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Oxalic acid production by *Aspergillus niger*: an oxalate-non-producing mutant produces citric acid at pH 5 and in the presence of manganese

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The external pH appeared to be the main factor governing oxalic acid production by *Aspergillus niger*. A glucose-oxidase-negative mutant produced substantial amounts of oxalic acid as long as the pH of the culture was 3 or higher. When pH was decreased below 2, no oxalic acid was formed. The activity of oxaloacetate acetylhydrolase (OAH), the enzyme believed to be responsible for oxalate formation in *A. niger*, correlated with oxalate production. OAH was purified from *A. niger* and characterized. OAH cleaves oxaloacetate to oxalate and acetate, but *A. niger* never accumulated any acetate in the culture broth. Since an *A. niger acuA* mutant, which lacks acetyl-CoA synthase, did produce some acetate, wild-type *A. niger* is apparently able to catabolize acetate sufficiently fast to prevent its production. An *A. niger* mutant, *prtF28*, previously isolated in a screen for strains deficient in extracellular protease expression, was shown here to be oxalate non-producing. The *prtF28* mutant lacked OAH, implying that OAH is the only enzyme involved in oxalate production in *A. niger*. In a traditional citric acid fermentation low pH and absence of Mn^{2+} are prerequisites. Remarkably, a strain lacking both glucose oxidase (*goxC*) and OAH (*prtF*) produced citric acid from sugar substrates in a regular synthetic medium at pH 5 and under these conditions production was completely insensitive to Mn^{2+}.

Keywords: oxalic acid, citric acid, oxaloacetate acetylhydrolase, acetyl-CoA synthase, *A. niger*

INTRODUCTION

Oxalic acid is produced by a variety of fungi, including saprophytic and phytopathogenic species (excellently reviewed by Dutton & Evans, 1996). In phytopathogenic fungi it is believed to play a role in pathogenesis, facilitating plant cell wall degradation. In saprophytic species, such as *Aspergillus niger*, the role of oxalic acid production is less clear, but may also be related to mobilizing substrates from plant cell wall polysaccharides, e.g. pectin (Tanaka & Nonaka, 1981). Both through acidification and via its chelating properties oxalic acid may increase availability of metal ions such as iron and calcium (Dutton & Evans, 1996). On the other hand, Murphy & Levi (1983) have suggested that formation of copper oxalate crystals resulted in a higher copper tolerance for several fungi, including *A. niger*. Alternatively, oxalic acid formation by *A. niger* may be involved in biological competition (Shanta & Rati, 1990). The biosynthesis of oxalic acid seems to fit the general strategy of *A. niger* and other fungi to acidify their environment via an extracellular process involving glucose oxidase, by secreting organic acids which first accumulate intracellularly, or by a combination of these processes. *A. niger* is a very efficient oxalic acid producer, which can be illustrated by the findings of Van de Merbel *et al.* (1994), who reported production of 13 g oxalic acid l^{-1} from 20 g sugar l^{-1} in 45 h. Production of 38 g oxalic acid l^{-1}, which is close to the solubility of sodium oxalate, was reported by Strasser *et al.* (1994) using a fed-batch process at pH 6 with sucrose as the carbon source.

Several pathways have been described for oxalic acid

Abbreviations: ACS, acetyl-CoA synthase (EC 6.2.1.1); OAH, oxaloacetate acetylhydrolase (EC 3.7.1.1).

production. In *A. niger* (Hayashi *et al*., 1956; Müller, 1975; Lenz *et al*., 1976) and in a number of other fungi (Dutton & Evans, 1996), as well as in some *Streptomyces* species (Houck & Inamine, 1987), the current evidence favours production of oxalic acid by a Mn^{2+}-dependent enzyme, oxaloacetate acetylhydrolase (EC 3.7.1.1) (OAH). In *A. niger* this enzyme is localized in the cytoplasm (Kubicek *et al*., 1988), where it catalyses the following reaction:

$$\text{oxaloacetate} + H_2O \rightarrow \text{oxalate} + \text{acetate}$$

Cleland & Johnson (1956) postulated that a second pathway should exist in *A. niger* which generates oxalate from pentoses via glycolate and glyoxylate as intermediates. This route requires operation of glyoxylate dehydrogenase as the final step to oxidize glyoxylate to oxalate, but attempts to measure glyoxylate-oxidizing enzymes in extracts of *A. niger* that produced oxalate were unsuccessful (Müller, 1975).

The physiology of oxalic acid production has been studied to some extent (Kubicek, 1987), but some details are lacking, e.g. a proper investigation of the effect of pH has not been performed. Production of oxalate has been reported to be optimal in the pH range of 5–8 (Cleland & Johnson, 1956; Lenz *et al*., 1976; Kubicek *et al*., 1988). In most cases information about oxalate biosynthesis has only been obtained under conditions also leading to the synthesis of other organic acids, in particular gluconic acid. In this study we followed another approach using an *A. niger* mutant lacking glucose oxidase.

In several processes employing *A. niger*, such as citric acid production or production of enzymes, oxalate may arise as an unwanted by-product for a number of reasons. First, it may decrease yield of the intended product. Another reason is that oxalate complicates recovery of the product because additional steps are required to remove the acid. Since oxalic acid is toxic, its removal is particularly important from products that have applications in food or medicine. In this report we describe a number of features of oxalic acid production by *A. niger* and, in addition, we present data on an *A. niger* mutant that is unable to produce oxalic acid and demonstrate its use in citric acid production.

