PRODUCTION OF ORGANIC ACIDS

F. Sanchez-Riera
Bio-Technical Resources, Wisconsin, USA

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Summary

The microbial production of several organic acids of industrial importance is discussed. Organic acids constitute a significant portion of the fermentation market in the world, and microbiological production is an important economic alternative to chemical synthesis for many of them.

Acids are treated individually. The most remarkable aspects of the biochemistry of the producing organisms, most common processes and key process parameters are discussed in each case. Information about the most recent advances presented in patents and published studies are also included.

Citric, lactic, gluconic, and itaconic acid are currently produced by fermentation. Microbial production of acetic acid is important for food applications, in the form of vinegar. Propionic, succinic, and pyruvic acid are not currently being produced commercially by fermentation, but have significant potential for future manufacture by this route.

1. Introduction

Organic acids are broadly distributed in nature, and humans have used them in their natural sources since early ages. Fermentation processes that involve the production of acetic and lactic acid have been used in food preparation for centuries. Industrial production with the microbiological route was started when the acids were identified as the main product in known fermentation processes. During the first half of the twentieth century, advances in chemical synthesis offered new manufacturing procedures that became economically competitive and replaced many fermentation processes. The situation changed in the 1990s, when further developments in the biotechnology field, environmental pressures and the vertical integration of the fermentation and corn processing industries resulted in much improved economics for the biological route.

The production of organic acids discussed here includes those which are currently manufactured by fermentation in large quantities or which offer potential for future developments.

2. Citric Acid

2.1 Historical Development

Citric acid (2-hydroxy-1,2,3 propanetricarboxylic acid, $C_6H_8O_7$) is widely distributed in plant and animal tissues and fluids. It occurs in rather high concentrations in citrus fruits, but is ubiquitous in nature as an intermediate in the Krebs cycle, whereby carbohydrates are oxidized to carbon dioxide.

It was first isolated from lemon juice in 1784, and its commercial production started in England in 1826 from Italian made calcium citrate. At the beginning of the twentieth century, Italy had a virtual monopoly with a production of about 10 000 tons. In 1893, Wehmer observed that citric acid was produced by some species of *Penicillium* when

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grown on sugar solutions, but his attempts to establish a manufacturing process by fermentation were unsuccessful. The 1917 discovery of citric acid producer strains of *Aspergillus niger*, which could grow at pH values around 2.5 to 3.5, led to the first commercial production by fermentation.

Plants were opened in Belgium in 1919 and the US in 1923. Several plants using the surface process were started in other countries, and in about 10 years around 80% of the citric acid was produced by fermentation.

Intense research led to the development of the submerged process, which was applied industrially after the Second World War. More efficient strains, cheaper medium and better control conditions have resulted in great process improvements, but research in the field continued through the years.

Several yeast strains have been identified for the production of citric acid from both carbohydrates and hydrocarbons. However, production from the latter has not been economically viable.

By 1997 the global capacity was estimated at 840 000 tons per year; demand has been increasing at a 5% rate from 1988 to 1997 and an average 3.5% growth is expected until 2002.

In the last few years the citric acid business has been mostly consolidated in large producers back-integrated to raw materials. While production has also increased in China and India, their capacities are still much lower than that of international companies.

The main application of citric acid (70%) is in the food and beverage industry, as the most versatile and widely used acidulant. Its pleasant taste, high water solubility, and property of enhancing flavors have affirmed its application in this market, which has been largely responsible for the increases in citric acid demand.

A buffering capacity and the ability to form complexes with heavy metals have driven the application of citric acid and several of its salts in the pharmaceutical and chemical processing industry as sequestering agents. The remaining demand is distributed as follows: 18% in detergents and cleaners, 6% in pharmaceuticals and cosmetics and 6% in industrial and chemical processing uses.

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2.2 Fermentation Basics

The excretion of citric acid is not a normal phenomenon for microorganisms, but it can accumulate in very large quantities when certain bacteria, yeast, and fungi are grown under particular constraints.

Appreciable accumulation is the result of severe irregularities in the metabolism caused by genetic deficiencies or by metabolic imbalances. The definition of the appropriate conditions to maximize citric acid yield and concentration varies widely depending on the strain in use and the main carbon source.

