

BIOTECHNOLOGICAL PRODUCTION OF CITRIC ACID

Belén Max¹; José Manuel Salgado¹; Noelia Rodríguez¹; Sandra Cortés¹; Attilio Converti²; José Manuel Domínguez^{1,*}

¹Department of Chemical Engineering, Sciences Faculty, University of Vigo (Campus Ourense), As Lagoas s/n, 32004 Ourense, Spain; ¹Laboratory of Agro-food Biotechnology, CITI-Tecnópole, Parque Tecnológico de Galicia, San Cibrao das Viñas, Ourense, Spain; ²Department of Chemical and Process Engineering, University of Genoa, Via Opera Pia 15, 16145 Genoa, Italy.

Submitted: April 16, 2010; Approved: May 24, 2010.

ABSTRACT

This work provides a review about the biotechnological production of citric acid starting from the physicochemical properties and industrial applications, mainly in the food and pharmaceutical sectors. Several factors affecting citric acid fermentation are discussed, including carbon source, nitrogen and phosphate limitations, pH of culture medium, aeration, trace elements and morphology of the fungus. Special attention is paid to the fundamentals of biochemistry and accumulation of citric acid. Technologies employed at industrial scale such as surface or submerged cultures, mainly employing *Aspergillus niger*, and processes carried out with *Yarrowia lipolytica*, as well as the technology for recovering the product are also described. Finally, this review summarizes the use of orange peels and other by-products as feedstocks for the bioproduction of citric acid.

Key words: citric acid, fermentation, review, *Aspergillus niger*

INTRODUCTION

Citric acid (2-hydroxy-propane-1,2,3-tricarboxylic acid) derives its name from the Latin word *citrus*, a tree whose fruit is like the lemon. Citric acid is a tricarboxylic acid (Figure 1) with a molecular weight of 210.14 g/mol, which contains three carboxylic functional groups with three different values of pK_a (3.1, 4.7, and 6.4). It is a primary metabolic product formed in the tricarboxylic acid (or Krebs) cycle and is found in small quantities in virtually all plants and animals, being isolated from lemon juice in 1784.

Citric acid was first commercially-produced in England around 1826 from imported Italian lemons (lemons contain 7-9% citric acid). Lemon juice remained the commercial source

of citric acid until 1919, when the first industrial process using *Aspergillus niger* started in Belgium. Currently, the extraction of citric acid is limited to some small factories in Mexico and Africa.

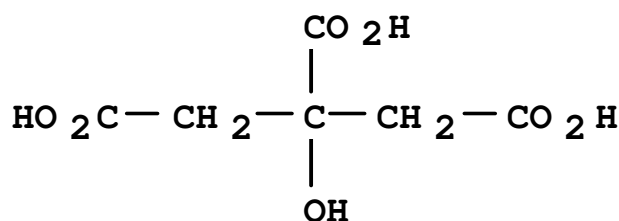


Figure 1. Chemical structure of citric acid.

*Corresponding Author. Mailing address: Department of Chemical Engineering, Sciences Faculty, University of Vigo (Campus Ourense), As Lagoas s/n, 32004 Ourense, Spain.; E-mail: jmanuel@uvigo.es

Citric acid was synthesized from glycerol by Grimoux and Adams (21) and later from symmetrical dichloroacetone. Other routes have been published from various synthetic materials since then, but chemical methods have so far proved uncompetitive.

Wehmer (106) was the first to demonstrate that *Citromyces* (now *Penicillium*) accumulated citric acid in a medium containing sugar and inorganic salts. Since then, many organisms have been found to accumulate citric acid: *A. niger*, *Aspergillus awamori*, *Aspergillus nidulans*, *Aspergillus fonsecaeus*, *Aspergillus luchensis*, *Aspergillus phoenicus*, *Aspergillus wentii*, *Aspergillus saitoi*, *Aspergillus flavus*, *Absidia* sp., *Acremonium* sp., *Botrytis* sp., *Eupenicillium* sp., *Mucor piriformis*, *Penicillium janthinellum*, *Penicillium restrictum*, *Talaromyces* sp., *Trichoderma viride* and *Ustilina vulgaris* (73).

Currie (15) found that some strains of *A. niger* were able to grow in a medium containing sugars and salts at an initial pH of 2.5-3.5. Throughout their growth, these strains excreted large amounts of citric acid, which established the basis for industrial production.

Besides fungi, it is known that several yeasts produce citric acid from *n*-alkanes and carbohydrates (63), especially species belonging to the genera *Candida*, *Hansenula*, *Pichia*, *Debaromyces*, *Torula*, *Torulopsis*, *Kloeckera*, *Saccharomyces*, *Zygosaccharomyces* and *Yarrowia*. During the '60s and '70s oil was cheap, and citric acid was produced industrially from this source by *Candida* sp., including *C. tropicalis*, *C. catenula*, *C. guilliermondii* and *C. intermedia* (73). Today, this production is not economical. As a disadvantage, the fermentation by yeasts led to the formation of large quantities of isocitric acid as an unwanted byproduct, so mutant strains with low aconitase activity should be used.

Although many microorganisms can be employed to produce citric acid, *A. niger* is still the main industrial producer. In fact, specific strains that are able to overproduce citric acid in different types of fermentation processes have been developed. The theoretical yield is 112 g of anhydrous citric acid per 100 g of sucrose. However, in practice, due to

losses during trophophase, the yield of citric acid from these strains often does not exceed 70% of the theoretical yield on carbon source. Despite a long and successful history of producing citric acid, there is not unanimous explanation of the biochemical basis of the process.

FACTORS AFFECTING CITRIC ACID FERMENTATION

The conditions for citric acid fermentation were established during the '30s and '40s when the effects of various components of the fermentation media were evaluated.

The accumulation of citric acid is strongly influenced by the composition of the medium, especially in submerged fermentation processes. However, with the exception of early studies by Currie (15), there were no other systematic studies on the composition of the medium until the 40s (92, 93). These authors developed a medium that was the basis for further research on the production of citric acid. It was shown that the factors mainly affecting the citric fermentation are the type and concentration of carbon source, nitrogen and phosphate limitation, pH, aeration, oligoelements concentration, and morphology of the producing microorganism. Certain nutrients have to be in excess (such as sugars, protons or oxygen), other at limiting levels (such as nitrogen and phosphate) and others below well-established threshold values (such as trace metals, particularly manganese).

Carbon source

The carbon source for citric fermentation has been the subject of many studies, especially regarding the use of polysaccharides. In general, only the sugars that are quickly assimilated by the microorganism allow high final yield of citric acid (62).

Polysaccharides are a useful raw material for fermentation only if the microorganism possesses hydrolytic enzymes highly effective at the low pH values required for fermentation. In general, sucrose is preferable to glucose (24, 30, 42, 110), as *A. niger* has an extracellular mycelium-bound invertase that is

active at low pH.

The most widely used carbon sources in industrial fermentations are glucose syrups from starch hydrolysis, sugar beet molasses and low quality-sugarcane byproducts that, in general, are contaminated by high levels of cations from previous processes. Cations usually come from insoluble residues formed by precipitation with potassium ferrocyanide. Due to the complexity of these pretreatments, a lot of research has been conducted using refined sugars, mainly glucose or sucrose.

The concentration of carbon source is also crucial for citric fermentation. The final yield of citric acid increases with initial sugar concentration in batch processes or glucose feeding rate in chemostat, while the specific growth rate has an opposite behaviour (29, 76, 79, 80, 92, 93, 110). The highest productivities are usually achieved using 14-22% sugar, because such high concentrations of the carbon source lead to suppression of α -ketoglutarate dehydrogenase (30). On the other hand, at low glucose levels the size of the mycelium is reduced, and its shape is also affected (76, 79, 80).

