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A Review

Aspergillus niger acidogenic metabolism: A biased view from the C and N interface

NS Punekar*

Department of Biosciences and Bioengineering, Indian Institute of Technology Dharwad, Dharwad-580 007, Karnataka, India

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Aspergillus niger is the major citric acid producer and this has matured into an industrial process through fermentation. Despite a century of research to elucidate the underlying mechanisms of acidogenesis, clear and definitive answers have eluded us. The voluminous literature ranges from fungal physiology to genomics. Much of it focuses on carbon stoichiometry between glucose and citric acid and has overlooked the extant close interactions between the fungal carbon and nitrogen metabolism. Because the regulation at the carbon-nitrogen interface is important, several approaches were taken to discern the role of *A. niger* nitrogen metabolism in acidogenesis. An enhanced GABA shunt operates while the carbon flux exits TCA cycle at citrate. The NADP-glutamate dehydrogenase bridges TCA cycle and GABA shunt. The allosteric response of this enzyme to 2-oxoglutarate could control the carbon flux at the interface of carbon-nitrogen metabolism. While Mn[II] limitation favours citric acid production, arginase (metalloenzyme with bound Mn[II]) does not directly affect the fungal citrate overflow. The acidogenic growth appears to mimic nitrogen starvation and is associated with an extended autophagic state of *A. niger*. The *Δatg* strains are compromised in citrate overflow thereby directly implicating autophagy in the process. Autophagy-associated movement of relevant enzymes during fermentation suggests that the entire citrate synthesis machinery could be operating insidethe fungal vacuole.

Keywords: Acidogenesis, *Aspergillus niger*, Autophagy, Mycelial morphology, Nitrogen metabolism, Nitrogen starvation

Introduction

Very few microbes are endowed with unique predisposition towards acidogenic metabolism and citric acid secretion, in particular[1]. Over the last century *Aspergillus niger* has contributed immensely to the field of process biotechnology[2,3]. Of these, citric acid production by *Aspergillus niger* is an excellent example of industrial exploitation of overflow of fungal carbon metabolism. While the citric acid fermentation as a process has been well perfected, we are yet to understand the mechanism(s) responsible[4,5]. Wehmer first discovered (in 1893) that molds belonging to the group *Penicillium* produced citric acid from sugar. Subsequently, the American food chemist James Currie showed that certain strains of *Aspergillus niger* were efficient citric acid producers[6]and within a couple of years Pfizer began its industrial-level production of citrate. Over a thousand publications have addressed aspects of fungal morphology, physiology, metabolism, biochemistry, enzymes, genes, *etc*., over the years. Yet, acidogenesis by *A. niger* as a paradigm, continues to invite the attention of researchers both from industry and academia. This review summarizes key aspects of this literature with a focus on the interface of carbon and nitrogen metabolism of this fungus. Considering the voluminous literature (numerous book chapters and a few books!) on citric acid fermentation a selection of reviews and pointed references are included for the sake of brevity.

Fermentation conditions and parameters

Aspergillus niger is an aerobic, saprophytic, filamentous fungus with coenocytic cellular architecture. It grows best on semi-solid or solid substratum of the decaying organic matter in nature. However, for the purposes of fermentation, this fungus can be grown as a koji (solid substrate) culture or on liquid medium either as surface culture or as submerged culture. All three modes of growth have found place in the fermentation industry, but bulk of citric acid is currently produced through the submerged process. Much of the morphological, physiological and biochemical studies

*Correspondence:
E-mail: nsp@iitb.ac.in, nsp@iitdh.ac.in

List of Abbreviations: AOX, alternative oxidase; DOT, Dissolved oxygen tension; EGFP, Enhanced green fluorescence protein; GABA, 4-Aminobutyrate; GDH, Glutamate dehydrogenase; SHAM, Salicylhydroxamic acid; TCA, Tricarboxylic acid