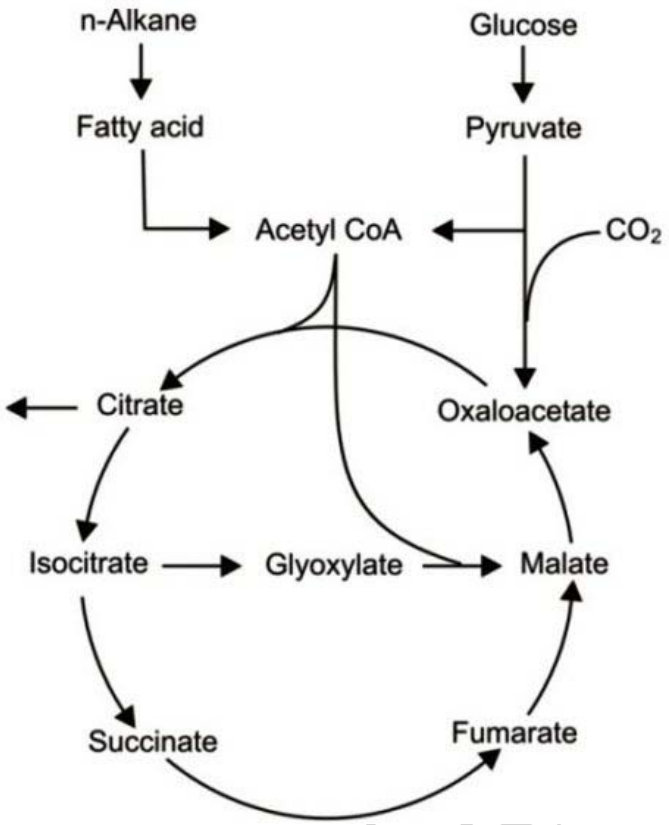

Figure 1. Metabolic relationships of citrate metabolism from carbohydrates or n-alkanes.

The basic metabolic sequences related to citrate metabolism are well established, as shown in Figure 1. However, the regulation of the pathway and the main points that control citrate accumulation are still subject to debate, because different conclusions have been reached in numerous studies. Among the reasons for this is the fact that many studies have used different strains and conditions, and that there is evidence that indicates that more than one set of conditions can lead to citric acid accumulation.

A.niger forms citric acid from glucose viaglycolytic catabolism to two moles of pyruvate and their subsequent conversion to oxaloacetate. Citrate synthase then condenses oxaloacetate with acetyl CoA to form citrate. The presence of the enzymes of the tricarboxylic acid cycle has been demonstrated in this organism under various conditions. For citric acid to accumulate, an imbalance between the metabolic flux in the glycolytic pathway and the tricarboxylic cycle must occur. This imbalance has been detected as an increase in the flux through glycolysis with high activity of the glycolytic enzymes due to culture conditions. A key step in the process is the synthesis of oxaloacetate by an anaplerotic carbon dioxide fixation catalyzed by pyruvate carboxylase, which is induced in the presence of high carbohydrate concentrations. This enzyme and citrate synthase produce an accumulation in the cellular concentration of citric acid. Some authors suggest that in the stages of production of citric acid, the tricarboxylic cycle is restricted at isocitrate dehydrogenase. But the nature of the regulation of overproduction and accumulation of citric acid is still unclear, because several different mechanisms have been proposed and the existing evidence is not enough to confirm a single one. When yeasts produce citric acid from alkanes, these

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compounds are converted by ß-oxidation to acetyl CoA, which combines with oxaloacetate to produce citrate. The anaplerotic glyoxylate cycle replenishes oxaloacetate (see *Cell Thermodynamics and Metabolism*).

Even though a large number of microorganisms can produce citric acid, the fungus *Aspergillus niger* has remained as the organism of choice for commercial production. In the last few years, production with the yeast *Yarrowia lipolytica* (asexual form *Saccharomycopsis*, formerly *Candida*) has increased significantly. Some other organisms include *A. wentii*, *A. carbonarius, A. aculeatus, A. awamori, Candida tropicalis, C. oleophila, C. guilliermondii*, and *C. citroformans*. A significant effort has been devoted to obtain strains with higher yields and better production efficiency, but the strains actually used in manufacture and the selection methods for strain improvements have been kept secret, and information about them is not generally available. Cell improvements for citric acid production through gene cloning and expression are not straightforward because, in general in these long pathways, the contribution of a single enzyme has very limited success in increasing overall productivity. Limitations and improvements are rarely based on a single step, and the regulatory enzymes may be less important to the overall productivity of the organism than the processes of uptake of substrate or excretion of the acid.