Nitrogen and phosphate limitations

Some complex media (such as molasses) are rich in nitrogen and rarely need to be supplemented with a nitrogen source. The highly-pure media used in laboratory scale-research are usually supplemented with ammonium salts, particularly ammonium nitrate and sulfate, which in turn leads to a decrease in pH that favors fermentation (62). Other sources of nitrogen such as urea and yeast/malt extract have also been employed successfully (110).

Conflicting reports have also been cited regarding the effects of nitrogen and phosphate limitation. According to Shu and Johnson (92, 93), phosphate needs not be limiting for citric acid production; however, when metal levels are not limiting, additional phosphate leads to secondary reactions and promotes biomass growth (76, 79, 80). However, Kubicek and Rohr (40) demonstrated that citric acid accumulated when phosphate was limiting, even when nitrogen was not. Instead, Kristiansen and Sinclair (37), using a continuous culture, came to the

conclusion that the limitation by nitrogen is essential for the production of citric acid.

pH of culture medium

The pH of the medium is important in two stages of the process. All fermentations start from spores and their germination requires $\text{pH} > 5$. The absorption of ammonia by germinating spores causes release of protons, thus lowering the pH and improving the production of citric acid. The low pH value during the production phase ($\text{pH} \leq 2$) reduces the risk of contamination by other microorganisms and inhibits the production of unwanted organic acids (gluconic and oxalic acids), which makes the product recovery easier.

Aeration

Industrial producers of citric acid have long known that variations in the rate of aeration can have a detrimental effect on performance. If the aeration rate is too high, the partial pressure of dissolved CO_2 in the broth may be too low. Carbon dioxide is important as a substrate for pyruvate carboxylase that replenishes the supply of oxaloacetate for citrate synthase. Sufficient CO_2 is produced by the reaction catalyzed by pyruvate decarboxylase, but excessive aeration leads to some losses. On the contrary, high levels of CO_2 in the gas are detrimental for the final concentrations of citrate and biomass (65). The effect of dissolved O_2 has been studied in some detail. Even short periods of reduced O_2 tension cause irreversible changes in the citric acid productivity (44).

Trace elements

A. niger requires certain trace metals for growth (62). However, a limitation by other trace elements is necessary for citric acid production (92, 93), especially in the submerged fermentation. The metals that should be in limiting concentrations are Zn, Mn, Fe, Cu and heavy metals.

Shu and Johnson (92) demonstrated that the optimal levels of Zn and Fe are 0.3 and 1.3 ppm, respectively. Whereas Clark *et al.* (11) observed that Mn concentrations as low as 3 mg/l drastically reduced the citric acid yield, Matthey and Bowes (64)

reported that the addition of 10 mg/l Mn^{2+} leads to a reduction in citric acid accumulation by 50%. Researches by Clark *et al.* (11) and Kissler *et al.* (35) confirmed the regulatory role of Mn^{2+} ions. The anabolism of *A. niger* is affected by manganese deficiency and/or limitation by nitrogen and phosphate. The breakdown of proteins under Mn deficiency results in high intracellular concentration of ammonium, which contrasts the citric acid inhibition of phosphofructokinase and hence glycolysis. Moreover, the combination of high concentrations of glucose and ammonium represses the synthesis of α -ketoglutarate dehydrogenase, hence inhibiting the catabolism of citric acid in the Krebs cycle and favoring its overproduction.

Manganese has also been proven important in many cell functions, particularly in the cell wall synthesis, sporulation and production of secondary metabolites (93). Therefore, great care must be taken when choosing the ingredients of the medium, including the bioreactor construction materials, to ensure that traces of manganese do not reduce the yield of fermentation.

Morphology of the fungus

The relationship between morphology of the fungus and productivity in submerged fermentations has been established long time ago. Despite disagreement on whether pellets or filamentous forms are more convenient for citric acid production, in all cases, the mycelium of acidogenic *A. niger* contains branches of short hyphae with swollen tips (94).

In submerged cultures, the morphology of fungi varies between pellets and free filaments, depending on growth conditions and genotype. The discussion of the factors influencing the morphology of *A. niger* in submerged cultures has to distinguish between macro and micro-morphology. Contrarily to Moyer (68), Snell and Schweiger (94) and Takahashi *et al.* (99) reported that citric acid production requires the formation of compact aggregates or pellets (< 0.5 mm in diameter). Image analysis studies showed that, under appropriate conditions, yields exceed 85% when the macro-morphological form is constituted by stable particles of

intertwined filamentous around a small nucleus, while the micro-morphology is characterized by branches of short hyphae with swollen tips (76, 77, 78, 79, 80).

The main factors affecting the morphology of *A. niger* in submerged cultures and then influence the process performance are the agitation intensity, medium pH, growth rate, nutritional factors and inoculum type and concentration.

Although intense agitation leads to the development of short, thick and highly branched filaments, excess shear stress can cause fragmentation of filaments, and the new fragments generated give rise to new filaments overproducing citric acid (80). The preferred morphology (i.e., small aggregates of short filaments) associated with an increase in production was obtained at pH values around 2.0 ± 0.2 . At pH 1.6, the morphological development was abnormal and production fell sharply, while at pH 3.0 aggregates had longer perimeters and oxalic acid formation was observed (76).

Regarding those trace metals that greatly affect the fermentation, only the concentration of manganese has been shown to affect the morphology of *A. niger*. Manganese ions in concentration below 10^{-7} M resulted in an abnormal morphological development (35), while the addition of 30 mg/l of Mn to a Mn-free medium led to morphological changes accompanied by a 20% reduction in citric acid yield (76). Thus, it is evident that optimum citric acid production is only possible within a strict Mn concentration range.

The spore inoculum level is another parameter that influences the citric acid fermentation. Papagianni and Mattey (75) found, through digital image analysis, a clear transition from pellets to dispersed forms of *A. niger* by varying the inoculum level from 10^4 to 10^9 spores/ml.

BIOCHEMISTRY OF CITRIC ACID PRODUCTION

To review the biochemistry of citric acid formation is an immense task. Shortly, it can be said that the overproduction of citric acid requires a unique combination of unusual nutritional conditions (excess of carbon source, hydrogen ions and dissolved oxygen, and suboptimal concentrations of certain

trace metals and phosphate), which synergistically influence the fermentation performance (36).

According to Habison *et al.* (26) and Rohr and Kubicek (87), a deficiency of manganese, or phosphate and nitrogen limitation, inhibits the *A. niger* anabolism, and the resulting degradation of proteins leads to increased ammonium ion concentration. This increase is able to counterbalance the inhibition exerted by citric acid on phosphofructokinase (26), being a positive end-effector (6, 25). High concentrations of NH_4^+ and glucose also repress the synthesis of α -ketoglutarate dehydrogenase, inhibiting the citric acid catabolism via the Krebs cycle, leading to its accumulation (87). Therefore, one of the reasons for the accumulation of citric acid is the result of high speed flow of income and a reduction in outflow velocity (Figure 2). But, other observations appear to contradict these assumptions (81).

Other smaller issues are of interest to other enzymes: invertase (9, 89), hexokinase (91, 97), glucose oxidase (18, 28, 67, 86), phosphofructokinase (26, 32, 46, 55, 62), other enzymes of the pentose phosphate pathway (56), pyruvate kinase (16, 17, 66, 90, 100, 101), and citrate synthase (41, 58).