on this fungus have been made with the submerged fermentation. Further, the media conditions are significantly tweaked to achieve better citric acid production rather than produce biomass. The medium composition for good growth (minimal medium containing: 20.0 g/L glucose, 3.0 g/L KH_2PO_4, 6.0 g/L Na_2HPO_4, 0.5 g/L $MgSO_4.7H_2O$, 2.25 g/L NH_4NO_3, 10 mg/L $ZnSO_4.7H_2O$, 3.0 mg/L $MnSO_4.7H_2O$, 1.5 mg/L $Na_2MoO_4.H_2O$, 20.0 mg/L $FeCl_3.6H_2O$ and 1.0 mg/L $CuSO_4.H_2O$. The pH is adjusted with 0.1 N HCl to 5.5–6.0) is significantly different from the medium prescribed for optimal acid production (acidogenic medium containing: 140.0 g/L sucrose, 1.0 g/L KH_2PO_4, 0.1 g/L $Fe(NH_4)_2(SO_4)_2$, 2.25 g/L NH_4NO_3 and 0.25 g/L $MgSO_4.7H_2O$. The pH is adjusted between 2.0-2.5)[7,8]. The salient features of the fermentation medium conducive to good citric acid yields are listed in (Table 1). The different components/conditions are known to synergistically influence the yield of citric acid.

The conversion of glucose to citrate is a highly aerobic process where partial oxidation of glucose takes place. The fermentative growth of the fungus takes place in two distinct phases namely, the initial growth phase (trophophase) followed by the long citric acid production phase (idiophase)[9-13]. The conversion of sugar to citric acid by this fungus is very efficient (0.95 g per g sugar against the expected theoretical yield of 1.067 g per g sugar) in a successful fermentation. After the initial investment of the carbon source for biomass and energy (during trophophase), the remaining sugar is stoichiometrically converted to citrate (in the idiophase, the production phase). The sugar taken up by the fungus goes through glycolysis to produce two pyruvate molecules. While one of them is decarboxylated to acetyl CoA, the other one is carboxylated to form oxaloacetate. In the final step, the two are condensed by citrate synthase, the first Krebs cycle enzyme,to produce citrate (Fig. 1). The result is a deceptively simple carbon stoichiometry where glucose a C-6 sugar is partially oxidized to citrate a C-6 metabolite.

Table 1 — Conditions for a successful citric acid fermentation

Media component	Requirement
Carbon source	Preferably sucrose, at 15-20% (*w/v*)
Aeration	Uninterrupted vigorous aeration (high DOT)
Trace elements	Deficiency of Mn[II] most prominent
pH	Acidic pH between 1.0-3.0, with low buffering
Nitrogen availability	Suboptimal, acid ammonium salts preferred
Phosphate availability	Limiting amounts required

Chemical conversion:

$$C_6H_{12}O_6 + 3/2\ O_2 \rightarrow C_6H_8O_7 + 2\ H_2O$$

Biochemical conversion:

$$C_6H_{12}O_6 + 3\ NAD^+ + H_2O \rightarrow C_6H_8O_7 + 3\ NADH + 3\ H^+$$

For this very reason, most of the research literature is dedicated to *A. niger* carbon metabolism, to understand the mechanism(s) of acidogenesis. The relevance of fungal nitrogen metabolism in citric acid fermentation is largely overlooked. This review attempts to bring that aspect into focus.

Morphology, physiology and biochemistry

The fermentative growth of *A. niger* is an imbalanced state of growth with significant alterations in the fungal physiology. The unusual medium composition dictates this situation, and the key factors include a very high carbon to nitrogen ratio of the medium, intentional poor buffering, and trace metal deficiency. Several attempts to discern relevant changes have been made over the decades. The earliest insight was the need for Mn[II] deficiency to achieve good citric acid yields[14,15]. However, the cellular targets of this deleterious Mn[II] effect on the citrate yields are yet to be identified. Several significant alterations in the fungal mycelial morphology and metabolism were recorded during fermentation (Table 2). Beginning with the uptake/transport of the carbon source, both glycolysis and Krebs cycle have received much attention. Sucrose (*viz*., from molasses) is a better carbon source than glucose for this fermentation. Almost all the enzymes of glycolysis have been characterized for their role in fungal acidogenesis. The regulatory

