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A Century of Citric Acid Fermentation and Research

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Summary

Citric acid fermentation is one of the largest biotechnical industries with a history dating back about a century. It may be assumed that around two thousand scientific contributions or more, and several hundred patents are related to this endeavour. The present article wants to collect some, more or less, known historical data to review how our knowledge has increased over these hundred years, and to add some thoughts, perhaps superfluous – perhaps stimulating.

Keywords: citric acid, fermentation, *Aspergillus niger*

In the very beginning (1891/93) there was Wehmer *(1)*, who described oxalic acid production and excretion in several strains of *Aspergillus*, esp. *Aspergillus niger*. Since he had observed that acid formation was followed by almost complete decomposition, he added calcium carbonate to collect and preserve the acid salt as formed. Besides this he observed citric acid formation in other strains of fungi *(2,3)*, some of which he designated as a new genus, *Citromyces*, now known as belonging to *Penicillium*. Being aware of the practical potential of this finding (French Patent 228,554, 1893; Ger. Patent 72957, 1894; United States Patent 515,033, 1894), attempts were made to run a plant at Fabrique de Produits Chimiques de Thann et de Mulhouse, which failed however due to inevitable contaminations since single production runs had to be conducted for periods of several weeks. Operation of this plant was finally terminated in 1919.

In 1913 Zahorsky *(4)* took out a patent for a citric acid production process using *Sterigmatocystis nigra*, claimed to differ from *Aspergillus niger*, but later regarded as identical.

Of pivotal importance, however, is the work of Currie (1916/1917). Currie, working as a dairy chemist in the Bureau of Animal Industry in Washington, D.C., with the famous mycologist, Ch. Thom, of the Bureau of Chemistry, had observed *(5)* that a great number of strains of black Aspergilli, but also related fungal strains from all over the world, were more or less active oxalic acid producers. In the course of this work, it was found that several strains regarded as *Aspergillus niger* produced some acid in excess to oxalic acid, which was identified as citric acid *(6)*. In his famous study *(7)*, Currie disproved the belief that the culture fluid would have to be neutralized with the onset of acid production. In contrast, he demonstrated that without the addition of calcium carbonate, citric acid was formed abundantly, much more rapidly and with considerably reduced risk of contamination due to the low pH (approx. 2) of the culture medium.

In addition, he elaborated optimum conditions for the production of citric acid, finding low nitrogen supply, high concentrations of sugar, and nitrogen supplied as ammonium salts rather than as nitrates, as the prerequisites enabling citric acid production with yields of more than 50% based on sugar consumed. Remarkably he stated that »it is clear that the highest yield of fermentation (by)products will be had when the development of mycelium is restricted and not when stimulated«. His formulation of a fermentation medium can still be regarded as standard.

Currie left the Bureau to join Chas. Pfizer Inc. in New York, where a plant was established which went into production in 1923. The technology to be developed was a surface culture using shallow pans stacked in air-

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conditioned chambers, in which the fungus formed a wrinkled mycelial felt that reached into the liquid thus forming a high specific contact area facilitating diffusion and exchange of substrate and product. Interestingly, no further papers were published and patenting was not attempted.

Within a few years, this production became markedly competitive to the mainly Italian manufacturers of citric acid from citrus fruits, who produced and exported calcium citrate which was processed to citric acid at its destinations, foremost England, France and the United States. The fact that Italy as well as the United States imposed elevated taxes to exports and imports, respectively, greatly facilitated conditions for fast development of a citric acid fermentation industry. Wells and Herrick *(8)* have given a rather detailed description of events, especially the various governmental measures to protect the respective domestic producers. According to these authors, beginning with the years after 1927, the United States not only became independent of foreign supplies but could also export citrate, mainly to England. Exports of about 3,600 t of calcium citrate to England in 1933, however, decreased sharply when one of the main producers of citric acid from calcium citrate, J. and E. Sturge Ltd. in England, had started their own citric acid fermentation based on patents taken out by Fernbach *et al.* in 1927 and 1932 *(9,10)*.