METHODS

***A. niger* strains, isolation of mutants, linkage analysis and transformation of *A. niger*.** All strains used were descendants from *A. niger* N400 (CBS 120.49). N573 (*cspA1 bioA1*) and NW228 (*cspA1 bioA1 prtF28*) have been described (Van den Hombergh *et al*., 1995). NW136 (*fwnA1 argH12 pyrA6 cspA1 lysA7 hisC3 pheA1 pdxA2 nicB2 trpB2*) (Van den Hombergh *et al*., 1995) was used as a tester strain in genetic analysis. NW253 (*cspA1 bioA1 acuA3*) and NW254 (*cspA1 bioA1 acuA5*) are two acetate non-utilizing mutants derived from N573 (see Results). NW255 (*cspA1 fwnA1 pyrA6 lysA7 acuA5*) is a recombinant obtained from a cross between NW254 and NW136. NW131 (*cspA1 goxC17*) has been described before (Ruijter *et al*., 1997). NW185 (*cspA1 fwnA1 goxC17 prtF28*) was constructed in this study and contains the *goxC17* (glucose-oxidase-negative) and the *prtF28* (oxalate-non-producing) mutations. Acetate-non-utilizing mutants (*acu*) were isolated on medium containing propionate as described by Sealy-Lewis (1994). Conidiospores (10^6) of strain N573 were irradiated with UV (60% survival) and plated on minimal medium (see below) containing 1% (w/v) glucose and 0·5% (w/v) sodium propionate. Initially 100 colonies were picked and tested for growth on acetate. Linkage analysis was performed as described by Bos *et al*. (1988). Co-transformation of *A. niger* was performed essentially as described by Kusters-van Someren *et al*. (1991), using the *pyrA* gene (Goosen *et al*., 1987) as a selective marker. Plasmid pRAS7, which contains the *Aspergillus nidulans facA* gene, encoding acetyl-CoA synthase (ACS) (Sandeman & Hynes, 1989), was obtained from the Fungal Genetics Stock Center.

Media and culture conditions. Conidiospores were propagated on complete medium (Pontecorvo *et al*., 1953) solidified with 1·5% (w/v) agar and containing 50 mM glucose. Spores were harvested from the agar slopes with 0·05% (w/v) Tween 80. Cultures were inoculated with spores to a final concentration of 10^6 ml^{-1}. For isolation and analysis of *acu* mutants mycelium was cultured on minimal medium (MM) (Pontecorvo *et al*., 1953) containing 0·02% (v/v) of a trace metal solution (Vishniac & Santer, 1957) and appropriate carbon sources. For plate tests MM was solidified with 1·5% (w/v) agar. Production of acids was studied in 3 litre jacketed stirred tank reactors (Applikon) using two different media. PM medium contained, per litre: 1·2 g $NaNO_3$, 0·5 g KH_2PO_4, 0·2 g $MgSO_4.7H_2O$, 0·5 g yeast extract, 0·04 ml of a trace metal solution (Vishniac & Santer, 1957) and carbon source as indicated in the legends of tables and figures. Unless indicated otherwise the culture pH was 5. Culture pH was controlled by automatic addition of either 2 M HCl or 5 M NaOH. Cultures were sparged with 0·2 v.v.m. air, while addition of pure oxygen was used to keep the dissolved oxygen tension above 30% air saturation. A medium optimized for citric acid production (CAF) was described earlier (Ruijter *et al*., 1997). In bioreactor cultures 0·5 ml 30% polypropylene glycol in alcohol was added per litre of medium as antifoam. Where necessary media were supplemented with, per litre, 4 μg biotin, 0·2 g arginine, 0·2 g phenylalanine, 0·1 mg pyridoxine, 1 mg nicotinamide, 0·2 g tryptophan, 0·2 g histidine, 0·365 g lysine and 1·22 g uridine. In all experiments *A. niger* was cultured at 30 °C.

Preparation of cell extracts and enzyme assays. Mycelium was collected from a culture sample by filtration under vacuum, washed three times with approximately 50 ml 10 mM potassium phosphate buffer pH 7·0 and frozen in liquid nitrogen. For each sample approximately 0·5 g of the frozen mycelium was powdered using a micro-dismembrator (B. Braun Biotech) and suspended in 1 ml extraction buffer at 0 °C. For ACS, extraction was done in 100 mM potassium phosphate pH 7 containing 1 mM EDTA, whereas OAH was extracted in 50 mM potassium phosphate pH 7 containing 0·5 mM EDTA, 5 mM 2-mercaptoethanol, 5 mM $MgCl_2$, 10% (v/v) glycerol. Following centrifugation at 15000 ***g*** for 5 min enzyme activities were assayed in the resulting supernatant. Enzyme assays were performed at 30 °C using a Shimadzu UV2501 spectrophotometer. Biochemicals were from Boehringer or Sigma. ACS activity was assayed in 75 mM triethanolamine pH 8·4 containing 7·5 mM $MgCl_2$, 20 mM malate, 0·4 mM NAD^+, 2·5 mM ATP, 0·17 mM CoA, 1 U citrate synthase ml^{-1}, 100 U malate dehydrogenase ml^{-1}, 20 mM acetate. OAH activity was measured using direct optical determination of oxaloacetate at 255 nm as described by Lenz *et al*. (1976).

