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Mechanisms of Citric Acid Fermentation by *Aspergillus niger*

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Fermentation is the major method for producing citric acid. Overflow of fungal metabolism forms the basis of the citric acid industry[1]. Over the years, many reviews have covered the physiology and the state of art of this commercially important process[2-4]. Extensive studies have been carried out to maximize the yield and to pinpoint the nature of regulatory mechanisms operating during fermentation. A major raw material used as a carbon source for citric acid production by *A. niger* is molasses obtained as a byproduct of the sugar industry[5]. Optimization of citric acid fermentation[6,7] has become mandatory, because the raw material (molasses) is being channelized to more profitable industries, such as alcohol production. Alternatively, newer ways of citric acid production from (i) low sugar cotton waste[8] and (ii) immobilized *A. niger*[9] are being employed. Production of citric acid from a variety of new materials, e.g. oils, fats, sugars, cellulose and *n*-paraffins by yeasts has gained much importance in recent years[10-18]. Such yeasts are able to convert n-paraffins to citric acid in extremely high yields. Nevertheless, production of citric acid by fermentation using *A. niger* continues to be a preferred commercial process.

1 Parameters Influencing Citric Acid Fermentation

A detailed account of many conditions controlling citric acid accumulation was given by Berry *et al.*[3]. A brief review of the conditions employed during fermentation is presented, as many of the physiological processes of the fungus are closely related to these parameters.

Commercially, the fungus is grown on a very high (up to 15%) concentration of the carbon source, either as surface cultures in shallow pans, or as submerged cultures in well aerated fermenters[19]. The fermentation continues in two distinct phases--an initial growth phase (trophophase), and a subsequent production phase (idiophase), in which limited growth of the organism occurs. Contaminants in the crude substrates, such as molasses, have been shown to interfere with citric acid production[20]. Phosphate and nitrogen sources are usually supplied at low levels. It has also been documented that citric acid production by *A. niger* is inhibited under conditions leading to excessive biomass production[21]. The relationship between growth conditions, the morphology of the mycelium and citric acid production has been discussed[7,22], and it was recently reported that germination of *A. niger* on high citric acid yielding medium led to the formation of bulbous cells[23].

A. niger is characteristically tolerant of low *p*H (between 2.0 and 3.0), which is also an important parameter controlling citric acid accumulation. This highly acidic *p*H is not only optimal for citric acid formation, but also reduces the risk of contamination by other microorganisms. Low buffering capacity of the fermentation medium (Table 1) is further responsible for the decreased *p*H during fermentation.

Citric acid production is highly sensitive to trace metals. Among these, manganese apparently exerts the maximal inhibitory effect on citric acid fermentation compared to similar concentrations of other ions, e.g. iron and zinc[24-26].

2 Glycolysis and Kreb's Cycle

Citric acid is a primary metabolic intermediate of the tricarboxylic acid cycle (TCA-cycle, Fig.1). The mechanism of citric acid fermentation is interesting not only because of its relevance to fungal physiology, but also in understanding metabolic regulation of cell growth and acidogenesis. Towards this objective, TCA-cycle has received considerable attention. Production of excessive amounts of citric acid during this fermentation has been attributed to its formation from acetyl CoA and oxaloacetate derived from the TCA-cycle[27]. A number of studies have revealed that almost all the TCA-cycle enzymes are present throughout the period of citric acid accumulation[26,28,29]. Many enzymes have been studied in detail to account for their contribution towards citric acid accumulation by this fungus.

2.1 Glycolytic enzymes

Glycolysis supplies the carbon fragments for the synthesis of citric acid. Studies on glycolytic enzymes[26]

Table 1—Conditions for Citric Acid Fermentation and for Normal Growth of *A. niger**

