Citric acid production

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Abstract. Citric acid is a commodity chemical produced and consumed throughout The World. It is used mainly in the food and beverage industry, primarily as an acidulant. Although it is one of the oldiest industrial fermentations, its World production is still in rapid increasing. Global production of citric acid in 2007 was over 1.6 million tones. Biochemistry of citric acid fermentation, various microbial strains, as well as various substrates, technological processes and product recovery are presented. World production and economics aspects of this strategically product of bulk biotechnology are discussed.

Keywords: citric acid biosynthesis, microbial strains, biochemistry, substrates, production processes, product recovery, economic aspects

Introduction

Citric acid is the main organic acid produced today by fermentation. The history of citric acid actually started in 1784 with W. Scheele [1] who first isolated it from the lemon juice as calcium citrate, which treated with sulphuric acid gave citric acid in the liquid phase.

In 1838, Libieg considered that citric acid is actually three carboxylic acid and in 1880 Grimoux and Adam [2] synthesized citric acid from glycerol-derived 1,3 dichloroacetone for the first time chemically [1].

Wehmer in 1893 was the first who observed the presence of citric acid as by-product of calcium oxalate produced by a culture of *Penicillium glaucum* fermenting sugar [1,2]. The result of this fermentation had encouraged him to patent the process for citric acid production [3]. On this base in 1894 the first industrial fermentation, using open-tray system was built. Ten years later the factory was closed, as the fermentation was considered too long and frequent contamination occurred [4].

After Wehmer several other researchers followed [5,6], but reasonable advance in citric acid production appeared with Zahorsky in 1913, who first patented a new strain – *Aspergillus niger* [7]. Following the fundamental investigations by Thom and Currie 1916 [8], Currie 1917 [9] opened the way for industrial citric acid fermentation using a new microorganism. His most important finding was that *Aspergillus niger* could grow well at low pH values

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BIOTECHNOLOGY ANNUAL REVIEW
VOLUME 13 ISSN 1387-2656
DOI: 10.1016/S1387-2656(07)13011-8

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around 2.5–3.5. This low pH prevented contamination which was common in Wehmer's process.

In 1928 beet molasses, as a cheap sugar source was first used in Czechoslovakia. Difficulties, however, were encountered with this source due to its trace metals content. Using a patent of Mezzadroli 1938 [10], this problem was effectively solved by using potassium hexacianoferrate as a chelating agent for the trace metals in beet molasses substrate.

Originally first industrial citric acid fermentations were carried out as surface cultures. The introduction of the submerged fermentation was a significant improvement. Among the studied preceding the commercial implementation of submerged fermentation the work of Perquin 1938 [11]; it should be mentioned as the first one for its shill and precision comparable to Currie's work on the surface process [9]. In Japan in the 1960s, a new process emerged by using *n*-alkanes as a carbon source. A yeast of the genus *Candida*, which produced appreciable amounts of citric and isocitric acid was used [12,13].

The world production of this '2-hydroxy-propan-1,2,3-three carboxy acid', by fermentation, is rapidly increasing. Although in South America, Mexico and Greece there still exists some factories where citric acid is isolated from unripe citrus fruits, today over 99 per cent of the world's output of citric acid is produced microbially by various fermentation processes, substrates and microorganisms.

The traditional method of preparing citric acid by extraction from the juice of lemons, limes and pineapple wastes is still in practice in the developing world, but its production is not significant, as it comprises less world production.

Various chemical syntheses of citric acid have appeared in the chemical and patent literature since the first one based on the reaction of glycerol-derived 1.3 dichloroacetone with cyanide by Grimoux and Adam in 1880 [2]. However none of these has reached a commercial status competitive with fermentation processes.

Uses and occurrence

60% of citric acid product is mainly used in the food and beverage industry, because of its general recognition as safe having pleasant taste, high water solubility and chelating and buffering properties. Citric acid is used extensively in carbonated beverages to provide taste and complement fruit and berry flavours. It also increases the effectiveness of antimicrobial preservatives. The amount of acid used depends on the flavour of the product. It may usually vary from 1.5 to 5 per cent [1–14].

In jam and jellies it is used for taste and for pH adjustment in the final product. For optimum gelation, pH has to be adjusted in very narrow limits [15]. Citric acid is usually added as a 50 per cent solution to assure good

distribution through the batch. In confectionery industry 0.5–2.0 per cent is used as flowing agent [16]. The chelating and pH adjusting properties of citric acid enable it to optimize the stability of frozen food products by enhancing the action of antioxidants and inactivating enzymes. It also helps to prolong the shellfire of frozen fish and shellfish [17].

Citric acid also inhibits colour and flavour deterioration in frozen fruit [18]. Amounts in concentration of 0.005–0.02 per cent citric acid are used as an antioxidant synergism in fats, oils and fat containing foods [16]. As a flavour adjunct, citric acid is used in sherbets and ice creams [16].

Temperament of total citric acid production is used in pharmaceutical industry as oral pharmaceutical liquids, elixirs and suspensions to buffer and maintain stability of active ingredients and to enhance the activity of preservatives. Addition of 0.02 per cent citric acid to liquid dosage forms complexes with trace iron and copper ions and retards degradation of active ingredients [19].

Citric acid is a standard ingredient in cosmetic formulations for pH adjustment, and in antioxidant systems as a metallic-ion chelator [20]. The detergent-building properties of citrate enables it to be used as a rapidly biodegradable environmentally acceptable phosphate substitute in non-phosphate detergent powders [21]. Citric acid-based metal cleaning formulations efficiently remove metal oxidation products from the surface of ferrous and non-ferrous metals [22]. Citrates have been reported to assist in platting of copper [23], nickel [24], chromium, lead [25] and various heavy metals [16].

Various other uses of citric acid and its salts and esters were reported also in photography as a component of printing plate emulsions in various bleaches, fixers and stabilizers [26] in oil well treatment and cements [16], in textile industry [27], in paper industry [28] and the tobacco industry [29]. Citric acid is also a preferred nucleating or blowing agent in polymeric foams for food and beverage use and its esters are used as plasticizers in the preparation of polymer compositions [16].

As the by-products of citric acid fermentation various enzymes (amylolytic, pectolytic, etc.) were referred [29].

Strains for citric acid production

Many strains excrete traces of citric acid as a metabolite of primary metabolism. It is a result of some severe irregularity of metabolism caused by genetic deficiency or by metabolic imbalances.

In the history of citric acid fermentation, in the last hundred years, various strains of genera fungi, yeast and bacteria were reported such as: *Penicillium luterum*, *P. purpurogenum*, *P. restrictum*, *P. janthinellum*, *P. citrinum*, *Paecilomyces divaricatum*, *Mucor piriformis*, *Trichoderma viride*, *Sacharomycopsis lipolitica*, *Arthrobacter paraffineus*, *Corynebacterium* sp. and others [30,31].

However, only mutants of *Aspergillus* and yeast genus *Candida* have almost exclusively been utilized.

Apart from *Aspergillus niger* the following species of *Aspergillus* have been reported: *Aspergillus niger*, *A. wentii*, *A. awamori*, *A. foetidus*, *A. fenicis*, *A. fonsecalus*, *A. fumaricus*, *A. luchensis*, *A. saitoi and A. usumii*.

From the genus *Candida* the following have to be mentioned: *Candida lipolytica*, *C. tropicalis*, *C. guilliermondii*, *C. intermedia*, *C. parapsilosis*, *C. zeylanoides*, *C. fibriae*, *C. subtropicalix*, *C. oleophila*. Mutants of *A. niger*, *A. wentii* and on paraffine substrats *Candida lipolitica* are used in industrial production [32].

For industrial citric acid production, filamentous fungus *A. niger* is far the most used microorganism. In the second-half of the 20th century, progress in life sciences and accumulating knowledge about metabolic events stimulated several research groups to study the biochemical basis of citric acid accumulation by *A. niger*. They investigated why, and under which circumstances, citric acid is accumulated so that productive strains might be improved further and yields increased. Although a number of biochemical events were found to be responsible for citric acid overflow, differences existed amongst individual high-producing strains. Research during the last two decades, has resulted in generally accepted theory that describes the metabolic pathways used and the regulation events that are significant during citric acid accumulation [33].

Biochemistry

There have been many theories proposed to explain the phenomena of citric acid accumulation by *A. niger* [34–42], but so far no complete explanation is available. It can be said that citric acid accumulates by an induced abnormality in the metabolism of the mould during the operation of the tricarboxylic acid cycle (TCA) postulated by Krebs in 1937, under its original name ''citric acid cycle'' [47] The TCA cycle is a cyclic sequence of reactions of almost universal occurrence in mitochondria in aerobic organisms. It is catalysed by multienzyme system, that accepts the acetyl group of acetyl-Co enzyme A as fuel and dismembers it to yield carbon dioxide and hydrogen atoms [40,43].

On each turnaround within the TCA cycle, one molecule of acetic acid (two carbon atoms) enters as acetyl-Co enzyme, condenses with a molecule of the four-carbon compound oxaloacetic acid to form citric acid, the six-carbon compound. Citric acid is then degraded through a reaction sequence, that yields two molecules of CO_2 and regenerates the four-carbon oxaloacetic acid. Another turn of the cycle may now start by the reaction of the oxaloacetic acid with another molecule of acetyl-Co enzyme A. Thus, in each turn of the cycle one molecule of acetic acid enters, two molecules of ATP and CO_2 are formed and a molecule of oxaloacetate is utilized to form citrate, but is regenerated at the end of the cycle [44].