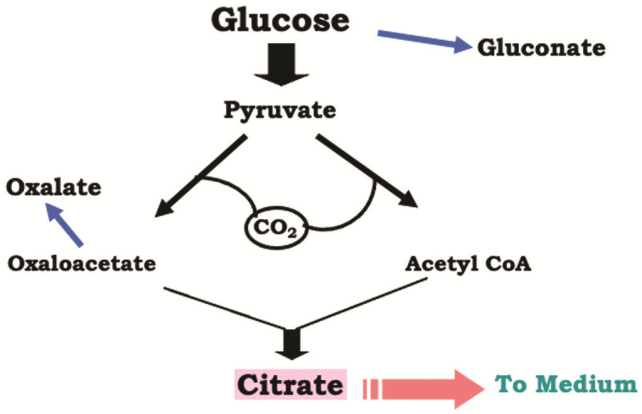

Fig. 1 — Schematic of the stoichiometric formation of citrate from glucose. The two other acids, gluconate and oxalate, that *A. niger* is capable of producing are also shown

Table 2 — Changes observed in the acidogenic *A. niger* mycelia

- Robust glycolytic flux at the expense of pentose phosphate pathway
- Impaired mitochondrial respiratory Complex I (CN^- sensitive)
- Operational alternate oxidase (SHAM sensitive)
- Accumulation of NH_4^+ and glutamate family amino acids
- Fate of NH_4^+ – conversion to glucosamine and reutilization?
- Elevated proteinase activity and protein degradation
- Increased monosome portion of ribosomes
- Elevated chitin and decreased β-1,3 glucan in cell wall
- Reduced rate of DNA synthesis
- Altered fatty acids composition - plasma membrane phospholipids
- Decreased cellular phospholipid content
- Crippled, branched mycelia, pellet morphology
- Giant vacuolated cells, no sporulation

*The information is summarized from the literature reviewed in Ref. 9-13

features of hexokinase and phosphofructokinase were analysed in detail. It is generally observed that an increased glycolytic flux occurs at the expense of pentose phosphate pathway. Among the Krebs cycle enzymes citrate synthase should be and is active throughout the course of fermentation[16]. The normal operation of the TCA cycle is abrogated as 2-oxoglutarate dehydrogenase becomes inactive[17]. The reducing equivalents generated are typically processed through mitochondrial electron transport (oxidative phosphorylation) in an aerobic organism. Citric acid fermentation by *A. niger* is a highly aerobic process and requires uninterrupted oxygen supply (high dissolved oxygen tension, DOT) throughout. However, the cyanide (CN^-) sensitive, mitochondrial respiratory Complex I is impaired during acidogenesis, and the alternate oxidase (AOX; the salicylhydroxamic acid (SHAM) sensitive electron transport) is operative[18-20]. This electron exhaust mechanism sustains the continued oxidation of glucose to citrate by recycling NADH and reducing oxygen to water.

The provision of acid ammonium salts (at suboptimal levels) results in certain physiological effects. The medium pH becomes more acidic as the mycelia take up ammonium. Intracellular NH_4^+ levels rise, and certain amino acids (particularly of the glutamate family) accumulate and are also found in the medium.

Several changes have been recorded at the cellular and morphological level. In the fermentation medium *A. niger* displays pellet morphology and the mycelia are crippled, branched with highly vacuolated giant cells. The fungus does not sporulate on the acidogenic medium. At the biochemical level, the mycelia have reduced phospholipid content with an altered fatty acid composition. The cell walls of these mycelia are thick and contain higher chitin content while their β-1,3 glucan levels area decreased. Similarly, these mycelia have reduced DNA synthesis, protein synthesis and increased protein degradation (Table 2).

Mechanisms proposed to explain citrate overflow in *A. niger*

A mechanistic understanding of this deceptively simple carbon to carbon conversion (while others two such examples include ethanol and lactic acid) is intellectually challenging yet with a direct biotechnological impact. Periodically, attempts have been made to understand and explain the mechanism(s) involved in the acidogenic metabolism of *A. niger* (Table 3)[4,5]. The usual suspects include and starting with the uptake/ transport of sugar from the fermentation medium. The transport (uptake and export) of citrate is reciprocally controlled by Mn[II] ions. Regulation of individual enzymes of glycolysis and Krebs cycle was implicated. Increased cytosolic levels of NH_4^+ allosterically activate phosphofructokinase and counter the citrate inhibition. Among the Krebs cycle enzymes, citrate synthase, 2-oxoglutarate dehydrogenase, and isocitrate dehydrogenase were implicated. Besides inactivation, the inhibition of 2-oxoglutarate dehydrogenase by the redox couple ($NADH/NAD^+$) and oxaloacetate was proposed[17]. A feedforward inhibition of isocitrate dehydrogenase by accumulating citrate was also considered a possibility.

