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# THE BIOCHEMISTRY OF CITRIC ACID ACCUMULATION BY *ASPERGILLUS NIGER*

## (A REVIEW)*

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Fungi, in particular *Aspergilli,* are well known for their potential to overproduce a variety of organic acids. These microorganisms have an intrinsic ability to accumulate these substances and it is generally believed that this provides the fungi with an ecological advantage, since they grow rather well at pH 3 to 5, while some species even tolerate pH values as low as 1.5. Organic acid production can be stimulated and in a number of cases conditions have been found that result in almost quantitative conversion of carbon substrate into acid. This is exploited in large-scale production of a number of organic acids like citric-, gluconic- and itaconic acid. Both in production volume as well as in knowledge available, citrate is by far the major organic acid.

Citric acid (2-hydroxy-propane-1,2,3-tricarboxylic acid) is a true bulk product with an estimated global production of over 900 thousand tons in the year 2000. Till the beginning of the 20$^{th}$ century, it was exclusively extracted from lemons. Since the global market was dominated by an Italian cartel, other means of production were sought. Chemical synthesis was possible, but not suitable due to expensive raw materials and a complicated process with low yield. The discovery of citrate accumulation by *Aspergillus niger* led to a rapid development of a fermentation process, which only a decade later accounted for a large part of the global production.

The application of citric acid is based on three of its properties: (1) acidity and buffer capacity, (2) taste and flavour, and (3) chelation of metal ions. Because of its three acid groups with pKa values of 3.1, 4.7 and 6.4, citrate is able to produce a very low pH in solution, but is also useful as a buffer over a broad range of pH values (2 to 7). Citric acid has a pleasant acid taste which leaves little aftertaste. It sometimes enhances flavour, but is

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also able to mask sweetness, such as the aspartame taste in diet beverages. Chelation of metal ions is a very important property that has led to applications such as antioxidant and preservative. Moreover, it is a "natural" substance and fully biodegradable.

**Keywords**: citric acid accumulation, *Aspergillus niger*

## Metabolism of citric acid biosynthesis

Early studies by Martin & Wilson [1] and Cleland & Johnson [2] showed that in *A. niger*, citric acid is mainly formed via glycolysis with subsequent condensation of a $C_4$ unit with a $C_2$ moiety. In short, citric acid biosynthesis involves uptake of the sugar substrate, glycolitic catabolism of glucose to 2 moles of pyruvate, their subsequent conversion to oxaloacetate and acetyl-CoA, condensation of these two presursors to citric acid and finally, excretions of citric acid from mitochondria and from mycelia, respectively (Fig. 1).

*Fig. 1.* Overview of pathways leading to citric acid production

*Extracellular catabolism of glucose*

The possession of glucose-oxidase is common within *A. niger* strains (Mischak et al. [3], Roukas & Harvey [4]). This enzyme is induced by high concentrations of glucose and strong aeration in the presence of low concentrations of other nutrients, conditions which are also typical for citric acid fermentation. Hence, glucose oxidase will inevitably be formed during the starting phase of fermentation and convert a significant amount of glucose into gluconic acid. However, due to the extracellular location of the enzyme, it is directly influenced by the external pH and will be inactivated at pH < 3.5 (Mischak et al. [3], Dronawat et al. [5]). Because of the pK-values for citric acid, pH of the culture filtrate is to decrease to pH 1.8, thereby inactivating glucose-oxidase. It is not known if – and by which mechanism – can gluconic acid be catabolized to citric acid during fermentation.

*Sugar uptake*

Kinetic analysis Torres et al. [6] shows that *A. niger* possesses at least two glucose transporters. High-affinity glucose transport ($K_m$ 0.3 mM) was detected after growth on low (1%) glucose concentration, whereas an additional low-affinity permease was only found in mycelium cultured in the presence of high (15%) glucose concentrations. The latter may therefore contribute to the increased glycolytic flux during growth on high glucose concentrations. Surprisingly, despite of the fact that the transport of fructose is also relevant for sucrose-based citric acid production, no data is available for *A. niger* in this respect.