Purification of OAH. OAH was purified from strain NW131 cultured for 28 h on PM medium (see above) containing 2% (w/v) glucose at pH 6. Harvest and disruption of mycelium was done as described under 'Preparation of cell extracts and enzyme assays'. Powdered mycelium (8 g) was suspended in 50 ml extraction buffer containing 50 mM Bistris pH 7·0, 5 mM $MgCl_2$, 0·5 mM EDTA, 5 mM 2-mercaptoethanol and 10% (v/v) glycerol. The resulting suspension was centrifuged at 10000 ***g*** for 10 min at 4 °C. To the supernatant, $(NH_4)_2SO_4$ was added to 40% saturation. Precipitation of protein was allowed to occur for 20 min at 4 °C with gentle mixing. To the supernatant obtained after centrifugation for 10 min at 10000 ***g*** and 4 °C, $(NH_4)_2SO_4$ was added to obtain 50% saturation. Following 20 min incubation at 4 °C and another centrifugation step the precipitated protein, which contained OAH, was dissolved in 3 ml extraction buffer and applied to a Sephacryl S-300 (Pharmacia Biotech) column (90 cm × 5 cm^2) which was pre-equilibrated with extraction buffer. Fractions containing OAH activity were pooled and applied to a 1 ml Resource Q (Pharmacia Biotech) column. Following rinsing of the column with extraction buffer, adsorbed protein was eluted with a 0–0·5 M NaCl gradient in extraction buffer over 20 column volumes. Fractions containing OAH activity were pooled and rechromatographed on Resource Q applying a 0–0·5 M NaCl gradient over 40 column volumes. Fractions having OAH activity were stored at −70 °C.

Analytical methods. Denaturing electrophoresis in 10% (w/v) polyacrylamide gels containing 0·1% (w/v) SDS was performed as described by Laemmli (1970) in a Mini-V system (Life Technologies). Molecular mass markers were phosphorylase *b* (92·5 kDa), BSA (68 kDa), ovalbumin (45 kDa) and carbonic anhydrase (29 kDa). For immunochemical detection, protein was blotted onto nitrocellulose filters and blots were then incubated with specific antisera, followed by staining with alkaline phosphatase labelled goat anti-mouse IgG as described by the manufacturer (Bio-Rad). Antibodies against *A. niger* OAH were prepared as described previously (Van der Veen *et al*., 1991). Sugars and organic acids were analysed by HPLC using an Aminex HPX-87H (Bio-Rad) column eluted with 25 mM HCl at 50 °C and using UV (210 nm) and RI (refractive index) detection. Sugars and citric acid were, in addition, determined enzymically according to Bergmeyer (1985). Fungal dry weight and protein were determined as described earlier (Ruijter *et al*., 1997).

RESULTS

Physiology of oxalic acid production by *A. niger*

To study production of oxalic acid, an *A. niger* N400 derivative lacking glucose oxidase (*gox*C), strain NW131, was used to prevent interference by gluconic acid production. At pH 6 in a minimal medium containing 14 mM $NaNO_3$ as the nitrogen source, oxalic acid was produced on all carbon sources tested, including D-glucose, D-fructose, sucrose, D-gluconate, D-xylose, acetate and glycerol. Molar yield [mol C(oxalate) per mol C(carbon source)] was in all cases 0·5–0·6.

In basidiomycetes, the type and concentration of nitrogen source is known to affect oxalate production (e.g. Micales, 1994; Kuan & Tien, 1993). Using 2% glucose as a carbon source, an increase in the $NaNO_3$ concentration from 6 to 60 mM resulted in an increase of molar yield from 0·5 to 0·54, with a concomitant increase in dry weight from 3·2 to 4·2 g l^{-1}. Using 28 mM of NH_4Cl instead of $NaNO_3$ the molar yield was 0·5 (dry weight 5·5 g l^{-1}). Thus, the type and concentration of the nitrogen source did not affect the molar yield of oxalic acid very much. These data are in accordance with the findings of Müller (1965), who reported production of 4·3–6·3 g oxalate l^{-1} from 50 g glucose l^{-1} in buffered medium when the KNO_3 content was varied between 12 and 100 mM.

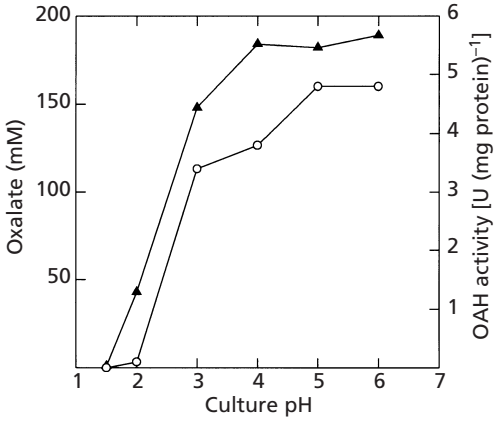

Fig. 1. Oxalate production and OAH activity as a function of culture pH. *A. niger* NW131 was cultured for 71 h on PM medium containing 2% (w/v) glucose at the pH values indicated on the *x*-axis. Since *A. niger* conidiospores did not germinate in PM medium at pH 1·5, the mycelium was pregrown for 15 h at pH 2 and the pH was then adjusted from 2 to 1·5. The final level of oxalate in the cultures is shown (▲), whereas the OAH activity in cell extracts (○) was determined after 24 h of culturing. Data are means of two experiments.