Obviously, the cellular compartmental distribution of enzymes was also brought into focus. It was noted that the fungus displays a constitutive pyruvate carboxylase thereby permitting the stoichiometric formation of oxaloacetate and acetyl CoA from

Table 3 — Mechanisms proposed for citrate overflow in *A. niger*

- Low affinity glucose transporter and/or passive transport at high glucose concentrations
- Decreased flux through glucose oxidase and oxaloacetate hydrolase
- Increased glycolytic flux as NH_4^+ activates phosphofructokinase and overcomes citrate inhibition
- Constitutive pyruvate carboxylase and stoichiometric formation of oxaloacetate and acetyl CoA from pyruvate
- Critical, constant supply of oxaloacetate and a poorly regulated citrate synthase
- Inhibition of 2-oxoglutarate dehydrogenase by oxaloacetate and [NADH/NAD^+]
- Feed forward inhibition of isocitrate dehydrogenase by citrate
- Cytosolic malate synthesis facilitating citrate↔malate exchange across mitochondrial membrane
- Uptake and export of citrate reciprocally regulated by Mn[II] ions
- Impaired respiratory complex I leading to catabolic overflow of citrate
- Alternative oxidase permits recycling of NADH without ATP synthesis

*The information is summarized from the literature reviewed in Ref. 9-13

pyruvate. A critical, constant supply of oxaloacetate and a poorly regulated citrate synthase combine to continuously produce citrate. Further, the ability for a total cytosolic malate synthesis provides for citrate↔malate exchange across the mitochondrial membrane. Replacement of the defective respiratory Complex I by a functional alternative oxidase (AOX) in the idiophase facilitates recycling of NADH without the associated ATP synthesis[18-20].

None of the mechanisms proposed above could fully account for the catabolic overflow of citrate during acidogenesis. As is often the case in biological systems, it may be that several of these factors partly contribute to the unique physiological state of *A. niger*. The genomes of a few *A. niger* strains were sequenced in the last 2-3 decades[21-25]. Sequence analysis of all and the functional annotation of majority of the genes identified to be potentially relevant to acidogenesis are done. Few interesting features have emerged. Important genes (*viz*., alternative mitochondrial oxidoreductases and citrate synthase, albeit putative) are represented in more than one copy. These may be targeted to and expressed in different cellular compartments as gleaned through their sequence (putative signal sequences *etc*.) information[23]. Additionally, data from subtractive hybridization and transcriptome analysis are available to distinguish features of acidogenic versus normal growth of this fungus[26,27]. There is a flurry of activity to identify key targets with the availability several genome sequences. More potential targets and possible physiological explanations are forthcoming. One active area of research is the role of citrate transporters in the acidogenic process. However, currently there is no consensus on the mechanism(s) of acidogenesis and subsequent secretion of citrate into the fermentation medium.

Genetic manipulations to engineer better citrate yields

The genetic engineering tools became available for *A. niger* in the later part of the last century. Several attempts were made towards the genome manipulation of this fungus. Genes for many enzymes were either over-expressed (by copy number increase) or disrupted/silenced to improve carbon flux to citrate and/or its export (Table 4). Almost all these manipulations were based on simplistic assumptions on how metabolism functions. Expectedly, this drew the comment 'look before you clone'[28]. Metabolic engineering at the periphery is easy. Disruption of glucose oxidase and oxaloacetate acetyl hydrolase eliminated the flow of carbon towards gluconic acid and oxalic acid, respectively (Fig. 1). Similarly, genes for better nutrient utilization are easy targets. In most cases, the immediate phenotypes corresponding to over-expression of an enzyme or deletion/down-regulation of an enzyme were observed, yet the effects on citrate yields were marginal at best. Among the few genetic manipulations, that positively improved citric production are the deletion of *t6ps* gene (trehalose-6-phosphate synthase; relief of hexokinase inhibition resulting in shorter fermentation time), citrate transporter over-expression (facilitating transport of citrate across mitochondrial and plasma membranes), and silencing the *chsC* gene (chitin synthase; morphology better suited for acid production). Availability of several Aspergillus genome sequences (and from a few of the *A. niger* workhorse strains) set the stage for capturing the near complete metabolism of this fungus into in silico models[29-30]. Such genome scale metabolic modelling (using multi-omics data sets) is identifying additional targets and encouraging further metabolic engineering efforts[1,31-33]. Citrate being an important primary