An important aspect concerns the need that the Krebs cycle can be completed to support the continuous production of citric acid. To address the lack of cycle intermediates consequent to the metabolic dysfunction responsible for the accumulation of citric acid, pyruvic acid produced from glucose is not only decarboxylated to acetyl-CoA by the pyruvate dehydrogenase complex, but it is also partially carboxylated to oxaloacetic acid during the idiophase (45), by the action of pyruvate carboxylase (8, 13, 19, 107, 108). This reaction, which requires ATP and is dependent on K^+ and Mg^{2+} , is not the only anaplerotic reaction used to replenish the Krebs cycle. Depending on the organism, more oxaloacetic acid can be produced from phosphoenolpyruvic acid and CO_2 by phosphoenolpyruvate carboxykinase, phosphoenolpyruvate carboxytransphosphorylase, and phosphoenolpyruvate carboxylase, and from pyruvic acid by the NADPH-dependent malic enzyme (60).

Finally, it must be remembered that breaking of isocitric acid by isocitrate lyase is the first reaction of glyoxylic acid cycle, a pathway that leads to the synthesis of L-malic acid from isocitric acid and acetyl-CoA, via oxalic acid, a toxic byproduct (69) that can accumulate under suboptimal conditions. Kubicek *et al.* (43) demonstrated that this pathway, which helps to restore the Krebs cycle intermediates, is poorly active from sugars, meanwhile isocitrate lyase and malate synthase, the second enzyme of the glyoxylic acid cycle, are induced when acetate or *n*-alkanes are used as carbon sources (60).

ACCUMULATION OF CITRIC ACID

It has been proposed that the accumulation of citric acid requires deactivation of the Krebs cycle enzymes responsible for its degradation, aconitase and/or isocitrate dehydrogenase.

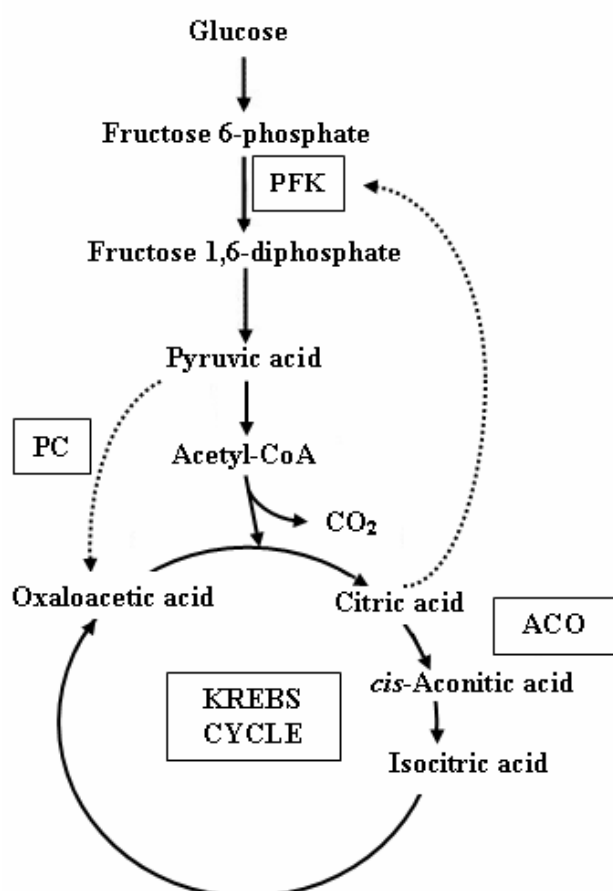


Figure 2. Schematic representation of the main metabolic reactions involved in the production of citric acid by *A. niger* (Manzoni, 2006). PFK = phosphofructokinase, PC = pyruvate carboxylase, ACO = aconitase.

But there are evidences that during the production of citric acid, the Krebs cycle is active in the production of intermediates required for biomass formation (3, 32). Therefore, as stressed by Kubicek (39), citric acid accumulation may more likely be the result of enhanced (deregulated) biosynthesis rather than inhibited degradation.

An alternative hypothesis to explain the accumulation of citric acid is associated to tricarboxylate transporter activity (38), which competes with aconitase for citric acid. Under conditions in which its affinity for citric acid is greater than that of aconitase, this enzyme ejects citric acid out of the mitochondria without inhibition of enzymes of the cycle.

Finally, there are three different isozymes of isocitrate dehydrogenase in *A. niger*, a NAD^+ -dependent form present in mitochondria at low concentrations, and two NADP^+ -dependent forms, one in the mitochondria and the other in the cytoplasm (40, 51). All require Mg^{2+} or Mn^{2+} and the NADP^+ -dependent form is inhibited by citric and α -ketoglutaric acids (40, 61), which ensures the accumulation of citric acid.

INDUSTRIAL PRODUCTION OF CITRIC ACID

About 99% of world production of citric acid occurs via microbial processes, which can be carried out using surface or submerged cultures. The product is sold as an anhydrous or monohydrate acid, and about 70% of total production of 1.5 million tons per year (52) is used in food and beverage industry as an acidifier or antioxidant to preserve or enhance the flavors and aromas of fruit juices, ice cream, and marmalades. 20% is used, as such, in the pharmaceutical industry as antioxidant to preserve vitamins, effervescent, pH corrector, blood preservative, or in the form of iron citrate as a source of iron for the body as well as in tablets, ointments and cosmetic preparations. In the chemical industry, which uses the remaining 10%, is employed as a foaming agent for the softening and treatment of textiles. In metallurgy, certain metals are utilized in the form of citrate. Citric acid is also used in the detergent industry as a phosphate substitute, because of less eutrophic effect, and in the cement one to slow down the

hardening of cement.

Although many strains of bacteria produce citric acid, only a few mutants of *A. niger* and *A. wentii*, which are closely related species, are used for industrial production.

All enzymes are expressed during the idiophase, except α -ketoglutarate dehydrogenase. The activity of citrate synthase increases by 10 times, whereas those of the aconitase and isocitrate dehydrogenase are reduced slightly compared to the trophophase. During the trophophase, glucose is mainly used for biomass production and oxidized to CO_2 via respiration, whereas during the idiophase losses by breath are minimal and the substrate is almost entirely converted to organic acids.

The carbon sources employed in industrial fermentation are sugars in solutions at concentrations ranging from 15 to 25%. Since the microorganisms used industrially have amylases and invertases, which make them capable of hydrolyzing polysaccharides and sucrose, simultaneous saccharification and fermentation processes can be carried out (2) using as substrates potato starch, sugarcane and sugarbeet molasses, among other residues (7). If glucidic hydrolysates are used as culture medium, a pretreatment with precipitating agents or ion exchange resins is necessary to remove cations and then promote the metabolic dysfunction responsible for citric acid accumulation. Alternatively, molasses are often treated with calcium hexacyanoferrate to precipitate heavy metals.

Strains are selected according to the carbon source. To evaluate the optimal cultivation conditions for molasses, no general methods of analysis have been developed; hence, any work cycle should be preceded by a preliminary test of fermentation. Companies using molasses often optimize fermentation conditions in 30 m³ pilot plant before moving to production.

Yields can be maximized using Cu^{2+} , Mg^{2+} , Mn^{2+} , Fe^{2+} , Zn^{2+} and Mo^{2+} ions in concentrations in the order of ppm, above which the process is negatively affected. For instance, iron, one of the cofactors of aconitase, plays a crucial role, favoring biomass growth at concentrations higher than 2 ppm, or citric acid overproduction at concentrations of only 0.05-0.5

ppm depending on the substrate. Interestingly, Cu reverses the effect of Fe. Anyhow, sensitivity of microorganisms decreases with decreasing temperature.

pH is another crucial parameter, which is set at around 5 at the beginning of the trophophase, drops to 3 within the first 48 h of trophophase as a result of the nitrogen metabolism, and is then kept at this value during the idiophase to inhibit the formation of oxalic and gluconic acids.