Medium components	Normal growth[a]	Fermentation[b]
Carbon source	Sugars, e.g. glucose (1-5%)	Molasses (sucrose) (about 15%)
Nitrogen source	NH_4Cl, KNO_3, etc. (5g/litre)	NH_4NO_3 (2g/litre)
Phosphate and buffer capacity	$Na_2HPO_4.2H_2O$ (6g/litre) and KH_2PO_4(3g/litre)	KH_2PO_4 (1g/litre)
*p*H	5.0-6.0	2.0-2.5
Micronutrients, mg/litre	$MgSO_4.7H_2O$(500) $FeCl_3.6H_2O$(20) $ZnSO_4.7H_2O$(10) $Na_2MoO_4.2H_2O$(1.5) $CuSO_4.5H_2O$(1.0)	$MgSO_4.7H_2O$(250) $Fe(NH_4)(SO_4)_2.12H_2O$ (0.1)
Manganese	$MnSO_4.H_2O$ (3mg/litre)	Deficient (≤2 ppb)

*The data in this table were compiled from different references given in this review.

a—On normal growth medium *A. niger* sporulates within 3-4 days.

b—No sporulation occurs even after 10 days.

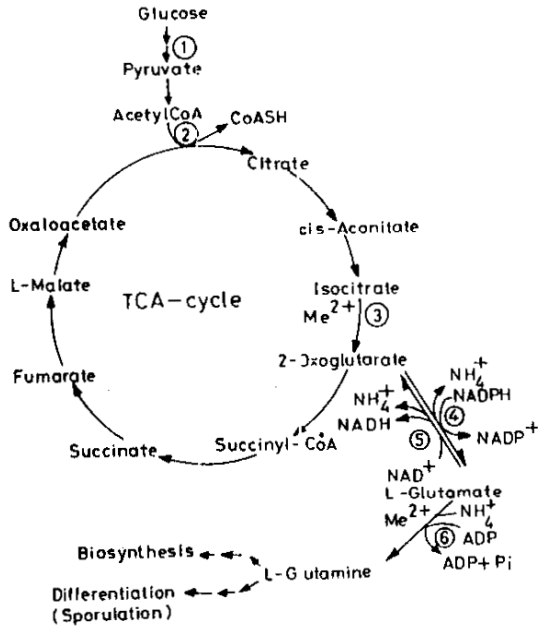

Fig. 1—Interlock of carbon (energy) metabolism with nitrogen metabolism. The important enzymes are: (1) Phosphofructokinase (2) Citrate synthase, (3) Isocitrate dehydrogenase, (4) NADP-Glutamate dehydrogenase, (5) NAD-Glutamate dehydrogenase, (6) Glutamine synthetase. Me^{2+}-step where involvement of Mn^{2+} has been implicated

have shown that they are unaffected during acidogenesis, and, in fact, an unimpaired glycolysis is thought to play an important role. Also, citrate synthase (EC 4.1.3.7), a key enzyme for citric acid formation, closely resembles glycolytic enzymes in its activity pattern. Phosphofructokinase (EC 2.7.1.11), an important regulatory enzyme of glycolysis, was shown to retain its regulatory properties in citric acid producing *A. niger*[30]. Elevated amino acid pools were reported under similar conditions in this fungus. Recent studies on the regulation of partially purified phosphofructokinase from *A. niger* indicated that some of its properties were similar to those of the yeast enzyme and some others resembled the mammalian enzyme[31]. Simulation of *in vivo* conditions supports the hypothesis that the apparent insensitivity of the enzyme to citrate during the accumulation of citric acid in this fungus is due to elevated NH_4^+ pools, which counteract the citrate inhibition of phosphofructokinase.

2.2 Tricarboxylic acid cycle and the enzymes

A mechanism to explain citric acid production on a biochemical rationale was first proposed by Ramakrishnan *et al.*[32]. Citric acid accumulation by *A. niger* was attributed by these workers to the loss of aconitase (EC 4.2.1.3) and isocitrate dehydrogenase (EC 1.1.1.42) activity. The presence of these enzymes, even during fermentation, was clearly demonstrated later by La Nauze[28]. Several workers have also looked at the regulation of isocitrate dehydrogenase with respect to its role in citric acid fermentation[33,34]. Based on these studies, it was concluded that acidogenesis in *A. niger* could be attributed to the inhibition of isocitrate dehydrogenase by citric acid. Further studies on this enzyme, and the role of

manganese ions in the production of citric acid, led Bowes and Mattey[35,36] to implicate mitochondrial NADP-specific isocitrate dehydrogenase (EC 1.2.4.2) (Fig.1) as one of the key enzymes involved in this fermentation. Other workers, however, reported that 2-oxoglutarate dehydrogenase (Fig.1) is the key enzyme involved in citric acid accumulation by *A. niger*[37], and it is believed that the role of isocitrate dehydrogenase may be important only during the later stages of citric acid production.