In 1928 a plant was established in Kaznèjov near Plzen in the Czech Republic which for the first time utilized molasses as a cheap carbon source based on Montan- und Industrialwerke, vorm. J. D. Starck, 1924 *(11) and* Szücs *(12)* patents. Molasses presents some advantages due to its contents of growth stimulants and assimilable substances. On the other hand, the presence of ash components, especially higher amounts of heavy metals, has to be counteracted appropriately. This, according to Leopold and Valtr *(13)* was first achieved in the plant in Kaznèjov by pretreating molasses using hexacyanoferrate as a complexant and precipitant of these metal ions. Apparently kept secret, this method was published ten years later by Mezzadroli, 1938 *(14)*.

Other plants were erected in Belgium (La Citrique Belge S.A.), in the former Soviet Union, and in several other countries. By 1933, this industry already contributed 85% (Europe, 5,100 t and United States, 3,500 t) of the world citric acid production of 10,400 t, leaving only small amounts to Italy and other citrus processing countries.

At the same time, citric acid fermentation became an object of study by several academic groups which were actively engaged in optimizing the process and in elucidating the biochemical mechanisms leading from the sugar substrate to citric acid. An amazing number of hypotheses trying to explain the conversion of a straight chain form of glucose *via* diverse intermediates to a branched chain six-carbon acid were published until citric acid was identified in 1949 as the principal metabolite of the tricarboxylic acid cycle (see *e.g.* Foster, *(15)* and Walker, *(16)*). Among the first of such research groups was that of Bernhauer at Prague. Particularly his work on strain development was of great help for the plant at Kaznèjov, as well as later for the German plant of Joh. A. Benckiser GmbH at Ladenburg, which was ready for production in 1944. Bernhauer's experience in 1936 also offered first opportunities to introduce specific experiments for the training of students *(17)*. Another group (Doelger and Prescott) was from MIT, the Massachusetts Institute of Technology *(18)* in the United States. Workers from Poland (*e.g.* Chrzaszcz) and the former Soviet Union (*e.g.* Butkewitsch) add to this list. The famous group of Raistrick, in England, apparently was only marginally involved, although there is reference published in 1936 in a review of Clutterbuck *(19)* that he developed a production process claiming extraordinary high yields, which apparently was never disclosed (cf. Miall, *(20)*).

Details of one of the successfully operating plants were made accessible through the reports prepared by Waller *(21)* and Ellis and Gresford *(22)*, when at the end of World War II members of the staff of the Ladenburg plant of Joh. A. Benckiser were interrogated.

With respect to the utilization of molasses in surface fermentation, work of the group of Leopold at Kaznéjov should be mentioned, which disclosed a great number of problem solutions connected with the use of this type of raw material in a large series of papers starting after World War II. Other communications from his group were devoted to strain selections etc.

In 1933, Kluyver and Perquin *(23)* invented the so-called shake-flask technique for a novel fermentation process, submerged fermentation. In his excellent dissertation *(24)*, Perquin laid the foundation for a submerged process of citric acid fermentation. As a matter of fact, there had been several earlier publications describing submerged citric acid production, which apparently were neglected. Noteworthy examples are a patent from 1926 by Bleyer *(25)*, and a paper from 1930 by Amelung (1930) *(26)*. In the latter, however, yields were reported to be smaller than in surface culture. On the other hand, it was stated in 1939 by Wells and Ward *(27)* that submerged culture would not work at all as a feasible technology of industrial citric acid production. Perquin in 1938 elaborated the principal laboratory technique, showing *e.g.* that the fermentation was rather sensitive to heavy metals, especially iron ions. He found that phosphate limitation was important indicating that it was necessary to elaborate the nutritional requirements more carefully as compared with the surface process.

With a short intermission due to the World War II, the first patents on industrial submerged fermentation processes appeared. Once again it was Szücs *(28)* with a patent of 1944 in which *Aspergillus niger* was pre-grown in a simple synthetic medium (growth solution) and the mycelial mass transferred to a medium lacking phosphate (fermentation solution) where citric acid production »took place substantially«.

In an attempt to supersede the existing technologies, *e.g.* that of Pfizer, Waksman and coworkers at Rutgers University found another potent producer strain, *Aspergillus wentii*, an organism closely related to *Aspergillus niger*, but differing sufficiently from the latter, *e.g.* in color (brown). In the respective patent *(29)*, assigned to Merck & Co., Inc., a similar technique as that of Szücs consisting of a pre-culture and a fermentation medium low in phosphate ($\gamma(P) = 0.018$ g/L) was described (see

also Karow and Waksman *(30)*). The organism had been isolated from soil in 1915 by Waksman. Interestingly, it had first been described by Wehmer as a »technical fungal species« from Java in 1896.