*Sugar phosphorylation*

The first metabolic step after uptake is the phosphorylation of the sugar. Two hexose phosphorylating enzymes exist in *A. niger*, a hexokinase and a glucokinase (Panneman et al. [7, 8]). Both enzymes are present under citric acid producing conditions, but their contribution to the phosphorylation of glucose and fructose is different. The hexokinase is able to phosphorylate both glucose and fructose, whereas glucokinase has a very low, physiologically irrevelant affinity for fructose and a very high ($K_m$ 0.06 mM) affinity for glucose. Hexokinase is strongly inhibited by trehalose 6-phosphate, an intermediate of trehalose biosynthesis (Panneman et al. [8]). By removing this inhibition glycolytic flux could successfully be increased (Arisan-Atac et al. [9]) and by disrupting the gene encoding trehalose 6-phosphate synthase, a higher rate of citric acid production from sucrose in the early phase of accumulation was observed. Since glucokinase is not sensitive for trehalose 6-phosphate inhibition, this approach may not be successful when glucose is the carbon source of choice.

From the level of glucose 6-phosphate, carbon metabolism can either proceed via the glycolytic or the penthose-phosphate pathway. In earlier stages of the fermentation pentose-phosphate pathway dominates and provide the cell with building blocks and NADPH, while later it accounts only for a minor fraction and carbon is mainly metabolized via glycolysis. It was speculated that this may be due to the inhibitory effect of citrate on 6-phosphogluconate dehydrogenase, but evidence for this is lacking. It should be noted that both arabitol and erythritol are accumulated as byproducts until late stages of fermentation (Röhr et al. [10]), and hence a complete blockage of the pentose phosphate pathway is obviously not taking place.

Citrate is one of the most reputed inhibitors of glycolysis, and the ability of *A. niger* to overproduce citrate by an active glycolytic pathway has attracted biochemical interest for a long time. The explanation in short for this phenomenon is that under appropriate nutrient conditions citrate inhibition is heavily counteracted by the accumulation of various positive effectors, and this feedback does not occur (Habison et al. [11], Arts et al. [12]).

Phosphofructokinase, a key enzyme for glycolysis is regulated by a large number of metabolites, both inhibitory (citrate, phosphoenol-pyruvate [PEP], ATP) and stimulatory ($NH_4^+$, AMP, fructose 2,6 bisphosphate [F2,6 $P_2$]). Allosteric activation of phospho-fructokinase by F2,6 $P_2$ is probably the most relevant factor in the regulation of the enzyme (Arts et al. [12]). Under physiological conditions phosphofructokinase is not even active in the absence of F2,6 $P_2$ (Ruijter et al. [13]). In high-producer mutants, phosphofructokinase and hexokinase activities were 2-fold higher than in the parental strain (Schreferl et al. 14]). However, amplification of phosphofructokinase, individually or in combination with pyruvate kinase, did not increase the rate of citric acid production (Ruijter et al. [13]). Interestingly, recombinant strains with increased phosphofructokinase activity consistently had a 40% lower intracellular concentration of F2,6 $P_2$. *In vitro* measurements mimicking intracellular conditions, assuming an intracellular pH of 6.8, revealed that such a reduction in the F2,6 $P_2$ concentration could significantly decrease the specific activity of phosphofructokinase in the cell. The level of the enzyme producing F2,6 $P_2$ (6-phosphofructo-2-kinase) was comparable in parent and recombinant strains, suggesting regulation of its activity. Thus, the fungus seems to adapt to overexpression of phosphofructokinase by decreasing the specific activity of the enzyme through a reduction in the level of F2,6 $P_2$.

