

Research review paper

# Advances in citric acid fermentation by *Aspergillus niger*: Biochemical aspects, membrane transport and modeling

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## Abstract

Citric acid is regarded as a metabolite of energy metabolism, of which the concentration will rise to appreciable amounts only under conditions of substantive metabolic imbalances. Citric acid fermentation conditions were established during the 1930s and 1940s, when the effects of various medium components were evaluated. The biochemical mechanism by which *Aspergillus niger* accumulates citric acid has continued to attract interest even though its commercial production by fermentation has been established for decades. Although extensive basic biochemical research has been carried out with *A. niger*, the understanding of the events relevant for citric acid accumulation is not completely understood. This review is focused on citric acid fermentation by *A. niger*. Emphasis is given to aspects of fermentation biochemistry, membrane transport in *A. niger* and modeling of the production process. © 2007 Elsevier Inc. All rights reserved.

**Keywords:** Citric acid; *Aspergillus niger*

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## 1. Introduction

Citric acid (2-hydroxy-propane-1,2,3-tricarboxylic acid) derives its name from the Latin word *citrus*, the citrus tree, the fruit of which resembles a lemon. The acid was first isolated from lemon juice in 1784 by Carl Scheele, a Swedish chemist (1742–1786). Citric acid is a tricarboxylic acid with a molecular weight of 210.14 Da. In view of its three carboxylic acid functional groups, it has three  $pK_a$  values at pH 3.1, 4.7, and 6.4. Citric acid is a nearly universal intermediate product of metabolism and its traces are found in virtually all plants and animals.

Citric acid was first produced commercially in England around 1826 from imported Italian lemons. As its commercial importance increased, Italian lemon growers started producing it to establish a virtual monopoly during the most of the nineteenth century. Lemon juice remained the commercial source of citric acid until 1919 when the first industrial process using *Aspergillus niger* began in Belgium.

Citric acid had been synthesized from glycerol by Grimoux and Adams (1880) and later from symmetrical dichloroacetone. Several other synthetic routes using different starting materials have since been published, but chemical methods have so far proved uncompetitive with fermentation mainly because the starting materials are worth more than the final product.

Wehmer (1893) first showed that a “*Citromyces*” (now *Penicillium*) accumulated citric acid in a culture medium that contained sugars and inorganic salts. Many other microorganisms have since been found to accumulate citric acid including strains of *A. niger*, *A. awamori*,

*A. nidulans*, *A. fonsecaeus*, *A. luchensis*, *A. phoenicis*, *A. wentii*, *A.saitoi*, *A. flavus*, *Absidia* sp., *Acremonium* sp., *Botrytis* sp., *Eupenicillium* sp., *Mucor piriformis*, *Penicillium janthinellum*, *P. restrictum*, *Talaromyces* sp., *Trichoderma viride* and *Ustilina vulgaris*.

Currie in 1917 discovered that some strains of *A. niger* grew abundantly in a nutrient medium that had a high concentration of sugar and mineral salts and an initial pH of 2.5–3.5. While growing, these strains excreted large amounts of citric acid. This laid down the basis for industrial production of citric acid. Prior to this, *A. niger* was a known producer of oxalic acid, but the low pH of Currie’s work suppressed both the formation of oxalic and gluconic acids. Currie’s discovery formed the basis of citric acid production established by Pfizer in the 1923 in the United States.

In addition to molds, several yeast strains are now known to produce large amounts of citric acid from *n*-alkanes and carbohydrates (Matthey, 1999). During the 1960s and 1970s hydrocarbons were relatively cheap and citric acid production from yeasts was used commercially. Industrial production of citric acid from *n*-alkanes is no longer economical but yeasts are used to make citric acid from carbohydrate feedstocks. Yeasts that are known to produce citric acid from various carbon sources include: *Candida*, *Hansenula*, *Pichia*, *Debaromyces*, *Torula*, *Torulopsis*, *Kloeckera*, *Saccharomyces*, *Zygosaccharomyces*, and *Yarrowia*. From these, the *Candida* species, including *C. tropicalis*, *C. catenula*, *C. guilliermondii*, and *C. intermedia* have been used industrially. As a disadvantage, yeast fermentations produce substantial quantities of isocitric

acid, an unwanted byproduct. Selection for mutants with very low aconitase activity has been used in attempts to reduce production of isocitric acid.

Although many microorganisms can be used to produce citric acid, *A. niger* remains the main industrial producer. Specific strains that are capable of overproducing citric acid have been developed for various types of fermentation processes. The yield of citric acid from these strains often exceeds 70% of the theoretical yield on the carbon source. Despite a long and successful history of production of citric acid, there is still no single explanation for the biochemical basis of the process to consistently explain the observed behavior of this fermentation. This review discusses the biochemical factors that contribute to citric acid accumulation in *A. niger* fermentations.

## 2. Fermentation conditions

The biochemical mechanism by which *A. niger* accumulates citric acid has attracted much interest. Fermentation conditions were established during the 1930s and 1940s when the effects of various medium components were evaluated. The simple surface culture methods used in the first commercial production plants were labor intensive by modern standards, and although submerged culture techniques had been developed before 1940, some old-style surface culture plants apparently still thrive (Mattey, 1992).

Since 1930s, various explanations have been proposed for the accumulation of citric acid. Citric acid only accumulates when several nutrients are present either in high concentrations (i.e. sugar, acidity, dissolved oxygen) or at suboptimal levels (i.e. trace metals, nitrogen, phosphate). Many biochemical events likely jointly contribute to the observed overproduction. In view of the nature of production biochemistry, influence of individual factors cannot always be assessed without influencing the other factors. Moreover, some of the literature studies have used low- or moderately overproducing strains, or used conditions that were not optimal for accumulation of citric acid. This has made a comparison of data difficult and adversely affected the prospects for creating a better overall picture of the commercial citric acid fermentation. Although much basic biochemical research has been carried out with *A. niger*, the fundamental understanding of factors leading to citric acid accumulation remains poor.

### 2.1. Submerged fermentation

Accumulation of citric acid is strongly influenced by the composition of the fermentation medium. This is

especially so in submerged fermentation processes. Other than the early studies by Currie (1917), further systematic studies relating to medium composition did not occur until the 1940s (Shu and Johnson, 1948a,b). Shu and Johnson (1948a,b) developed a medium that provided the foundation for subsequent research on citric acid production. The following main factors were found to affect citric acid fermentation: type and concentration of the carbon source, nitrogen and phosphate limitation, pH, aeration, concentration of trace elements and the morphology of the producer organism. Certain nutrients needed to be in excess (i.e. sugar, protons, oxygen), others had to be limiting (i.e. nitrogen, phosphate) and some otherwise common feed components had to remain below defined limits (i.e. trace metals, especially manganese).

#### 2.1.1. Carbon source

The carbon source for the citric acid fermentation has been the focus of much study, frequently with a view to the utilizing polysaccharide sources. In general, only sugars that are rapidly taken up by the fungus allow a high final yield of citric acid (Mattey, 1992). Polysaccharides, unless hydrolyzed, are generally not a useful raw material for citric acid fermentation because they are broken down too slowly to match the high rate of sugar catabolism required for citric acid production. The slow hydrolysis of polysaccharides is due to the low activity of the hydrolytic enzymes at the low pH that is necessary for producing citric acid. According to Kubicek and Röhr (1989) sucrose is preferable to glucose as *A. niger* has a potent extracellular mycelium-bound invertase that is active at low pH and rapidly hydrolyzes sucrose. Superiority of sucrose over glucose and fructose was demonstrated by Gupta et al. (1976), Hossain et al. (1984) and Xu et al. (1989).

The carbon source used in industrial fermentations is typically beet molasses, although cane molasses, fruit pulp, polysaccharides and sugars are used if local conditions permit their economic use. Such low-grade carbon sources are waste products and generally contaminated with high levels of cations from prior processes. Cations are typically made insoluble by precipitation with potassium ferrocyanide or they are removed by using cation exchange resins. Activated carbon, bone char and bauxite have also been proposed for removing the ions (Prescott and Dunn, 1959). In view of the complexity and messiness of pretreatment of crude carbon sources, most of the published research has been done using refined sugars, generally glucose or sucrose.

Both the type of carbon source and its concentration are critical to citric acid fermentation. The final yield

of citric acid increases when the initial sugar concentration is increased and maximal production rates are usually achieved at 14–22% of sugar (Shu and Johnson, 1948a,b; Honecker et al., 1989). Xu et al. (1989) studied the effect of carbohydrate concentration on citric acid yield in submerged fermentation. The sugars maltose, sucrose, glucose, mannose and fructose were tested and highest yields were observed at sugar concentration of 10% w/v, with the exception of glucose where 7.5% gave the best results. No citric acid was produced in media that contained less than 2.5% sugar. Xu et al. (1989) noted that an increase in sugar concentration from 1% to 14% increased the duration of the lag phase of growth from 12 to 18 h and decreased the growth rate by around 20%. Hossain et al. (1984) suggested that high concentrations of appropriate carbon sources lead to repression of  $\alpha$ -keto-glutarate dehydrogenase, hence explaining the effect of the sugar concentration and source in terms of enzyme repression.