Important contributions came from the group of Johnson from the University of Wisconsin in 1946. In one of the first communications *(31)* it was found that cationic exchange treatment of commercial sugars could increase citric acid yields appreciably, whereas additions of certain heavy metal ions at appropriate concentrations (0.1 mg/L) had beneficial effects. This was the first application of ion exchange to remove excess of metallic ions from nutrient media including molasses *(32)*, and it was later transferred to industrial scale. From one out of a series of representative strains (now ATCC 1015), in part still from Thom's collection, a »high producer«, strain 72-4 (now ATCC 11414), was fortunately isolated, forming the basis of further studies.

Another important study was that of Shu and Johnson *(33–35)*. Although using small scale (shake flask) experiments, the authors were able to demonstrate that the most significant positive effect on citric acid accumulation in submerged culture was deficiency of manganese and iron.

Regarding manganese, they reported that traces of manganese detrimental to the fermentation could even be carried over to the fermentation medium from the preculture medium, if the latter had a manganese concentration of 0.1 mg/L.

With respect to phosphate, they showed that not the concentration of phosphate alone but rather the relative proportions of phosphate, manganese and zinc were the crucial factors.

In one of their communications they presented diagrams showing the time-courses of biomass, citric acid and sugar concentrations which, although containing only few datapoints over a fermentation period of 200 hours, were later used by Gaden Jr. *(36)* in 1955 to present his well-known concept of fermentation types.

In 1952 successful pilot plant fermentations in stirred and aerated 50-gallon tanks were reported by Buelow and Johnson *(37)* with acid yields exceeding 75% and productivities around 0.7 g $L^{-1}h^{-1}$ based on initial sugar concentrations of 100–160 g/L.

Concomitant with the work of the Wisconsin group regarding effects of traces of metals, Tomlinson *et al. (38,39)* investigated the influences of zinc, iron, copper, and manganese on citric acid production, finding that these metals, normally present only by chance as contaminants of medium constituents, would become limiting if purer chemicals for making up fermentation media were applied.

In the meantime, patents by Schweiger and Snell *(40)*, Snell and Schweiger *(41)* and Woodward and Snell *(42)*, from Miles Laboratories, Inc., Elkhart, U.S.A., had been taken out covering the use of less refined sugar sources, such as high-test (invert) molasses or corn starch hydrolysis products. These patents may be considered as another milestone in the development of submerged fermentation. The claims in these patents comprised: reduction of the iron content to values of about 1 mg/L by (if necessary two-fold) cation exchange, which was found to be prerequisite – thereby creating a specific morphology of the growing hyphal material characterized by »abnormally short, stubby, forked, bulbous mycelium; numerous swollen, oval to spherical-shaped cells well distributed throughout the mycelial structure; mycelial structures all showing granulation, and numerous vacuoles or refractile bodies; absence of normal reproductive bodies (vesicles or sterigmata); formation of compact aggregates having a gross granular appearance and of sizes under 0.5 mm in cross section and averaging about 0.1 mm«. In addition, application of ammonium carbonate as the nitrogen source sufficient for cell synthesis was found to be an improvement.

Another approach to counteract the deleterious action of heavy metals was described by Moyer *(43,44)*, who had observed that the addition of small amounts of low molecular weight alcohols, preferentially methanol, could increase the tolerance level of these metals in a fermentation. The nature of this effect has never been elucidated exactly. It seems, however, that a single causative event can be excluded (see, *e.g.* Maddox *et al., (45)*.

Analogous to practice in surface fermentation, the possibilities of utilizing molasses as a considerably cheaper raw material were studied by several groups. The varying compositions of molasses, especially the fact that cane and beet molasses display appreciable differences in quality, were posing considerable problems to workers in this field. It goes without saying that a great portion of all publications on citric acid fermentation has been dealing with a substrate yielding less reproducible results due to its ill-defined composition – and nowadays posing the additional problem of environmental pollution.