The regulation of phosphofructokinase by F2,6 $P_2$ may not be the only parameter regulating citrate accumulation. Inhibition of phosphofructokinase by citrate seems *in vivo* also to be antagonized by ammonium ions (Habison et al. [11]). This antagonism is functionally linked to the well-known effect of trace metal ions (e.g. particularly manganese ions) on citric acid accumulation, as one of the effects caused

by manganese deficiency is an impairment of DNA synthesis in *A. niger* (Kubicek et al. [15], Hockertz et al. [16]), which causes increased protein degradation (the effect of manganese deficiency can be mimicked by addition of hydroxyurea, an inhibitor of the enzyme ribonucleotide reductase that provides deoxyribonucleotides for DNA replication). As a consequence, mycelia accumulate elevated concentrations of $NH_4^+$. Mutants with partially citrate-insensitive phosphofructokinase exhibited a citrate accumulation more tolerable to the presence of $Mn^{2+}$ (Schreferl et al. [14]). Also, the precisely controlled exogenous addition of $NH_4^+$ during particular stages of citrate fermentation even stimulates the rate of citrate production (Yigitoglu & McNeil [17]).

A further mechanism of regulation of phosphofructokinase would involve the phosphorylation of the enzyme by a cyclic-AMP-dependent protein kinase (Legisa & Bencina [18]). They speculate that a high concentration of sucrose induces an increase in mycelial cyclic-AMP levels, which trigger the phosphorylation of phosphofructokinase, thereby converting an inactive (non-phosphorylated) form into an active (phosphorylated) one (Legisa & Gradisnik-Grapulin [19]). The support for their model is their observation that phosphofructokinase was inactivated by treatment with alkaline phosphatase (Legisa & Bencina [18]). Unfortunately, their paper does not indicate whether (and how) the alkaline phosphatase had been removed or inactivated prior to the phosphofructokinase assay. If this has not been done, the inactivation of phosphofructokinase may have been due to a removal of fructose-6-phosphate from the assay and thus be an artefact.

*The Cleland–Johnson reaction*

Catabolism of glucose via glycolysis yields 2 moles of pyruvate, which are subsequently converted to the precursors of citrate (oxaloacetate and acetyl-CoA). Cleland & Johnson [2] were the first to show that *A. niger* re-uses the $CO_2$ released in decarboxylation of pyruvate, to form oxaloacetate. This reaction is of utmost importance to the high (80%) citric acid yields commonly obtained, because oxaloacetate could otherwise only be formed by one turn of the Krebs–cycle. This would result in the loss of 2 moles of $CO_2$, with a concomitant maximum theoretical yield of 67%. The enzyme catalyzing this reaction is pyruvate carboxylase. Unlike the enzyme from several other eukaryotes, pyruvate carboxylase of *A. niger* is localized in the cytosol (Bercovitz et al. [20], Jaklitsch et al. [21]). Glycolytic pyruvate will therefore be converted to oxaloacetate and reduced to malate by the cytosolic malate dehydrogenase isoenzyme (Ma et al. [22]), thereby also regenerating half of the glycolytically produced NADH. It has been postulated (Kubicek [23]) that in analogy to higher eukaryotes, the cytosolic malate may serve as the co-substrate of the mitochondrial tricarboxylic acid carrier, which probably catalyzes transport of citrate

from the mitochondrion to the cytoplasm. The fixation of $CO_2$ does not seem to occur during the early phases of fermentation, since in the first 70 hours respiratory coefficient ($CO_2$ released / $O_2$ taken up) is close to 1, and it reaches the level predicted from the operation of the pyruvate carboxylase reaction (0.66) only at the producing phase. Hence, the Cleland–Johnson reaction is important in the citrate producing phase only, whereas initial accumulation takes place without anaplerotic $CO_2$ supply. Interestingly, sparging extra $CO_2$ into the fermenter to supply pyruvate carboxylase did not result in improved productivity (McIntyre & McNeil [24]).