Papagianni (1995) and Papagianni et al. (1999a,b,c) studied the influence of glucose concentration on growth and citric acid production by *A. niger* in batch and fed-batch cultures in which the concentration of glucose was maintained stable (glucostat). The level of glucose had a marked effect on citric acid production rate. The specific rate of citric acid production increased with increasing initial glucose concentration (batch culture) or increasing constant glucose level (glucostat culture). In both culture methods, the specific growth rate increased with decreasing glucose concentration for the first 48 h of fermentation. The size of mycelial clumps was reduced at low glucose levels and their shape was also affected as the ratio between the perimeter of the clump and the perimeter of its core increased as glucose concentration decreased.

### 2.1.2. Nitrogen and phosphate limitation

Complex media such as molasses are rich in nitrogen-containing compounds and rarely need to be supplemented with a nitrogen source. The high purity media that are used mainly in research laboratories are generally supplemented with ammonium salts, particularly ammonium nitrate and ammonium sulfate, to provide the necessary nitrogen. An advantage of using ammonium salts is that the pH declines as the salts are consumed and a low pH is a requirement of citric acid fermentation (Mattey, 1992). Other sources of nitrogen that have been used include urea and yeast/malt extract (Xu et al., 1989).

Reports concerning the effects of nitrogen and phosphate limitations have been contradictory. Accord-

ing to Shu and Johnson (1948a,b), phosphate does not need to be limiting for citric acid production; however, when trace metal levels are not limiting, additional phosphate results in side reactions and increased biomass growth (Papagianni et al., 1999a,b,c). Kubicek and Röhr (1977) showed that citric acid accumulated whenever phosphate was limited even when nitrogen was not. In contrast, Kristiansen and Sinclair (1979) using continuous culture concluded that nitrogen limitation was essential for citric acid production.

### 2.1.3. Broth pH

The pH of the medium is important at two different times in the fermentation for different reasons. Firstly, the spores require a pH > 5 in order to germinate. All fermentations are started from spores even if the culture is “scaled up” through seed fermenters before being transferred to production fermenters. Secondly, the pH for citric acid production needs to be low (pH  $\leq$  2). A low pH reduces the risk of contamination of the fermentation with other microorganisms. A low pH also inhibits the production of unwanted organic acids (gluconic acid, oxalic acid) and this makes the recovery of citric acid from the broth simpler. The uptake of ammonia by the germinating spores causes a corresponding release of protons that lower the pH to approximately the right level after spore germination has occurred. Increasing the pH to 4.5 during the production phase reduces the final yield of citric acid by up to 80% (Papagianni, 1995).

### 2.1.4. Aeration

Industrial producers of citric acid have known for a long time that variations in the aeration rate can have a detrimental effect on the yield of a batch. If the aeration rate is too high (a condition that occurs only at laboratory scales), the partial pressure of dissolved CO<sub>2</sub> in the medium can become too low. Carbon dioxide is important as a substrate for pyruvate carboxylase which replenishes the supply of oxaloacetate for citrate synthase. Sufficient CO<sub>2</sub> is produced by the pyruvate decarboxylase reaction to satisfy the stoichiometric demand of the pyruvate carboxylase reaction, but excessive aeration results in some loss. McIntyre and McNeil (1997) showed that elevated levels of CO<sub>2</sub> in the sparged gas actually had a detrimental effect upon the final citrate concentration and final biomass concentration.

The effect of dissolved oxygen has been studied in some detail. Even short periods of decreased dissolved oxygen tension (DOT) cause irreversible changes in the rate of citric acid production (Kubicek et al., 1980). *A. niger* is known to use two different respiratory

pathways during citric acid production. Synthesis of citric acid is dependent on the cyanide sensitive pathway during the final phase of the fermentation. Both growth and the start of citric acid production are dependent on salicylhydroxamic acid (SHAM)-sensitive respiration. A SHAM-sensitive bypass is produced late in trophophase, and an alternative ubiquinol oxidase component of this (Ruijter and Visser, 1999) has been discovered. The presence of these bypasses and their importance to citric acid production is not coincidental. The production of ATP at substrate level via glycolysis is probably sufficient for the cell's energy requirements, and so there is little need for much more ATP to be generated via oxidative phosphorylation of NADH. A high internal concentration of ATP would have an inhibitory effect on glycolytic enzymes, therefore, an alternative oxidase to recycle NADH for further glycolysis would seem to be an essential component for maintaining a high flux through the glycolytic pathway.

#### 2.1.5. Trace elements

*A. niger* requires certain trace metals for growth (Mattey, 1992). However, a limitation in other trace elements is necessary for production of citric acid (Shu and Johnson, 1948a,b), especially during submerged fermentation. The metals that must be limiting include Zn, Mn, Fe, Cu, heavy metals and alkaline metals.

Shu and Johnson (1948a) established the optimal levels of Zn and Fe at 0.3 and 1.3 ppm, respectively. Addition of Mn at concentrations as low as 3 µg/l has been shown to drastically reduce the yield of citric acid under otherwise optimal conditions (Clark et al., 1966). Mattey and Bowes (1978) reported that the addition of 10 mg Mn<sup>2+</sup> per liter reduced accumulation of citric acid by 50% relative to control culture. Investigations by Clark et al. (1966) and Kisser et al. (1980) confirmed the key regulatory role of Mn<sup>2+</sup> ions. The influence of Mn<sup>2+</sup> ions on protein synthesis was considered to be of major importance because cycloheximide, an inhibitor of de novo protein synthesis, was found to antagonize the effect of manganese addition (Kisser et al., 1980). Cellular anabolism of *A. niger* is impaired under manganese deficiency and/or nitrogen and phosphate limitation. The protein breakdown under Mn deficiency results in a high intracellular ammonium concentration which causes inhibition of the enzyme phosphofructokinase (PFK, an essential enzyme in the conversion of glucose and fructose to pyruvate) leading to a flux through glycolysis pathway and the formation of citric acid. The combination of high glucose and ammonium concentrations, on the other hand, strongly repress the formation of 2-oxoglutarate dehydrogenase, thus inhibiting the catabolism of

citric acid within the TCA cycle. Thus, citric acid is regarded as an “overflow end product” due to high flux rates upstream and reduced flux rates downstream of the accumulation point (Fig. 1).

Manganese has also been shown to be important in many other cell functions, most notably cell wall synthesis, sporulation and production of secondary metabolites (Shu and Johnson, 1948b). Care must therefore be taken when choosing broth ingredients and even the materials of construction of the bioreactor vessels, to ensure that traces of manganese do not reduce the yield from the fermentation.

#### 2.1.6. Fungal morphology

The relationship between fungal morphology and citric acid productivity in submerged fermentation has long been established. Despite disagreement over whether the pelleted or filamentous form is more desirable for citric acid production, in all cases, the mycelium of acidogenic *A. niger* has been found to conform to the morphological type described by Snell and Schweiger (1951), that is, short, swollen hyphal branches that may have swollen tips.

In submerged culture, the morphology of filamentous fungi varies between pellets and free filaments, depending on culture conditions and the genotype of the strain. Discussion of the factors influencing *A. niger* morphology in submerged culture needs to distinguish between macro- and micro-morphology although a number of similarities exist for both in relation to citric acid production and responses to the environment. Snell and Schweiger (1951) reported the formation of compact aggregates or pellets (<0.5 mm diameter) as being necessary for citric acid production. Takahashi et al. (1958) also found the pellet growth to be superior to the diffuse filamentous form for acid accumulation. In contrast, Moyer (1953) reported that pellet formation was undesirable for citric acid production. Papagianni et al. (1994, 1998, 1999a,c) using image analysis studies, reported a strong relationship between morphology and productivity in various bioreactors while the strain used grew in the form of “clumps”. Mycelial clumps are stable particles of filaments intertwined around a small core. They lack the characteristic compact structure of pellets, but they represent the main morphological type for many filamentous fungal fermentations. Under appropriate conditions, citric acid yields exceeded 85% with this particular macro-morphological form, while micro-morphology was characterized by short, swollen branches with swollen tips (Fig. 2).