The environmental factor that influenced production of oxalic acid most was pH (Fig. 1). Using *goxC* strain NW131 oxalic acid production was similar between pH 4 and 6 (approx. 190 mM oxalate from 110 mM glucose). Below pH 4 the amount of oxalic acid produced decreased. At pH 2, 40–50 mM was still being produced from 110 mM glucose, but at pH 1·5 oxalic acid production was very low (< 1 mM). Production of oxalic acid correlated qualitatively to the OAH activity measured in cell extracts (Fig. 1). At culture pH 3–6 the OAH activity was 3·5–5 U mg^{-1}, whereas below pH 3 the activity decreased markedly. At culture pH 1·5 OAH activity was not detectable.

Properties of OAH

Our data corroborate published reports that OAH is responsible for oxalic acid formation in *A. niger*. We found that the enzyme was present as long as the culture pH was ⩾3, but some studies have found a somewhat different regulation of OAH biosynthesis by pH. Kubicek *et al*. (1988) reported that OAH is not produced at or below pH 4 in *A. niger* strain B60, and Lenz *et al*. (1976) described that biosynthesis of OAH in *A. niger* strain ATCC 1015 is only induced upon a pH shift from

Table 1. Purification of *A. niger* OAH

OAH was purified as described in Methods. Data from a representative purification are given.

Step	Volume (ml)	Protein (mg)	Total activity (U)	Specific activity (U mg^{-1})	Recovery (%)
Extract	60	210	903	4·3	100
$(NH_4)_2SO_4$ 50% saturation	3·4	32	593	19	66
Sephacryl S-300	58	5·6	215	38	24
Resource Q	5	1·1	64	58	7

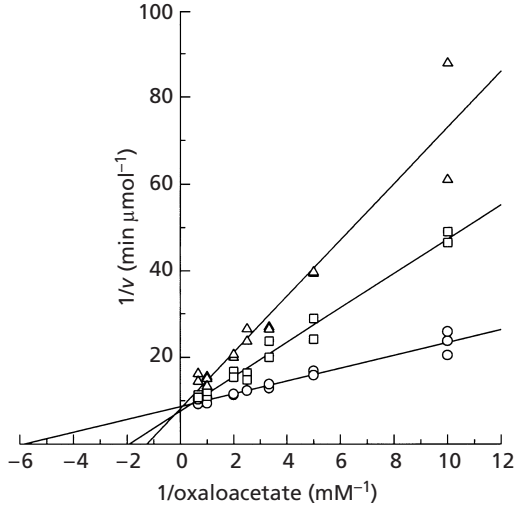

Fig. 2. Inhibition of OAH activity by oxalate. A double-reciprocal plot of OAH activity versus the concentration of oxaloacetate is shown at the following concentrations of oxalate: no oxalate (○), 0·05 mM (□) and 0·1 mM (△). Assay conditions are described in Methods. In all assays 0·1 U OAH was used.

6 to 8 with Na_2CO_3 and not with NaOH. Some enzymic and physico-chemical properties of OAH from *A. niger* ATCC 1015 have been described in the past (Lenz *et al.*, 1976), but to rule out strain differences and to establish the properties of *A. niger* N400 OAH we purified the enzyme. In three steps we obtained a partially purified enzyme (Table 1). SDS-PAGE of the final Resource Q preparation showed three protein bands of molecular mass 29–33 kDa (data not shown). Since the enzyme could not be further purified by a number of different techniques (including phenyl-Sepharose and chromatofocusing) we decided to characterize the partially purified enzyme. Gel filtration chromatography showed a molecular mass of approximately 250 kDa, suggesting that the native enzyme is a multimeric protein. Lenz *et al.* (1976) reported a molecular mass of 420 kDa, which is somewhat larger than the value we determined, but also indicates that the enzyme is a rather large complex. At present, it cannot be ruled out that the complex contains more than one type of subunit. Oxaloacetate was converted to equimolar concentrations of oxalate and acetate by OAH. OAH was dependent on the presence of Mn^{2+} and maximal activity was obtained at 0·2 mM $MnCl_2$. The activity did not vary much between pH 5·5 and 9 in MES, PIPES, Tris and triethanolamine, but increased approximately twofold when the pH was increased from 6·5 to 7·5 in imidazole buffer. Optimal activity was obtained in imidazole buffer at pH 7·6, which was used in all other experiments. A K_m of 0·17 mM was found for oxaloacetate. Oxalate competitively inhibited hydrolysis of oxaloacetate by OAH (Fig. 2) with a K_i of 0·03 mM, whereas acetate (100 mM) did not inhibit hydrolytic activity of OAH. Thus, although regulation of OAH biosynthesis by extracellular pH seems to be different in *A. niger* strains ATCC 1015 and NW131, the enzymic and physicochemical properties of OAH from these strains are similar.