A detailed analysis of the role of TCA-cycle in citric acid accumulation by *A. niger* showed that citrate synthase parallels sugar uptake rate, whereas the other enzymes of TCA-cycle were repressed at the beginning of the idiophase[26,37,38]. However, it was also reported that citrate synthase played no regulatory role in citric acid accumulation by *A. niger*[39]. Similar studies on molecular and regulatory properties of two isozymes of NADH-malate dehydrogenase[40] (EC 1.1.1.37) rule out the possibility of their involvement. A correlation between pentose-phosphate pathway, leading to decreased biosynthetic potential and citric acid fermentation, was made by Kubicek and Rohr[26]. The decreased activity of hexose monophosphate shunt pointed to a decreased biosynthetic potential. Formation of gluconic acid at the cost of citric acid at low *p*H values, under special conditions of fermentation[41], was also a pointer in this direction.

Involvement of glyoxylate cycle in the accumulation of citric acid appears to be unclear. The presence of glyoxylate cycle enzymes in this fungus was reported[29] and elevated levels of pyruvate carboxylase (EC 6.4.1.1), isocitrate lyase (EC 4.1.3.1) and oxaloacetic hydrolase (EC 3.7.1.1) during oxalic acid and citric acid production were reported[42]. On the contrary, isocitrate lyase was reported to be absent[26] during fermentation, thus ruling out the possible involvement of glyoxylate cycle. Both pyruvate carboxylase and phosphoenolpyruvate carboxykinase were reported in *A. niger*[43], supporting the role for the reactions fixing CO_2 in replenishing the supply of organic acids, e.g. oxaloacetate.

Intracellular pools of citric acid cycle intermediates have been monitored during various stages of fermentation by *A. niger*[37]. Citrate, malate and 2-oxoglutarate levels were elevated during acidogenesis. Also, under the conditions of manganese deficiency, significant increases in the concentrations of pyruvate and oxaloacetate were observed. Based on these studies, Kubicek and Rohr[37] proposed that "the lack of 2-oxoglutarate dehydrogenase activity and the concomitant increase in the concentrations of TCA-cycle intermediates to extremely high values" were responsible for citric acid production by *A niger*.

2.3 Other studies

The energy metabolism in *A. niger* was the subject of a study carried out with the hope of getting some insight into the mechanisms of acidogenesis. The mycelia were more sensitive to oxygen deficiency at the beginning of the idiophase. Studies on the effect of dissolved oxygen tension on citrate accumulation showed the absence of adenine nucleotide involvement in this phenomenon[44]. Using specific inhibitors of oxidative respiratory systems, an alternative pathway of respiration was detected in *A. niger*[45]. Citric acid synthesis was highly sensitive towards salicylhydroxamic acid during active growth and was more sensitive to antimycin A during idiophase, emphasizing the importance of redox potential in submerged citric acid fermentation[46]. The metabolic activity of the fungus appeared to play a significant role during fermentation. The 'catabolic reduction charge' [NADH/(NAD$^+$ + NADH)] was higher during citric acid accumulation. The 'anabolic reduction charge' [NADPH/(NADP$^+$ + NADPH)], on the other hand, was unaffected[47]. It is very likely that citric acid formation occurs with glycolysis as the major energy (ATP) producer. Consequently, the reduced pyridine nucleotides formed are oxidized by the processes which are uncoupled from mitochondrial functions. This explains the pathways for alternative respiration during citric acid fermentation.

In summary, the important enzymes of carbon metabolism that have been implicated in the regulation of acidogenesis are phosphofructokinase, isocitrate dehydrogenase and 2-oxoglutarate dehydrogenase. Regulatory aspects of citric acid production have been recently reviewed by Rohr and Kubicek[4].