The degree of involvement of TCA cycle during the accumulation is indeed controversial. Shu *et al.* in 1954 found that 40 per cent of the citric acid was formed from recycled dicarboxylic acid [45]. In contrast to that other researchers were able to demonstrate some degree of recycling [46,47]. It was also considered [48] that the citric acid accumulated came from the disappearance of aconitase and isocitric dehydrogenase, since prior to the accumulation of citric acid all enzymes of TCA cycle were present.

Citric acid is excreted from the cells in response to unfavourable intracellular condition caused by increased levels of tricarboxylic acids (TCA). A crucial prerequisite for overflow of citric acid from *A. niger* cells is therefore increased at the level of Krebs cycle intermediates caused by anaplerotic reactions. Extensive studies have revealed that there are three main metabolic events that replenish TCA intermediates and predispose the cell to product overflow.

- Fast uptake of glucose based on simple diffusion.
- Unrestricted metabolic flow through glycolysis, making precursors for synthesis of the TCA cycle intermediates readily available.
- Uncoupled NADH re-oxidation resulting in lower levels of ATP and therefore decreased anabolic reaction.

Only the activities of certain enzymes of individual *A. niger* cells can lead to such intracellular conditions.

Glucose uptake rate has been identified as an important factor in the rate of citric acid production [49,50]. By using mathematical modelling, it was shown that glycolytic reactions of *A. niger* are limited by the supply of the initial substrate and the removal of the final product. Two glucose carriers have been identified, the first, a high-affinity carrier that is expressed at all times, and the second, a low-affinity carrier that is expressed only in the presence of high concentrations of glucose [51]. However, Mischak *et al.* [52] and Torres *et al.* [51] reported that both glucose carriers are inhibited by citric acid under production conditions. If the entry of glucose is primarily via the glucose carriers, the effect of citric acid concentration on the observed glucose uptake should be pronounced. By contrast, a simple diffusion model fits all the observed data under citric acid excretion conditions, explaining the observed relationship between specific uptake rate and glucose concentration, which would not exist under carrier-saturated conditions [53]. Finally, because simple diffusion is an inevitable physical process, it is not capable of being regulated directly by the organisms; this may, in itself, account for dramatic overproduction of citric acid under the conditions used in this process. The simple nature of this mechanism also explains the similarity of the uptake relationship from the different sources, despite the use of different strains and growing conditions.

However, the de-regulated metabolic flux through glycolysis is a prerequisite for rapid synthesis of citric acid. In glycolysis, the reactions catalysed by hexokinase, phosphofructokinase and pyruvate kinase are virtually irreversible. The activities of these enzymes are regulated by reversible binding of allosteric effectors or by covalent modification. Normally in eukaryotic organisms, the phosphofructokinase is the most important control element. However, in *Aspergillus niger*, during the growth on high sugar concentrations that are needed for rapid citric acid formation, the control of glycolysis is shifted from 6-phosphofructo-1-kinase level to the glyceraldehyde step [54]. In the literature, two attempts can be found to influence the efficiency of this pathway by genetic modification of the enzymes involved. Firstly, by disrupting trehalose-6-phosphate synthase gene (ggsA), the synthesis of trehalose-6-phosphate, a potent inhibitor of glycolysis was prevented, yet citric acid accumulation improved only slightly [55]. Secondly, moderate overexpression of the key regulatory enzymes, 6-phosphofructo-1-kinase and pyruvate kinase did not enhance acid production [56].

De-regulated glycolysis leading to strong anaplerosis is characteristic for a productive phase of *A. niger* cells, however, significant physiological changes are taking place in the cells during the early stages of growth in high initial sucrose or glucose medium that have crucial impact on overall productivity and yield. In fact, there could be no citric acid detected in the substrate during the first 24 h of growth [57], while a relatively slow accumulation rate initiates only during the second day of fermentation, followed by a sudden increase in specific productivity afterwards [57–59].

By flux distribution experiments pentose phosphate (PP) pathway was found to be predominant during the germination of spores, followed by a switch to glycolysis [60,61]. Initial phases of growth are characterized also by polyol formation and glucosamine accumulation. Polyols especially glycerol, whose intracellular concentration can reach up to 175 mM, may play an important role as an osmoregulator in *A. niger* cells during the growth in high sucrose medium [61].

Because the enhanced glycolytic flux is a prerequisite for increased anaplerosis, understanding the mechanism of the switch in carbohydrate metabolism from the PP pathway to glycolysis is of crucial importance. Although the initial inhibition at the level of TCA cycle enzymes has been extensively studied in the past, some authors in their recent reviews on citric acid accumulation by *A. niger* exclude the hypothesis of an inhibition of TCA cycle in the phase where acid accumulation starts [62].

However by measuring intracellular citrate concentration in the cells, low levels of citrate were recorded in germinating spores followed by a constant rise up to 10 mM before 24 h of fermentation [61].

An inhibition of NADP-dependent isocitrate dehydrogenase (ICDH) by glycerol was initially proposed to trigger an increase of intracellular citrate [63] on the basis of kinetic measurements performed on un-purified enzyme in

the homogenate [63,64]; however, later tests on partially purified enzyme showed no inhibition by glycerol [64]. NADP-specific isocitrate dehydrogenase was found to be predominantly located in mitochondrial compartment when glucose was used as sole carbon source [65], while only minor activities of NAD-dependent enzyme were detected in *A. niger* cells [66].

However, NADP-isocitrate dehydrogenase was found to be inhibited by citrate [66] and reduced metabolic flow at the early stages of fermentation through the TCA cycle at the stage of ICDH, could be predicted from studies based on the distribution of different marked C^{13} atoms in glucose by Peksel *et al.* [54]. At the early stages of citrate formation, their model indicated a substrate cycle of oxaloacetate to pyruvate was substantial in comparison to the flux of oxaloacetate to citrate. Simultaneously, a significant pyruvate/phosphoenolpyruvate substrate cycle was predicted. Later in the fermentation there was decreased operation of the pyruvate/phosphoenolpyruvate substrate cycle and a net flux to citrate. The question of what triggers the initial increase in citrate concentration remains unexplained. It might be that another substance structurally related to citrate and metabolically formed from glycerol could cause initial deactivation of mitochondrial TCA cycle enzyme or the mass action effect of intermediates from glucose might cause the increase.

The initial increase in intracellular citrate concentration could cause a decrease in glucose degradation through the PP pathway. It was shown that 6-phosphogluconate dehydrogenase, one of the regulatory enzymes of the oxidative step of PP pathway, is inhibited by citrate with apparent K_i value of 0.8 mM [67].

The direct conversion of hexoses to pyruvate via glycolysis becomes predominant during the productive phase of citric acid accumulation that starts after about 24 h and accelerates after 40–50 h of growth in a batch system. Regulation of the central part of hexose metabolism takes place at several levels: at the transcriptional level, by regulating the activity of allosteric enzymes by specific effectors and as revealed recently [68,69], even by post-translational modification.

In the glycolytic flux, 6-phosphofructo-1-kinase (EC 2.7.1.11) is the most important control element. It catalyses essentially irreversible reaction of glycolysis, the phosphorylation of fructose-6-phosphate using Mg-ATP to form fructose-1,6-bisphosphate and releasing Mg-ADP. Six organic allosteric ligands either increase or decrease substrate-binding affinity and concomitantly determine overall enzyme activity [70]. The enzyme attracted the interest of investigators, due to its ability to maintain a high glycolytic flux in spite of elevated intracellular concentrations of citrate, a well-known inhibitor of PFK1, which was reported to reach concentrations between 4 [57] and 10 mM [61].

Recently, another enzyme exhibiting PFK1 activity was isolated from *A. niger* mycelium with molecular mass of 49 kDa. A fragment of identical

size could also be obtained *in vitro* by the proteolytic cleavage of the purified native PFK1 with proteinase K, which regained its activity after the phosphorylation of the protein molecule by catalytic subunit of cAMP-dependent protein kinase. The native enzyme as a sole PFK1 enzyme could be isolated only from the early stages of growth on a minimal medium, while a 49 kDa fragment seemed to appear later and was activated concurrently with a sudden change in the growth rate. There is a strong evidence that the native PFK1 enzyme undergoes spontaneous posttranslational modification at the early stages of the fungal development.

By measuring kinetic parameters of both PFK1 forms found in *A. niger* cells, ATP proved to be a strong inhibitor of the short PFK1 fragment, but the negative effect of ATP seemed to be suppressed by physiological concentrations of fructose-2,6-bisphosphate. The same effector significantly increased the V_{max} and the affinity of the fragmented protein towards the substrate, while it does not affect the maximal velocity of the native protein [68]. In *A. niger* about 6 μM of fructose-2,6-bisphosphate were detected under citric acid excreting conditions [56]. The studies on fructose-2,6-bisphosphate formation showed that its synthesis is stimulated after the transfer of *A. niger* mycelium from low (1 per cent) to high (14 per cent) initial sucrose medium simultaneously with a rapid increase in cAMP level [71]. Other PFK1 stimulators, AMP and ammonium ions, increased the activity of the shorter fragment more intensely than the activity of the native protein, while citrate, a well-known allosteric inhibitor of eukaryotic PFK1 enzymes, showed moderate inhibition of the native enzyme, while no inhibition of the fragment could be observed by concentrations up to 10 mM [69]. Kinetic data so far obtained support the hypothesis that the posttranslational modification is needed for the formation of a highly active PFK1 enzyme insensitive to normal feedback control by citrate.