2.3 Fermentation Processes

Different fermentation processes have been developed for the different strains and carbon sources. *A. niger* and *Y. lipolytica* are used in commercial processes. Surface and submerged methods have been used with *A. niger* and either carbohydrates or n-alkanes can be the carbon source in processes using yeasts.

2.3.1 Production with A. niger

In order to achieve abundant excretion of citric acid, the growth of the strain must be restricted. High sugar concentrations favor high glycolytic flux, but limitations in either nitrogen or phosphorous are required as well as very low levels of certain heavy metals, including iron and manganese. Various media have been described in the literature, and the general requirements for production with *A. niger* can be summarized as follows: high level of a carbon source (60-240g l^{-1}), low level of a nitrogen source (2-3g l^{-1}), low level of a phosphate source (1.5-3.0g l^{-1}), deficiency of manganese (<0.000001g l^{-1}), low level of iron (<0.0013g l^{-1}), low level of zinc (0.00025g l^{-1}), high aeration and low pH (2 or 3).

Beet and sugar cane molasses and glucose syrups are the main carbon sources utilized industrially. The composition of the molasses is variable and testing and pre-treatments are required. The main problem is related to the levels of zinc, iron, magnesium, and manganese. The manganese concentration is of critical importance in the process as it has been demonstrated that this ion regulates several enzymes, including those related to citric acid export. Control of the level of trace metals is achieved by addition of sodium or potassium ferrocyanide. The optimum addition level must be above that needed to chelate the metals; these compounds may also contribute to a certain degree of growth inhibition. Trace metal levels can also be reduced by ion exchange, but this is only

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applicable to substrates with low salt concentrations, such as corn syrups. Optimum mineral combinations and strains resistant to metal level regulation have been pursued.

Either nitrogen or phosphate limitations favor citric acid production. Nitrogen sources such as ammonium sulfate and ammonium nitrate are commonly used in concentrations between 1 to 3g l^{-1}. The type and concentration of the nitrogen source affect cell growth and citric acid formation. Phosphate content is more important in fermentations where the trace metal concentration is not tightly controlled, but low phosphate levels are usually recommended. Magnesium is required both for growth and citric acid production. Other nutrients such as lower alcohols (methanol and ethanol) and lipid materials in vegetable oils have been shown to increase citric acid production. The effect of alcohols is related to trace metal concentrations, and they can be inhibitory when used with purified substrates.

The first industrial process was the surface one, which is still employed in some cases as it is simpler to operate, has lower energy costs than submerged processes and is less sensitive to the trace metals composition. However, it is much more labor intensive. The mycelium is grown as a surface mat in a large number of shallow trays (see *Microbial Cell Culture*). The carbohydrate source is diluted to 15%, pretreated, and the pH is adjusted to between 5 and 7 to allow the spores to germinate. The medium is then sterilized and pumped into the trays. The culture is inoculated with spore suspensions. Air is blown through the fermentation chamber, where temperature is maintained at 30°C and humidity between 40 and 60%. Aeration supplies the oxygen requirements and also removes the heat generated. As the fermentation progresses, the pH decreases to about 2.0. The fermentation is completed in between 7 and 15 days with yields around 70%. The fermented liquor is separated from the mycelium for further processing.

The submerged process is the most commonly used either in stirred tank reactors or tower fermentors. The process is run in batch. Fermentors are equipped with aeration systems capable of maintaining high dissolved oxygen levels, which is critical for high citric acid production. Medium preparation involves the dilution of the carbon source to between 15 and 27% and also the pre-treatment procedures to eliminate manganese and reduce the level of the other critical trace metals. Different combinations of metal concentration and pre-treatments have been described and patented, and there is not a unique set of acceptable conditions. Initial pH values are between 2.5 to 3.0; fermentation temperature is between 28 and 35°C; inoculation is performed with spores or with preculture mycelia in a 1:10 ratio. The characteristics of the inoculum and of the medium influence the culture morphology, which impacts citric acid yield. The formation of small pellets with a smooth and hard surface results in a broth with lower energy requirements and better mass transfer characteristics. The fermentation runs from 5 to 10 days depending on the process.

Fed-batch and even continuous processes have been proposed (see *Microbial Cell Culture*). As accumulation of citric acid is only partially growth associated, the continuous process is less efficient and fed-batch processes do not support significant enough gains to become standard. Because of the dissociation between growth and production, the use of immobilized mycelia has been attempted in several laboratories.