*The role of the Krebs–cycle*

Numerous workers claimed that inactivation of citrate-degrading enzymes (aconitase, isocitrate dehydrogenase) should be essential for the accumulation of citric acid. However, solid evidence proves that an intact Krebs–cycle is present throughout the fermentation, hence explanations based on this view are incorrect. The same is true for theories based on the inactivation of these enzymes due to metal ion deficiencies.

Inhibition of the NADP-dependent isocitrate dehydrogenase by glycerol was proposed to decrease flux through the Krebs–cycle and, because of the $K_{eq.}$ of aconitase, lead to accumulation of citrate (Legisa et al. [25]). However, studies of increased mycelial glycerol concentrations on the oxidation of 1,5-$^{14}$C-citrate indicated that the appearance of labeled $CO_2$ (which, because of the labeling position applied can only be released during the metabolic conversion of citrate to 2-ketoglutarate) was unaffected by high glycerol concentrations (Arisan-Atac & Kubicek [9]). Moreover, highly purified NADP-specific isocitrate dehydrogenase was found not to be inhibited by citrate (Arisan-Atac et al. [9]).

A reasonable, although so far neglected hypothesis is that a mitochondrial tricarboxylate transporter enzyme is responsible for the accumulation of citrate. This carrier would compete directly with aconitase for citrate and may be able to pump it out of the mitochondria without any necessity for a bottleneck in the Krebs–cycle downstream of citrate. As the tricarboxylate carrier of mammalian tissues and yeast operates by countertransporting with malate (Evans et al. [26]), such a situation is envisagable when its counterion malate accumulates in the cytosol. In fact, malate accumulation has been shown to preceed citrate accumulation (Röhr et al. [27]). Nevertheless, the mitochondrial citrate carrier of *A. niger* has not yet been investigated, rendering this intriguing theory a speculation thus far.

*The alternative respiratory pathway*

Formation of citric acid is dependent on strong aeration, and dissolved oxygen tensions higher than those required for vegetative growth of *A. niger* stimulate production. On the other hand, sudden interruptions in the air supply cause an irreversible impairment of citric acid production without any harmful effect on mycelial growth (Kubicek et al. [28]). An explanation for these observations is that *A. niger* forms a cyanide-resistant, salicylhydroxamic acid sensitive alternative respiratory pathway under conditions that promote citrate production (Fig. 2). In the course of the fermentation the activities of the cytochrome-dependent respiratory enzymes decrease whereas the activity of the alternative oxidase increases. The alternative oxidase does not pump protons concomitantly with the electron transport, and its physiological function is thought to be the removal of the reducing equivalents in excess. The almost quantitative conversion of hexose to citric acid results in net production of 1 mol of ATP and 3 moles of NADH. Since there is not much growth in the stage of citric acid production, the cells do probably not require much ATP, and a switch from cytochrome-dependent respiration to the alternative one would enable the fungus to re-oxidize its NADH without concomitant ATP production. Recently, a cDNA encoding an alternative oxidase was cloned (Kirimura et al. [29]), but it is still not known whether citric acid production rates are affected by the overexpression or disruption of this gene.

The existence of the cyanide-resistant alternative respiratory pathway is known for more than seven decades now, yet its regulation is not fully understood. In plants the intensity of the alternative route will depend on the amount of oxidase present, on the redox status of the redox-sensitive disulphide bond between neighbouring subunits, on the intracellular concentration of organic acids (particularly of pyruvate), and also on the ratio of reduced ubiquinone to the total ubiquinone pool. A conserved cysteine residue near the N-terminus of the protein is responsible for both disulphide bond formation and organic acid activation of the alternative oxidase. Replacing this cystein with a serine residue results in a permanently monomeric enzyme that is specifically stimulated by succinate at concentrations of 1–5 mM. These concentrations are likely to be physiologically relevant, as succinate is produced within the mitochondrial matrix where it may become concentrated.

It was just recently established that in contrast to plant enzymes, fungal alternative oxidases are permanently monomeric and are not activated directly by α-keto acids. It was also revealed that a domain of about 40 amino acids responsible for the establishment of the disulphide bond in the plant enzyme is missing in these sequences.