Table 4 — Genetic manipulations in *A. niger* attempted to improving citric acid yields

Enzyme (gene)	Change anticipated	References
Deletion or silencing		
Glucose oxidase (*gox*)	Elimination of gluconate (byproduct)	34
Oxaloacetate acetyl hydrolase (*oah*)	Elimination of oxalate (byproduct)	34
α-1,4-Glucosidase (*agdA*)	Improve substrate utilization (reduce transglycosylation)	35
Trehalose-6-phosphate synthase (*t6ps*)	Reduce feedback inhibition	36
ATP citrate lyase (*acl*)	Optimising precursor provision	37,38
NADH oxidase (*aox*)	Essential for recycling NADH	18,19
Citrate transporter (*cexA*)	Main citrate transporter at the plasma membrane	39,40
Citrate transporters (*ctpA* to *ctpF*)	Citrate export from mitochondria to cytoplasm	41,42
Citrate-oxoglutarate exchanger (*cocA*)	Mitochondrial citrate-oxoglutarate shuttle protein	43
Chitin synthase (*chsC*)	Regulation of fungal morphology	44
Over-expression		
Glucoamylase (*glaA*)	Better substrate utilization	35
Phosphofructokinase (*pfk*)	Enhanced glycolysis	45
Phosphofructokinase (*pfkA*)	Enhanced glycolysis (citrate inhibition resistant, highly active enzyme)	46
Pyruvate kinase (*pki*)	Enhanced glycolysis	45
Citrate synthase (*citA*)	Increased flux to citrate	47
Fumarate reductase (*frds1*)	Enhancing precursor provision	48
Fumarase (*fumR*)	Enhancing precursor provision	48
NADH oxidase (*aox*)	Enhanced recycling of NADH without ATP generation	19,20
Citrate transporter (*cexA*)	Increased citrate efflux to medium	39,40

metabolite, it is not trivial to engineer metabolism and overcome the control architecture. The compartmental distribution of the pathway from sugar to citrate makes it challenging.

There has been slew of publications on the discovery of citrate transporters from *A. niger*[39-43,49]. These include transporters that function at the mitochondrial membrane as well as the plasma membrane. A citrate-oxoglutarate antiport is also described. LaeA (loss of aflR expression A) is a global transcriptional regulator of secondary metabolism and is reported to control citric acid production through regulation of the citrate exporter-encoding *cexA* gene[50]. An Alg3 (dolichyl-P-Man: Man(5)GlcNAc(2)-PP-dolichylmannosyl transferase) gene-deleted *A. niger* strain and with an increased expression of LeaA was patented for improved citric acid production[51].

This overview of accumulated literature on acidogenesis, including the most recent findings, clearly shows that carbon metabolism has received almost all the attention. The next section brings into focus the role of nitrogen metabolism and the interplay between C and N metabolism of this fungus, particularly as relates to acidogenesis.

Fungal nitrogen metabolism and C and N interface

It is generally believed that citric acid fermentation is a nitrogen limited state of growth of *A. niger*. The skewed ratio of carbon and nitrogen sources set up in the fermentation medium is the first pointer in this direction (Table 1). Acid ammonium compounds, when included as N source, contribute to reduction in the pH as a function of growth. This is due to uptake/ utilization of NH_4^+ ions during the initial increase in biomass (trophophase or the growth phase). Subsequent accumulation of citrate in the medium acidifies the medium and fungal growth is further limited. Due to these unusual growth conditions, it appears that the organism faces apparent nitrogen starvation despite the presence of as similable nitrogen in the medium[4]. The initial rapid uptake (and disappearance!) of NH_4^+ from the fermentation medium was thought to result in the stoichiometric formation of glucosamine[52,53]. However, a recent detailed study suggests otherwise and finds no transient accumulation of glucosamine[54]. *A. niger pyrG* was serendipitously identified as a promising genetic lead for generating citric acid hyper-producing strains. The *pyrG* disruption enhanced the glycolysis flux and significantly improved abundance of citrate and its precursors[55]; but no molecular basis for this effect was suggested.