Another phenomenon taking place at the early stages of *A. niger* growth in a high citric acid yielding medium seems to be relevant for the development of high citric acid yielding mycelium. Namely, the shorter fragment is inactive immediately after proteolytic cleavage and must be phosphorylated to regain activity [68,72]. PKA was found to be capable of the appropriate phosphorylation, which led to the re-activation of 49 kDa fragment [68]. Kinases are normally under the tight control of specific regulatory subunits and cyclic AMP is known to induce PKA enzyme. In *A. niger* strain A60, a spontaneous increase in the concentration of cyclic AMP was recorded after 24 h of growth in a citrate yielding medium. Further analyses have shown that the amount of cAMP formed depends on the initial concentration of sucrose in the medium. Under higher sucrose conditions the cAMP peak appeared earlier and was higher, while in lower sucrose media a flattened peak was observed later in fermentation [73]. A spontaneous increase in cyclic AMP concentration could be caused by intracellular acidification since a drop of intracellular pH is known to stimulate the RAS-adenylate cyclase signalling

pathway in a number of fungal species, including *Saccharomyces cerevisiae* [74].

In the citrate accumulating strain (A60) grown in a high sucrose medium, intracellular acidification was indeed recorded at the early stages of growth [61,75], while in another *A. niger* strain (NW131) no change in cytoplasmic and vacuolar pH could be detected by P^{31} NMR technique during the growth of immobilized cells [76]. Significant differences in membrane H^+-ATPase activities of both strains were described. In the A60 (NRRL 2270; ATCC 11 414) strain, the activity of proton pumps was a quarter of that in A158 (CBS 120,49; N400), a strain which is related to the NW131 strain [75]. Moreover, under identical growth conditions strain A158 extruded protons more rapidly into the medium when ammonium ions were used as a sole nitrogen source than the A60 strain, indicating that proton pumps of the latter strain perhaps cannot extrude all the protons that are released into the cytosol after initial increase in intracellular citric acid concentration and ammonium assimilation [75]. Citric acid, which can reach a concentration of up to 10 mM in the cells [61], dissociates at neutral pH values ($pK_3 = 5.4$) releasing two protons. However under citric acid accumulating conditions, the ammonium salts are the preferred source of nitrogen. *A. niger* consumes ammonium very rapidly and it is normally depleted from the medium between 40 and 50 h of fermentation, which is well before the fungus stops growing [58]. The amount of protons excreted from the biomass appeared to be directly related to the initial ammonium concentration [58]. It is worth noting that ammonium ions are taken up by an uniport mechanism, however after the incorporation of NH_4^+ ion as an amino group, two protons are released which must be pumped back to the medium by H^+-ATPases to maintain electroneutrality in the cells. Stoichiometric modelling of the early stages of the fermentation revealed that ammonium ions combine with a carbon-containing metabolite inside the cells in a ratio 1:1, to form an organic nitrogen compound, which is immediately excreted by the mycelium. The compound was proven to be glucosamine [58]. Characteristically, the maximal rate of acid overflow was recorded only after the depletion of ammonium from the medium, although increase in dry biomass was observed at later phases of growth as well. The enzyme responsible for glucosamine formation must be glucosamine-6-phosphate deaminase, which catalyses amination of fructose-6-phosphate to produce glucosamine-6-phosphate. Since the enzyme competes for the same substrate (fructose-6-phosphate) as PFK1, rapid accumulation of glucosamine must significantly decrease the metabolic flux through glycolysis at the early stages of growth; however, better understanding of the phenomenon must await more detailed characterization of deaminase kinetics.

Aspergillus niger is well known for its strong extra and intracellular proteolytic activity [77]. Although proteases are normally strictly compartmentalized in the cells and are activated from their pre-pro forms only in the vacuoles, some leakage through the tonoplast into the cytosol must occur,

since cleavage of the native PFK1 enzyme takes place. In *A. niger* cells increased protein degradation was reported under the manganese-deficient conditions, which was also reflected by increased intracellular proteinase activities [78,79].

For efficient citric acid fermentation, lack of trace metal ions, particularly Mn^{2+} ions from the medium is of major importance. Much has been speculated about the principal physiological role of manganese ions in citric acid overflow in past, which seem to affect metabolism on various levels [62]. Whether manganese ions are somehow involved in increased cytosolic protease activities, and concomitantly, in the posttranslational modification of PFK1, will have to await further investigation.

During the idiophase, the phase of maximal product formation [80], no significant inhibition of the TCA cycle could be observed and an increased level of all tricarboxylic acids, with an exception of 2-oxo-acids, can be detected in the mycelium [81]. The only plausible explanation of the phenomenon is accelerated glucose metabolism, which was confirmed also by testing mutants with increased citric acid productivity in respect to the parental strain [82]. However, another two enzymatic reactions that appear in *A. niger* cells play a noteworthy role in citric acid overflow. Cytosolic pyruvate carboxylase [83] and malate dehydrogenase isoenzyme [84] are catalysing the conversion of pyruvate first into oxaloacetate and finally into malate. In the late phase of fermentation, carbon dioxide fixation by pyruvate carboxylase became an important anaplerotic reaction [85], while increased concentration of malate in the cytosol finally serves as a counter ion for citrate export from the mitochondrial compartment by a tricarboxylic acid carrier [86].

The formation of citric acid is dependent on strong aeration; dissolved oxygen tensions higher than those required for the vegetative growth of *A. niger* stimulate citric acid fermentation [87,88]. The biochemical basis of this observation is related to the presence of an alternative, cyanide-resistant respiratory pathway, which is required for the re-oxidation of glycolytically produced NADH, when high oxygen tension is maintained. The enzyme responsible for the additional respiratory pathway is an alternative oxidase, which catalyses reduction of oxygen to water without the translocation of protons across the inner mitochondrial membrane, and thus functions as a non-energy-conserving member of the respiratory electron chain.

The alternative respiration seems to be constitutively present in citric acid producing strains [89,90]. The alternative oxidase is synthesized in the cytosol and translocated into the mitochondria [91]. Although *aox*-1 gene encoding alternative oxidase from *A. niger* cells has been isolated, cloned and characterized [92], no transformants carrying multiple gene copies or strains with disrupted gene were prepared and tested for intracellular ATP concentrations and/or citric acid overflow. However, it is generally accepted that the presence of uncoupled NADH re-oxidation results in lower levels of ATP and therefore decreases anabolic reactions.

After citrate first accumulates in the cytosol, it must pass the plasma membrane to be excreted into the substrate. It was assumed that citrate, a charged metabolite cannot cross the lipid bilayer without support of the transport protein and an active, pH driven, H^+-symport-dependent system was proposed that was functional only under the manganese deficient growth conditions [93]. However, recent thermodynamic calculations presented for citrate overflow from *A. niger* cells at the pH value 3 of the substrate suggest that a passive transport step suffices for citrate excretion [94].

Future perspectives

In the last several decades enough knowledge on biochemical mechanisms leading to citric acid overflow have accumulated to generally understand the phenomenon, however many details still remain unexplained. Recently, several genomes of *Aspergillus* species have been fully sequenced and the information published: *A. fumugatus* [95], *A. nidulans* [96], *A. oryzae* [97], while the information of several other genomes including *A. niger* will be released in near future. On the basis of data from sequence analyses, and physiological information published from *A. niger* and related filamentous fungi, *in silico* model of the central carbon metabolism of *A. niger* has been constructed [98] and is regularly updated. Application of the stoichiometric model together with recent discoveries on the posttranslational modification of the key regulatory enzyme of glycolysis will present a powerful tool for further improvement of the primary metabolism in *A. niger* that will result in stronger anaplerosis and increased productivity.

Influence of the trace metals

In citric acid technology, absence of iron and manganese in the fermentation substrate plays the most crucial role [99,100]. Trace element nutrition is specially highlighted by the fact that an optimal nutrient medium for citric acid fermentation will not allow high production unless the trace elements content is carefully controlled [101]. However, if the trace element nutrition is correct, other factors (sugar concentration, phosphate and the others) have only less pronounced effects [102].

Iron ions in higher concentration than 1.5 mg/l strongly affect cellular morphology, by promoting unproductive filamentous mycelial growth form [103,104]. In further insights into the importance of metal ions, the presence of manganese ions to citric acid fermentation was reported by Clark *et al.* [105]. As little as 1 μg/l of manganese could completely ruined the production yield and caused organism's morphology to switch from microbial pellets, known as citric acid productive form, to unproductive filamentous growth.

In contrast, the most recent research by Berovic *et al.* [106] found that when fungal biomass reaches its stationary phase even in a case when fed

media contains unusually high amounts of manganese ions up to 200 μg/l, the presence of heavy metal ions do not affect on mycelial growth nor citric acid biosynthesis.