Based on knowledge obtained from the reports of the work at Ladenburg at the end of World War II, scientists of the National Research Council of Canada at Ottawa started their work using readily available Canadian beet molasses as raw material. It is interesting to note that parallel to this work aiming at the development of an indigenous process, valuable studies concerning the biochemical mechanisms and enzymes involved were undertaken within this group.

In 1952 Clement *(46)*, using a strain from Ladenburg (Benckiser XXII = ATCC 10577), succeeded in establishing a satisfactory submerged fermentation of beet molasses treated with hexacyanoferrate. Subsequent work by Martin and collegues *(47–50)* established a working technology for the production of citric acid from molasses, mainly beet molasses, whereas cane blackstrap molasses, the worst of these raw materials, was shown to be hardly suitable. In the course of this work, using now the Wisconsin strain 72-4, it was observed that even a slight excess of free hexacyanoferrate had an inhibiting effect on fungal growth, thus being beneficial with respect to citric acid production *(51)*. This effect was confirmed also by other groups. Treatment with hexacyanoferrate was optimized empirically, and a method for the determination of free hexacyanoferrate was elaborated by Marier and Clark *(52)*, which is well-known to all workers in this field. In several papers it was shown that a special type of pellet morphology can be attained by appropriate adjustment of hexacyanoferrate concentrations in the course of the fermentations

(53–55), enabling also moderate scale-up *(56)*. Attempts at elucidating the effects of hexacyanoferrate additions on the composition of molasses by spectrometric analyses by Clark, *et al. (57)* could not clear the problem sufficiently. Appropriate control of hexacyanoferrate concentrations, however, was helpful in achieving high yields with cane molasses which had been found unsuitable in earlier experiments *(58)*. In a further investigation *(59)* it was observed that the addition of as little as 2 μg/L of manganese to ferrocyanide-treated molasses during fermentation reduced citric acid yield by 10%, with concomitant change in the morphology of the mycelium from the normal pellet form to filamentous outgrowth.

Altogether, it may be seen that problems in the fermentation of molasses of varyous origin and quality could be overcome by common purification procedures combined with hexacyanoferrate treatment and appropriate additions. Nowadays, with modern methods of sugar manufacturing, molasses have lost their attraction as a cheap raw material, especially when considering that molasses confer enormous loads of inorganic material into waste-water streams of a citric acid plant.

Reciprocal to this, relatively purer raw materials such as more or less refined starch hydrolyzates or cheaper surplus sucrose, which can be purified by ion exchange, have gained increased appreciation. In 1959, Schweiger *(60)* working with Miles Laboratories, Inc. took out a patent claiming that the addition of small amounts of Cu(II)ions to fermentation media counteracts the possible contaminations by Penicillia. Soon after he claimed *(61,62)* that the analogous addition of Cu(II)ions shows a pronounced antagonistic action with respect to deleterious concentrations of iron in fermentation media, making removal of iron, as claimed in an earlier patent, unnecessary. Such addition produces the same type of mycelial morphology as described above bringing about high citric acid yields and productivities. Apparently these achievements are the special features of one of the most effective, though highly sophisticated, technologies in the citric acid area.

Similar ideas, *i.e.* counteracting the influence of heavy metals in fermentations with pure carbon sources were followed by claims that the addition of rather small amounts of hexacyanoferrate as compared to practice with molasses would likewise bring about the physiological conditions necessary for abundant citric acid production, as reported in 1973 by Kabil *(63)*.

In view of the large number of communications being published during the last thirty years, it is extremely difficult to specify and acknowledge all the achievements that have accumulated within this period. Perhaps restriction to a few major points will be appropriate: studies on the **regulation of citric acid production** as a prerequisite for genetic and genetic engineering approaches to strain improvement (several contributions to this item have been published by the group of the present author together with C.P. Kubicek); the advent of **novel citric acid producers**, yeasts; and **downstream processing**.

At this point it should be mentioned that a great number of published reports are dealing with work under conditions not really resembling industrial practice, *e.g.* with respect to substrate concentrations, productivities and yields, respectively. In addition, as mentioned above, the use of molasses as a carbon source precludes precise scientific conclusions in many instances.

Regulation of citric acid accumulation: When, at the end of the 1960s, the mechanisms and pathways leading to citric acid formation had been elucidated, interest obviously was focussed on the regulatory mechanisms of the citric acid forming system in *Aspergillus niger*, and later in yeasts. A great number of papers of the last three decades are devoted to this subject.