Surface cultures

Citric acid can be produced in surface or submerged cultures. The simple surface culture methods used during the first commercial production required intensive labor. Although submerged culture techniques were developed before 1940, some plants of the first type are apparently still working (62).

According to the substrate employed, surface methods are divided into solids and liquids, the latter being of higher economic importance.

Surface liquid cultures are usually carried out batchwise, using fermentation broths prepared from wheat bran, potato starch, glucose syrup or molasses in concentrations around 160 g/l. With this technology *A. niger* strains are less sensitive to oligoelements. The first attempts were made using surface liquid nutrients that still provide 20% of world production, even by some major producers of citric acid (39). Despite these plants being less sensitive to interference by trace metal ions and variations in the dissolved oxygen tension and requiring low capital investment, low energy for cooling and relatively simple technology (39), their maintenance cost is higher, compared to submerged processes, due to the heavy labor required for cleaning pipes, trays and reactor walls.

After steam sterilization, the nutrient medium contained in aluminum trays to a depth of 3-200 cm, depending on their size (39, 59), is inoculated at 30-40 °C by injecting $2\cdot5\cdot10^7$ dry spores/m² or by spraying a spore suspension, and is then incubated at 28-30 °C (60). 24 hours after inoculation, germinated spores form a continuous mycelium on the surface and pH drops from 6.0-6.5 to 1.5-2.0. Temperature is usually maintained constant by a stream of sterile air, since avoiding

contamination is particularly important in surface fermentations. Such a ventilation is also important to remove CO₂, which otherwise would inhibit the production of citric acid in concentrations higher than 10% while nutrient solutions are sparged through a distribution system on trays. To accelerate the growth, α -amylase can be added, even though it is not strictly necessary.

The idiophase begins after about 30 hours. If too much iron is present, recovery can be hampered due to the formation of oxalic acid and yellow pigments. Temperature is kept constant thanks to the evaporation of the solvent and the volume is reduced by 30-40%. The total duration of the cycle is 7-15 days (39) and the daily production 1.2-1.5 kg citric acid monohydrate/m², which corresponds to a yield of 70-80%, while heat release is 12,500 kJ/g product (60). For recovery, the mycelium must be washed thoroughly with boiling water and eventually pressed.

Submerged cultures

The industrial production of citric acid in submerged culture was already reviewed in detail (14, 39, 59, 60). Depending on the type of agitation, submerged processes (74) can be divided in mechanically mixed or agitated by air circulation. Most of them are carried out batchwise in conventional or airlift fermenters with 120-250 m³ or 900 m³ volume, respectively.

Spores germinate at 32 °C in a prefe fermenter, using a molasses solutions containing 150 g/l of sugar in the presence of cyanide ions to induce the formation of mycelium in granular form. Addition of cyanide in insufficient amount favors biomass growth, but affects the subsequent production of citric acid. Granules with 0.2-0.5 mm diameter are formed within 24 hours and then used for the inoculum (10% v/v); after this period the pH drops to 4.3 (29, 60).

The mode of production of spores and the morphology of the mycelium during the trophophase largely influence the conversion rate, the efficiency and then the success of the process. If the mycelium is thin and wiry, with few branches and no chlamydospores, citric acid is hardly produced during

idiophase. Pellets of sufficient consistency (1 mm in diameter) must be formed to increase citric acid production, and this can be done acting also on the Fe/Cu ratio in the culture medium.

80% of world production of citric acid is obtained by submerged cultures, which have the advantage of lower investment and maintenance costs compared with surface cultures. The disadvantages are the high cost of energy and a more sophisticated technology, which in turn requires specialized staff. The main substrates are solutions of sucrose, molasses or glucose from cornstarch with 160 g/l of total sugars, 25% of which are consumed during the trophophase (pH 5 and duration of 20-40 h) and 75% during the idiophase (pH 2-3 and duration of 4-6 days). For best control, the two phases can be separated by lowering the pH below 2 after the end of growth. Another advantage of the strong acidity is the low risk of contamination.

Two additional factors are of particular importance for citric acid production in submerged cultures: the type of material used to build the fermenter and the aeration. Fermenters must be resistant to acidity and are made of stainless steel, because ordinary steel can be dissolved at pH 1-2, inhibiting the fermentation. In fermenters with capacity less than 1 m³, due to increased surface/volume ratio, corrosion becomes significant, and even steel chambers must be protected with a layer of plastic material.

Because *A. niger* is quite unstable genetically, only a small number of stages can be used for the development of the inoculums, and in some applications the spores are even inoculated directly into the fermenter. *A. niger* requires relatively little oxygen, but it is very sensitive to its total absence, and even brief interruptions can cause an immediate cessation of production. A rate of aeration of 0.2-1 vvm is often used during the production phase to avoid dissolved oxygen levels lower than 20-25% of the saturation concentration. Thanks to the low viscosity of cultures, in some cases no mechanical agitation is used or airlift reactors are employed.

Compared with surface cultures, submerged cultures are somewhat less sensitive to changes in the composition of

media, which is an advantage when using molasses having a highly variable composition. On the other hand, a typical problem of submerged cultures is the formation of foam, which can be avoided using antifoam agents and chambers with volume of up to one third of the total fermenter volume. The most common antifoams are animal or vegetable fats or mechanical systems. The final yield after 7 to 10 days is 70-90%, corresponding to 110-140 g/l of citric acid and 10-15 g/l of dry biomass (39, 60).

Process with yeasts from hydrocarbons

In addition to *A. niger*, several yeasts, among which *Candida catenula*, *Candida guilliermondii*, *Yarrowia lipolytica* and *Candida tropicalis*, have been described that form large amounts of citric acid from *n*-alkanes or –even though with lower yield– from glucose (39). When this process was successfully used before the world oil crisis of 1973/1974, the culture medium usually consisted of C9-C23 *n*-alkanes in a concentration of 100 g/l, and the best results were obtained with *Y. lipolytica* in batch (duration of 5-6 days) or fed-batch submerged systems at pH 5.0-6.5 and 1.0-1.5 vvm aeration (60). The yield is higher than with carbohydrates (1.30-1.45 grams of acid per gram of paraffin) (14), but there are difficulties associated with low water solubility of alkanes and the presence of up to 50% of isocitric acid among the products, which has to be separated during recovery (59). Therefore, mutants with very low aconitase activity were selected using monofluoroacetate resistance as a selection criterion (39).

BIOPRODUCTION OF CITRIC ACID FROM INDUSTRIAL BY-PRODUCTS

Its low cost, high carbohydrate content and high susceptibility to fermentation make the processing of citrus by-products an attractive, environmentally-friendly, alternative way for the biotechnological production of chemicals (23).

The recombinant strain *Escherichia coli* KO11 was shown to ferment arabinose and galacturonic acid obtained from enzymatic hydrolysates of orange peel to acetic acid, ethanol, carbon dioxide and small amounts of lactic acid (10, 72). Due

to the presence of inhibitors such as limonene, the fermentation of these hydrolysates by the yeast *Saccharomyces cerevisiae* did not give interesting results (53, 54, 70, 98, 105). After removal by filtration, the yeast was shown to ferment well only hexoses such as glucose, fructose and galactose released by enzymatic hydrolysis of the polysaccharides contained in the peel (22), but not pentoses (arabinose and xylose) or galacturonic acid (96).

Liquors extracted from orange peel have been used as substrates for citric acid production by fungi (12, 50). Aravantinos-Zafiris *et al.* (5) utilized to this purpose three different, commercially-available strains of *A. niger*, specifically NRRL 599, 364 and 567, being the first one the best producer of citric acid. The most favorable conditions for citric acid production by *A. niger* NRRL 599 resulted to be pH 5.8 in the presence of 40 ml/kg of methanol, without additional nutrients. Under these conditions, the concentration of citric acid after 12 days was 30 g of citric acid per kg of liquor from orange peels washing, corresponding to a yield of 630 g/kg based on total sugars consumed. The yield increased up to 730 g/kg when the liquor was previously subjected to a thermal pretreatment.