The main factors that affect *A. niger* morphology in submerged culture and subsequently influence the process outcome are the following: the levels of applied

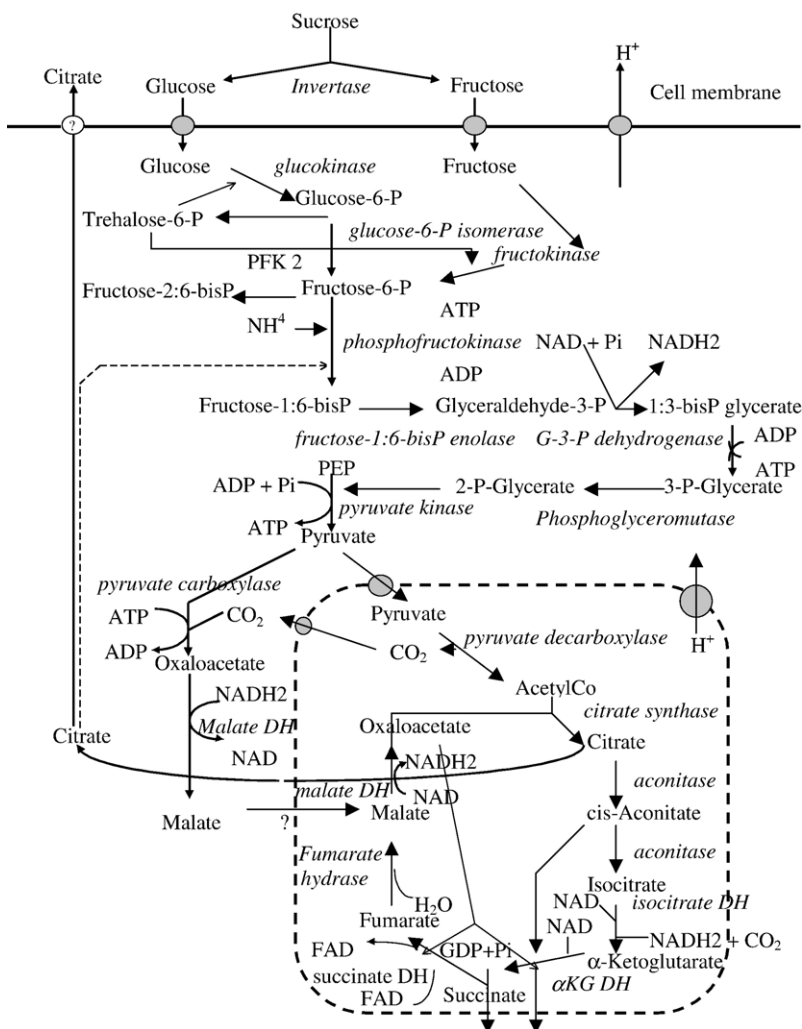


Fig. 1. Schematic representation of the metabolic reactions involved in citric acid production, the enzymes (*italics*), the known feedback loops (dashed lines) and their locations within the cellular structure of *A. niger*.

agitation; the pH of the broth; the growth rate of the microorganism; nutritional factors; and the type and concentration of inoculum.

Intensive agitation is associated with development of short, thick, and highly branched filaments that overproduce citric acid (Fig. 3). However, high shear stress can cause breakage of filaments. A cycle of mycelial fragmentation and regrowth has been observed in citric acid producing *A. niger* cultures. Fragmentation and regrowth are beneficial to the citric acid production because the mycelia that are most susceptible to fragmentation are the old and heavily vacuolated parts of the filaments that are metabolically inactive; the new tips generated from fragmentation give rise to new filaments (Papagianni et al., 1999c).

Culture pH can have a profound effect on citric acid production by *A. niger* because certain enzymes within the TCA cycle are pH sensitive. Maintenance of a low pH during fermentation is vital for a good yield of citric acid and it is generally considered necessary for the pH to fall to around 2.0 within a few hours of the initiation of the fermentation. Failing this, the yields are reduced (Mattey, 1992). Quantitative data on the effect of pH on *A. niger* morphology and productivity has been reported (Papagianni, 1995; Papagianni et al., 1999a). The preferred morphology (i.e. small aggregates and short filaments) associated with increased citric acid production was obtained at pH values around 2.0±0.2. At pH 1.6, morphological development was abnormal (bulbous hyphae) and citric acid production was reduced

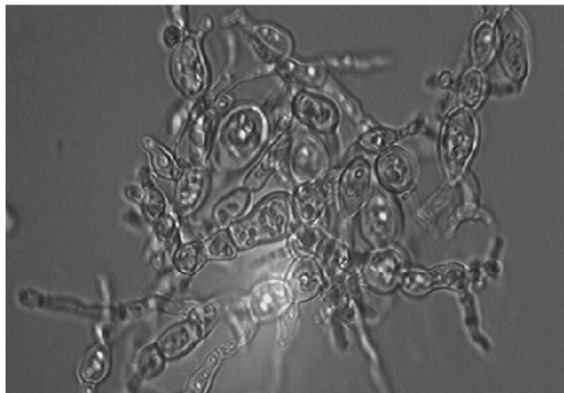


Fig. 2. Typical acidogenic mycelium of *A. niger* grown in manganese deficient medium in a stirred tank bioreactor under intensive agitation conditions (i.e. the average value of impeller power levels, expressed as energy dissipation/circulation function, was  $29.0 \pm 1.0 \text{ kW} \cdot \text{m}^{-3} \cdot \text{s}^{-1}$ ) and pH controlled at 2.0.

dramatically. At pH 3.0, the aggregates had longer perimeters and oxalic acid formation was evident.

Of the trace metals that greatly affect the citric acid fermentation, only manganese concentration has been shown to influence *A. niger* morphology. Kissler et al. (1980) studied morphology and cell wall composition of *A. niger* under conditions of manganese sufficient and deficient cultivation in an otherwise citric acid producing medium. Omission of manganese ions (less than  $10^{-7} \text{ M}$ ) from the nutrient medium resulted in abnormal morphological development that was characterized by increased spore swelling and squat, bulbous hyphae. Manganese ions influenced the frequency of mycelial branching and chitin synthesis (Kissler et al., 1980). Morphological changes, that included prevention of clumping, absence of swollen cells and reduced diameters of filaments, accompanied by a 20% reduction in citric acid yield, have been reported following addition of 30 mg/l Mn to an Mn-free medium (Papagianni, 1995; Papagianni et al., 1999a).

Spore inoculum level is another parameter that influences citric acid fermentation. Papagianni and Mattey (2006) studied the development of germinating spores and the morphology of *A. niger* in a stirred tank bioreactor under citric acid production conditions, as a function of the spore inoculum level that ranged from  $10^4$  to  $10^9$  spores per ml. Morphological features, evaluated by digital image analysis, were classified using an artificial neural network (ANN). The input layer consisted of the morphological features and cultivation time, while the output layer consisted of four object types: globular and elongated pellets, clumps and free mycelial trees. The significance of the

particular morphological features and their combination was determined by cluster analysis. Cell volume fraction analysis for the various spore inoculum levels tested revealed that by raising the spore inoculum level from  $10^4$  to  $10^9$  spores per ml, a clear transition from pelleted to dispersed forms occurred. Glucosamine formation and release by the mycelium in the fermentation broth appears to be related to spore inoculum level. Maximum concentrations were detected in fermentations inoculated with  $10^4$  and  $10^5$  spores/ml, where pellets predominated. At much higher inoculum levels ( $10^8$  and  $10^9$  spores/ml), lower dissolved oxygen levels during the early fermentation phase were associated with slower uptake of ammonium ions and significantly lower glucosamine concentrations in the broth while the mycelium developed in dispersed morphologies.

### 3. Biochemistry of citric acid formation

Citric acid overproduction requires a unique combination of several unusual nutrient conditions, i.e. excessive concentrations of carbon source, hydrogen ions, and dissolved oxygen, and suboptimal concentrations of certain trace metals and phosphate, that synergistically influence the yield of citric acid (Kristiansen and Sinclair, 1978). Citrate is one of the best-known inhibitors of glycolysis, and the ability of *A. niger* to overproduce citrate by an active glycolytic pathway has therefore attracted substantial interest. Under particular nutrient conditions citrate inhibition is counteracted because of the accumulation of various positive effectors of the phosphofructokinase gene (*pfk 1*) (Habison et al.,

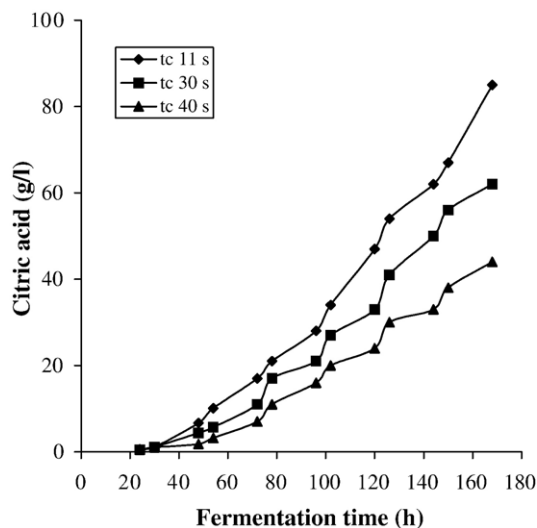


Fig. 3. The effect of agitation: the relationship between circulation time (tc) and citric acid production by *A. niger* in a tubular loop bioreactor.