In the opinion of the present author, citric acid formation and excretion is a typical example of metabolic overflow due to nutrient limitation and subsequent starvation. The theoretical foundations of this concept have been laid with the works of Foster *(15)* and Pirt *(64,65)* and were further developed by *e.g.* Neijssel and Tempest *(66–69)*. According to these sources, metabolic overflow or metabolic over-production may be brought about by either withdrawal of an essential nutrient, *i.e.* starvation, or by inhibition of growth by a chemical inhibitor or physically adverse conditions resulting in starvation as well. If one considers the kind of measures that have been exerted on the citric acid producer, *Aspergillus niger*, it becomes evident that several kinds of such limitation have been induced, namely withdrawal of nitrogen, phosphorus or heavy metals, or additions of chemicals such as hexacyanoferrates or copper ion. The actions of the later is probably manyfold: in the case of hexacyanoferrate it is withdrawal of heavy metals by complexing/precipitation, and inhibitory action on growth associated metabolism while in the case of copper it is ion antagonism and again inhibitory action on growth metabolism which is important. However the metabolic effects as such are not sufficiently understood so far. Seehaus *et al. (70)* have shown that Cu(II)ion antagonize the negative effect of manganese ions on citric acid production by interfering with a specific manganese uptake system of *Aspergillus niger*. Quite recently, Netik *et al. (71)* have reported that manganese ions are involved in the regulation of uptake and excretion of citric acid in *Aspergillus niger*.

What are the experimental results that can be put forward in relation to this concept? It may be seen from the work of many groups, including the one of the present author, that growth is limited to an extent that the biomass (dry mater) concentration in a fermentation batch is low, usually around 10 g/L. In experiments correlating growth and citric acid formation by Roehr, Zehentgruber and Kubicek *(72)*, it was shown that after a period of rather vigorous growth with little citric acid production, growth during active citric acid production is rather scarce. It was suggested that as a kinetic model this can be described by a modification, proposed by Brown and Vass *(73)*, of the well-known equation of Luedeking and Piret. Brown and Vass introduced a so-called maturation time, which in the work on *Aspergillus niger* may be interpreted as citric acid being formed mainly by the almost non-growing subapical part of the mycelium. Usually the duration of this production phase is 100–200 hours, and this would imply a transition to the non-growing state as indicated by Trinci and Thurston *(74)*.

Although conflicting results regarding almost every step in the long series of reactions and enzymes must be considered, a simple or perhaps simplified picture of events and their regulation can be offered (for reviews see Milsom and Meers *(75)*; Milsom *(76)*; Mattey *(77)*; Bigelis and Arora *(78)*; Roehr, Kubicek and Kominek *(79, 80)*).

One of the prerequisites for abundant citric acid production known since Currie (1917) is to provide high concentrations of a readily assimilable carbon source, thus creating a high substrate flux through the system. In 1987 Kubicek *(81)* has tried to apply flux control analysis to the system which poses the problem that in a pathway with a larger number of steps it becomes difficult to identify enzymes with pronounced regulatory properties. Kubicek identified the hexokinase reaction as the »rate limiting« step. In the meantime Torres *(82,83)* has refined and corroborated this interpretation applying more sophisticated mathematical methods including multisite optimization procedures. *Aspergillus niger* has only one hexokinase (cf. Steinböck *et al. (84)*) which is increasingly formed at high sugar concentrations. Hexokinase reaction is followed by phosphofructokinase (PFK). The known fact that PFK is a potent regulator of glycolysis has been confirmed for *Aspergillus niger (85–87)*. It is activated by AMP, NH_4^+ ions and fructose-2,6-bisphosphate, inhibited by PEP, citrate and ATP, and there is some evidence for its regulation by a cAMP dependent protein kinase (Legisa and Bencina *(88)*). High concentrations of sugar give rise to elevated concentrations of fructose-2,6-bisphosphate (Kubicek-Pranz *et al. (89)*), which is only observed with carbon sources readily converted to citric acid. PFK activity is thus indirectly regulated by its substrate, fructose-6-phosphate, via fructose-2,6-bisphosphate, catalysed by a poorly regulated enzyme, phosphofructokinase 2, which is mainly regulated by the availability of its substrate, fructose-6-phosphate *(90)*.