The yields obtained with this substrate are comparable or even higher than those obtained using other raw materials such as oil, starch and molasses (95). These authors reported a citric acid concentration of 27 g/l with a yield of 45% when using wood hemicelluloses hydrolysates, while Adham (1) reached a maximum concentration of 8.6 g/l (9.8% conversion) using beet molasses.

Zhang (111) used solid wastes from a factory producing orange juice as a substrate for citric acid production by *A. niger* 999. It was necessary to add again 2% of methanol to the culture broth, and the fermentation lasted 4 days at 30 °C. Kang *et al.* (33) produced citric acid from tangerine skins using *A. niger*, finding the highest production in semisolid state cultures. Furthermore, the production of citric acid was enhanced by the addition of 0.2% NH_4NO_3 , 0.1% of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 2.5% methanol or 1.5% of ethanol. Under optimal conditions, the maximum yield of citric acid was

80.4% of the theoretical value.

Kumagai *et al.* (47), who studied the factors influencing the semisolid citric acid production by *A. niger* from dry and concentrated liquid tangerine wastes, found that the fermentation of the former residue required the addition of molasses juice to face the negative effect of large amounts of impurities. Thus, the maximum concentration of citric acid was achieved in a medium consisting of 6 g of tangerine skin and 11 ml molasses juice (14% sugar content) that ensured fermentation yields in the range 55-65% at 30 °C after 3 days. Hang *et al.* (27), using the skin of kiwi and *A. niger* NRRL 567, produced about 100 grams of citric acid/kg in the presence of 2% methanol at 30 °C after 4 day-solid-state fermentation, with a yield higher than 60% based on consumed sugars.

Vandenberghe *et al.* (104), who evaluated three different agro-industrial wastes, specifically sugarcane bagasse, coffee husk and cassava bagasse, to produce citric acid by a culture of *Aspergillus niger*, found the highest CA yield (88 g/kg dry matter) when the fermentation was carried out with cassava bagasse as a substrate. Finally, Flores *et al.* (20), after 5 day-solid-state fermentation of dry prickly pear skin by *A. niger*, achieved a maximum production of 380 g of monohydrate CA per kg at 30 °C and 86% humidity, corresponding to a yield on initial sugars of 68% and to a maximum volumetric productivity of 0.539 g/lh.

Methanol can stimulate citric acid fermentation. Wastes from pineapple shells were also used to produce citric acid by *A. niger* ATCC 1015 (57). The highest production (132 g/kg of residue) was achieved after 6 days of fermentation at 29 °C in medium containing 4% methanol. This residue was also employed by Tran *et al.* (102) with three strains of *A. niger* and *Aspergillus foetidus*. Among these, *A. niger* ACM 4992 ensured the highest citric acid production (194 g/kg), corresponding to a yield of 0.74 g citric acid/g sugar consumed. The optimal conditions were 65% initial moisture, 3% methanol, 30 °C, unadjusted initial pH of 3.4, a particle size of 2 mm and 5 ppm of Fe^{2+} . Citric acid production was carried out more effectively by using Erlenmeyer flasks in batch fermentations, while yields decreased when trays or rotating

drums bioreactors were used. The same stimulating effect of methanol was observed by Kumar *et al.* (48, 49) using a mixture of different fruit wastes and bagasse for *A. niger* DS1 solid-state fermentation. The use of 4% methanol (v/w) was also reported for the submerged fermentation of orange peel hydrolysates (84) and the solid-state fermentation of sugarcane molasses (85), that led to citric acid productions of 9.2 g/l and 445.4 g/kg, respectively. Roukas (88) observed an increase in citric production from 176 to 264 g/kg when the concentration of methanol was increased up to 6% (w/w) in dry pod solid-state fermentation. Although the role of methanol stimulation is not completely known, it was related to a) removal of the adverse effect of trace metals (84), b) changes in the mycelium morphology consequent to variations in phospholipid composition (83), c) increase in cell membrane permeability, thereby making the excretion of citric acid easier (111), or d) some influence on growth and sporulation (31).

Conversely, some authors observed detrimental effects when methanol was added, since the microorganism is not able to assimilate it. For instance, CA production by solid-state fermentation of kiwifruit peel by *A. niger* ATCC 9142 significantly decreased after supplementation with 0.74 mmol methanol/l (27), and Tsay and To (103) reported that methanol inhibited both *A. niger* TMB 2022 growth and CA production. Similar findings were reported by other authors (4, 71, 109).

CITRIC ACID RECOVERY

The first step of citric acid recovery involves the precipitation of oxalic acid, possibly in the form of calcium oxalate at low pH, and subsequent separation from the medium containing the mycelium through rotating filters or centrifuges. Citric acid is then precipitated at pH 7.2 and 70-90 °C and recovered by filtration and drying. If a purer product were desired, it is dissolved with sulfuric acid, treated with charcoal or ion exchange resins, and again crystallized as anhydrous citric acid (above 40 °C) or as a monohydrate (below 36.5 °C) (60). In surface processes, the mycelium is sometimes squeezed to increase the recovery yield.

Pinacci and Radaelli (82) have proposed a process of electrodialysis with bipolar membranes for the recovery of citric acid from fermentation media, while Kılıç *et al.* (34) an extractive fermentation, in which the steps of citric acid production by *A. niger* and separation occur simultaneously, using corn oil and Hostarex A327 in oleic alcohol.

In the process carried out with *Y. lipolytica*, the recovery of citric acid after filtration of the exhausted medium includes the precipitation of oxalic acid at low pH in form of calcium salt followed by citric acid precipitation at pH 7.2 and 70-90 °C.

REFERENCES

1. Adham, N.Z. (2002). Attempts at improving citric acid fermentation by *Aspergillus niger* in beet-molasses medium. *Bioresour Technol.* 84, 97–100.
2. Aghdam, M.G.; Taherzadeh, M. (2008). Production of citric acid by solid state fermentation. *J Biotechnol.* 136, S464.
3. Ahmed, S.A.; Smith, J.E.; Anderson, J.A. (1972). Mitochondrial activity during citric acid production by *Aspergillus niger*. *Trans. Br. Mycol. Soc.* 59, 51–61.
4. Ali, S.; Ashraf, H.; Ikram, U. (2002). Enhancement in citrate production by alcoholic limitation. *J. Biol. Sci.* 2, 70-72.
5. Aravantinos-Zafiris, G.; Tzia, C.; Oreopoulou, V.; Thomopoulos, C. D. (1994). Fermentation of orange processing wastes for citric acid production. *J. Sci. Food Agric.* 65, 117–120.
6. Arts, E.; Kubicek, C.; Röhr, M. (1987). Regulation of phosphofructokinase from *Aspergillus niger*: effect of fructose-2,6-bisphosphate on the action of citrate, ammonium ions and AMP. *J. Gen. Microbiol.* 133, 1195–1199.
7. Bizukojc, M.; Ledakowicz, S. (2004). The kinetics of simultaneous glucose and fructose uptake and product formation by *Aspergillus niger* in citric acid fermentation. *Proc. Biochem.* 39, 2261-2268.
8. Bloom, S.J.; Johnson, M.J. (1962). The pyruvate carboxylase of *Aspergillus niger*. *J. Biol. Chem.* 237, 2718–2720.
9. Boddy, L.M.; Berges, T.; Barreau, C.; Vainstain, M.H.; Johnson, M.J.; Ballance, D.J.; Peberdi, J.F. (1993). Purification and characterisation of an *Aspergillus niger* invertase and its DNA sequence. *Curr. Genet.* 24, 60–66.
10. Burchhardt, G.; Ingram, L.O. (1992). Conversion of xylan to ethanol by ethanologenic strains of *Escherichia coli* and *Klebsiella oxytoca*. *Appl. Environ. Microb.* 58 (4), 1128-1133
11. Clark, D.S.; Ito, K.; Horitsu, H. (1966). Effect of manganese and other heavy metals on submerged citric acid fermentation of molasses.