1979; Arts et al., 1987). One of the positive effectors is ammonium ( $\text{NH}_4^+$ ). Citrate inhibition of *pfk* 1 seems *in vivo* to be antagonized by ammonium ions (Habison et al., 1983) and this antagonism is functionally linked to the well-known effect of trace metal ions, particularly manganese ions, on citric acid accumulation (Wolschek and Kubicek, 1999). A critical role in citric acid fermentation has been attributed to manganese ions. Influence of manganese ions on protein synthesis was considered to be of major importance because cycloheximide, an inhibitor of *de novo* protein synthesis, was found to antagonize the effect of manganese addition (Wolschek and Kubicek, 1999). Cellular anabolism of *A. niger* is impaired under manganese deficiency and/or nitrogen and phosphate limitation. According to Habison et al. (1983) and Röhr and Kubicek (1981), the protein breakdown under manganese deficiency results in a high intracellular  $\text{NH}_4^+$  concentration (the “ammonium pool”), that causes inhibition of the enzyme phosphofructokinase, an essential enzyme in the conversion of glucose and fructose to pyruvate. This leads to a flux through glycolysis and the formation of citric acid. The high glucose and  $\text{NH}_4^+$  concentrations, strongly repress the formation of 2-oxoglutarate dehydrogenase and thus inhibit the catabolism of citric acid within the tricarboxylic acid cycle (Röhr and Kubicek, 1981).

Recent studies on the early stages of citric acid accumulation by *A. niger* (Papagianni et al., 2005) contradict the existence of an intracellular ammonium pool that has been claimed to be responsible for inhibition of the enzyme phosphofructokinase. Investigating the fate and role of ammonium ions in the early phase of the citric acid fermentation process, Papagianni et al. (2005) came to the conclusion that ammonium ions are not simply deposited inside the cell to make an ammonium pool but they enter the cell to combine with glucose and form glucosamine. Glucosamine is immediately released in the fermentation broth. The slightly acidic intracellular pH, the extremely low ammonium ion concentrations inside the cell (about 1% of the external concentration), and the synthesis and release of glucosamine show that the inhibition of phosphofructokinase is certainly not due to an increased concentration of ammonium ions. The long-held relationship between high glucose and ammonium ion concentrations and the enzymes phosphofructokinase, 2-oxoglutarate dehydrogenase, and the synthase of glucosamine within the TCA cycle certainly needs further investigation.

### 3.1. Invertase, hexokinases, and glucose oxidase

The pathway of reactions leading from sucrose to citric acid starts outside the cell, with a membrane bound

invertase hydrolyzing sucrose to fructose and glucose for transport into the cell (Boddy et al., 1993; Rubio and Maldonado, 1995). High temperatures of the sterilization process also breakdown sucrose in the medium into its component sugars. The availability of glucose from sucrose is not thought to have any influence upon the rate of citric acid production.

*A. niger* has several hexokinases. One of the hexokinases has an affinity for glucose that is 1000-fold higher than its affinity for fructose. This glucokinase has been found to be inhibited non-competitively by citrate (Steinböck et al., 1994). Experiments with *A. niger* mutants indicate that the level of trehalose-6-phosphate is important and regulates the flux from glucose into glycolysis (Arisan-Atac et al., 1996). The citrate inhibition of glucokinase was found to be due to chelation of the  $\text{Mg}^{2+}$  that is required to bind ATP, and is most probably irrelevant under physiological conditions where  $\text{Mg}^{2+}$  is present in excess.

Schrefel-Kunar et al. (1989) produced *A. niger* mutants with a reduced lag time for growth on high sucrose concentrations. These mutants were found to possess enhanced hexokinase and phosphofructokinase activities. Another class of *A. niger* mutants that were resistant to 2-deoxyglucose and had an increased glucose metabolizing ability, produced citric acid at a high rate (Kirimura et al., 1992), but the precise stage of glucose metabolism (probably likely to be at the start of glycolysis) was only poorly defined.

*A. niger* also possesses the ability to oxidize glucose using glucose oxidase (Hayashi and Nakamura, 1981). The enzyme is induced under conditions that are otherwise typical for the citric acid fermentation, i.e. by high concentrations of glucose and strong aeration in the presence of low concentrations of other nutrients (Mischak et al., 1985; Rogalski et al., 1988; Dronawati et al., 1995). Glucose oxidase is formed early in fermentation and converts a significant amount of glucose into gluconic acid. However, its effect is limited, as the enzyme is inactivated when the accumulation of protons in the broth causes the pH to fall below 3.5 (Mischak et al., 1985; Roukas and Harvey, 1988). It is not known whether *A. niger* can utilize gluconic acid to make citric acid later in the fermentation.

### 3.2. Phosphofructokinases

The first step in the pathway is the phosphorylation of fructose-6-phosphate by one of two phosphofructokinases (PFK1 and PFK2). PFK1 is the best known of these enzymes. It phosphorylates the  $\text{C}_1$  position to



make fructose-1:6-bisphosphate. PFK1 is known to be inhibited by high concentrations of ATP, manganese and citrate and is activated by  $\text{NH}_4^+$ ,  $\text{Zn}^{2+}$ ,  $\text{Mg}^{2+}$  and fructose-2:6-bisphosphate, the product of the PFK2 reaction (Kubicek-Pranz et al., 1990). A study by Legisa and Bencina (1994) showed that *A. niger* possesses a cAMP dependent protein kinase that activates PFK1 and, possibly, also PFK2. PFK1 is not regulated at the transcription stage as inactive PFK1 has been isolated from mycelia early in the batch fermentation process (Legisa and Bencina, 1994).

Schreferl et al. (1986) isolated a mutant with a citrate insensitive PFK2 enzyme. This strain was more tolerant of manganese when accumulating citric acid, showing that the inhibitory effect of  $\text{Mn}^{2+}$  on PFK1 is antagonized by fructose-2:6-bisphosphate. Citrate inhibition of PFK1 is antagonized by  $\text{NH}_4^+$  ions. Several researchers have shown that citrate accumulation does not occur unless a certain amount of  $\text{NH}_4^+$  ions is taken up at the start of the batch fermentation. The overall effect of activators and inhibitors on PFK is small. Torres (1994a,b) has shown that in steady-stage fermentation, PFK enzyme step has no great effect on the flux through glycolysis. The combination of the levels of inhibitors and activators in productive cytoplasm (Habison et al., 1983; Jernejc et al., 1992; Mattey, 1992) simply permit the accumulation of citric acid through glycolysis.

### 3.3. Glycolytic and the pentose phosphate pathway

The well-known tracer studies by Cleland and Johnson (1954) and Martin and Wilson (1951) showed that citric acid is mainly formed via the reactions of the glycolytic pathway, as shown in Fig. 1. *Aspergillus* sp. use glucose and other carbohydrates for biosynthesis and maintenance by channeling glucose into the reactions of the glycolytic and pentose phosphate pathways. The pentose phosphate pathway accounts for only a small percentage of the carbon metabolized during citric acid production and this decreases further during prolonged cultivation (Legisa and Mattey, 1986).

### 3.4. Pyruvate kinase

Pyruvate kinase was considered to be an important regulatory stage in the synthesis of citric acid until Meixner-Monori et al. (1984) showed that the purified enzyme was only slightly affected by metabolic levels of inhibitors. When Ruijter et al. (1996) amplified the genes encoding phosphofructokinase 1 (pfkA) and pyruvate kinase (pkiA) individually and in combination, the rates of citrate accumulation by a moderately citric

acid producing strain were not increased. This supported calculations of Torres (1994a,b) who concluded that it would be necessary to increase the enzyme activities at seven different glycolytic steps to increase the effective flux through the pathway. A 17-base pair sequence was found upstream of the *A. niger* pkiA genes that may act as upstream regulating sequence (De Graaff et al., 1992). It has also been proposed that a cis-acting element initiates transcription of pyruvate kinase during growth on glycolytic carbon sources (De Graaff et al., 1988).

### 3.5. Fixation of carbon dioxide

The catabolism of glucose via glycolysis leads to two moles of pyruvate, that are subsequently converted by two separate reactions into the precursors of citrate (i.e. oxaloacetate and acetylCoA). Cleland and Johnson (1954) were the first to show that in these reactions, *A. niger* uses a quantity of carbon dioxide for the formation of oxaloacetate that equals the quantity of  $\text{CO}_2$  released during acetylCoA formation. This is important for producing high citric acid yields because the only alternative way of producing oxaloacetate would be a complete turn of the tricarboxylic acid cycle, with the associated loss of two moles of  $\text{CO}_2$ . If that were to happen, only two-thirds of carbon source would accumulate as citric acid and at least a third would be wasted.

Fixation of carbon dioxide does not seem to occur during the early phases of fermentation (Kubicek et al., 1979). Continuous analysis of  $\text{CO}_2$  and oxygen in the exit air of a pilot plant citric acid fermentation showed that during the first 70 h of the batch fermentation the respiratory coefficient (i.e.  $\text{CO}_2$  released/ $\text{O}_2$  taken up) was close to unity. Respiratory quotient then began to decrease and reached the level predicted from the operation of the pyruvate carboxylase reaction (i.e. respiratory quotient=0.66) only at stages where citrate accumulation was already taking place at a constant rate (e.g. <120 h into fermentation). The Cleland and Johnson reaction may therefore be important only at later stages of fermentation, with the initial phase of citric acid accumulation taking place without any overall carbon dioxide uptake. The enzyme catalyzing Cleland and Johnson reaction was shown to be pyruvate carboxylase (Woronick and Johnson, 1960; Bloom and Johnson, 1962), and has been characterized by Feir and Suzuki (1969) and Wongchai and Jefferson (1974). Unlike the enzyme from several other eukaryotes, the pyruvate carboxylase of *A. niger* is located in the cytosol (Bercovitz et al., 1990; Jaklitsch et al., 1991).