3 Role of Manganese in Acidogenesis

The concentrations of metal ions have a profound influence on citric acid production[3]. However, the exact role that the metal ions play has not been clear. Among a number of trace metals tested, manganese appears to have the most inhibitory influence on citric acid accumulation[24,25,48]. Although Zn^{2+} ions have been implicated by Wold and Suzuki[49] in acidogenesis by *A. niger*, the role of manganese appears to be overwhelming[26]. For these reasons, the medium is made essentially free of manganese by treating molasses with ferrocyanide, which reduces the physiologically active manganese content below 2 ppb[25]. Many other heavy metals have been employed to antagonize the inhibitory effect of Mn^{2+}; one such metal ion is copper[50].

The role of manganese appears to be indirect. Manganese deficiency was shown to result in severely reduced lipid levels in acid producing mycelia[51]. In addition, definite changes in cell wall composition and

cell morphology were reported[52], indicating possible alterations in membrane integrity and hence the transport of citric acid to the outside.

To-date, a direct role of manganese in enzyme activity has been suggested only for $NADP^+$-dependent isocitrate dehydrogenase[35]. An interplay of citrate inhibition, and its dependence on the nature of metal ion, e.g. Mg^{2+} or Mn^{2+}, has led to the suggestion of isocitrate dehydrogenase as one of the control points (Fig.1) for citric acid accumulation. Manganese deficient growth has also been correlated with the decrease in the concentrations of several enzymes connected with anabolism at the end of the growth phase[26].

4 Nitrogen Metabolism in Relation to Citric Acid Fermentation

It is generally accepted that citric acid fermentation is a nitrogen limited condition for the growth of the fungus[3]. Although the carbon source is provided in large excess, the fermentation medium usually contains suboptimal concentrations of the nitrogen source. Possible involvement of manganese deficiency in amino acid and protein metabolism has been suggested by Kubicek *et al.*[53] based on the findings that manganese deficiency resulted in high intracellular NH_4^+ pools[30] in *A. niger*. Manganese deficiency and hence fermentation growth led to the accumulation of amino acids; there is also a change in the composition of this amino acid pool. These studies indicated multiple role for Mn^{2+} ions in amino acid metabolism. Experiments using cycloheximide suggested that this amino acid accumulation was due to impaired protein synthesis. There was an excessive accumulation of amino acids derived from glutamic acid[53].

Very few studies have been carried out to assess the role of enzymes of nitrogen metabolism in acidogenesis. In fact, the only report is that of Kubicek and Rohr[26] which deals with levels of NADH-glutamate dehydrogenase (EC 1.4.1.2), NADPH-glutamate dehydrogenase (EC 1.4.1.3), glutamate-oxaloacetate transaminase and glutamate-pyruvate transaminase. Levels of all these enzymes paralleled nitrogen uptake by the fungus and were independent of manganese deficiency or fermentation status of the mycelia.

Occurrence of excessive amounts of glutamic acid and metabolites derived from it[53] clearly showed that synthesis of this amino acid and hence of glutamate dehydrogenase (Fig.1) was probably not blocked during fermentation. A build up of glutamate even when enough NH_4^+ was available[30] strongly suggests a block at glutamine synthetase (EC 6.3.1.2) step, indicating a possible link between carbon and nitrogen metabolism.

The decreased levels of glutamine synthetase activity under fermentation conditions (Punekar *et al.*, communicated to *J. Bact.*) also support the generalization of Kubicek and Rohr[26] that the biosynthetic potential of the fungus was decreased during citric acid fermentation. Glutamine synthetase from *A. niger* appears to be a manganese (II) enzyme, and its activity is markedly inhibited under fermentation conditions. A block in the activity of glutamine synthetase may result in poor utilization of the nitrogen source and delinking of nitrogen metabolism from carbon supply. This block results in the back up of TCA-cycle leading to the accumulation of citric acid, which is conveniently excreted into the medium by the fungus. Thus, citric acid fermentation may be a case of altered regulation at glutamine synthetase by free manganese (II).