The acidogenic system is complicated in terms of extant metabolic redundancy, regulation, and possible sub-cellular compartmental effects. Modeling of carbon metabolic flux was attempted with limited success and did not seriously consider the nitrogen metabolism[1,31-33].

Significant connections between carbon and nitrogen metabolism do exist and are highlighted in (Fig. 2). These include the interconversions of pyruvate↔alanine, oxaloacetate↔aspartate, and 2-oxoglutarate↔glutamate. Glutamate, and hence glutamine, provide the major flow of carbon flux to biomass (that includes proteins, nucleic acids, phospholipids, and chitin).

That the distribution of carbon flux at the 2-oxoglutarate node is important during acidogenesis is borne out by certain observations (Tables 2 & 3, and discussion above). Protein synthesis, DNA synthesis andthe chitin content of the cell wall are all affected. Amino acids of the glutamate family accumulate and are put out in the medium during acidogenesis. *A. niger* mycelia during idiophase display glutamate decarboxylase activity[56], which is usually found during conidiospore germination. But there is no sporulation in acidogenic growth and possibly the enzyme remains in the mycelia. 4-Aminobutyrate (GABA) also accumulates as both glutamate and the glutamate decarboxylase coexist. The fungus also shows GABA transaminase and succinic semialdehyde (SSA) dehydrogenaseactivities[57,58]. Along with NADP-glutamate dehydrogenase (NADP-GDH), these three activities combine to constitute the GABA bypass (Fig. 2); that circumvents the metabolic block at the 2-oxoglutarate dehydrogenase step (Table 3). However, the bypass is energetically less efficient than the regular steps of Krebs cycle.

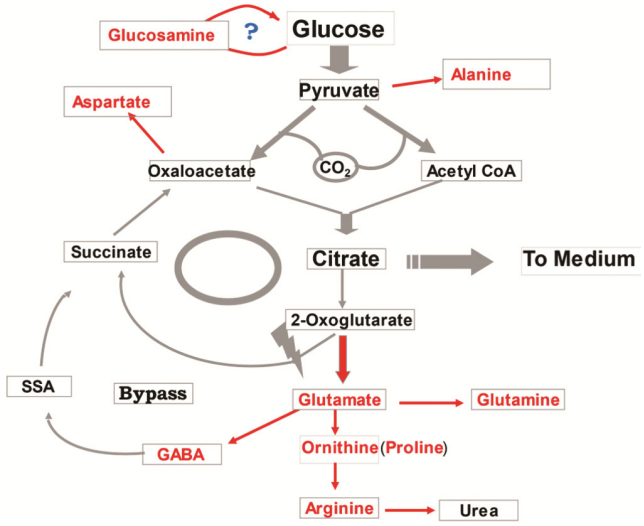

Fig. 2 — The interface between the carbon and nitrogen metabolism of *A. niger*. The carbon skeletons of the glutamate, aspartate and alanine are directly drawn from the central carbon metabolism. Chitin draws from fructose 6-phosphate and many nitrogen containing metabolites are built using the amide nitrogen of glutamine. Glutamate and GABA also form part of GABA shunt

Classical Krebs cycle:

2-Oxoglutarate + NAD^+ + GDP + P_i⇒ Succinate + CO_2 + NADH + H^+ + GTP

GABA bypass:

2-Oxoglutarate + NAD^+ + H_2O⇒ Succinate + CO_2 + NADH + H^+

A radiotracer study (using ^{14}C-glucose and ^{14}C-glutamate) established that the GABA in this fungus exclusively arises from the decarboxylation of glutamate. Also, there is an enhanced carbon flux through GABA bypass while the flux through Krebs cycle goes to zero during idiophase growth of *A. niger*[58]. This is consistent with the report that Krebs cycle is blocked after the 2-oxoglutarate step (Table 3)[17].