Glycolytic pyruvate can therefore be converted to oxaloacetate without being transported into the mitochondria, and can be converted further to malate by the cytosolic malate dehydrogenase isoenzyme (Ma et al., 1981). The enzyme in *A. niger* is inhibited by aspartate, but not by acetylCoA or  $\alpha$ -ketoglutarate, both of which inhibit the analogous enzyme in the closely related *A. nidulans*.

### 3.6. Glyoxylate cycle

Breakdown of isocitrate by isocitrate lyase is the first stage in the glyoxylate shunt, a pathway that forms malate from isocitrate and acetylCoA to regenerate the TCA cycle precursors during growth. It was once thought possible that this route formed malate and oxaloacetate during acidogenesis. The glyoxylate cycle has also been implicated in the biosynthesis of oxalic acid from citrate as a toxic byproduct of citric acid fermentation (Müller, 1875). However, Kubicek et al. (1988) have shown that isocitrate lyase is absent during citrate production, and therefore all malate and oxaloacetate must originate from the carboxylation of pyruvate during this phase. Oxalic acid biosynthesis originates from glucose via the hydrolysis of oxaloacetate by oxaloacetate hydrolase, an enzyme located in the cytosol. The reaction appears to act in competition with citrate overproduction as a mechanism by which excess carbon can be channeled into an energetically neutral pathway (Kubicek et al., 1988).

### 3.7. Tricarboxylic acid cycle

A large amount of work has been concerned with identifying bottlenecks in the tricarboxylic acid (TCA) cycle to explain the accumulation of citric acid. Several investigators have claimed that inactivation of a citrate degrading enzyme (e.g. aconitase or isocitrate dehydrogenases) as being essential for the accumulation of citric acid. However, the presence of a complete set of citric acid cycle enzymes during citric acid fermentation has been demonstrated (Ahmed et al., 1972). Furthermore, although the internal levels of free amino acids do fall significantly during the citric acid accumulation phase, the initial levels of these amino acids cannot explain the considerable observed increase in cell protein level during the fermentation process (Jernejc et al., 1992). This suggests that an active TCA cycle is producing the necessary intermediates for biomass formation.

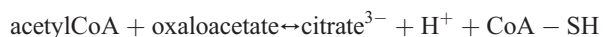
Mitochondrial AMP, cis-aconitate, oxaloacetate, NADH/NAD and NADPH/NADP ratios are all known to affect enzymes in the TCA cycle (both of the

mitochondrial isocitrate dehydrogenases,  $\alpha$ -ketoglutarate dehydrogenase, and succinate dehydrogenase) but their effect *in vivo* has not been determined (Chan et al., 1965; Meixner-Monori et al., 1985, 1986). Malate accumulation has been shown to precede citrate accumulation (Ma et al., 1981; Röhr and Kubicek, 1981). However, research on mitochondrial citrate carriers of *A. niger*, which probably would explain citrate accumulation, has not been carried out.

A hypothesis for the accumulation of citric acid is associated with the activity of the tricarboxylate transporter (Kubicek, 1988). Tricarboxylate transporter competes directly with aconitase for citrate. If its affinity for citrate is shown to be much higher than that of aconitase, then tricarboxylate transporter would pump citrate out of the mitochondria without the necessity for any inhibition in one of the TCA cycle enzymes. The tricarboxylate carriers of mammalian tissue and yeasts export citrate from the mitochondria by counter transport with malate (Evans et al., 1983). Such a situation can be envisaged in *A. niger* when malate is produced from oxaloacetate in the cytosol by malate dehydrogenase (Kubicek, 1988).

### 3.8. Citrate synthase

Citrate synthase catalyzes the reversible condensation reaction between oxaloacetate and acetylCoA:



This equilibrium reaction favors the production of citrate because of the thioester hydrolysis that occurs as a part of the reaction (Lowenstein, 1969). The rate of the reaction is controlled *in vivo* by the availability of the substrates and not by any inhibitors. Oxaloacetate has the most effect on reaction rate because the concentration of oxaloacetate affects the  $K_m$  value of the enzyme for the substrate acetylCoA (Kubicek and Röhr, 1980). The reaction follows an ordered mechanism, with oxaloacetate binding to the enzyme first and acetylCoA binding subsequently. *In vitro*, both ATP and coenzyme A inhibit the enzyme that catalyzes the above reaction; both these compound compete with acetylCoA for the active site. There is no or little inhibition of the reaction at the concentrations of metabolites seen *in vivo*.

### 3.9. Isocitrate dehydrogenases

There are three different isocitrate dehydrogenase isoenzymes in *A. niger*. The difference between them is the recipient that they use for the protons that are removed from isocitrate. These enzymes are referred to

as NAD<sup>+</sup>-dependent and NADP<sup>+</sup>-dependent isocitrate dehydrogenases. The NAD<sup>+</sup>-dependent form exists only in mitochondria. One of the two NADP<sup>+</sup>-dependent forms exists in mitochondria and the other exists in the cytoplasm. The NADP<sup>+</sup>-dependent form is present in greater quantities compared with the NAD<sup>+</sup>-dependent enzyme (LaNauze, 1966; Kubicek and Röhr, 1977).

The isocitrate to  $\alpha$ -ketoglutarate is a two step process. Protons are removed first and CO<sub>2</sub> is produced in the second step as shown in Fig. 4. Divalent metal ions such as Mg<sup>2+</sup> and Mn<sup>2+</sup> are required for the reaction, and are bound by the enzyme at the active site. The metal ion has two functions: it is involved in the binding of the substrate to the enzyme and has an important role in stabilizing the reaction intermediate, oxalosuccinate. Mg<sup>2+</sup> and Mn<sup>2+</sup> can be used interchangeably in the enzyme without influencing enzyme activity. Citrate and  $\alpha$ -ketoglutarate have both been shown to act as inhibitors of the NADP<sup>+</sup>-dependent form of the enzyme (Mattey, 1977; Kubicek and Röhr, 1977) and this helps to perpetuate the accumulation of citric acid once the process has started.

### 3.10. Glutamine synthase

Punekar et al. (1984) have suggested that the roles of ammonia, pH and manganese ions in citric acid accumulation are linked with glutamine synthase. Glutamine synthase has two forms, depending on whether the enzyme has Mg<sup>2+</sup> or Mn<sup>2+</sup> ion in its structure. Only the Mg<sup>2+</sup> form can exist during growth on Mn<sup>2+</sup>-free medium used for citric acid accumulation. As the pH in the medium falls to low levels, the intracellular pH also falls to between 6.0 and 6.5. The activity of the Mg<sup>2+</sup> form of glutamine synthase is greatly reduced under these conditions, leading to the accumulation of free glutamic acid, TCA cycle intermediates and NH<sub>4</sub><sup>+</sup> ions.

### 3.11. NADH regeneration and oxidative phosphorylation

Strong aeration and dissolved oxygen tensions higher than those required for vegetative growth of *A. niger* are

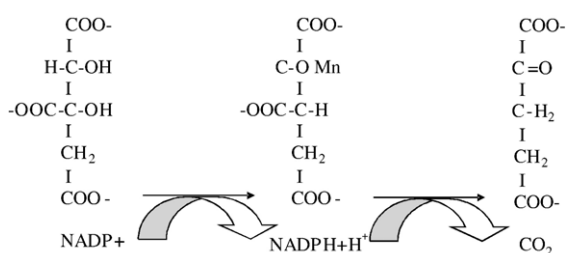


Fig. 4. The conversion of isocitrate to  $\alpha$ -ketoglutarate.

necessary for citric acid overproduction (Clark and Lentz, 1961; Kubicek et al., 1980; Dawson et al., 1988a). Sudden interruptions in aeration cause an irreversible impairment of citric acid production without affecting growth (Kubicek et al., 1980; Dawson et al., 1988b). The biochemical basis for this observation lies in the induction of an alternative respiratory pathway that is required for re-oxidation of the glycolytic NADH (Zehentgruber et al., 1980; Kubicek et al., 1980; Kirimura et al., 1987, 1996).

The activity of the proton pumping NADH:ubiquinone oxidoreductase (complex I) in the mycelium was found to be proportional to the levels of Mn<sup>2+</sup> in the growth medium by Wallrath et al. (1992) further suggested that complex I is at least partly inactive in citric acid producing conditions. A high yielding producer of citric acid (*A. niger* strain B60) was observed to lose its entire complex I activity at the onset of citric acid production (Wallrath et al., 1991). Strain B60 has the normal peptide sequence in complex I, but the assembly of the complex appears to be different (Schmidt et al., 1992). In citric acid production conditions, excess NADH is oxidized by an alternative oxidase that does not pump protons. This enzyme is produced constitutively, but does not normally have any effect on metabolism as its affinity for NADH is only 1% of the affinity of complex I for NADH (Prömper et al., 1993; Schmidt et al., 1992).