Manganese deficiency lower than 10^{-7} M raised chitin and reduced β-glucan production. Manganese levels also affect lipid synthesis, which in turn affects cell membrane composition [107]. It also exhibits effects on DNA synthesis of *A. niger*. Under manganese limitation, DNA formation was not inhibited but RNA synthesis was impaired [78]. On the other hand manganese deficiency in *A. niger* cultivation also results in significantly lower lipid levels due primarily to reduction of triglycerides and with little effect on free acids and sterols [108]. In anyway the influence of manganese ions on *A. niger* is very complex and it represents the most critical metal ion in citric acid fermentation [101,104].

In recent articles, the attention to *A. niger* metal ion tolerance was related to action of elevated manganese ion concentration and effects of copper and zinc antagonism to iron and manganese [109,110] and to various genetic manipulation for metal resistance strain improvement [111–113].

Substrates

Most processes are based on molasses, although the use of cleaner sources is gaining ground. Whatever the source, its cost and preparation to permit optimal fermentation conditions are two important aspects of the technology in citric acid production. The basic substrate for citric acid fermentation in plants using the surface method of fermentation is beet or cane molasses. Plants using submerged fermentation can use not only beet or cane molasses, but a substrate of higher purity such as hydrolysed starch, technical and pure glucose, refined or raw sugar, purified and condensed beet or cane juice. This is because use of a pure substrate may result in increase in yield, or reduction in fermentation time [114].

Molasses

Molasses is a widely used substrate, coming in a variety of qualities. High-quality molasses is usually demanded for citric acid production. Cane and beet molasses are not identical in composition; often one type will be preferred to the other. They are sometimes mixed to take advantage of the additional nutrients arising from the differences in composition.

Besides substrate type (sugar beet, sugar cane), the chemical composition of molasses depends on many factors such as soil and climate conditions, fertilization type, crop method, time and conditions of storage, production technology, technical equipment of plant, etc. [114].

Beet molasses

Beet molasses consists of about 65–80 per cent dry substance and 20–25 per cent water. The main ingredient of molasses is sucrose, 44–54 per cent by weight. Other sugars (carbohydrates), which can be found in higher amounts are inverted sugar 0.4–1.5 per cent, raffinose 0.5–2.0 per cent and kestose and neokestose 0.6–1.6 per cent. Raffinose is a natural part of sugar beet, while kestose is the result of microbial action during sugar beet treatment. Other sugars in molasses are arabinose, xylose and mannose in amounts of 0.5–1.5 per cent. All sugars (except sucrose) are included in the non-nitrogen organic substances of molasses. Products of chemical and thermal sugar decomposition (melanoidines, caramel) and organic acids also belong to this group. Caramel consists of sugar anhydride and colouring matters; mela-noidines are made in hot solution as the result of a reaction between reducing sugars and amino acids. In addition to the non-volatile dark coloured compounds, there are about 40 volatile compounds as aliphatic aldehyde, methylglyoxal, diacetyl, acetoin, acetone, oxymethylfurfurol and others [114].

The non-volatile organic acids present in molasses are glutaric, malonic, succinic, aconitic, malic and lactic acid; the remainder are oxalic, citric and tartaric acid. These can all react with calcium to form insoluble salts that can influence the precipitation and recovery of the citric acid crystals. Molasses contain such volatile acids as formic, acetic, propionic, butyric and valeric acid. Almost all organic acids, volatile and non-volatile, are from potassium or calcium salts. Molasses containing higher amounts (over 1 per cent) of volatile acids are normally too dark to be used as feedstock for the citric acid fermentation.

Nitrogen compounds contained in molasses are mostly betaine (about 60–70 per cent of total nitrogen), amino acids (20–30 per cent of nitrogen), protein (3–4 per cent of nitrogen) and traces of nitrogen in ammonium nitrate and amide. The amino acids content in molasses depends on the soil and climate conditions and beet cultivation. Betaine comes from beet and is not used by microorganisms as a nitrogen source. The content of mineral substances in beet molasses amounts to 8.5–14.0 per cent [114].

Besides these factors, one of the most relevant parameters for high yielding citric acid fermentation is also the amount of particular microelements in different molasses.

The pH of molasses depends on the sugar extraction technology. It was considered that a neutral, or slightly alkaline molasses gave the best citric acid yields. Citric acid production needs molasses with low buffer ability, to make possible the required rapid fall of medium pH during fermentation [114].

Cane molasses

Cane molasses differs from beet molasses in its chemical composition. It contains less sucrose and more inverted sugar, has lower content of nitrogen and raffinose, more intensive colour and lower buffer capacity.

Beet and cane molasses can also contain other substances, which appear in small amounts, but are often crucial in deciding whether the molasses are suitable for use in citric acid biosynthesis. These are pesticides, fungicides and herbicides used in beet and cane cultivation and also substances used for defoaming in sugar production process. All have mostly toxic properties and negatively affect molasses usability. In general beet molasses is more suitable for citric acid fermentation than cane molasses. It is especially relevant in submerged fermentation where the quality of the substrate is more important for productivity and fermentation yield.

The microflora of molasses can be an agent of negative influence on yield and productivity of fermentation. Molasses will always contain a certain number and type of microorganisms, sometimes the count can be higher than $10{,}000\,g^{-1}$ of molasses. The most common microorganisms in molasses are species of *Bacillus*, sometimes yeasts of *Candida* species, and very rarely, moulds of *Penicillium*, *Aspergillus* and other species [114].

The basic operation in molasses preparation is a treatment for heavy metal ions removal. Potassium ferrocyanide or other complex compounds are commonly used. Another compound complexing with heavy metals is the sodium salt of ethylene-diamineacetic acid (EDTA). Other heavy metal complexing compounds can also be used, *e.g.*, sodium polyphosphates, potassium rhodanate, 2,4-dinitrophenols and 8-oxyquinoline. Molasses media are sometimes purified by ionites, especially on cation exchanger. Not all microelements should be removed during this process, as some of them are necessary for growth of the *Aspergillus niger* mycelium [114].

Sucrose

Refined sugar of beet or cane is almost pure sucrose, which *Aspergillus niger* strains ferment very well [115]. Preparation of a refined sugar solution as a fermentation medium is based on its diluting with water to a concentration of 15–22 per cent, adding necessary nutrients (NH_4NO_3, KH_2PO_4, $MgSO_4$) and acidifying with sulphuric acid to pH 2.6–3.0 [116]. Batch medium is sterilized in the fermentation vessel. All the ingredients of the fermentation medium are added straight into the bioreactor or are prepared separately by diluting in hot water (85–95°C) and then pumped into the bioreactor. In this case, sugar is diluted to 50–60 per cent concentration and pumped into the fermenter that has had an exact amount of sterile water added, resulting in a total sugar concentration of 15–22 per cent.

Syrups

Syrups of beet or cane sugar can also be used as basic substrate for the submerged citric acid fermentation. The great advantage with this substrate is its purity; however, the quality of the syrups deteriorates rapidly during

storage. Because of this they can only be used during the sugar campaign season and only if the citric acid plant is not too far from the sugar factory because of the large transport costs.

Preparation of the syrups for fermentation entails dilution with water to a sugar concentration of 15–20 per cent, addition of necessary nutrients (NH_4NO_3, KH_2PO_4, $MgSO_4$, $(NH_4)_2C_2O_4$), acidification with hydrochloric or sulphuric acid to pH 4–5 and sterilization at 121°C for 0.5–1 h [117].

Starch

The production of citric acid from sources of starch such as corn, wheat, tapioca and potato is widely used. The suitability of these substrates for citric acid fermentation depends on their purity and method of hydrolysis. Acid hydrolysis, enzymatic hydrolysis, or a combination of the two, are used. Preparation of starch substrates for fermentation is based on their enzymatic liquefaction and saccharification to a defined hydrolysis level. Additional nutrients are added, depending on which starch is used. The pH is adjusted to 3–4 using hydrochloric or sulphuric acid and the medium is sterilized at 121°C for 0.5–1 h.

Good citric acid yields have been also obtained using dextrose syrup, obtained by enzymatic hydrolysis of starch. This method is now employed also in industrial scale. In this case it is especially important to restrict the amount of heavy metals below critical levels; heavy metals should therefore be removed by ion exchange.

When using an *Aspergillus niger* strain resistant to higher concentrations of heavy metals, practically the same yield may be obtained on decationized and non-decationized dextrose syrup [118].

Hydrol

This is a paramolasses obtained as a by-product during crystalline glucose production from starch. Because of the high glucose content (40–45 per cent) and high purity coefficient it is a very good substrate for citric acid production. Preparation of hydrol for fermentation involves dilution to a sugar concentration of 15–18 per cent, addition of necessary nutrients and adjustment of pH with hydrochloric or sulphuric acid to 3.0–4.0. The solution is sterilized at 121°C for 0.5 h and cooled to 32–35°C [119].

Alkanes

The low price of alkanes, coupled with the ability of many organisms to utilize them, produced major changes in the fermentation industry during the 1960s and 1970s. Citric acid production, using *Candida lipolytica*, is a typical example and has been the subject of many patents [120,121]. There are few

industrial citric acid processes that are based on alkanes. In these processes, isocitric acid would also be produced at concentrations that would cause product recovery problems, as well as reduced citric acid yields [122]. A fourfold increase in price since 1973 no longer makes alkanes a cheap substrate.