- Biotechnol. Bioeng.* 8, 465–471.
12. Clark, D.S.; Lentz, C.P. (1961). Submerged citric acid fermentation of sugar beet molasses: effect of pressure and recirculation of oxygen. *Can. J. Microbiol.* 7, 447–445.
 13. Cleland, W.W.; Johnson, M.J. (1954). Tracer experiments on the mechanism of citric acid formation by *Aspergillus niger*. *J. Biol. Chem.* 208, 679–692.
 14. Converti, A. (2009). *Aprovechamiento de residuos agroindustriales para la generación de aditivos alimentarios: Tratamiento de las materias primas y estudios metabólicos*. Vigo, Spain, 240p. (PhD Dissertation. Faculty of Sciences. Vigo University).
 15. Currie, J.N. (1917). The citric acid fermentation of *A. niger*. *J. Biol. Chem.* 31, 5.
 16. De Graaff, L.; van den Broeck, H.; Visser, J. (1988). Isolation and characterisation of the pyruvate kinase gene of *Aspergillus nidulans*. *Curr. Genet.* 13, 315–321.
 17. De Graaff, L.; van den Broeck, H.; Visser, J. (1992). Isolation and characterisation of the *Aspergillus niger* pyruvate kinase gene. *Curr. Genet.* 22, 21–27.
 18. Dronawat, S.N.; Svihla, C.K.; Hanley, T.R. (1995). The effects of agitation and aeration on the production of gluconic acid by *Aspergillus niger*. *Appl. Biochem. Biotechnol.* 51/52, 347–354.
 19. Feir, H.A.; Suzuki, I. (1969). Pyruvate carboxylase of *Aspergillus niger*: kinetic study of a biotin-containing enzyme. *Can. J. Biochem.* 47, 697–710.
 20. Flores, J.L.; Gutierrez-Correa, M.; Tengerdy, R.P. (1994). Citric acid production by solid state fermentation of prickly pear peel with *Aspergillus niger*. *Agro-Food-Industry Hi-Tech* 5(1), 18–20.
 21. Grimoux, E.; Adams, P. (1880). Synthèse de l'acide citrique. *C. R. Hebd. Seances Acad. Sci.* 90, 1252.
 22. Grohmann, K.; Cameron, R.G.; Buslig, B.S. (1995). Fermentation of sugars in orange peel hydrolyzates to ethanol by recombinant *Escherichia coli* KO11. *Appl. Biochem. Biotechnol.* 51/52, 423–35.
 23. Grohmann, K.; Cameron, R.G.; Buslig, B.S. (1995). Fractionation and pretreatment of orange peel by dilute acid hydrolysis. *Bioresour Technol.* 54, 129–141.
 24. Gupta, J.K.; Heding, L.G.; Jorgensen, O.B. (1976). Effect of sugars, hydrogen ion concentration and ammonium nitrate on the formation of citric acid by *Aspergillus niger*. *Acta Microbiol. Acad. Sci. Hung* 23, 63–67.
 25. Habison, A.; Kubicek, C.P.; Röhr, M. (1979). Phosphofructokinase as a regulatory enzyme in citric acid accumulating *Aspergillus niger*. *FEMS Microbiol. Lett.* 5, 39–42.
 26. Habison, A.; Kubicek, C.P.; Röhr, M. (1983). Partial purification and regulatory properties of phosphofructokinase from *Aspergillus niger*. *Biochem. J.* 209, 669–676.
 27. Hang, Y.D.; Luh, B.S.; Woodams, E.E. (1987). Microbial production of citric acid by solid state fermentation of kiwifruit peel. *J. Food Sci.* 52, 226–227.
 28. Hayashi, S.; Nakamura, S. (1981). Multiple forms of glucose oxidase with different carbohydrate compositions. *Biochim. Biophys. Acta* 657, 40–51.
 29. Honecker, S.; Bisping, B.; Yang, Z.; Rehm, H.J. (1989). Influence of sucrose concentration and phosphate limitation on citric acid production by immobilized cells of *Aspergillus niger*. *Appl. Microbiol. Biotechnol.* 31, 17–24.
 30. Hossain, M.; Brooks, J.D.; Maddox, I.S. (1984). The effect of the sugar source on citric acid production by *Aspergillus niger*. *Appl. Microbiol. Biotechnol.* 19, 393–397.
 31. Ingram, L.O.; Buttke, T.M. (1984). Effects of alcohols on microorganisms. *Adv. Microb. Physiol.* 25, 253.
 32. Jernejc, K.; Perdih, A.; Cimerman, A. (1992). Biochemical composition of *Aspergillus niger* mycelium grown in citric acid productive and non-productive conditions. *J. Biotechnol.* 25, 341–348.
 33. Kang, S.K.; Park, H.H.; Lee, J.H.; Lee, Y.S.; Kwon, I.B.; Sung, N.K. (1989). Citric acid fermentation from mandarin orange peel by *Aspergillus niger*. *Sanop. Misaengmul Hakhoechi* 17, 510–518.
 34. Kılıç, M.; Bayraktar, E.; Ate, S.; Mehmetoglu, Ü. (2002). Investigation of extractive citric acid fermentation using response-surface methodology. *Proc. Biochem.* 37, 759–767.
 35. Kissler, M.; Kubicek, C.P.; Röhr, M. (1980). Influence of manganese and cell wall composition of *Aspergillus niger* during citric acid fermentation. *Arch. Microbiol.* 128, 26–33.
 36. Kristiansen, B.; Sinclair, C.G. (1978). Production of citric acid in batch culture. *Biotechnol. Bioeng.* 20, 1711–1722.
 37. Kristiansen, B.; Sinclair, C.G. (1979). Production of citric acid in continuous culture. *Biotechnol. Bioeng.* 21, 297–315.
 38. Kubicek C.P., The role of the citric acid cycle in fungal organic acid fermentations, *Biochem Soc Symp* 54 (1987) 113–126.
 39. Kubicek, C.P. (2001). Organic acids. In: Ratledge, C.; Kristiansen, B. (eds.), 2nd Edition, Chapt. 14. *Basic Biotechnology*. Cambridge, UK. Cambridge University Press, pp. 305–324.
 40. Kubicek, C.P.; Röhr, M. (1977). Influence of manganese on enzyme synthesis and citric acid accumulation in *Aspergillus niger*. *Appl. Microbiol. Biotechnol.* 4, 167–175.
 41. Kubicek, C.P.; Röhr, M. (1980). Regulation of citrate synthase from the citric acid producing fungus *Aspergillus niger*. *Biochim. Biophys. Acta* 615, 449–457.
 42. Kubicek, C.P.; Röhr, M. (1989). Citric acid fermentation. *Crit. Rev. Biotechnol.* 4, 331–373.
 43. Kubicek, C.P.; Schrefel-Kunar, G.; Wöhrer, W.; Röhr, M. (1988). Evidence for a cytoplasmic pathway of oxalate biosynthesis in *Aspergillus niger*. *Appl. Environ. Microbiol.* 54, 633–637.
 44. Kubicek, C.P.; Zehentgruber, O.; El-Kalak, H.; Röhr, M. (1980). Regulation of citric acid production by oxygen: effects of dissolved oxygen tension on adenylate levels and respiration in *Aspergillus niger*. *Eur. J. Appl. Microbiol. Biotechnol.* 9, 101–116.
 45. Kubicek, C.P.; Zehentgruber, P.; Röhr, M. (1979). An indirect method