Mutant strains of *A. niger* that lack complex I activity have been prepared by Schmidt et al. (1992) and Prömper et al. (1993). Deletion of complex I in *A. niger* B60 (Schmidt et al., 1992) reduced this strain's ability to form ATP via oxidative phosphorylation to 60% of that of the wild type. The mutant strain accumulated a high concentration of intracellular citrate, but appeared unable to excrete significant amounts of it into the medium. The mutant actually secreted less citrate than the wild type strain. Schmidt et al. (1992) postulated that this could be due to a higher alternative oxidase activity in the high yielding strain, which oxidized all of the excess NADH that could not be used by complex I. As the mutant strain possessed only the wild type alternative oxidase, it was unable to recycle enough NADH to maintain the high glycolytic flux that is essential for citric acid production.

During citric acid accumulation, the quantitative conversion of citric acid to glucose yields 1 molecule of ATP and 3 molecules of NADH. Unless a significant proportion of the NADH pool can be re-oxidized without the formation of ATP by the alternative respiratory pathway described above, the yield of ATP will probably exceed the cell's maintenance requirement. Wolschek and

Kubicek (1999) have suggested that the ATP could be consumed by the membrane bound ATPase which maintains the pH gradient (Mattey et al., 1988), and that the inhibition of the alternative pathway would therefore inhibit glycolysis by causing an accumulation of NADH.

#### 4. Membrane transport aspects in *A. niger*

Both the transport of sugars and ammonia into the cell and the export of citrate ions from the cell are crucial to an understanding of the overproduction of citric acid by *A. niger*. Membrane transport is also the subject of much disagreement. Theoretical calculations by Torres (1994a,b) suggest that a major part of the control of citric acid production must occur at hexose uptake and/or phosphorylation. That is, if the rate of glucose uptake can be increased, the rate of citrate production will increase. Gupta and Sharma (1995) demonstrated this without influencing the overall yield. Gupta and Sharma (1995) showed that an increase in the rate of glucose transport reduced the lag time between ammonia uptake and citrate production. However, in fed-batch experiments by Papagianni (1995), maintaining the concentration of glucose at specific levels during production of citric acid, did not affect the yield of citric acid, but its rate of production strongly correlated with glucose concentration.

At least two glucose transporters have been identified in *A. niger*. Torres et al. (1996a) showed that a high glucose concentration (>50 g/l) was a prerequisite for the formation of an alternative low-affinity glucose transporter that was capable of providing the high flux of glucose required for citrate production. With such high levels of glucose in the medium, there is a potential for their diffusive transport through the membrane at significant rates. This idea is supported by Mischak et al. (1984), but it appears to have been mostly overlooked. Mischak et al. (1984) clearly showed that small quantities of extracellular citric acid inhibited the uptake of glucose (at 0.5 mM concentration) in mycelium that had been grown under citric acid producing conditions. Inhibition of the glucose transporter was not affected by metal ions (Mischak et al., 1984), indicating that citrate affected the carrier directly, and not by chelating essential divalent metal ions. Under these conditions, the low affinity glucose transporter should theoretically be present (Torres et al., 1996a), and so it must be concluded that the high flux transport mechanism is inhibited under producing conditions and that some other mechanism is responsible for the transport of glucose into the cell.

Wayman and Mattey (2000) identified simple diffusion as the primary mechanism for glucose uptake

during the production phase of the *A. niger* citric acid process. Models for the known glucose transporters in *A. niger* and for simple diffusion of glucose through the hyphal membrane were prepared and the simulations from these models were compared with published fermentation data. The purely physical and uncontrolled process of diffusion was found to satisfactorily explain the specific rate of glucose uptake observed during the production phase in several different types of fermentations (Papagianni and Mattey, 2004).

The only publications on uptake of ammonium ions by *A. niger*, are those of Papagianni et al. (2005) (see Section 3) and Mattey et al. (1988). The effect of metabolic inhibitors on the rate of uptake implies that the ammonium uptake requires energy either directly or indirectly. Published studies of ammonium uptake in other microorganisms all focus on active uptake when ammonium ions are present in low concentrations.

Mattey and co-workers (Mattey, 1992; Kontopidis et al., 1995) explained the export of citrate through the plasma membrane in terms of the large pH gradient between the cytosol and the extracellular medium. Citrate exists in different ionic states in the cell, which has a pH between 6.0–7.0, and the medium that is at pH 2.0 or lower. The cell membrane has been proposed to be permeable to citrate<sup>2-</sup> (Mattey, 1992; Kontopidis et al., 1995), the prevalent form of citrate at the intracellular pH. Thus, citrate<sup>2-</sup> is postulated to diffuse out of the cell rapidly. If this assumption is correct, a low pH would be necessary to keep the external concentration of citrate<sup>2-</sup> negligible. The rate of citrate export at different external pH levels would also be related to the ratio of the different ionic forms of citrate at the external pH.

According to Wolschek and Kubicek (1999), intracellular citrate is excreted from the cell by an active process that requires ATP to pump citrate into the medium. The authors showed that citrate export would not occur at pH 5.0 unless the mycelium was Mn<sup>2+</sup> deficient. This particular type of transport was also sensitive to metabolic inhibitors, implying an active process. At a pH of 5.0, the rate of citrate export was reduced to 50% of the rate at pH 2. In a different study (Papagianni, 1995), the export of citrate in producing mycelium at pH 4.0 was reduced to 42% of the rate at pH 2.0. The transporter implicated by Wolschek and Kubicek (1999), continued to export citrate at pH 7.0 at 25% of the rate at pH 2.0, but at pH 7 citric acid export generally does not occur. This suggests that the transporter system investigated by Wolschek and Kubicek (1999) is not the main means of citrate export in *A. niger* under producing conditions.

Unpublished studies referenced by Mischak et al. (1984), suggest that citrate is not taken up at pH 2.0 by

hyphae of *A. niger*. Furthermore,  $Mn^{2+}$  deficient hyphae do not take up citrate at pH 3.3 (Netik et al., 1997). Therefore, it is conceivable that *A. niger* possesses an active transport system, but either does not use it, or does not need to use it when the external pH is low.

In contrast, Kontopidis (1997) showed that cells take up radioactive citrate when the addition of a buffered citrate solution causes a small, local rise in the pH. Citrate is not taken up under the same conditions if the citrate solution is buffered to the pH of the broth. The most prevalent form of citrate at the broth pH is the undissociated acid. One of the ionic forms will be most prevalent at higher pH values. This suggests that the membrane contains a carrier for a dissociated form of the acid that can be reversed, if the external concentration of the dissociated ions rises as a consequence of a rise in pH. This reversibility is not a feature of active transport and demonstrates that facilitated diffusion proteins are available for the transport of at least one specific citrate ion across the membrane.

## 5. Modeling citric acid production by *A. niger*

### 5.1. Kinetic modeling by Röhr, Zehntgruber and Kubicek (1981)

Kinetics of citric acid production by *A. niger* growing on sucrose in a pilot plant were investigated by Röhr et al. (1981). A typical growth curve for *A. niger* under citric acid producing conditions shows an initial rapid growth phase followed by a phase of slow growth. Röhr et al. (1981) subdivided cell growth and product formation into several phases, each described by a simple deterministic model (Fig. 5). The best fitting model equations in the various phases were identified. The growth phases identified were: the hyphal growth phase ( $B_x$ ), pellet growth phase ( $C_x$ ), restricted growth phase ( $D_x$ ), transition period between trophophase and idiophase ( $E_x$ ) and idiophase growth ( $F_x$ ). The logarithmic, cube root and linear equations described by Trinci (1970) were used to describe the growth in each phase, as follows:

$$\text{Log of growth linear with time, i.e.} \\ \ln X_t = \ln X_0 + \mu t \quad (1)$$

$$\text{Cube root of growth linear with time, or} \\ X_t^{1/3} = X_0^{1/3} + K_c t \quad (2)$$

$$\text{Growth linear with time, i.e. } X_t = X_0 + K_t t \quad (3)$$

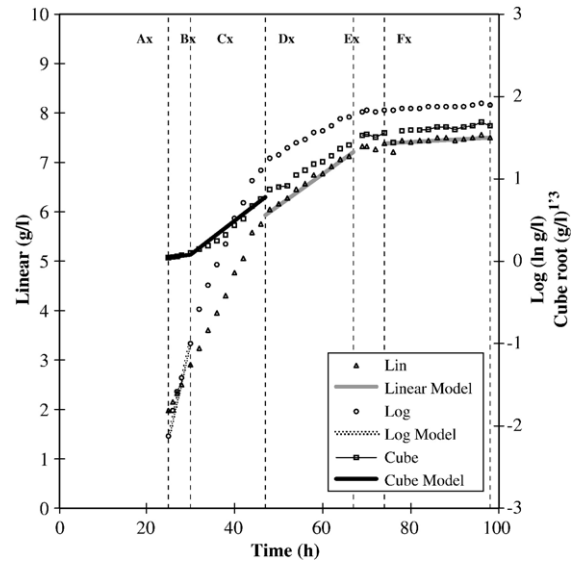


Fig. 5. Biomass versus time during a citric acid fermentation (Röhr et al., 1981). Lines added subsequently show interpretation of kinetics by Röhr et al. (1981). Presence of lines implies that the model equations closely agree with the data. Absence of lines implies that the model did not agree closely with the measured data.