Oils and fats

For citric acid production, oils are now being used as principal carbon source in a manner analogous to the previous use of alkanes. With palm oil as carbon source, a yield of citric acid of 145 per cent using a mutant of *Candida lipolytica* has been reported [123]. There are examples of oil being added in small concentrations to *Aspergillus niger* fermentation [124] and even being used as a sole carbon source for *Aspergillus niger* fermentation. It was found that citric acid could be produced on these substrates with good yield [125]. These oils and fats may replace alkanes in several fermentations, but it is unlikely that they will remain at their current low prices.

Production processes

Although in citric acid, industrial scale production in past surface or emerged production in earlier years of twenty centuries dominated over traditional method of preparing citric acid by extraction from various juices; at the present time a much greater emphasis is placed on the use of submerged culture production. Batch techniques in stirred tank or airlift bioreactors are in general use. Very promising results were obtained in fed-batch process [38–40] and by continuous fermentation [126–129] where various kinds of bioreactors as stirred tank reactors [99,102,105], airlift reactors [130,131], external loop reactors [132,133], magnetic drum contactors [134], reciprocated jet reactors, biodisc reactor [135], deep jet reactors [136,137], in hollow fibre [138] or by use of fix bed reactor [133].

Several report of citric acid fermentation using immobilized *A. niger* cells on various kinds of carriers as glass [139], polyurethane foams [140], entrapment in calcium alginate beds [141–143] polyacrylamide gels [144,145] agar [146] agarose [147] cellulose carriers [148,149] metal screens and polyester felts [150,151].

The traditional method of preparing citric acid by extraction from the juice of lemons, limes and pineapple wastes is still in practice in the developing World, but its production is not significant, as it comprises less World production [152,153].

Various chemical syntheses of citric acid have appeared in the chemical and patent literature since the first one based on the reaction of glycerol-derived 1.3 dichloroacetone with cyanide by Grimoux and Adam [2]. However, none

of these has reached a commercial status competitive with fermentation processes.

Surface process on liquid substrate

The surface fermentation process, using liquid substrate, is the oldest production method and accounts for 5–10 per cent of the World supply of citric acid.

This process is still in use because of low investment, and energy cost for the cooling and heating system, and due to simple technology, despite to the higher labour costs as compared to submerged fermentation. The system consists of fermentation rooms in which a large number of trays are mounted one over the other in stable racks. The trays are generally made of high purity aluminium or special stainless steel. Their size varies from $2 \times 2.5 \times 0.15$ m to $2.5 \times 4 \times 0.15$ m, with usage liquid depths of 0.08–0.12 m. Provision is made for continuous filling and draining by appropriate overflow devices. Aeration is provided by climatized sterile air circulation, which serves the purpose of temperature regulation and only to a lesser extent that of supplying oxygen and controlling humidity. Air is introduced in to the fermentation chamber in an almost laminar flow manner [27].

Molasses substrates are generally employed as substrates containing 15–20 per cent of sucrose, added nutrients, various natural polymers [42,43], acidified with, *e.g.*, phosphoric acid to a pH 6.0–6.5 and heated at temperature 110°C for 15–45 min. Subsequently, potassium hexacyanoferrate is added to the hot substrate, to precipitate or complex trace metals [Fe, Mn, Zn] and to act in excess as a metabolic inhibitor restricting growth and promoting acid production [43]. For some molasses combined treatment with tricalcium phosphate, hydrochloric acid and Sephadex was used [46].

Inoculation is performed in two ways, as a suspension of conidia added to the cooled medium, or as a dry conidia mixed with sterile air and spread as an aerosol over the trays [27]. The temperature is kept constant at 30°C during the fermentation by means of air current. Ventilation is also important for gas exchange because the rate of citric acid production drops if carbon dioxide in the atmosphere increases over 10 per cent. Within 24 h after inoculation, the germinating spores start forming a 2–3 cm cover blanket of mycelium floating on the surface of the substrate. As a result of the uptake of ammonium ions, the pH of the substrate falls to 2.0. After 30 h the idiophase begins. If too much iron ions are present, oxalic acid is produced and a yellowish pigment is formed, which later complicates the recovery process. The fully developed mycelium floats as a thick white layer on the nutrient solution. Through evaporation, the temperature can be maintained constant, but the culture loses 30–40 per cent of its original volume. The fermentation process stops after 8–14 days.

For recovery, the mycelium and nutrient solution are removed from the chambers. Owing to its volume, the mycelium must be carefully washed in sections. On some cases, mechanical presses are also used to obtain more citric acid from the cells.

Solid state fermentation

Surface process employing solid substrate may use fibrous residues from apple [44], grape pommace [45], wheat bran or rice starch containing residual pulps from starch manufacture [46], potato [46] and sweet potato [47].

In this process, based on the traditional koji process know-how, the *Aspergillus niger* strains are not sensitive to trace elements as in surface fermentation with liquid substrate or in submerged process [48].

On the solid-state fermentation process, the solid substrate is soaked with water up to 65–70 per cent of water content. After the removal of excess water, the mass undergoes a steaming process. After sterilization, sterile starch paste is inoculated by spreading *Aspergillus niger* conidia in the form of aerosol or as a liquid conidia suspension on the substrate surface [27].

The pH of the substrate is about 5–5.5, and incubation temperature 28–30°C. Growth can be accelerated by adding α-amylase, although the fungus can hydrolyse starch with its own α-amylase. During the citric acid production pH dropped to values below 2 [40].

The solid-state surface process takes 5–8 days at the end of which the entire is extracted with hot water. On other cases, mechanical passes are also used to obtain more citric acid from the cells.

Using cane bagasse as the substrate by solid-state fermentation citric acid was obtained in 6 days [154]. Total World production of citric acid by solid-state fermentation was in 1990 about 350,000 tons [152,153].

Submerged fermentation

An effective alternative to surface fermentation processes is the submerged process. Although taking a longer fermentation time it has several advantages: lower investment by a factor of 2.5, 25 per cent lower total investment and labour costs, more effective process control and sterility. The disadvantages are the higher energy costs and more sophisticated control, which require more highly trained personal.

Three main factors especially important for high yielding citric acid production in submerged processes are [40]:

- quality of the stainless steel for the construction of the bioreactor,
- mycelium structure, and
- oxygen transfer.

Batch techniques in stirred tank or airlift bioreactors are in general use. Very promising results were obtained in fed-batch process [38–40] and by continuous fermentation [126–129] where various kinds of bioreactors as stirred tank reactors [99,102,105], airlift reactors [130,131], external loop reactors [132,133], magnetic drum contactors [134], reciprocated jet reactors, biodisc reactor [135], deep jet [136,137] hollow fibre [138] or by use of fix bed reactor [133].

Several report of citric acid fermentation using immobilized *A. niger* cells on various kinds of carriers as glass [139], polyurethane foams [140], entrapment in calcium alginate beds [141–143] polyacrylamide gels [144,145] agar [146] agarose [147] cellulose carriers [148,149] metal screens and polyester felts [150,151]. Bioreactors for citric acid production must be either protected from acids or constructed of special stainless steel. At pH value 2, the heavy metals leached from ordinary steel fermenter walls can inhibit the formation of citric acid [40].

Various substrates as beet [43,155–158] and cane molasses [159–161], media [162–164], starch hydrolysates [165–169], C9-C23 paraffins [28,29] and consume oil [170–172] have been reported. The concentration of carbon source in fermentation substrate is of great importance. Maximum citric acid production is usually achieved at carbon concentrations as high as 14–27 per cent.

Submerged fermentation using A. niger

In the case of beet molasses substrate, the reducing sugar content is usually 12–15 per cent. The row molasses is previously clarified by sulphuric acid and neutralized. Potassium hexacyanoferrate is added to the preparatory substrate for the purpose of suppressing, by means of complex formation, any detrimental effect of metal ions, particularly iron and to prevent a too rapid growth of the mycelium.

Nutritive salts, such as ammonium nitrate or potassium dihydrogen phosphate may be added. For substrate preparation common tap water can be used. Owing to its content of salts, it is generally more suitable than deionized water, pH of the substrate should be adjusted to 5.5–5.9, which is most suitable for the germinated conidia aggregation. Substrate is sterilized by heat, mostly by continuous sterilization [165].

In the case of the relatively pure sucrose containing substrates, fermentation is generally run at the medium sucrose concentration of 15–27 per cent. After ion exchange of the cations, the filtered solution is sterilized subsequently by heating and after cooling to 50°C fed to bioreactor. The bioreactors filled up to its working volume and nutrients are added. The pH is adjusted to an initial value of 2.5–3.0 [27].

The process can usually run in one or two stages, using hydrophilic spores suspensions [40] or germinated conidia from the propagator stage [165]. The use of germinated conidia may shorten the fermentation cycle from 12 to 24 h

[27]. Amounts of spores are 5–25 × 10^6 per litre of substrate [27] and conidia 10^{10} per litre [165]. It has been proved useful to incubate the spore suspension for 6–8 h in saline solution with added surface active agents prior to inoculation, thus shortening the fermentation cycle for 12 h [162].