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Mycelium has been immobilized in alginate beads, collagen, and polyurethane foam. Productivity rates are still too low and the process is too cumbersome to be of industrial interest. The increase in the available knowledge about fermentation conditions, and the development of more accurate models that support better controlled fermentations, have resulted in more significant improvements than these process configurations.

The simplest process for the production of citric acid is the solid state one known as Koji process (see *Food Fermentation and Processing*). It was initially developed for the use with solid raw materials (sweet potato fibrous residues, rice, and wheat bran). Several new studies have applied the process to different waste materials such as fruit pomace and sugar cane residues. The carbohydrate source is soaked with water to about 70% moisture, sterilized, placed in trays, and inoculated with conidia of *A. niger*. The starch is hydrolyzed by amylase produced by the fungus and converted to citric acid in from 4 to 5 days. This process is primarily considered to satisfy small demands for citric acid in decentralized economic systems.

2.3.2 Production with Yeasts

Production of citric acid with yeasts is also applied commercially. Yeast cells can tolerate higher initial concentration of sugar, have a higher fermentation rate and are insensitive to metals, which reduces the pre-treatments required. Several processes using different strains have been patented. Production with yeast is always performed in a submerged culture. The original process, patented in 1968, controlled the pH of the fermentation above 5.5 with addition of calcium carbonate. In some later processes, the pH was allowed to fall to values between 3 and 4 after cell growth. Several yeast genera were proposed for citric acid production, with most emphasis in various species of *Candida*. The main disadvantage of the use of yeasts is the formation of isocitric acid as a by-product, but various additives have been tested to reduce its production. The use of halogen-substituted alkanoic mono or di-substituted acids, lead acetate and other substances has been patented, and many mutant strains with reduced isocitrate production have been selected. An osmophilic strain that can convert sugar concentrations as high as 28% without pre-treatment of the molasses has also been patented.

The production of citric acid from n-alkanes was developed as an alternative when hydrocarbons were a cheap carbon source. A process using *Yarrowia lipolytica* was able to achieve more than 200g l^{-1} citric acid using C_{14}-C_{16} n-paraffins. A final pH between 2 and 3 allowed for the recovery of the free acid without the need to produce a salt. The fermentation was completed in about 6 days. The system is not being used commercially because it cannot compete economically with the utilization of carbohydrates.

2.4 Product Recovery

Citric acid cannot be recovered directly from the fermented liquor by crystallization because of excess of impurities. In the classic protocol, citric acid is precipitated as calcium citrate by the addition of lime. The washed precipitate is treated in aqueous

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suspension with H_2SO_4, yielding gypsum as a by-product. The citric acid solution is concentrated by vacuum evaporation and crystallized at low temperatures.

Solvent extraction can also be applied with the advantage of avoiding the formation of gypsum. Different solvents can be used; the extraction step is usually carried out at low temperature and the solvent is stripped with hot water. A mixture of n-octyl alcohol and tridodecylamine has been recommended for citric acid used in food and drug applications.

Some other recovery techniques have been studied and patented. They include the combination of membrane filtration with adsorption resins, the use of different anionic exchange resins and the application of electrodialysis. The main objective of these studies has been the development of economic recovery processes that eliminate the formation of gypsum and reduce the environmental impact of citric acid manufacture.

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Biographical Sketch

Fernando Sanchez Riera received his chemical engineering degree from the Universidad Nacional de Tucumán, Argentina, in 1980. He became a fellow of the National Research Council (CONICET) working at PROIMI (Planta Piloto de Procesos Industriales Microbiológicos) where he started his specialization in fermentation processes, while focusing on anaerobic treatment of agroindustrial wastes for the production of methane. From 1986 to 1988 he was a postdoctoral fellow at the Chemical Engineering Department at MIT (Massachusetts, USA) where his research efforts focused on fermentation control. Upon return to PROIMI as an Assistant Researcher, he worked on ethanol production with Zymomonas and its integration with waste utilization. In 1991, after a short period as a Visiting Scientist at the University of Wisconsin (Wisconsin, USA), he moved to Bio-Technical Resources (Manitowoc, Wisconsin, USA). There he has been involved in the development and optimization of several fermentation processes including the production of lactic acid, a subject on which he co-authored a book chapter, and biotransformations for the production of chemicals by fermentation and enzymatic processes.

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