The next essential step is the formation of oxalacetate as one of the two substrates of citric acid synthesis. Most remarkably it was found that in *Aspergillus* species including *Aspergillus niger*, pyruvate carboxylase is exclusively located in the cytosol (Osmani and Scrutton *(91)*; Jaklitsch, Kubicek and Scrutton *(92)* Bercovitz *et al. (93)*). This offers a consistent interpretation of citric acid excretion: cytosolic oxalacetate may enter the mitochondrion after reduction to malate; after reoxidation of malate, citric acid is formed in the mitochondrion and can leave the mitochondrion in the same amount as the inflowing malate via the known malate/citrate shuttle. Thus anaplerotic formation of oxalacetate from the glycolytic pathway is specifically related to citric acid formation and excretion (Kubicek, *(94)*). Pines *et al. (95)* have shown that in *Saccharomyces cerevisiae* overexpression of cytosolic malate dehydrogenase causes a 6- to 16-fold increase in cytosolic malate dehydrogenase activity resulting in a more than 3-fold increase in L-malic acid accumulation in the fermentation medium. Overexpression of this enzyme with concomitant malic acid overproduction is always accompanied by increased citric acid formation providing evidence that cytosolic malate is the precursor for citric acid production.

Several ideas have been put forward to account for the fact that citric acid is excreted instead of being metabolised subsequently in the tricarboxylic acid cycle. They all postulate that the activities of some enzyme(s) following citric acid synthesis should be diminished: aconitate hydratase, isocitric acid dehydrogenase (NAD), isocitric acid dehydrogenase (NADP), and 2-oxoglutarate dehydrogenase. There is evidence that aconitate hydratase is not inhibited under conditions of citric acid production (Kubicek and Roehr, *(96)*). Inhibition of NADP-specific isocitrate dehydrogenase by citrate has been reported by Mattey *(97)* and Bowes and Mattey *(98)* as well as that of NAD-specific isocitrate dehydrogenase by the ratio NADH/(NAD+NADH), *i.e.* the catabolic reduction charge (Kubicek *(99)*). 2-oxoglutarate dehydrogenase is inhibited by oxaloacetate, cis-aconitate and NADH (Meixner-Monori *et al. (100)*).

Returning to the above-mentioned concept of nutrient limitation with concomitant starvation, the triggering events investigated are limitation in nitrogen, phosphate or trace metals iron and manganese. Nitrogen limitation has been found as the triggering event for citric acid production by yeasts on *n*-alkanes (Marchal, Vandecasteele and Metche *(101)*; Aiba and Matsuoka, *(102)*; Behrens, and Weissbrodt and Lehmann *(103)*). Several studies on citric acid formation by yeasts on glucose media came to the same conclusion (*e.g. (103, 104)*). McKay, Maddox and Brooks *(105)* have studied citric acid production by *Yarrowia lipolytica* under conditions of nitrogen, sulfur, magnesium and phosphorus limitations, respectively. The authors found that highest yields could be achieved when nitrogen or sulfur was limiting. Kristiansen and Sinclair *(106,107)* suggested nitrogen limitation as decisive in batch and continuous culture of *Aspergillus foetidus*. Pines *et al. (95)* reported that in their experiments with yeast and filamentous fungi organic acid production is correlated with nitrogen depletion in the medium (cf. Battat *et al. (108)* for malic acid production).

In contrast to yeasts, nitrogen limitation in mold fermentations does not seem to have been exploited on an industrial scale. In the Miles process ammonium ions are used to adjust the pH in the course of the fermentation. As mentioned above, in this process another reasoning is applied, namely limitation by decreasing the levels of iron. Obviously this applies to manganese as well, as reported in a great number of contributions by other workers. It is extremely difficult to define those steps that are sensitive to higher concentrations of iron or manganese, since these metals are involved in a great number of enzyme catalyzed reactions. With respect to iron, it was suggested that the iron requirement of aconitate hydratase would affect its activity. It has been shown, however, that aconitase activity does not change in the course of citric acid fermentation *(96)* irrespective of the absence or presence of iron. In yeast fermentations, on the other hand, significant decline of the activities of aconitate hydratase and both NAD- and NADP-linked isocitric acid dehydrogenases with the exhaustion of the nitrogen source could be observed *(109)*.