In *A. niger*, the NADP-glutamate dehydrogenase (NADP-GDH) is largely responsible for the assimilation of NH_4^+ into glutamate[59]. Blocking this enzyme by an *in vivo* inhibitor mimics the state of nitrogen starvation[8,60]. The enzyme is present throughout the trophophase (Fig. 3)[61] and it responds allosterically to the levels of 2-oxoglutarate and NH_4^+ions[59,62]. This is crucial in deciding how much of 2-oxoglutarate flux is committed to biomass (glutamate synthesis) versus energy metabolism (Krebs cycle).

We noted earlier that presence of trace metal ions, Mn[II] in particular, negatively affect outcome of citric acid fermentation. Several attempts have been made to overcome this drawback *viz*., pre-treating the raw materials used for fermentation. A subtractive hybridization study (growth on media with and without Mn[II]) identified the gene for Brsa-25; whose down-regulation facilitated pelleted growth and enhanced citric acid production in the presence of Mn[II] ions[26]. A few reports exist where for citric acid fermentation, *A. niger* strains not sensitive to Mn[II] were identified/isolated[63]. Nevertheless, the issues of trace metal interference in optimal fermentations remains. A survey of literature suggests that there are very few proteins/enzymes that exclusively require Mn[II] ions for their activity[64]. The two enzymes of nitrogen metabolism, that operate within the glutamate family,are glutamine synthetase (activated by Mn[II] ions) and arginase (which is a Mn[II] metalloenzyme). *A. niger* glutamine synthetase activity is influenced by micromolar levels of Mn[II] ions and its activity is minimal or absent during the idiophase[61,65]. This is consistent with the fact that the overall biosynthetic potential of the fungus is compromised during the idiophase of the fermentation.