Acid production by an ACS (*acuA*) mutant

A striking observation during oxalate production is that acetate is never detected in the culture broth. If OAH is the only enzyme involved in oxalate production, the acetate formed must be very efficiently reconsumed. To investigate acetate consumption by *A. niger* we isolated acetate-non-utilizing (*acu*) mutants. Following UV mutagenesis we selected colonies that were resistant to propionate on minimal medium plates containing glucose as the carbon source. Four of these propionate-resistant mutants grew less well than the parental strain on acetate medium and belonged to two different complementation groups. The *acuA5* mutation was localized on chromosome VI by linkage analysis. One other mutant was also *acuA*, whereas the two remaining mutants belonged to another complementation group, which was designated *acuB*. The *acuB* mutation was localized on chromosome VIII, but not further analysed. When pregrown on glucose and transferred to acetate medium, the *acuA* mutants lacked ACS activity, whereas the parental strain N573 had an acetate-induced ACS activity of 0·73 U mg^{-1}. When grown on glucose N573 had an ACS activity of 0·36 U mg^{-1}. This shows that ACS is present during growth on sugars and suggests that acetate metabolism is probably not very much repressed by glucose. Co-transformation of the *acuA pyrA* strain NW255 with pRAS7, containing the *A. nidulans facA* gene (encoding ACS), and pGW635,

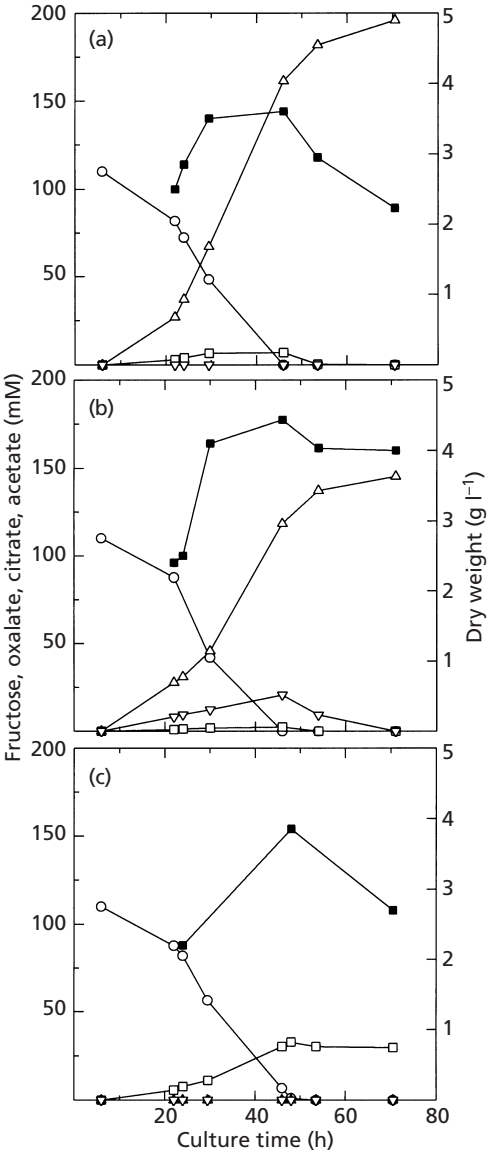

Fig. 3. Acid production by *A. niger* wild-type, an *acuA* mutant and a *prtF* mutant. Strains N573 (wild-type, a), NW254 (*acuA*, b) and NW228 (*prtF*, c) were cultured on PM containing 2% (w/v) fructose at pH 5. Data are means of two experiments. ○, Fructose; ■, dry weight; △, oxalate; □, citrate; ▽, acetate.

containing the *A. niger pyrA* gene, resulted in a number of $pyrA^+$ transformants. Of five transformants isolated, four were able to grow on acetate, showing that *acuA* encodes ACS. These results confirm data of Sealy-Lewis & Fairhurst (1998), who recently described *A. niger acu* mutants as well.

Strain N573 did not produce acetate from fructose, whereas approximately 200 mM oxalate is produced (Fig. 3a). In contrast, strain NW254, carrying *acuA5*, transiently produced acetate (Fig. 3b). During consumption of fructose, acetate slowly accumulated to a maximal concentration of 21 mM in the culture broth, but was reconsumed after exhaustion of the fructose. The rates of acetate consumption can be calculated from the data. Between 30 and 46 h of culturing, the *acuA5* mutant produced 1·06 mmol oxalate (g dry wt)$^{-1}$ h^{-1} and 0·12 mmol acetate (g dry wt)$^{-1}$ h^{-1}. With OAH producing equimolar oxalate and acetate, the difference is the acetate consumption by the mutant: 0·94 mmol (g dry wt)$^{-1}$ h^{-1}. Between 46 and 54 h there is net consumption of acetate [0·33 mmol (g dry wt)$^{-1}$ h^{-1}], but still production of oxalate and concomitantly acetate [0·57 mmol (g dry wt)$^{-1}$ h^{-1}]. The difference [0·9 mmol (g dry wt)$^{-1}$ h^{-1}] is again the acetate consumption rate, which closely matches the rate calculated for the period 30–46 h. Since ACS activity was not detectable, the remaining acetate catabolism probably proceeds via another pathway, which is unknown at present.

Identification of an oxalate-non-producing mutant

We previously described a number of *A. niger* mutants that exhibited reduced extracellular protease activity (Van den Hombergh *et al.*, 1995). Upon closer examination of these mutants it was found that one mutation, *prtF28*, resulted in decreased acidification of the culture broth under certain conditions. Analysis of the culture broth showed that the *prtF28* mutant did not produce any oxalate from fructose (Fig. 3c) under conditions that yield about 200 mM oxalate by the parental strain (Fig. 3a). Instead the mutant produced about 35 mM citric acid from 110 mM fructose. The simplest explanation for the absence of oxalate production was a defect in OAH and indeed this was found. Whereas strain N573 had 4·4 U mg^{-1} OAH activity, the *prtF28* mutant completely lacked OAH activity. Using an antiserum raised against partially purified OAH it was shown by Western blotting analysis that the OAH protein was absent in the *prtF28* mutant, but present in the wild-type (data not shown).