In recent years, more and more results suggested that this non-coupled respiration may contribute to the prevention of the generation of reactive oxygen species. These are formed under conditions when the cytochrome path is impaired, and can initiate lipid peroxidation and a subsequent damage in membrane structures. In plants, when substrate supply and hence the reduced ubiquinone-pool is too large in the cytochrome pathway, organic acids, especially pyruvate, accumulate and lower the $K_m$ value of the oxidase for ubiquinol in a classical feed-forward regulation. Should the capacity of the alternative route not be sufficient, active oxygen species themselves induce the expression of the alternative oxidase protein, similarly to the $H_2O_2$-mediated expression of the plant pathogenesis-related proteins. In the light of the recently proposed nature of the fungal alternative oxidase, however, reactive oxygen species might be the only signal towards activation (Karaffa et al. [30]). Whether or not the enhanced dissolved oxygen tensions, necessary for high citrate production rate affects the appearance of these molecules and concomitantly that of the alternative oxidase remains to be elucidated.

The alternative oxidase is growth rate dependent and seems functionally connected to the succinic dehydrogenase complex (Karaffa et al. [31, 32]). The latter was indicated by results where the addition of soybean oil had no effect on the total rate of respiration, but it increased the part of respiration which proceeded via the alternative, cyanide-insensitive part in the soybean supplemented cultures.

*Export of citric acid from mycelia*

Earlier it was suggested that export of citrate through the membrane is due to the large pH gradient between the cytosol and the extracellular medium, and was postulated that citrate efflux from the cell may occur by diffusion of the 2 (-) citrate anion, driven by a gradient (Kontopidis et al. [33]). It was recently shown, however, that citrate export requires ATP and its $V_{max.}$ is not strongly affected by the external pH, rendering the diffusion hypothesis rather unlikely (Netik et al. [34]). Citrate export is strongly increased in mycelia grown under manganese deficiency, which is consistent with previous observations, that intracellular concentration of citrate in manganese sufficient and deficient grown mycelia is not strongly different, despite the 5-7 fold higher extracellular levels under the latter conditions (Prömper et al. [35]). The reason for the requirement of manganese deficiency for citrate export may be related to its precisely opposite effect on citrate uptake – only the manganese-chelated citrate can serve as a substrate for the citrate permease (Netik et al. [34]). The reciprocal effect of manganese ions on citrate export and import may also be related to an other effect of manganese deficiency, which is the inhibition of triglyceride and phospholipid synthesis as well as a shift in the ratio of saturated to unsaturated fatty acids of whole

mycelial lipids. The ability of $Cu^{2+}$ ions to antagonize the harmful effect of $Mn^{2+}$ ions may also be related to citrate excretion, since $Cu^{2+}$ ions strongly inhibit the uptake of citric acid. An other possibility is that the effect of $Cu^{2+}$ ions is due to in the competitive inhibition of the $Mn^{+}$ ion uptake in *A. niger*.

*Fig. 2.* The alternative respiratory pathway

*Oxalic acid biosynthesis*

Although not directly within the topic of this lecture, for historical reasons it shall be noted that *A. niger* is also capable of accumulating another organic acid – oxalate – as a (toxic) byproduct of citric acid fermentation. The biosynthesis of this compound on glucose as a carbon source occurs by the hydrolysis of oxaloacetate catalyzed by oxaloacetate hydrolase, which is cytosolically located and appears to act as a valve by which the carbon overflow can be chanelled into an energetically neutral pathway and hence compete with citrate overproduction.

Interestingly, the basic discovery of the oxalate and citrate production by microbial means belongs to the same Austrian scientist. Wehmer also went on record as the founder of the Department of Microbial Physiology in 1898 at the Technical University of Vienna, a still active credle of scientists whose efforts greatly contributed to our knowledge on fungal citric acid production.

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