Comparative investigations of enzyme and metabolite levels under manganese deficient and sufficient conditions expecting to find pronounced sites of action was

the first activity within the group of the present author (Dissertation Kubicek, 1977). Although several points of action of manganese on citric acid production were identified in subsequent studies, a comprehensive interpretation is still lacking (for review, see Kubicek and Roehr *(110)*). As mentioned earlier, Netik *et al. (71)* have recently reported that manganese ions are involved in the regulation of uptake and excretion of citric acid in *Aspergillus niger.*

With respect to the iron/manganese problem it should be considered that manganese not only occurs as an impurity of macronutrient chemicals but also as a rather constant companion of iron, and it is thus difficult to discriminate between the actions of these metals.

The case of phosphate limitation is especially interesting. In almost all studies following the work of Currie (1917), but particularly beginning with studies in submerged fermentations, phosphate concentrations applied were low – in several cases pronounced limitation of phosphate was claimed by the respective authors to be beneficial (see above, *e.g.* Szücs, 1944 *(28)*; Waksman and Karow *(29)*; Karow and Waksman *(30)*; Shu and Johnson *(34,35)*; Martin and Steel *(111)*; Baras *(112)*; Kubicek and Roehr *(113)*; Dawson, Maddox and Brooks *(114)*; Honecker *et al. (115)*; Omar, Honecker and Rehm *(116)*; Pintado *et al. (117)*; Chen *(118)*). In many cases »limitation« by lowering both the concentrations of phosphate and heavy metal ions was advised or unattemptedly applied. It is thus difficult in many cases to identify the accurate nature of limitation. This question is currently studied in the author's laboratory. In yeast fermentations Fukuda *et al. (119)* have claimed high citric acid yields by maintaining a ratio of carbon to phosphorus of 1 : 500–10000.

If one adopts the concept of starvation due to limitation of essential nutrients, a common interpretation would be that excess of a readily metabolized, energy generating carbon source in relation to low concentrations of growth promoting elements such as nitrogen, phosphorus and heavy metal ions will cause an energy surplus which the organism cannot cope with. Nature has provided futile cycles to dispose of energy surplus. One of these could be the formation of polyols which have been observed by several authors during citric acid production *(115,120–124)*. The other more effective one seems to be the existence of an alternative respiration pathway which has been demonstrated in *Aspergillus niger* under conditions of active citric acid production (Zehentgruber, Kubicek and Roehr, *(125)*; Kubicek *et al. (126)*, and Kirimura, Hirowatari and Usami *(127)*). Thus the lack of heavy metal ions and phosphate starvation could well be considered as responsible for citric acid accumulation as a »waste« material with concomitant operation of energy cycling reactions. Wallrath *et. al. (128)* have shown that respiratory chain NADH:ubiquinone reductase (complex I) is lost concomitant with citric acid accumulation in *Aspergillus niger* (see also *(129)*) and have claimed this defect to be the causative event in citric acid accumulation; unfortunately this has only been studied in one strain of *Aspergillus niger.* Variations of the respiratory state in a fermentation process could probably account also for significant changes in the redox potential which has been proposed for controlling and scaling up in citric acid fermentation practice as reported by Berovic and Cimerman *(130, 131)*.

Although research on the regulation of citric acid accumulation has been successful in defining distinct targets/regulatory steps, effective conversion into process improvements by genetic techniques has not been achieved yet. Recently, Ruijter *et al. (132)* have overexpressed PFK and pyruvate kinase (3–5)-fold over the wild type without being able to observe significant increases in citric acid production.

On the other hand, as mentioned above, overexpression of cytosolic malate dehydrogenase could also enhance citric acid accumulation (Pines *et al. (95)*) and the authors have defined pyruvate carboxylase as their next target.

Likewise it would be of interest to learn whether optimization procedures such as those outlined by Torres *et al. (133)* can be transferred to industrial practice.

Novel citric acid producers – yeasts: Yeasts exhibiting abundant citric acid production are mainly species of *Candida*, especially *C. lipolytica* and its sexual form, *Yarrowia (Saccharomycopsis) lipolytica* (cf. Barth and Gaillardin *(134)*.