- for studying fine control of citric acid accumulation by *Aspergillus niger*. *Biotechnol. Lett.* 1, 47–52.
46. Kubicek-Pranz, E.M.; Mozelt, M.; Röhr, M.; Kubicek, C.P. (1990). Changes in the concentration of fructose 2,6-bisphosphate in *Aspergillus niger* during stimulation of acidogenesis by elevated sucrose concentration. *Biochim. Biophys. Acta* 1033, 250–255.
 47. Kumagai, K.; Usami, S.; Hattori, S. (1981). Citric acid production from mandarin orange waste by solid culture of *Aspergillus niger*. *Hakko Kagaku Kaishi* 59(5), 461–464.
 48. Kumar, D.; Jain, V.K.; Shanker, G.; Srivastava, A. (2003). Citric acid production by solid state fermentation using sugarcane bagasse. *Proc. Biochem.* 38, 1731–1738.
 49. Kumar, D.; Jain, V.K.; Shanker, G.; Srivastava, A. (2003). Utilisation of fruits waste for citric acid production by solid state fermentation. *Proc. Biochem.* 38, 1725–1729.
 50. Kundu, S.; Panda, T.; Majumdar, S.K.; Guha, B.; Bandyopadhyay, K.K. (1984). Pretreatment of Indian cane molasses for increased production of citric acid. *Biotechnol. Bioeng.* 26(9), 1114–1121.
 51. La Nauze, J.M. (1966). Aconitase and citric acid dehydrogenases in *Aspergillus niger* in relation to citric acid accumulation. *J. Gen. Microbiol.* 44, 73–81.
 52. Lancini, G. (2008). Parte I – L'uso industriale dei microrganismi. Storia e campi di applicazione. In: Donadio, S.; Marino, G. (eds.). *Biotechnologie Microbiche*, Casa Editrice Ambrosiana, Milan, p. 5–35.
 53. Lane A G. (1980). Production of aromatic acids during anaerobic digestion of citrus peel. *J. Chem. Technol. Biot.* 30(6), 345–40.
 54. Lane A G. (1983). Removal of peel oil from citrus peel press liquors before anaerobic digestion. *Environ. Tech. Lett.* 4(2), 65–72.
 55. Legiša, M.; Benčina, M. (1994). Evidence for the activation of 6-phosphofructo-1-kinase by cAMP-dependent protein kinase in *Aspergillus niger*. *FEMS Microbiol. Lett.* 118, 327–334.
 56. Legiša, M.; Matthey, M. (1986). Glycerol as an initiator of citric acid accumulation in *Aspergillus niger*. *Enzyme Microb. Technol.* 8, 607–609.
 57. Lima, V.L.A.G.; Stamford, T.L.M.; Salgueiro, A.A. (1995). Citric acid production from pineapple waste by solid-state fermentation using *Aspergillus niger*. *Arq. Biol. Technol.* 38, 773–783.
 58. Lowenstein, J.M. (1969). *Citric Acid Cycle — Control and Compartmentation*, Dekker, London.
 59. Manzoni, M. (2006). *Microbiologia Industriale*, 1th Edn., Casa Editrice Ambrosiana, Milan.
 60. Marzona, M. (1996). *Chimica delle Fermentazioni & Microbiologia Industriale*, 2nd Edn., Piccin, Padua, Italia.
 61. Matthey, M. (1977). Citrate regulation of citric acid production by *Aspergillus niger*. *FEMS Microbiol. Lett.* 2, 71–74.
 62. Matthey, M. (1992). The production of organic acids, *Crit Rev Biotechnol.* 12, 87–132.
 63. Matthey, M. (1999). Biochemistry of citric acid production by yeasts. In: Kristiansen, B., Matthey, M., Linden, J. (eds). *Citric Acid Biotechnology*, Taylor and Francis, London, p. 33–54.
 64. Matthey, M.; Bowes, I. (1978). Citrate regulation of NADP⁺-specific isocitrate dehydrogenase of *Aspergillus niger*. *Biochem. Soc. Trans.* 6, 1224–1226.
 65. McIntyre, M.; McNeil, B. (1997). Dissolved carbon dioxide effects on morphology, growth and citrate production in *Aspergillus niger* A60. *Enzyme Microb. Technol.* 20, 135–142.
 66. Meixner-Monori, B.; Kubicek, C.P.; Röhr, M. (1984). Pyruvate kinase from *Aspergillus niger*: a regulatory enzyme in glycolysis?. *Can. J. Microbiol.* 30, 16–22.
 67. Mischak, H.; Kubicek, C.P.; Röhr, M. (1985). Formation and location of glucose oxidase in citric acid producing mycelia of *Aspergillus niger*. *Appl. Microbiol. Biotechnol.* 21, 27–31.
 68. Moyer, A.J. (1953). Effect of alcohol on the mycological production of citric acid in surface and submerged culture, I, Nature of the alcohol effect. *Appl. Microbiol.* 1, 1–7.
 69. Müller, H.M. (1975). Oxalate accumulation from citrate by *Aspergillus niger*. I. Biosynthesis of oxalate from its ultimate precursor. *Arch. Microbiol.* 103, 185–189.
 70. Murdock, D.I.; Allen, W.E. (1960). Germicidal effect of orange peel oil and d-limonene in water and orange juice I. Fungicide properties against yeast. *Food Technol-Chicago* 14(9), 441–445.
 71. Navaratnam, P.; Arasaratnam, V.; Balasubramaniam, K. (1998). Channelling of glucose by methanol for citric acid production from *Aspergillus niger*. *World J. Microbiol. Biotechnol.* 14(4), 559–563.
 72. Ohta, K.; Beall, D.S.; Mejía, J.P.; Shanmugam, K.T.; Ingram, L.O. (1991). Genetic improvement of *Escherichia coli* for ethanol production: chromosomal integration of *Zymomonas mobilis* genes encoding pyruvate decarboxylase and alcohol dehydrogenase II. *Appl. Environ. Microb.* 57(4), 893–900.
 73. Papagianni, M. (2007). Advances in citric acid fermentation by *Aspergillus niger*: Biochemical aspects, membrane transport and modeling. *Biotechnol. Adv.* 25, 244–263.
 74. Papagianni, M.; Matthey, M. (2004). Modeling the mechanisms of glucose transport through the cell membrane of *Aspergillus niger* in submerged citric acid fermentation processes. *Biochem. Eng. J.* 20, 7–12.
 75. Papagianni, M.; Matthey, M. (2006). Morphological development of *Aspergillus niger* in submerged citric acid fermentation as a function of the spore inoculum level. Application of neural network and cluster analysis for characterization of fungal morphology. *Microbial Cell Factories* 5, 3.
 76. Papagianni, M.; Matthey, M.; Berovic, M.; Kristiansen, B. (1999). *Aspergillus niger* morphology and citric acid production in submerged batch fermentation: effects of culture pH, phosphate and manganese levels. *Food Technol. Biotechnol.* 37, 165–171.
 77. Papagianni, M.; Matthey, M.; Kristiansen, B. (1998). Citric acid production and morphology of *Aspergillus niger* as functions of the mixing intensity in a stirred tank and a tubular loop bioreactor. *Biochem.*

