et al. [117] studied the interspecific hybridization between A. terreus and A. asperellus through protoplast fusion. Although A. terreus has amylase activity, the activity is not strong enough to produce itaconic acid from starch di-
rectly. On the other hand, *A. usamii*, an *A. niger* related species, shows high productivity of glucoamylase which retains high activity under conditions of low pH (2–4) during fermentation. The resulting soluble starch was able to ferment soluble starch to accumulate 36 g/l itaconate from 120 g/l soluble starch after 6 d incubation. This is, perhaps, the first report of the use of an interspecific protoplast fusion between two different species of *Aspergillus* for the production of itaconic acid from soluble starch.

Itonic acid production using immobilized cells has also been studied (see [54] for pertinent reference list). The highest productivity of 0.33 g itaconic acid/g of carrier/d was obtained on glucose in a packed-bed reactor which was operated continuously for 4.5 months [118].

9 Conclusions

The organic acids discussed in this chapter are either those manufactured in large volumes or offer vast potential for future development. The production of these acids by fermentation represents a potential route to the production of commodity chemicals from renewable feedstock. The biosyntheses of many of the organic acids by mycelial fungi discussed in this chapter utilize simple sugars through the reductive reactions of the TCA cycle that involves incorporation of carbon dioxide and, theoretically, can result in a net increase in mass relative to the substrate. "Multifunctional" reactive organic acids can be utilized as the feedstock for "biodegradable" polymers or "green" fine chemicals. Many current technologies for the biological conversion of renewable biomass are still in the experimental stage. With the advance of biotechnology through the understanding of biological and biochemical regulations, better biocatalysts can be developed to overcome some of the shortcomings of the existing ones. By experimentation, better bioreactors can be designed and used for the enhancement of productivity and, through better design, more efficient product recovery systems can be obtained. Through relentless research, agricultural residues, food wastes, and municipal wastes can be efficiently converted into valuable products. Overall, the production of organic acids from renewable biomass offers a tremendous opportunity to recycle and reuse natural resources.

References