In the 1960s workers of British Petrol in France invented a novel process of removing the *n*-alkane fraction of crude oil by growing special types of yeasts in aerated emulsions of nutrient solutions and crude oil, thereby combining deparaffination with the production of single cell protein (SCP) for animal nutrition. Due to problems in purifying the biomass obtained according to animal health regulations, this process had to be abandoned. It turned out, however, that the use of pure C_9 to C_{30} fractions of *n*-paraffines would improve this process. Later it was found that several species of yeast were able to convert C_9–C_{20} alkanes almost quantitatively to citric acid *(135,136)*. Due to the fact that after the world oil crisis of 1973/74 *n*-paraffines could no longer be regarded a cheap carbon source, this interesting process again had to be abandoned. In the meantime, however, it had been observed by several Japanese workers that the same yeasts that had been found to utilize paraffines could also convert glucose to citric acid with similar yields. This line was followed by Miall and Parker at Chas. Pfizer, Inc. *(137)*, describing also the use of molasses, and by a group in the former German Democratic Republic *(103, 138)*. Activities of the latter group were terminated in the course of the German reunion.

The advantages of using yeasts as unicellular organisms are obvious. Disadvantages, such as *e.g.* the concomitant formation of isocitric acid, have been overcome to a large extent. In fact, it is difficult to predict the future in relation to conventional fungal technology, especially in view of the fact that citric acid manufacture and manufacturers may be regarded as rather conservative.

Downstream processing: The occurrence of citric acid as an initially low concentration by-product of hydrocarbon utilization by yeasts, as mentioned above, probably stimulated work on the possibilities of recovering and purification of citric acid by solvent extraction. Liming, on the other hand, is known to produce equal amounts of gypsum per amount of citric acid, which has to be disposed of. In many locations this is performed in

waste deposits causing environmental pollution. In the last three decades a large number of patents have been taken out covering various methods of extractive recovery and purification of citric acid (reviewed *e.g.* by Roehr, Kubicek and Kominek *(80)*). There is still some controversy as to which of the two principal methods can meet both economical and ecological demands as well. According to Milsom *(76)* the solvent used by Miles Laboratories *(139,140)* has been approved by the U.S. Food and Drug Administration (FDA).

Other techniques of citric acid purification, such as ion exchange or the application of liquid membranes have been developed and are ready for evaluation on an industrial scale. Likewise, direct crystallization of citric acid or citrates from purer or purified fermentation fluids has been exploited. Needless to say, the choice of purification procedures and the choice of raw materials influence each other in specific ways.

As mentioned at the beginning, citric acid fermentation as one of the main industrial fermentation processes has always been considered as a most sophisticated technology. Complete knowledge of the triggering mechanisms leading to abundant accumulation and excretion of citric acid by the producer organism is still lacking and being studied by several research groups.

With respect to the economic situation, this may be characterized as follows: producing a high-volume/low-price product with a significant dependence on the availability of cheap raw materials, citric acid manufacturers face the common problem of competition in a tremendously increasing market. This results in tendencies to enlarge and concentrate production capacities at the expense of smaller facilities as has been the case within the last decades. Survival of smaller units can only be observed in situations where local demands can be satisfied in smaller somehow protected economic areas. In addition, problems of environmental pollution have increasingly influenced the choice of suitable process variants (including raw materials), as well as that of size and location of the establishment. It can be noticed, however, that worldwide production still tends to increase slightly.

Conclusion

Currie, in his paper published 80 years ago, cited a German scientist, Felix Ehrlich, who in 1914 predicted that »... in time we will have a great fermentation industry in which many substances will be prepared which are now manufactured by expensive methods ...«. It is not an overstatement to say that this has come true – with citric acid production being a prominent example. And as we see, active work by a most dedicated community of citric acid researchers is still under way, as will be documented convincingly in the following contributions.

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Stoljeće istraživanja i dobivanja limunske kiseline vrenjem

Sažetak

Fermentacija limunske kiseline jedna je od najvećih biotehnoloških proizvodnji koja je započela pred skoro jedno stoljeće. O toj problematici postoji više od 2.000 znanstvenih radova i nekoliko stotina patenata. Rad obuhvaća više ili manje poznate povijesne podatke i prikazuje stogodišnji tijek razvoja naših spoznaja o vrenju limunske kiseline, iznoseći možda suvišne, a možda i stimulirajuće zamisli.