On the two-stage fermentation process, germinated conida are produced in the first stage at pH 5.8, with absence of phosphate, at low dissolved oxygen level and at a sugar concentration of 7–9 per cent at temperature of 32°C [166].

For citric acid production, the spherical mycelia pellet growth form is widely used [170,171]. An effective pellet formation is preferably performed by a higher shear stress affected by aeration and agitation of the substrate. The development of the hyphae and the aggregation generally requires a period from 9 to 25 h at temperature of 32°C. The first two or three days of fermentation, *i.e.*, the period of initial mycelial growth and pellet formation, are decisive for the success of the fermentation. Heavy metals in the medium (Fe, Mn), exceeding concentration of iron ions higher than 1.5 mg/l and manganese 1 μg/l strongly affect cellular morphology, by promoting unproductive filamentous mycelial growth form [27].

The production of citric acid starts after 24 hours of inoculation. Mycelial aggregates and spherical pellets, the productive form, can be detected at the first and the second maximum of the redox potential curve [172,173].

The start of citric acid production is followed by an excessive foaming, therefore an effective foam control system is essentially needed [174].

The additions of silicone antifoam agents can reduce the dissolved oxygen concentration [175,176] or influence increasingly the pseudoplastic rheological behaviour of the fermentation broth [171]. On the production phase the aeration is set from 0.3 v.v.m., in germination phase to 1 v.v.m. The change of pH in this phase is from 5.5 to 3.5, for beet molasses substrate, and to 2.2 for the sucrose substrate. The pH of 4.5–3 is also characteristic for the fed batch fermentation production phase, where bioreactor is only filled to 40 per cent of its working volume with the propagation substrate containing 7 per cent of sucrose content, and fulfilled with citric acid production substrate of 17 per cent sucrose content [27].

The temperature in the production phase is from 28°C to 32°C. Temperature change from 32°C to 28°C is also the base of some industrial processes and patents [177].

Submerged citric acid fermentation using starch hydrolysates as a carbon source is also an effective alternative to standard processes on beet molasses or sucrose substrate. An advanced step in this technology is LIKO process based on various starch hydrolysates [166].

On the first step starch is treated by thermostabile α-amylase and temperature of 103°C. Often additional enzymatic treatment by combination of pullulanase and fungal α-amylase is needed. Nutrient salts were added to the starch hydrolysate and substrate is after continuous sterilization used in propagation and production stage.

For the separation of the product and waste biomass tangential flow filtration is used. Compared to classic Ca citrate precipitations with 6.0 per cent bases, only 28 per cent of the citric acid was lost. The benefit of this process are simpler isolation more pure product and no calcium sulphate.

Submerged citric acid fermentation on wheat flavour hydrolysates designed is inexpensive technology with more pure product has a lot of perspectives in the future production Comparing to using beet molasses and sucrose substrates, this is also a cheaper process.

Probably one of the most perspective submerged citric acid technology on sucrose substrate by Lešniak *et al*. (88–90 per cent) yields on industrial 150 m^3 reactors [38]. As an alternative to batch and fed-batch process is *Aspergillus niger* continuous citric acid fermentation developed by B. Kristiansen and co-workers [126,127].

Submerged fermentation using yeasts

Candida strains are also used novel process that permits production of citric acid from C9 to C20 normal paraffins. Citric acid yields up to 95 per cent were claimed. On 1974 Pfizer patented a continuous process for fermentation by *Candida lipolitica* using a single bioreactor to which paraffin was continuously added and fermented broth continuously withdrawn [168].

On citric acid fermentation stirred tank bioreactors, with usual capacities from 50 to 150 m^3 and airlift bioreactors up to 220 m^3, are used. The fermentation is a growth-associated process which lasts from 6 to 8 days [190,191].

Submerged fermentation using immobilization of microorganisms

It is worth noting that some of the problems arising in the downstream processing of citric acid produced by submerged cultivation, especially in a continuous process, might be minimized by immobilization of microorganisms in the bioreactor. The successful application of immobilized microorganisms as living biocatalysts, involving more careful handling and often having higher production rates than free microorganisms, has prompted a rapid development of this technique. Citric acid production by immobilized *A. niger* has been performed on a laboratory scale with the use of calcium alginate gel [143,178], polyacrylamide gel [147,179], polyurethane foam [142,180,181] and cryopolymerized acrylamide [182]. The profitable effect of the immobilization of *A. niger* mycelium in view of the citric acid recovery from the fermentation broth depends on the type of the support material and process conditions.

Solid-state fermentation

The solid culture process is completed within 96 h under optimal conditions (8). The most common organism used in solid-state fermentation is *A. niger*.

However, there have also been reports with yeasts. The strains with large requirements of nitrogen and phosphorus are not ideal microorganisms for solid culture due to lower diffusion rate of nutrients and metabolites occurring at lower water activity in solid-state process. The presence of trace elements may not affect citric acid production so harmfully as it does in submerged fermentation, thus, substrate pretreatment is not required. This is one of the important advantages of the solid culture [183,184].

Product recovery

On completion of the citric acid fermentation, the obtained solution contains, besides the desirable product, mycelium and varying amounts of other impurities, *e.g.*, mineral salts, other organic acids, proteins, etc. The method of citric acid recovery from the fermentation broth may vary depending on the technology and raw materials used for the production [185]. Separation of biomass from fermentation broth takes place in first step of the recovery process. Separated mycelia retain about 15 per cent of the citric acid formed during fermentation. The mycelia are then washed and pressed in filter presses dried and often used as a protein-rich feed for cattle. If oxalic acid is formed as a side product due to suboptimal fermentation control, it can be precipitated as calcium oxalate at pH below 3.0 [186].

Surface process

In the surface process, the fermentation fluid is drained off the trays and hot water is introduced to wash out the remaining amount of citric acid from the mycelial mats. Although it is a relatively simple procedure in the case of surface fermentation, where biomass is in the form of 2–3 cm cover blanket on the substrate surface. Thorough washing at this stage is necessary, because the mycelium retains about 15 per cent of the product formed in the fermentation. In this vessel the mycelium is heated to about 100°C by steam. The solution containing 2–4 per cent of citric acid is added to the fermentation fluid, whereas the filtration cake, containing not more than 0.2 per cent of citric acid, is dried to yield a protein-rich feedstuff [186].

Submerged fermentation

In the submerged fermentation the mycelium is far more difficult to separate from the fermentation broth. After the fermentation process is completed the mycelium-containing broth is heated to a temperature of 70°C for about 15 min, to obtain partial coagulation of proteins, and then filtered. Rotating vacuum drum or belt discharge filters or in centrifuges are used in this case [40]. If the mycelium is to be used as a feedstuff, the filter aid must also be digestible, *e.g.*, from cellulosic materials. If during the fermentation process

oxalic acid is formed, it has to be removed from the broth. This is usually achieved by increasing the pH of the fermentation fluid with the calcium hydroxide to pH = 2.7–2.9 at a temperature of 70–75°C. Calcium oxalate thus precipitated may be removed from the solution by nitration or centrifugation, and the citric acid remains in solution as the mono-calcium citrate.

Recovery of citric acid from pretreated fermentation broth may be accomplished by several procedures: classical method of precipitation, solvent extraction, ion-exchange and some more sophisticated methods such as electrodialysis, ultra- and nanofiltration or application of liquid membranes [186].

Precipitation

The standard method of citric acid recovery has involved precipitating the insoluble tri-calcium citrate by the addition of an equivalent amount of lime to the citric acid solution. Successful operation of the precipitation depends on citric acid concentration, temperature, pH and rate of lime addition. To obtain large crystals of high purity, milk of lime containing calcium oxide (180–250 kg/m^3) is added gradually at a temperature of 90°C or above and pH below, but close to, 7. The concentration of citric acid in the solution should be above 15 per cent. The process of neutralization usually lasts about 120–150 min. The minimum loss of citric acid due to solubility of calcium citrate is 4–5 per cent. Calcium citrate is then filtered off and subsequently treated with concentrated sulphuric acid (60–70 per cent) to obtain citric acid and the precipitate of calcium sulphate (gypsum). After filtering off the gypsum a solution of 25–30 per cent of citric acid is obtained. The filtrate is treated with activated carbon to remove residual impurities or may be purified in ion-exchange columns. The purified solution is then concentrated in vacuum evaporators at temperature below 40°C (to avoid caramelization), crystallized. A conventional crystallization scheme consists of a batch vacuum-pan evaporator or a forced circulating evaporator coupled with auxiliary tankage and appropriate centrifuge equipment. Within these systems, the crystals formed are separated by centrifugation and the mother liquor is fed back to the activated carbon stage. Both batch and continuous units have been employed in this cooperation depending of process adaptability and economics [40].

The drying of citric acid monohydrate is usually performed in conventional rotary drying equipment or in fluidized bed dryers. As anhydrous citric acid is hygroscopic, care must be taken to achieve the final moisture specification during drying and to avoid storage in areas of high temperature and humidity [186].

The disadvantage of this technology is the large amount of lime required for citric acid neutralization and of sulphuric acid for calcium citrate decomposition. Moreover, it results in the formation of large amounts of liquid

and solid wastes (solution after calcium citrate filtration and gypsum). For 1 ton of citric acid, 579 kg of calcium hydroxide, 765 kg of sulphuric acid and 18 m^3 of water are consumed and approximately 1 ton of waste gypsum is produced [186].