A recombinant strain was constructed that combined the *acuA5* and *prtF28* mutations. This strain did not produce any acetate from fructose, suggesting that the acetate produced by the *acuA5* mutant is associated with oxalate production, i.e. formed by OAH.

Citric acid production by a *prtF* mutant

The finding that the *prtF28* mutant produced a reasonable amount of citric acid from 2% (w/v) fructose at pH 5 prompted us to investigate citric acid production by this strain. For this purpose a recombinant, NW185, was constructed that lacked both glucose oxidase (*goxC17*) and OAH (*prtF28*). In a traditional citric acid production process (i.e. pH < 2, no Mn^{2+}) strain NW185 performed comparably to a strain not carrying the *prtF* mutation (NW131) using glucose as the substrate (Table 2). However, at pH 5 in a minimal medium containing a mixture of trace metals (PM), NW131 produced a large amount of oxalic acid in addition to citric acid, whereas NW185 produced only citric acid (Table 2). The amount of citric acid produced by NW185 in PM at pH 5 is even slightly higher than observed in the traditional citric

Table 2. Major components of the fermentation broth during acid production on two different media by *A. niger* strains NW131 and NW185

NW131 (*goxC17*) and NW185 (*goxC17 prtF28*) were cultured for 4 d either on a traditional citric acid production medium (CAF) or on a minimal medium containing yeast extract and trace metals (PM) (for exact composition of the media, see Methods). In all cases the carbon source was 14% (w/v) glucose. Data are means (±SD) and are given in g l^{-1}. The numbers in parentheses are the number of independent fermentations for which the components were determined.

	CAF NW131	CAF NW185	PM NW131	PM NW185
Glucose	83·5±4·5 (10)	84·6±0·6 (3)	66·4±0·3 (2)	74·6±2·1 (2)
Dry weight	11·2±1·0 (10)	11·2±0·1 (3)	6·1±0·3 (2)	10·9±0·8 (2)
Citric acid	30·7±1·5 (10)	28·4±0·7 (3)	18·4±2·0 (2)	32·6±0·4 (2)
Oxalic acid	0·0	0·0	34·0±0·7 (2)	0·0

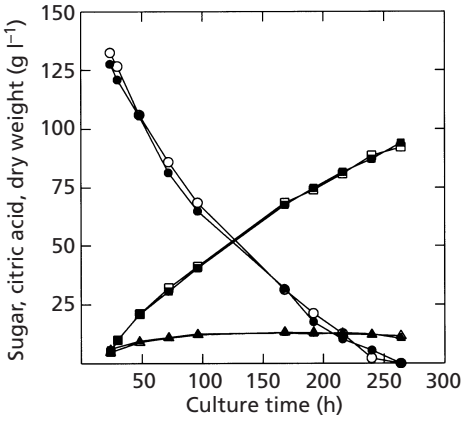

Fig. 4. Citric acid production by *A. niger goxC prtF* mutant NW185 at pH 5 and in the presence of manganese. NW185 was cultured in PM medium containing 14% (w/v) sucrose at pH 5 in the absence (open symbols) and presence (filled symbols) of 50 μM $MnCl_2$. Data are means of two experiments. ○, ●, Sugar (sucrose + glucose + fructose), □, ■, citric acid; △, ▲, dry weight.

acid fermentation. We tested a number of different conditions (e.g. using glucose, fructose or sucrose as the substrate, regulation of pH at 5 or no pH regulation) and found that production was optimal from sucrose at pH 5. Under these conditions NW185 produced approximately 90 g citric acid from 140 g sucrose in 10 d (Fig. 4). PM contains 1 μM Mn^{2+} (55 p.p.b.), which is well above the concentration recommended in citric acid fermentation (<1 p.p.b.; Mattey, 1992). To test whether NW185 was sensitive to Mn^{2+} we again performed fermentation in PM at pH 5 using sucrose, but 50 μM $MnCl_2$ (2·7 p.p.m.) was now added. Surprisingly, citric acid production was almost identical to that in the fermentation with 1 μM $MnCl_2$ (Fig. 4). Thus, it appears that with the *prtF* mutation citric acid can be produced at relatively neutral pH and production is then completely insensitive to the presence of Mn^{2+}. Like a *goxC* strain, a *goxC prtF* strain does not produce any citric acid in a traditional citric acid fermentation medium to which 50 μM $MnCl_2$ was added (data not shown). This implies that the requirement for Mn^{2+} deficiency is related to the specific conditions in a traditional citric acid fermentation and not to the *prtF* mutation. Only at relatively neutral pH is the absence or presence of Mn^{2+} no longer relevant.