In these equations,  $X_t$  is the biomass concentration at time  $t$ ,  $X_0$  is the initial biomass concentration,  $\mu$  is the specific growth rate, and  $K_c$  and  $K_t$  are constants.

Product formation was related to the growth rate by a modified Luedeking–Piret equation, which relates the formation of products ( $r_p$ ) to either the biomass concentration ( $x$ ) or the rate of biomass accumulation ( $r_x$ ):

$$r_p = (\alpha \cdot r_x) + (\beta \cdot x) \quad (4)$$

Although the similar relationships applied to both growth and acid formation (Fig. 5), the acid formation kinetics were usually different from the growth kinetics by a term that represented the lag time. This lag time, also known as the maturation time, is the period during which the culture taken up all the ammonium ions but has not yet started producing citric acid. The rate of product formation was then said to be proportional to the rate at which the cells entered the maturation state. The rate of product formation was expressed as follows:

$$r_{Pt} = kr_{X(t-t_m)} \quad (5)$$

where  $r_{Pt}$  is the product formation rate at time  $t$ ,  $r_{X(t-t_m)}$  is the rate of biomass growth at time  $t$  beyond the

maturation time  $t_m$ , and  $k$  is the product formation rate constant, respectively.

One problem with Eq. (5) is that  $k$  is actually not constant but increases in value during the fermentation. This was resolved by assuming that there were at least two different types of cells within the mycelium and these cells had different productivities. A production term (Table 1) for each type of cells was described in the form of Luedeking–Piret equation, and Eq. (5) was modified to the following equation:

$$r_{Pt} = k_1 r_{X(t-t_m)} + k_2 X(t-t_m) \quad (6)$$

where  $k_1$  is a growth-associated constant and  $k_2$  is a non-growth associated constant. The constants  $k_1$  and  $k_2$  were determined by a computer-based optimization procedure following the determination of  $t_m$ . The values of the various constants are shown in Table 1.

Fig. 6 compares the experimental values of the rate of citric acid production and the values of the rate calculated by Eq. (6). The model does closely follow the experimental data (Röhr et al., 1981). Nevertheless, this model has two problems. Firstly, it is difficult to decide which specific equation should be used at any particular time, as more than one equation may appear suitable. This is of particular importance near the start and end of each phase. The second problem with the model is that the different phases for product and biomass formation occur at different times as a consequence of the deterministic rather than mechanistic nature of the model. The phase changes used in the model cannot therefore give a precise indication of changes in the metabolism regulating the production of citric acid.

## 5.2. Metabolic modeling by Krzystek, Gluszczyk and Ledakowicz (1996)

Theoretical and experimental mass and energy balances for specific system inputs and outputs were made for the *A. niger* fermentation, by Krzystek et al. (1996). Measurements were made in an external-loop airlift bioreactor of 220 l working volume. The sole

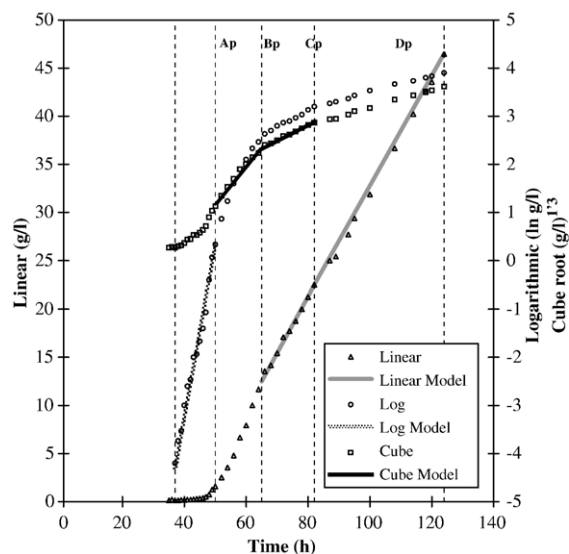


Fig. 6. Citric acid concentration versus time during a citric acid fermentation (Röhr et al., 1981). Lines added subsequently show interpretation of kinetics by Röhr et al. (1981). Presence of lines implies that the model equations closely agree with the data. Absence of lines implies that the model did not agree closely with the measured data.

carbon source used was sucrose at an initial concentration of 100 g/l. A simplified metabolic model of *A. niger* was developed using many of the main reactions inside the cell. In the calculating biomass and product yields and maintenance coefficients, mechanistic values of parameters (e.g. true biomass and citric acid yields  $Y_{ATP, X(P)}^{\max}$  on ATP; specific ATP consumption due to maintenance processes,  $m_{ATP}$  (subscripts:  $X$ , biomass;  $P$ , product) were used.

Fig. 7 illustrates the possible combinations of product yield  $Y_{PS}$  and a dimensionless biomass yield  $Y_{SX}/Y_{CX}$  (subscripts:  $S$ , substrate;  $C$ , concentration in mol carbon per l). Values above the diagonal line representing the carbon balance from  $Y_{PS}=Y_{CP}=1$  to  $Y_{SX}/Y_{CX}=1$  were forbidden because of the following equation:

$$\frac{Y_{SP}}{Y_{CP} \text{ production}} + \frac{Y_{SX}}{Y_{CX} \text{ growth}} \leq 1 \quad (7)$$

The permissible values below the diagonal line depended on the value of the ratio  $Y_{EX}/Y_C$ , that suggest that energy limits the cell growth.  $Y_E$  (dimensionless) is the upper limit of the yield factor  $Y_{ij}$  for a compound  $j$  on a compound  $i$  based on energy availability. Similarly  $Y_C$  (dimensionless) is the upper limit of  $Y_{ij}$  based on carbon availability. The highest

Table 1

Calculated values of the growth-associated and non-growth associated constants  $k_1$  and  $k_2$  (Röhr et al., 1981)

Parameter	Microorganism	
	<i>A. niger</i> B60/B3	<i>A. niger</i> B60/B5b
$t_m$ (h)	15	24
$k_1$	1.9	2.0
$k_2$	0.090	0.038

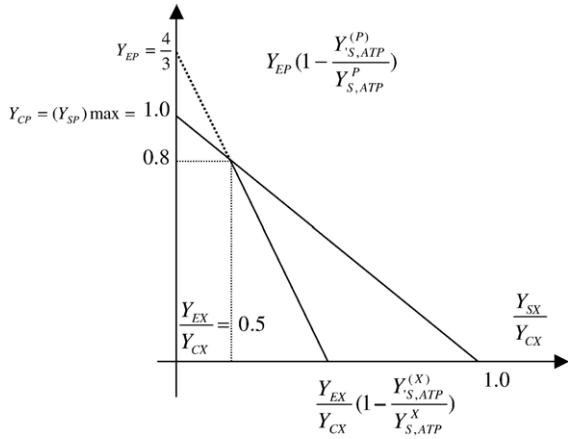


Fig. 7. Biomass and product yields during citric acid production according to Krzystek et al. (1996).

biomass and product yields that can be expected in practice correspond to the point where the energy balance line for the value of  $Y_{EX}/Y_{CX}=0.50$ , crosses the diagonal line representing the carbon balance, considering Eq. (7) and ignoring cell maintenance:

$$\frac{(Y_{SP})_{\max}}{Y_{CP}} \leq 1 - \frac{Y_{SX}}{Y_{CX}} \leq \frac{1 - Y_{EX}/Y_{CX}}{1 - Y_{EX}Y_{CP}/Y_{CX}Y_{EP}} \quad (8)$$

The maximum yield  $(Y_{SP})_{\max}$  for citric acid is 0.80 mol C (citric acid) per mol C (sucrose), and the corresponding maximum yield  $(Y_{SX})_{\max}$  for biomass is 0.18 mol C (biomass) per mol C (sucrose).

The yield coefficient  $Y_E$  was calculated taking into account the ATP requirement for maintenance, as follows:

$$Y_E = \frac{Y_C}{1 - Y_{S,ATP}/Y_{S,ATP}} \quad (9)$$

Substrate level phosphorylation was ignored and the overall energy balance of ATP and available electrons was given in the following form:

$$\frac{Y_{SP}}{Y_{EP}} + \frac{Y_{SX}}{Y_{EX}} = 1 - \frac{Y'_{S,ATP}}{Y_{S,ATP}} + \frac{Y^P_{S,ATP}}{Y_{S,ATP}} \quad (10)$$

production      growth                      growth                      production

The experimentally obtained yield coefficients relative to cell growth and citric acid production were 96%

and 83% of real maximum theoretical values, respectively. Calculated true biomass and citric acid yield coefficients closely agreed with the values estimated from mass balance analysis of stoichiometric equations. Calculated value of specific maintenance requirements  $m_{ATP}$  was 0.015 mol ATP per mol C (dry mass) per hour.