With the aim of decreasing the amount of lime and sulphuric acid by about one–third, [187] has proposed recovery of citric acid by precipitation of di-calcium acid citrate. An additional advantage of this method is that di-calcium acid citrate has a definite crystalline structure and washes cleaner than the amorphous tri-calcium citrate.

Solvent extraction

An alternative method of citrate-free recovery of citric acid from a fermentation broth is extracted by means of a selective solvent, which is insoluble or only sparingly soluble in the aqueous medium [188–190]. The solvent should be chosen so as to extract the maximum amount of citric acid and the minimum amount of impurities. The citric acid can then be recovered from the extract either by distilling off the solvent or by washing the extract with the water. From the aqueous solution purified citric acid is subsequently crystallized by concentration.

Various organic solvents which are partly or wholly immiscible with water, such as certain aliphatic alcohols, ketones, ethers or esters [190,191], organophosphorus compounds, such as tri-*n*-butylphosphate (TBP) [192] and alkylsulphoxides [193] and water-insoluble amines or a mixture of two or more of such amines are used [194–196].

Ion exchange

The efficiency of the ion-exchange separation process may be greatly enhanced by applying a simulated moving bed counter-current flow system. It consists of at least two static beds, connected with appropriate valving so that the feed mixture is passed through one adsorbent bed while the desorbent material can be passed through the other. Progressive changes in the function of each ion-exchange bed simulate the counter-current movement of the adsorbent in relation to liquid flow. In such a system, the adsorption and desorption operations are continuously taking place, which allows both continuous production of an extract and a raffinate stream and the continual use of feed and desorbent streams [197].

The disadvantage of the ion-exchange method may be seen in the fact that elution of citric acid from the adsorption bed may require a large amount of desorbent, due to the tailing effect known in chromatography, causing considerable dilution of the resulting citric acid solution. The periodical regeneration of the ion-exchange resins by inorganic bases may also be a source of unwanted effluent wastes.

Liquid membranes

Liquid membranes containing mobile carriers consist of an inert, microporous support impregnated with a water-immiscible, mobile ion-exchange agent. The mobile carrier, which is held in the pores of the support membrane by capillarity, acts as a shuttle, picking up ions from an aqueous solution on one side of the membrane, carrying them across the membrane and releasing them to the solution on the opposite side of the membrane [198]. For citric acid separation by liquid membranes, the tertiary amines which give the best results also in solvent extraction can also be used.

Recently, more sophisticated methods of citric acid separation with the application of liquid membranes are being developed [199–201].

Microporous hollow fibres

Microporous hollow fibres have been employed by Basu and Sirkar [202]. In this case the permeator consists of two sets of identical hydrophobic microporous hollow fibres. One set carries the feed solution of citric acid and the other the strip solution flowing in the lumen. The organic liquid membrane is contained in the shell side between these two sets of hollow fibres. This technique has been shown to be promising for citric acid separation even in the large scale, as the extent of citric acid recovery of up to 99 per cent was linear with the membrane area, suggesting easy scale-up [186].

Electrodialysis

This process enables separation of salts from a solution and their simultaneous conversion into the corresponding acids and bases using electrical potential and mono- or bipolar membranes. Bipolar membranes are special ion exchange membranes which, in an electrical field, enable the splitting of water into H^+ and OH^{2-} ions [203]. By integrating bipolar membranes with anionic and cationic exchange membranes, a three- or four-compartment cell may be arranged, in which electrodialytic separation of salt ions and their conversion into base and acid takes place [204].

Before the fermentation solution comes to the electrodialysis, some pretreatment steps are normally necessary: filtration of the broth, removal of ionogenic substances (especially Ca^{2+} and Mg^{2+} ions) and neutralization by means of sodium hydroxide. In the subsequent electrodialytic step the sodium citrate solution is converted into base and citric acid, which is simultaneously concentrated and for the most part purified. The produced NaOH may be reused for the neutralization [205].

The energy consumption (excluding pumping) for the separation of 1 kg of citric acid using bipolar membranes is in the range of 6.1×10^3–7.2×10^3 kWs

[206]. Owing to low mass transfer at low pH values, it is advantageous to adjust the pH of the feed acid stream to 7.5 [207,208].

Ultrafiltration

Continuous separation and concentration of citric acid may be also achieved by ultra and/or nanofiltration, verified in a laboratory scale a two-stage membrane process for citric acid recovery from the broth obtained in *A. niger* cultivation on sucrose. Polysulphone membrane with cut-off 10,000 used in the first stage allowed the product to pass through to the permeate stream, while the retentate stream contained most of the peptides and proteins from the broth. The rejection coefficient for the product in this step was 3 per cent, for the reducing sugars 14 per cent and for the proteins 100 per cent. Tighter nanofiltration membrane with cut-off 200 in the second stage rejected approximately 90 per cent of citric acid and 60 per cent of reducing sugars (mono-saccharides). A similar two-stage membrane technique was adapted by Bohdziewicz and Bodzek [209] for simultaneous separation and concentration of pectinolytic enzymes and citric acid from a fermentation broth.

Recovery of citric acid via calcium salt precipitation is a complex process. In this process calcium citrate is formed in further by adding a lime slurry at a neutral pH. After sufficient reaction time, the slurry is filtered and the precipitate washed free of soluble impurities. The resulting calcium citrate is then acidified with sulphuric acid. This reaction converts calcium citrate to calcium sulfate and citric acid in the presence of free sulphuric acid. Calcium sulphate is then filtered and washed free of citric acid solution. Both the calcium citrate and calcium sulphate reactions are generally performed in agitated reactors and filtrated commercially available filtration equipment.

The aqueous citric acid solution is demineralized at this step by strong cation exchange resin in the H^+ form (Dowex 50) and an anion exchange resin of medium strength. The purified citric acid solution is subsequently evaporated in a multi-stage evaporator at temperature of 40°C to avoid caramelization [27].

The clear citric acid solution undergoes a series of crystallization steps to achieve the physical separation of citric acid from the remaining trace impurities. A conventional crystallization scheme consists of a batch vacuum-pan evaporator or a forced circulating evaporator coupled with auxiliary tankage and appropriate centrifuge equipment. Within these systems, the crystals formed are separated by centrifugation and the mother liquor is fed back to the activated carbon stage. Both batch and continuous units have been employed in this cooperation depending of process adaptability and economics [40]. The drying of citric acid monohydrate is usually performed in conventional rotary drying equipment or in fluidized bed dryers. As anhydrous citric acid is hygroscopic, care must be taken to achieve the final

moisture specification during drying and to avoid storage in areas of high temperature and humidity [27].

Solvent extraction is an alternative recovery process, which involves the extraction of citric acid from fermentation brutish using hydrocarbons such as: *n*-octanol, C10 or C11 isoparaffin, benzene, kerosene; ethers: *n* or isobutyleter; esters: *n*-butylacetate; ketones: methyl isobutylketone [104] or various amines: trilaurylamine [105]. The recovery process by solvent extraction consists of selectively transferring citric acid via a solvent from an aqueous solution containing various by-products to another aqueous solution in which the citric acid is more concentrated and contains substantially less by-products. The final processing steps begin with a different wash of the aqueous solution by the hydrocarbon solvent, followed by the passage of the acid solution through a conventional sequence of evaporator-crystallizer steps to complete the manufacturing process.

Anhydrous citric acid and its monohydrate can be stored in dry form without difficulties; however, high humidity and elevated temperatures should be avoided to prevent caking. Therefore, the use of packing materials with a desiccant is suggested [106].

The citric acid recovery process leads to considerable accumulation of waste products. More than 60 per cent of it belongs to gypsum (calcium sulphate). Which still contains potassium hexacyanoferrate, charcoal and organic compounds from molasses making it so unsuitable as a building material. The waste mycelium from submerged and surface fermentation can be dried and used as an animal protein-rich feed or alternatively as fertilizer.

Economic aspects

Although the surface production process is from the viewpoint of energy requirements, a less expensive, there are a lot of disadvantages in it. This involves larger space requirements for production and isolation, higher steam requirement and higher sterility requests. One of the greatest problems of this production process sterility. Main advantages of the submerged fermentation process are: shorter fermentation time (6–7 days), higher level of process sterility and control of process parameters, simpler process operations, lower space requirements, process reproducibility and higher yields.

Schierholt [210] compared the economy of surface and submerged fermentation process for the citric acid production.

Capacities of 300 m^3 and 150 m^3 in 9 days of fermentation time at productivity 72 tons and 12 tons per day were compared. On his work he concludes that the building investment costs connected with the surface fermentation process are 2.5 times higher than those connected with the submerged fermentation. Contrary to this, the expenses on equipment are considerably higher at submerged fermentation, and more than 60 per cent of those expenses consist of complicated component as are bioreactors and

more sophisticated instrumental control, which are subject to relatively high wear.

The total investment costs for the submerged process are about 25 per cent lower for higher capacities and 15 per cent lower for smaller capacities than for surface fermentation. The more favourable total investment costs for the submerged process are in contrast to considerably higher production costs for any capacity. Especially evident is the high consumption of electric energy, which is about 30 per cent higher as much as that required at surface fermentation. The labour costs in highly developed countries are for surface fermentation considerably higher.