DISCUSSION

Most of the data available on oxalic acid production by *A. niger* are in favour of OAH being the only enzyme responsible for oxalate production. (1) Label distribution patterns in experiments using $^{14}CO_2$ showed that oxalate probably originates from hydrolysis of oxaloacetate (Kubicek *et al*., 1988). In earlier experiments, using a number of ^{14}C-labelled carbon sources, Cleland & Johnson (1956) concluded, also on the basis of product labelling, that for most carbon sources oxalate was produced via oxidation of glyoxylate or glycolate. However, as already mentioned by Kubicek *et al*. (1988), Cleland & Johnson (1956) used long labelling times. In these experiments the acetate, produced also by OAH, is probably reconsumed and also converted to oxalate (see below). This severely complicates interpretation of the labelling data and may explain why these authors concluded that oxalate could not be produced by hydrolysis of oxaloacetate from most of the substrates tested, but should arise via oxidation of glyoxylate. Müller (1975) could not detect any activity of glyoxylate-oxidizing enzymes in extracts of *A. niger* mycelium that produced oxalate, whereas OAH activity was readily identified. Although the failure to detect any glyoxylate-oxidizing activity could be due to unfavourable extraction and/or assay conditions, the results taken together strongly suggest that this pathway is not involved in oxalate formation. (2) Our data show that oxalate production correlates with OAH activity when the culture pH is varied. In addition, Kubicek *et al*. (1988) reported that in a pH-shift experiment OAH biosynthesis correlated with formation of oxalic acid. These data justify the conclusion that OAH is responsible for oxalate formation and also that production of the acid is controlled by expression of the gene(s) encoding OAH. (3) It has been shown by us and previously also by Lenz *et al*. (1976) with partially

purified OAH that the enzyme produces equimolar amounts of oxalate and acetate. However, wild-type *A. niger* did not produce any acetate. This might be explained by involvement of another enzyme, such as glyoxylate dehydrogenase, exclusively leading to oxalate production. We have shown here that some acetate is transiently produced by an *acuA* mutant, which lacks ACS. After depletion of fructose the acetate formed is slowly consumed again, indicating that some acetate catabolism was still possible by the *acuA* mutant. These results suggest that wild-type *A. niger* is able to catabolize acetate sufficiently fast to prevent its production and explains why acetate is never observed during oxalate production. (4) With the properties of the *prtF28* mutant we now present strong evidence that OAH is the only enzyme involved in oxalate production in *A. niger*. The *prtF28* mutant lacks OAH activity and does not produce oxalate. It can be argued that *prtF* is a mutation that has broader effects than merely the absence of OAH, for instance it may also result in absence of other enzymes potentially involved in oxalate production, but the simplest explanation is a mutation in the gene encoding OAH. *prtF* was originally described as a mutation that decreased extracellular protease activity (Van den Hombergh *et al*., 1995). None of the biochemical defects of the *prt* mutations was known at that point, but the *prtF* mutation now appears to cause a defect in OAH. Because the strain does not produce oxalic acid, the reduced acidification of the environment probably leads to lower induction of extracellular proteases expressed under acidic conditions.

In traditional citric acid production processes two very important conditions to obtain a good fermentation are low pH (<2) and absence of Mn^{2+}. Low pH is important mainly to prevent production of gluconic acid and oxalic acid. A low starting pH of the medium might easily be obtained with relatively clean substrates, such as glucose syrups, but is more difficult to achieve in crude media, such as molasses, which have a high buffering capacity at pH 5–6. A mutant lacking both glucose oxidase (*goxC*) and OAH (*prtF*) produced citric acid at relatively neutral pH and could therefore be particularly advantageous in production processes making use of molasses. An additional advantage is that it is no longer necessary to remove metal ions by, for example, potassium ferrocyanide treatment or cation-exchange treatment of the substrate, because, in the media tested, the mutant is completely insensitive to the presence of Mn^{2+}. Although we have shown here the usefulness of an oxalate non-producing strain for citric acid production, it is clear that such a strain will also be valuable in other processes employing *A. niger*, such as production of enzymes. In particular, downstream processing may be simplified when oxalate is no longer produced. Besides applying mutagenesis and selection of oxalate-negative mutants, it is obvious that cloning of the *oah* gene and subsequent gene disruption provides a straightforward approach to eliminate oxalate production.

The main conclusions from the work described in this paper are: (1) The external pH was the main factor controlling oxalic acid production by *A. niger*. (2) An oxalate non-producing *A. niger* mutant lacked OAH, implying that OAH is the only enzyme involved in oxalate production in *A. niger*. (3) A strain lacking both glucose oxidase (*goxC*) and OAH (*prtF*) produced citric acid from sugar substrates in a regular synthetic medium at pH 5 and under these conditions production was completely insensitive to Mn^{2+}.

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REFERENCES

Bergmeyer, H. U. (1985). *Methods of Enzymatic Analysis*, vols VI and VII. Deerfield Beach FL: VCH.

Bos, C. J., Debets, A. J. M., Swart, K., Huybers, A., Kobus, G. & Slakhorst, S. M. (1988). Genetic analysis and the construction of master strains for assignment of genes to six linkage groups in *Aspergillus niger*. *Curr Genet* **14**, 437–443.

Cleland, W. W. & Johnson, M. J. (1956). Studies on the formation of oxalic acid by *Aspergillus niger*. *J Biol Chem* **220**, 595–606.

Dutton, M. V. & Evans, C. S. (1996). Oxalate production by fungi: its role in pathogenicity and ecology in the soil environment. *Can J Microbiol* **42**, 881–895.

Goosen, T., Bloemheuvel, G., Gysler, C., de Bie, D. A., Van den Broek, H. W. J. & Swart, K. (1987). Transformation of *Aspergillus niger* using the homologous orotidine-5′-phosphate-decarboxylase gene. *Curr Genet* **11**, 499–503.