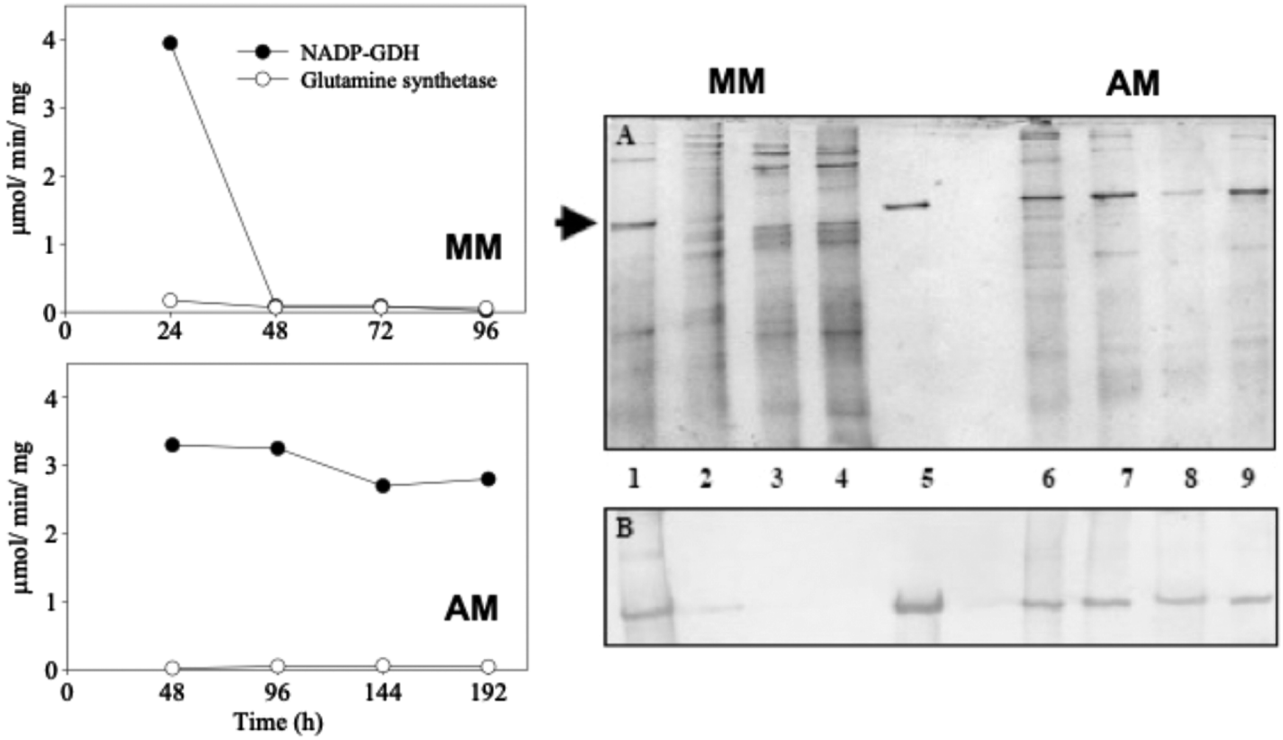

Fig. 3 — Mycelial NADP-glutamate dehydrogenase and glutamine synthetase levels during normal (MM) and acidogenic (AM) growth of *A. niger*. NAD-Glutamate dehydrogenase could not be detected in either case. (A) Native PAGE gel showing NADP-GDH protein bands from MM (Lanes 1 to 4 correspond to mycelia from 24 h, 48 h, 72 h, and 96 h, respectively) and AM (Lanes 6 to 9 correspond to mycelia from 48 h, 96 h, 144 h and 196 h, respectively) growth. Equal protein (5.5 µg) was loaded in each of these lanes. Lane 5 shows the pure NADP-GDH from *A. niger*; and(B) Western blot of the same gel probed using *A. niger* NADP-GDH antibodies [61]

Arginase accounts for a substantial amount mycelial Mn[II] ions bound to the soluble protein pool[64]. The absence of arginase protein (achieved through *aga* gene disruption) or its over-expression (multi-copy expression drive by the constitutive *PcitA*) did not significantly influence acidogenesis by *A. niger*. The cellular targets where Mn[II] effects may manifest continue to remain elusive.

The unique features of the fermentation medium push the fungus towards unusual pellet and mycelial morphology. Similarities between autophagy and acidogenic growth were recognized at several levels[8]. Nitrogen starvation (directly or imposed through the *in vivo* inhibition of NADP-GDH) induces autophagy response in *A. niger*. The cytosolic EGFP (as a reporter) relocates to vacuoles during N-starvation induced autophagy and the same is observed during the transition from trophophase to idiophase transition. The acidogenic growth appears to mimic nitrogen starvation and is associated with an extended autophagic state of *A. niger*. The autophagy compromised strains (both *Δatg1* and *Δatg8* strains) are also compromised in citrate overflow, thereby directly implicating autophagy in the acidogenic process[8]. Autophagy-associated movement of several relevant enzymes to vacuoles during fermentation was monitored. These include (besides α-mannosidase as the vacuolar marker) the glycolytic (hexokinase and glyceraldehyde 3-phosphate dehydrogenase) and the mitochondrial (citrate synthase and succinate dehydrogenase) enzymes as well as NADP-GDH the key enzyme of nitrogen assimilation. Like the relocation of cytosolic EGFP to the vacuoles, all these enzyme activities moved to the idiophase fungal vacuoles (unpublished observations). These observations suggest that the entire citrate synthesis machinery could be operating from inside the fungal vacuole. It is an interesting proposition that, due to an extended autophagy state, acidogenesis may possibly occur in the large vacuoles of oldermycelia that are in suspended animation! It however remains to be tested whether the actual carbon flux from glucose to citrate indeed is observed in these vacuoles.

Conclusion

Over a century of research has attempted to elucidate the underlying biochemical mechanisms of acidogenesis. This effort is spurred by the fact that

knowledge gained from this successful biotechnology could be repurposed and exploited for other processes/products. Much has been learnt but definitive answers toward the mechanisms involved have been elusive. Clearly, a simplistic focus on carbon stoichiometry between glucose and citric acid, that overlooks other important aspects of metabolism, physiology and cell biology, is inadequate. The extant close interaction between the fungal carbon and nitrogen metabolism is one such area. While attempting to circumvent the blocked Krebs cycle, GABA shunt comes into play. This is also linked to *A. niger* morphogenesis, conidiation, and development. The carbon flux split at the 2-oxoglutarate node prioritizes how to support fungal growth in terms of biomass and energy needs. NADP-GDH, key enzyme in the nitrogen metabolism, responds allosterically to 2-oxoglutarate levels. The apparent nitrogen limitation perceived by the fungus triggers a macro-autophagy response with the movement of the cytosolic enzymes to vacuoles. This opens up the interesting possibility that the site of acidogenesis may be the vacuoles of older idiophase mycelia.

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Conflict of interest

The author declares no conflicts of interest.

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