On countries where cooling water temperature exceeds 20°C, additional expenses for cooling the bioreactors are incurred by installation of cooling aggregates for submerged process. The submerged fermentation is sensitive to short interruptions or breakdowns in aeration, which results not only in loss of yield, but also in total breakdown of the respective batch. At surface fermentation, the resulting citric acid solution or fermentation broth is much more concentrated than at submerged fermentation, effected by higher evaporation rates during fermentation.

Production of citric acid by surface solid state or by isolation from citrus juices does not represent a significant percentage on the World scale. Although both processes are from all aspects very cheap, they are in use mostly in the countries with old traditions (Italy, Greece, Asia).

Citric acid World production

Development of citric acid fermentation industry during the nearly passed century has aroused a great deal of interest. Formerly, the raw material, calcium citrate, was produced almost entirely from citrus products, Italy being by far the largest producer. The bulk of the Italian production of calcium citrate was shipped to England, France and the United States. Because of the development of the fermentation process and the increased output of citrus materials, import in the United States has practically ceased since 1927. The fermentation process has to a large extent developed also in Europe. Large quantities of fermentation-based citric acid have been produced in England, Belgium and Czechoslovakia and probably Russia. The former dominant position occupied by the Italian producers of this commodity has thus been last through new methods introduced by scientific research.

The first successful commercial development of the citric acid fermentation process was achieved in the United States. Miles and later Charles Pfizer Company gradually developed in to the World's leading companies. The United States citric acid production in 1929 was 4,900 tons per year. While in 1978 the production by Miles was 29,000 tons and by Pfizer (U.S.) 42,500 tons, and raised in 1990 to 66,000 tons by Miles and 105,000 tons by

Pfizer (U.S.). Pfizer overall production, including the U.S. and other countries (Irish Republic, Nigeria, Taiwan, Argentina), takes about 30 per cent of the World's citric acid production. From 1978 until 1990 Pfizer increased its production by 34.5 per cent.

Although the World's greatest producers are in the United States, the World's greatest production continentally belongs to Europe with 250,000 tons per year, produced in 16 countries. The yearly production in North America was in 1990 about 215,000 tons followed by Asia with 66,000 tons, Africa 14,000 tons, Australia about 8,000 tons and South America with 7,000 tons. In Asia, citric acid production is characterized also by the use of traditional solid-state production on the food industry wastes and by submerged technologies based on various yeast strains (Table 1).

Citric acid is a commodity chemical produced and consumed throughout the World. It is used mainly in the food and beverage industry, primarily as an acidulant. It is estimated that over 65 per cent of the citric acid produced is consumed for food and beverages. Global production of citric acid in 2004 was about 1.4 million tons estimated by Business Communications Co. but in 2005 it was about 1,600 thousand metric tons. The majority of production capacity and consumption was in China, Western Europe and the United States. China is estimated to account for at least half of the global production capacity, while Western Europe and the United States combined account for about a third. Western Europe, the United States and China combined are estimated to account for 65–70 per cent of global citric acid consumption. The citric acid industry continues to be influenced by increased supply from China and abundant global capacity. In recent years, plant closures have occurred as a result of competition, and prices have continued to decline.

Table 1. The industrial World production of citric acid in 1990 [152].

The greatest World producers (tons)		
USA	Pfizer	105,000
	Miles	66,000
Belgium	Citrique Belge[a]	55,000
Austria	Jungbunzlauer	40,000
Ireland	Pfizer	36,000
Germ. Fed. Rep.	Biochemic Ladenburg	30,000
Italy	Biacor	25,000
Mexico	Quimica Mexama	19,000
Soviet Union	State Authority[a]	18,000
Great Britain	John & E. Sturge	14,000
Israel	Cadot Petroch	8,000
World production (1990)		598,000

[a]Surface production.

From the first industrial fermentations, the World's production has increased exponential from 5,000 tons in 1929 until the present production in 2006 of about 1,600,000 tons per year (Fig 1). [152,153].

In 2005, the top six producing companies accounted for about 53 per cent of the World's total capacity for citric acid. China's capacity was 800 thousand metric tons (50 per cent of World capacity), most of which is unrefined citric acid. The global production capacity of the World's six largest citric acid-producing companies, plus China (which produces mostly unrefined citric acid), is shown in Fig. 2.

Figure 3 shows World's consumption of citric acid by region in 2005.

Over half of the global consumption of citric acid is used for the beverage industry. The food industry consumes about 15–20 per cent, followed by detergent and soaps (15–17 per cent), pharmaceuticals and cosmetics (7–9 per cent) and industrial uses (6–8 per cent).

In the United States, the citric acid market will continue to grow mainly as a result of growth in the beverage market. New product introductions and continued use in diet colas, fruit-flavored waters, iced teas and sports drinks will lead to higher growth. Liquid detergent growth will also contribute to growing citric acid demand. New growth will also be seen in industrial applications, as renewable resources continue to grow. In Canada, citric acid use may increase significantly as a result of use in oil recovery.

In Europe, the market is impacted by price, which is driven down by a combination of strong competition from Chinese product and an abundance of global capacity. European producers are contending with imports from Chinese producers. While the average prices were declining, Chinese imports of citric acid to Western Europe grew from roughly 46 thousand metric tons in 1999 to 109 thousand metric tons in 2004. Chinese competition is mainly in

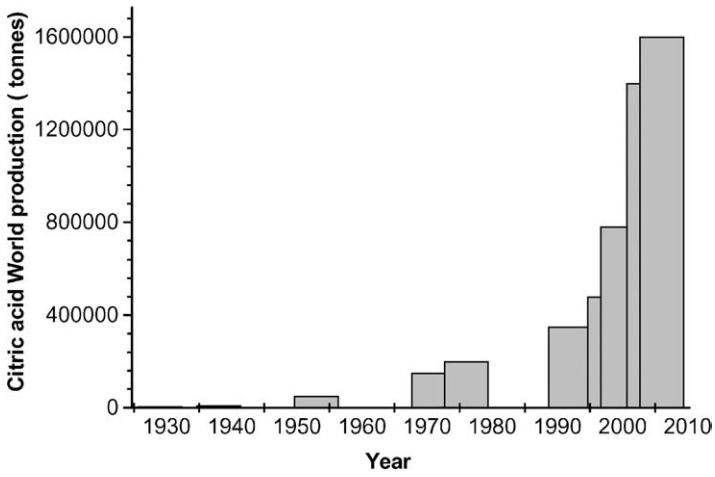

Fig. 1. Citric acid World production.

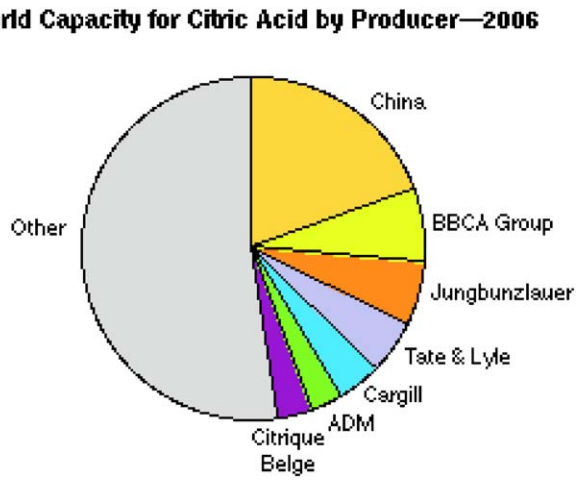

Fig. 2. World capacity for citric acid producer in 2006 [153].

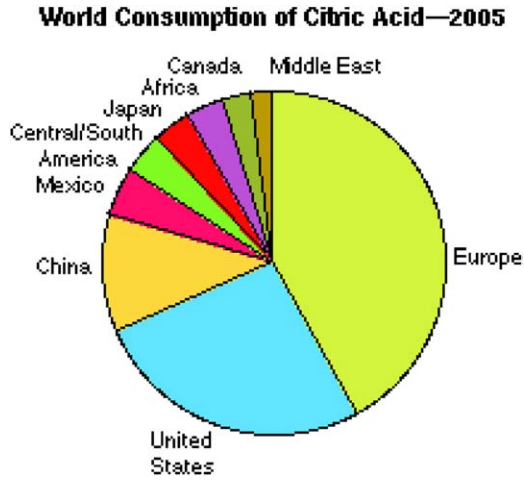

Fig. 3. World consumption of citric acid by region in 2005 [153].

citric acid monohydrate (solid form) and among citrates in sodium citrate, the most-used-form of citric acid salts. Chinese suppliers have started to adopt Western-pricing practices, which might lead to a more stabilized price. In the future, European manufacturers of citric acid and citrates might concentrate on the production of citric acid solutions (using solid form produced in-house or imported) and/or higher-value citrates.

The citric acid market continues to face pressure from Asian imports and increased global supply causing selling prices to decline. However, tight supplies from Europe caused by closures, and high energy and freight costs are

some of the factors leading to higher citric acid prices. The overall global market for citric acid is expected to grow at an average annual rate of 3.5–4.5 per cent in the next few years.

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