### 5.3. Work of Kristiansen and Sinclair (1979): expressions for differentiated states of cells

In the models mentioned above, no distinction was made for differentiated states of cells and differences in growth rates of cells in different metabolic states. A model with separate expressions for rates of growth of cells in different physiological states is likely to be more consistent with reality. Such a model was developed by Kristiansen and Sinclair (1979) for a single-stage ideally mixed continuous stirred tank reactor. The model used the following equations:

$$r_{X_b} = \mu_b X_b - k_t X_b - D X_b \quad (11)$$

$$r_{X_c} = \mu_c X_c + k_t X_b - k_d X_c + D X_c \quad (12)$$

$$r_{X_d} = k_d X_c - D X_b \quad (13)$$

where  $D$  is the dilution rate and subscripts  $b$ ,  $c$  and  $d$  refer to basic stage, citric acid-producing (carbon storage) stage and deactivated cells, respectively.

The dilution rate ( $D$ ) in these equations can be substituted by the overall growth rate ( $\mu$ ) for use in batch fermentation (Sinclair et al., 1987). The various constants in Eqs. (11)–(13), took the following forms:

$$\mu_b = \mu_{mb} \frac{N}{k_N + N} \quad (14)$$

$$\mu_c = \mu_{mc} \frac{S}{k_S X_c + S} \quad (15)$$

$$k_t = k_{tm} \frac{k_i}{k_i + N} \quad (16)$$

$$\mu_b = \mu_{mb} \frac{N}{k_N + N} \quad (17)$$

where  $\mu_b$  is the specific growth rate on a limiting substrate,  $\mu_c$  is the specific growth rate for citrate producing bulbous cells, and  $k_t$  is the rate of transformation of basic cells to storage cells.

#### 5.4. Mechanistic modeling by Torres, Voit and Gonzalez-Alcon (1996b)

A model developed by Torres et al. (1996a,b) described the optimum steady state flux through a twelve step metabolic pathway to oxaloacetate. The fermentation phase described in this model was the pseudo-steady state phase that occurred after the initiation of acidogenesis. Only optimum values for the levels of activators (such as  $\text{NH}_4^+$ ) and inhibitors (such as cytoplasmic citrate) were used in the model and the effect of pH was ignored. The model did not include biomass production and maintenance terms. The model was based on the S-system of metabolic flux (similar to the metabolic control theory) and, therefore, could not predict the conditions that lead to citric acid accumulation and are believed to be critical to the final yield of product. Torres et al. (1996a,b) claimed that there are no “rate limiting steps” in glycolysis during acid accumulation and that at least seven enzymes are jointly limiting. This concurs with the hypothesis that increases in productivity are likely to be gained by reducing the length of non-accumulating phases and increasing the rate of transport of citrate and sugars.

#### 5.5. Deterministic modeling by Ho, Kristiansen and Mattey (1994, 1999)

Ho et al. (1994) developed a model by plotting the results of batch fermentations and identifying phases in which simple expressions could be used to describe ammonium ( $N$ ) and glucose ( $S$ ) uptake, the drop in pH, and the accumulation of biomass ( $X$ ) and citric acid ( $P$ ) (Fig. 8). The model used four different Monod type growth rate equations with different values for  $\mu_{\max}$  and  $k_s$ . The concentrations of substrate and product influenced the overall growth rate by affecting the various growth rate equations. Available intracellular nitrogen was taken into account. The inputs and outputs of the model were derived from growth rates and yield coefficients calculated for various stages using the experimental data. The model agreed well with the experimental measurements (Fig. 8).

The modeling approach used by Ho et al. (1994) was similar to that of Röhr et al. (1981), but used more complex equations compared with the simple linear relationships used by Röhr et al. (1981). All the phases

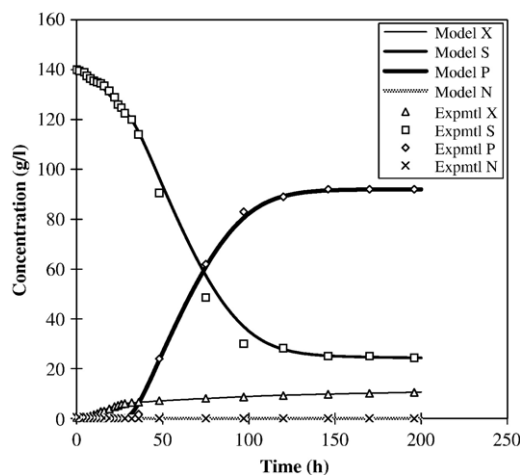


Fig. 8. Experimental points and modelled curves according to Ho (1994).

of fermentation linked together to achieve a degree of overlap during the batch. The expressions used for the growth rate employed different values for  $\mu_{\max}$  derived in different phases. Although the model allowed more than one rate expression to be valid at the same time, a situation in which all the expressions were valid was not likely to arise. The model was quite complex and it could not accurately simulate the experimental results for different batch conditions.

#### 5.6. Morphologically structured modeling by Bizukojc and Ledakowicz (2006)

Structured modeling of microbial processes implies that cells are not treated as a “black box” in which neither intracellular metabolic reactions nor microbial morphology are taken into account. The morphologically structured model for *A. niger* proposed by Bizukojc and Ledakowicz (2006) was based on the mathematical modeling framework formulated by Nielsen and Villadsen (1994). The model took into consideration six extracellular components that were detected in the fermentation medium. Hyphae were divided into four zones of different physiological and functional states. The model consisted of ten ordinary differential equations that balanced biomass in the different hyphal zones and included the effects of the most important nutrients and products (i.e. carbon sources, nitrogen source, citric acid). The model established a direct linear correlation between the physiological zone B (a less metabolically active and more vacuolated area) and citric acid excretion, confirming that this particular hyphal zone was responsible for acid excretion. A good agreement was found between the



model and experimental data obtained under various process conditions in two different reactors.

### 5.7. Stoichiometric modeling by Wayman (2001) and Papagianni, Wayman and Mattey (2005)

Wayman (2001) made a stoichiometric model that involved mass balances for biomass, glucose and ammonium ions during the first 30 h of the fermentation. For each measured data point, the expected glucose uptake rate was calculated from the rate of ammonium ion uptake  $r_N$ , the rate of biomass formation  $r_x$ , and the relevant yield coefficients ( $Y$ ). The following equations were used:

$$r_{s(x)} = \frac{-r_x}{Y_s} \quad (18)$$

$$r_{N(x)} = \frac{-r_x}{Y_N}, \quad (19)$$

$$r_{s(P)} = \frac{r_N - r_{N(x)}}{Y_{P/N}} \quad (20)$$

The calculated value for  $r_s$  was compared with the measured values of  $r_s$  obtained from the measured data. Biomass was assumed to have the generalized empirical formula  $\text{CH}_{1.8}\text{O}_{0.5}\text{N}_{0.2}$ . The fate of ammonium ions not incorporated into biomass could be altered by changing the yield of hypothetical product from nitrogen and carbon sources. The values for yield coefficients were as follows: 65% for biomass from glucose; 500% for biomass from ammonium ions (theoretical molar yield); and 100% for a simple hypothetical compound containing glucose and ammonium ions in equal molar amounts.

The experimental and calculated fermentation profiles agreed closely, indicating that ammonium ions combine with a carbon containing metabolite inside the cell. This good agreement further showed that the most likely ratio for this combination was one mole of glucose per mole of ammonia. Based on this stoichiometry, glucosamine was identified as a potential nitrogen storage compound (Papagianni et al., 2005). HPLC analysis identified glucosamine in the fermentation broth during the early stages of the fermentation. This was the first report of glucosamine being a by-product of the citric acid fermentation (Papagianni et al., 2005). Glucosamine synthesis and release into the broth can occur not only in the early part of the fermentation when nitrogen is provided with the other constituents of

the medium, but also later if glucose is available and further nitrogen is added. Glucosamine acts as a storage compound and it is used by the fungus during the course of the fermentation.

## 6. Concluding remarks

This review focused on citric acid fermentation by *A. niger*. Fermentation biochemistry, membrane transport in the producing microorganism and fermentation modeling were discussed. Findings concerning the role of ammonium ions (Papagianni et al., 2005) during the early stages of the citric acid fermentation lead to questions concerning the long-held theory of an “ammonium” pool within the cells. This pool has been regarded as the cause of inhibition of the enzyme phosphofructokinase, a consequent flux through glycolysis and formation of the acid. Clearly, the relationship between high concentrations of glucose and ammonium ion and the impact of this relationship on the various metabolic enzymes (phosphofructokinase, 2-oxoglutarate dehydrogenase, glucosamine synthase) deserves further investigation.

Concerning membrane transport in *A. niger*, the uptake of glucose under the high glucose conditions that saturate the known transporters, appears to be driven by pure diffusion that does not depend on any transporter proteins (Wayman and Mattey, 2000). The mediated transport systems previously described in the literature do not fit the observations reported in the literature. A fermentation model based on diffusive transport of glucose does explain well many of the previously unexplained observations.

Advances in image analysis technologies and their application to fungal fermentations have made available quantitative morphological data on *A. niger* for various stages of the fermentation. These developments have enabled quantitative investigation of the long-known empirical relationship between morphology and citric acid productivity. Fermentation models that are morphologically structured have been developed to better describe the behavior of this fermentation.

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