Hayaishi, O., Shimazono, H., Katagiri, M. & Saito, Y. (1956). Enzymatic formation of oxalate and acetate from oxaloacetate. *J Am Chem Soc* **78**, 5126–5127.

Houck, D. R. & Inamine, E. (1987). Oxalic acid biosynthesis and oxaloacetate acetylhydrolase activity in *Streptomyces cattleya*. *Arch Biochem Biophys* **259**, 58–65.

Kuan, L.-C. & Tien, M. (1993). Stimulation of Mn peroxidase activity: a possible role for oxalate in lignin degradation. *Proc Natl Acad Sci USA* **90**, 1242–1246.

Kubicek, C. P. (1987). The role of the citric acid cycle in fungal organic acid fermentations. *Biochem Soc Symp* **54**, 113–126.

Kubicek, C. P., Schreferl-Kunar, G., Wöhrer, W. & Röhr, M. (1988). Evidence for a cytoplasmic pathway of oxalate biosynthesis in *Aspergillus niger*. *Appl Environ Microbiol* **54**, 633–637.

Kusters-van Someren, M. A., Harmsen, J. A. M., Kester, H. C. M. & Visser, J. (1991). Structure of the *Aspergillus niger pel*A gene and its expression in *Aspergillus niger* and *Aspergillus nidulans*. *Curr Genet* **20**, 293–299.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.

Lenz, H., Wunderwald, P. & Eggerer H. (1976). Partial purification and some properties of oxalacetase from *Aspergillus niger*. *Eur J Biochem* **63**, 225–236.

Mattey, M. (1992). The production of organic acids. *CRC Crit Rev Biotechnol* **12**, 87–132.

Micales, J. A. (1994). Induction of oxalic acid by carbohydrate and nitrogen sources in the brown-rot fungus *Postia placenta*. *Mater Org* **28**, 197–207.

Müller, H.-M. (1965). Untersuchungen zum Saürestoffwechsel von *Aspergillus niger*. I. Mitteilung. Der Einfluss des C/N-Verhältnisses in der Ausgangs-nährlsung auf den pH-Wert und die Oxalsaüreanhäufung. *Arch Microbiol* **52**, 251–265.

Müller, H.-M. (1975). Oxalate accumulation from citrate by *Aspergillus niger*. I. Biosynthesis of oxalate from its ultimate precursor. *Arch Microbiol* **103**, 185–189.

Murphy, R. J. & Levi, J. F. (1983). Production of copper oxalate by some copper tolerant fungi. *Trans Br Mycol Soc* **54**, 165–168.

Pontecorvo, G., Roper, J. A., Hemmons, L. M., MacDonald, K. D. & Bufton, A. W. J. (1953). The genetics of *Aspergillus nidulans*. *Adv Genet* **5**, 141–238.

Ruijter, G. J. G., Panneman, H. & Visser, J. (1997). Overexpression of phosphofructokinase and pyruvate kinase in citric acid producing *Aspergillus niger*. *Biochim Biophys Acta* **1334**, 317–326.

Sandeman, R. A. & Hynes, M. J. (1989). Isolation of the *facA* (acetyl-CoenzymeA synthase) and *acuE* (malate synthase) genes from *Aspergillus nidulans*. *Mol Gen Genet* **218**, 87–92.

Sealy-Lewis, H. M. (1994). A new selection method for isolating mutants defective in acetate utilisation in *Aspergillus nidulans*. *Curr Genet* **25**, 47–48.

Sealy-Lewis, H. M. & Fairhurst, V. (1998). Isolation of mutants deficient in acetyl-CoA synthetase and a possible regulator of acetate induction in *Aspergillus niger*. *Microbiology* **144**, 1895–1900.

Shanta, T. & Rati, E. R. (1990). Isolation and characterization of an aflatoxin-inhibiting metabolite from *Aspergillus niger*. *Curr Sci* **59**, 326–327.

Strasser, H., Burgstaller, W. & Schinner, F. (1994). High-yield production of oxalic acid for metal leaching processes by *Aspergillus niger*. *FEMS Microbiol Lett* **119**, 365–370.

Tanaka, K. & Nonaka, F. (1981). Synergistic action of oxalic acid and pectinolytic enzyme on the rot of onion bulb caused by *Aspergillus niger*. *Ann Phytopathol Soc Japan* **47**, 166–174.

Van de Merbel, N. C., Ruijter, G. J. G., Lingeman, H., Brinkman, U. A. Th. & Visser, J. (1994). An automated monitoring system using on-line ultrafiltration and column liquid chromatography for *Aspergillus niger* fermentations. *Appl Microbiol Biotechnol* **41**, 658–663.

Van den Hombergh, J. P. T. W., Van de Vondervoort, P. J. I., Van der Heijden, N. C. B. A. & Visser, J. (1995). New protease mutants in *Aspergillus niger* result in strongly reduced *in vitro* degradation of target proteins; genetic and biochemical characterization of seven complementation groups. *Curr Genet* **28**, 299–308.

Van der Veen, P., Flipphi, M. J. A., Voragen, A. G. J. & Visser, J. (1991). Induction, purification and characterisation of arabinases produced by *Aspergillus niger*. *Arch Microbiol* **157**, 23–28.

Vishniac, W. & Santer, M. (1957). The thiobacilli. *Bacteriol Rev* **21**, 195–213.

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