5 Interrelationship between Carbon and Nitrogen Metabolisms

Different metabolic pathways may be linked to each other by subtle but nevertheless far reaching regulatory interactions[54]. It is possible that a regulatory superstructure of sophisticated interactions exerted *between* metabolic pathways acts to reinforce and modify the basic allosteric controls operating *within* specific pathways. Glutamine synthetase via glutamate dehydrogenase links TCA-cycle (carbon metabolism) and nitrogen metabolism (Fig.1). A derangement in nitrogen metabolism, especially a key enzyme such as glutamine synthetase, could reflect itself in an altered carbon metabolism. The integrated regulation of glutamine synthetase and acidogenesis in *A. niger* by manganese (II) may represent an example of such an interlock. The balance between functionally distinct metabolic processes depends upon the common intermediates involved. Production of 2-oxoglutarate by glutamate oxidation, or by isocitrate oxidation, is important in determining the relative contributions of the TCA-cycle to energy metabolism and nitrogen metabolism. Analogously, conversion of *n*-paraffins, oils and fats to citric acid in high yields by yeasts like *Candida lipolytica*[11,15–17] may represent an interlock between lipid metabolism and TCA-cycle.

A careful examination of the literature reveals that an alteration in this metabolic interlock may be one of the factors governing citric acid production. The alteration could be occurring by the inhibition of glutamine synthetase activity. This hypothesis is based on the following observations reported in the literature: (i) Maximal production of citric acid occurring under conditions of low *p*H and buffering capacity[3]; (ii) manganese deficiency being employed

for optimal acidogenesis[24-26]; (iii) accumulation of glutamic acid and products derived from it[53]; and (iv) elevated NH_4^+ pools[30] and TCA-cycle intermediates[37], e.g. citric acid and 2-oxoglutarate that can chelate divalent metal ions, conditions which either repress, strongly inhibit or inactivate glutamine synthetase *in vivo*. It is, therefore, hypothesized that glutamine synthetase is one of the major factors governing acidogenesis in *A. niger*.

The above hypothesis is supported by several recent experimental observations[55].

When the fungus was grown on an easily assimilable nitrogen source, such as NH_4^+ or glutamine, glutamine synthetase levels were very low, whereas on poor nitrogen sources like NO_3^- and glutamate, a 3-5-fold increase in the enzyme levels was obtained. These results suggested that the enzyme levels were regulated by the availability of nitrogen sources. The enzyme appeared to be repressed by NH_4^+, as evidenced by the low levels of glutamine synthetase activity when the fungus was grown on NH_4^+, and the enzyme content was dependent on the concentration of NH_4^+. At higher concentrations of NH_4^+ (100 mM), decreased levels of glutamine synthetase were observed. Glutamine synthetase obtained from cells grown on different nitrogen sources, e.g. NH_4^+, NO_3^-, glutamate and glutamine, was identical in its electrophoretic mobility, gel filtration profile on Biogel A5M, ratio of Mg^{2+}-dependent synthetase: Mn^{2+}-dependent transferase activity, suggesting that the enzyme in this organism was probably not regulated (Punekar *et al.*, communicated to *J. Biosci*) by covalent modification, as in the case of *Escherichia coli* enzyme[56], rapid *in vivo* inactivation and proteolysis[57,58] or by dissociation into oligomeric states[59,60], as observed in the case of bacteria and yeasts.

The *A. niger* enzyme purified to homogeneity catalyzes the Mn^{2+}- and Mg^{2+}-dependent synthetase and a Mn^{2+}-supported γ-glutamyl transferase reaction. Mg.ATP complex was the preferred substrate ($K_m = 1.5$ mM) at *p*H 7.8 and exhibited typical Michaelis(δ)Menten kinetics with no Mg^{2+} inhibition. Free ATP was, however, inhibitory to the enzyme ($K_{ATP} = 2.4$ mM). At varying concentrations of Mn^{2+} in the presence of different fixed levels of ATP, bell-shaped velocity curves (Mn^{2+} profiles) were obtained for Mn^{2+}-dependent synthetase reaction. This suggested that unlike Mg^{2+}, excess Mn^{2+} inhibited the enzyme activity. Using isovelocity method, kinetic constants for Mn^{2+} (0.6 mM), ATP (1.1 mM) and Mn-ATP (0.9 mM) were obtained from both Mn^{2+}- and ATP-profiles. Graphical analysis also provided the dissociation constant (K_d) value of 74 μM for the dissociation of the Mn-ATP complex. When Mn^{2+}-dependent synthetase activity was monitored at different equimolar Mn^{2+} and ATP concentrations, a sigmoid curve was obtained, showing that free ATP, Mn^{2+} and Mn.ATP complex were interacting with the enzyme. The results discussed above showed that *A. niger* glutamine synthetase was activated by either Mg^{2+} or Mn^{2+}; however, their mechanism of activation was quite distinct. Supporting evidence for the above-mentioned mechanisms was obtained by differential protection offered by these ligands against inactivation by N-ethylmaleimide and phenylglyoxal. The binding constant for Mg.ATP obtained by protection experiments (0.9 mM) agreed reasonably well with the kinetic constant (1.5 mM) for the ligand; the dissociation constant obtained for Mn^{2+} from protection experiments (14 and 52 μM) was at least an order of magnitude less than the value obtained from kinetic analysis (600 μM). This result suggested the presence of a high affinity site for Mn^{2+} on the enzyme and hence the possible *in vivo* occurrence of *A. niger* glutamine synthetase as an Mn(II) enzyme.