- Eng. J.* 2, 197–205.
78. Papagianni, M.; Matthey, M.; Kristiansen, B. (1994). Morphology and citric acid production of *Aspergillus niger* PM1. *Biotechnol. Lett.* 16, 929–934.
 79. Papagianni, M.; Matthey, M.; Kristiansen, B. (1999). Hyphal vacuolation and fragmentation in batch and fed-batch culture of *Aspergillus niger* and its relation to citric acid production. *Process Biochem.* 35, 359–366.
 80. Papagianni, M.; Matthey, M.; Kristiansen, B. (1999). The influence of glucose concentration on citric acid production and morphology of *Aspergillus niger* in batch and fed-batch culture. *Enzyme Microb. Technol.* 25, 710–717.
 81. Papagianni, M.; Wayman, F.M.; Matthey, M. (2005). Fate and role of ammonium ions during fermentation of citric acid by *Aspergillus niger*. *Appl. Environ. Microbiol.* 71, 7178–7186.
 82. Pinacci, P.; Radaelli, M. (2002). Recovery of citric acid from fermentation broths by electrodialysis with bipolar membranes. *Desalination* 148, 177–179.
 83. Pintado, J.; Torrado, A.; Miron, J.; Montemayor, I.; Gonzalez, P.; Murado, M.; Sanroman, A. (1995). Citric acid production from mussel processing wastes in solid state culture. Mededelingen - Faculteit Landbouwkundige en Toegepaste Biologische Wetenschappen (Universiteit Gent) 59(4B), 2429–27.
 84. Rivas, B.; Torrado, A.; Torre, P.; Converti, A.; Domínguez, J.M. (2008). Submerged citric acid fermentation on orange peel autohydrolysate. *J. Agric. Food Chem.* 56, 2380–2387.
 85. Rodrigues, C.; De Souza Vandenberghe, L.P.; Teodoro, J.; Pandey, A.; Soccol, C.R. (2009). Improvement on Citric Acid Production in Solid-state Fermentation by *Aspergillus niger* LPB BC Mutant Using Citric Pulp. *Appl. Biochem. Biotechnol.* 158, 72–87.
 86. Rogalski, J.; Fiedurek, J.; Szczodrak, J.; Kapusta, K.; Leonowicz, A. (1988). Optimisation of glucose oxidase synthesis in submerged cultures of *Aspergillus niger* G-143 mutant. *Enzyme Microb. Technol.* 10, 508–511.
 87. Röhr, M.; Kubicek, C.P. (1981). Regulatory aspects of citric acid fermentation by *Aspergillus niger*. *Process Biochem.* 16, 34–37.
 88. Roukas, T. (1999). Citric acid production from carob pod by solid-state fermentation. *Enzyme Microb. Technol.* 24, 54–59.
 89. Rubio, M.C.; Maldonado, M.C. (1995). Purification and characterisation of invertase from *Aspergillus niger*. *Curr. Microbiol.* 31, 80–83.
 90. Ruijter, G.J.; Panneman, H.; van den Broeck, H.C.; Bennett, J.M.; Visser, J. (1996). Characterisation of the *Aspergillus nidulans* fra1 mutant: hexose phosphorylation and apparent lack of involvement of hexokinase in glucose repression. *FEMS Microbiol. Lett.* 139, 223–228.
 91. Schrefel-Kunar, G.; Grotz, M.; Röhr, M.; Kubicek, C.P. (1989). Increased citric acid production by mutants of *Aspergillus niger* with increased glycolytic capacity. *FEMS Microbiol. Lett.* 59, 297–300.
 92. Shu, P.; Johnson, M.J. (1948). Citric acid production by submerged fermentation with *Aspergillus niger*. *Ind. Eng. Chem.* 40, 1202–1205.
 93. Shu, P.; Johnson, M.J. (1948). The interdependence of medium constituents in citric acid production by submerged fermentation. *J. Bacteriol.* 54, 161–167.
 94. Snell, R.L.; Schweiger, L.B. (1951). Citric acid by fermentation. British Patent 653 808. *Chem Abstr* 45 8719a.
 95. Soccol, C.R.; Vandenberghe, L.P.S.; Rodrigues, C.; Pandey, A. (2006). New perspectives for citric acid production and application. *Food Technol. Biotechnol.* 44, 141–149.
 96. Stabnikova, O.; Wang, J.-Y.; Ivanov, V. (2010). Value-added biotechnological products from organic wastes. In: Wang, L.-K.; Ivanov, V.; Tay, J.-H.; Hung, Y.-T. (eds.). *Environmental Biotechnology*, Vol. 10, Humana Press, Totowa-NJ, USA, p. 343–394.
 97. Steinböck, F.; Held, I.; Choojun, S.; Röhr, M.; Kubicek, C.P. (1994). Characterization and regulatory properties of a single hexokinase from the citric acid accumulating fungus *Aspergillus niger*. *Biochim. Biophys. Acta* 1200, 215–223.
 98. Subba M S, Soumithri T C, Rao R S. (1967). Antimicrobial action of citrus oils. *Journal of Food Science*, 32(2), 225–227.
 99. Takahashi, J.; Yamada, K.; Aasai, T. (1958). Studies on the effect of some physical conditions on the submerged mold culture: Part I. The process of pellet formation of *Aspergillus niger* under the shaking culture, and the effect of the inoculum size on the shape of pellet. *J. Agric. Chem. Soc.* 32, 501–506.
 100. Torres, N.V. (1994). Modeling approach to control of carbohydrate metabolism during citric acid production by *Aspergillus niger*: I. Model definition and stability of the steady state. *Biotechnol. Bioeng.* 44, 104–111.
 101. Torres, N.V. (1994). Modeling approach to control of carbohydrate metabolism during citric acid production by *Aspergillus niger*: II. Sensitivity analysis. *Biotechnol. Bioeng.* 44, 112–118.
 102. Tran, C.T.; Sly, L.I.; Mitchell, D.A. (1998). Selection of a strain of *Aspergillus* for the production of citric acid from pineapple waste in solid-state fermentation. *World J. Microbiol Biotechnol.* 14, 399–404.
 103. Tsay, S.S.; To, K.Y. (1987). Citric acid production using immobilized conidia of *Aspergillus niger* TMB 2022. *Biotechnol. Bioeng.* 19, 297–304.
 104. Vandenberghe, L.P.S.; Soccol, C.R.; Pandey, A.; Lebeault, J.-M. (2000). Solid-state fermentation for the synthesis of citric acid by *Aspergillus niger*. *Bioresour Technol.* 74, 175–178.
 105. Von Loesbeck, H.W.; Mottern, H.H.; Pulley, G.N. (1934). Preservation of orange juice by deaeration and flash pasteurization. *J. Ind. Eng. Chem.* 26, 771–773.
 106. Wehmer, C. (1893). Note sur la fermentation citrique, *Bull. Soc. Chem. Fr.* 9, 728.
 107. Wongchai, V.; Jefferson, Jr. W.E. (1974). Pyruvate carboxylase from *Aspergillus niger*: partial purification and some properties. *Fed. Proc.* 33, 1378.
 108. Woronick, C.L.; Johnson, M.J. (1960). Carbon dioxide fixation by cell-free extracts of *Aspergillus niger*. *J. Biol. Chem.* 235, 9–15.
 109. Xie, G.; West, T.P. (2009). Citric acid production by *Aspergillus niger*

110. ATCC 9142 from a treated ethanol fermentation co-product using solid-state fermentation. *Lett. Appl. Microbiol.* 48, 639-644.
111. Xu, B.D.; Madrit, C.; Röhr, M.; Kubicek, C.P. (1989). The influence of type and concentration of the carbon source on production of citric acid by *Aspergillus niger*. *Appl. Microbiol. Biotechnol.* 30, 553–558.
112. Zhang, Q. (1988). Utilization of citrus wastes in production of citric acid. *Shipin Kexue (Beijing, China)* 104, 21–24.