The specificity of the enzyme towards divalent cations, potent inhibition of Ca^{2+} and Zn^{2+} of the synthetase activity, the dependence of the reaction rate on the ratio of ATP to metal activator and the protection of the enzyme by free Mn^{2+} against inactivation suggested that these metal ions interact at a catalytic as well as at a regulatory site (Punekar *et al.*, unpublished results). Glutamine synthetase activity was probably not modulated by many of the end products of glutamine metabolism, such as CTP, GMP, tryptophan, anthranilic acid, NAD^+, glucosamine, etc. Metabolites which showed significant inhibition of *A. niger* glutamine synthetase include histidine, carbamyl phosphate, AMP and ADP. The inhibitions were more pronounced when Mn^{2+} was the metal ion activator and greater inhibition was obtained at lower *p*H values.

Citric acid fermentation was favoured by low *p*H, low buffering capacity, high concentration of the carbon source and manganese deficiency (<2 ppb, Table 1). Intracellular *p*H of *A. niger* cells was measured to determine whether Mn^{2+}- or Mg^{2+}-dependent activity was physiologically important. The bromophenol blue dye distribution method as well as the use of fluorescence probe showed that intracellular *p*H was acidic (6.0-6.5). *A. niger* glutamine synthetase was inactivated under these conditions, and Mn^{2+} ions protected it against this inactivation to some extent. The Mn^{2+}-supported synthetase activity was higher at acidic *p*H value of 6.0 compared to the Mg^{2+}-dependent synthetase activity. The amount of Mg^{2+} required to activate glutamine synthetase at *p*H 6.0 was very high and nonphysiological (>50 mM). Also, small amounts of Mn^{2+} inhibited the Mg^{2+}-dependent synthetase activity at *p*H 7.8. Both Mn^{2+}-

and Mg^{2+}-supported reactions were inhibited by citrate, 2-oxoglutarate, ATP as well as by ethylenediaminetetraacetate. These results suggested that inhibition by citrate, etc. was due to their ability to chelate metal ions rather than to any direct interaction with the enzyme.

The results discussed above showed that during citric acid fermentation, glutamine synthetase is almost completely inhibited. A block at glutamine synthetase leads to decreased biosynthetic potential and back up of tricarboxylic acid cycle, resulting in the excretion of large amounts of citric acid by the fungus.

Nowadays, citric acid is produced almost exclusively by strains of the mould *A. niger*[61]. Although for sometime fermentation of *n*-alkanes by yeasts (e.g. *C. lipolytica*) looked promising, it failed for a number of reasons, the increasing price of the raw material probably being the most important one.

Summary

Citric acid fermentation by *A. niger* is an excellent example of industrial exploitation of fungal overflow of metabolism. A number of conditions have been standardized for getting optimal yields of citric acid. Towards this end, extensive investigations were carried out on glycolysis, TCA-cycle and nitrogen metabolism of the fungus. Manganese appears to play a pivotal role in acidogenesis. A complex interplay of regulatory interactions between carbon and nitrogen metabolism due to the inhibition of glutamine synthetase appears to be responsible for the excretion of citric acid by this fungus.

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