Influence of medium components and metabolic inhibitors on citric acid production by *Penicillium simplicissimum*

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Penicillium simplicissimum excreted more than 100 mmol citric acid l⁻¹ [2.9 mmol (g dry wt)⁻¹; 9 d] if an industrial filter dust (> 50% ZnO) providing a high extracellular buffering capacity was present in the medium. A similar specific [2 mmol (g dry wt)⁻¹], but lower absolute (26 mmol l⁻¹), citric acid excretion occurred in the absence of an extracellular buffer and if amino acids or urea were used as nitrogen source. *P. simplicissimum* excreted no citric acid under conditions where *Aspergillus niger* produces citric acid (deficiency of trace elements, low pH and reduced biomass formation). Citric acid excretion by *P. simplicissimum* always paralleled biomass formation and occurred in a pH range between 4 and 7. This indicated that different imbalances of metabolism were responsible for citric acid excretion in *A. niger* and *P. simplicissimum*. However, provided a high extracellular buffering capacity was present, the response of the *Penicillium* system to different carbon and nitrogen sources was similar to the *Aspergillus* system. In contrast, the metals iron and copper had virtually no effect on citric acid excretion compared with *A. niger*. Estimation of intracellular citric acid, as well as the effects of the uncoupler 2,4-dinitrophenol, and the H⁺-ATPase inhibitor sodium orthovanadate, led to the conclusion that the buffer-stimulated citric acid efflux was dependent on metabolic energy and an energized plasma membrane, respectively. Despite similarities to the *Aspergillus* system, a different mechanism for buffer-stimulated citric acid excretion by *P. simplicissimum* seems probable.

Introduction

Organic acid excretion by fungi is a widespread, but still not completely understood phenomenon. In the best known case – citric acid production by *Aspergillus niger* – a high citric acid production rate is attributed to an ‘energy overflow’ metabolism, which occurs under conditions of high glycolytic activity (Kubicek & Röhr, 1986), and limited growth because of reduced respiration (Wallrath *et al.*, 1991, 1992). A decrease in respiration is caused in this system by a deficiency of trace elements, especially manganese, which – among other effects (Kubicek & Röhr, 1986) – impairs the function of complex I of the respiratory chain (Wallrath *et al.*, 1991, 1992). Additionally, a low extracellular pH contributes to the unfavourable growth conditions (Kubicek & Röhr, 1986).

Unlike the *Aspergillus* system, significant amounts of citric acid can also be excreted by fungi growing at normal rates, in a pH range between 4 and 7 and with

abundant trace elements. These characteristics apply to the enhanced citric acid excretion by *Penicillium simplicissimum* in the presence of an industrial filter dust containing more than 50% zinc oxide (Schinner & Burgstaller, 1989; Franz *et al.*, 1991). Leaching of metals from filter dusts using fungi is one possibility for recycling these materials (Burgstaller & Schinner, 1993). The addition of 2.5% (w/v) filter dust to the medium led to a high extracellular buffering capacity: $\text{ZnO} + 2\text{H}^+ \rightarrow \text{Zn}^{2+} + \text{H}_2\text{O}$ ($\beta = 0.38$). The high extracellular buffering capacity was proved to be one of the main reasons for an enhanced citric acid efflux (W. Burgstaller, A. Zanella & F. Schinner, unpublished results). In this paper, the *Penicillium* system is compared to the well known *Aspergillus* system.

Trace amounts of manganese, zinc and iron decrease citric acid production by *A. niger*, with manganese being the most inhibitory (Röhr *et al.*, 1983; Kubicek & Röhr, 1986). In *P. simplicissimum* citric acid excretion occurs in the presence of up to 200 mM-zinc (Schinner & Burgstaller, 1989) and millimolar amounts of other heavy metals (Burgstaller *et al.*, 1990; W. Burgstaller &

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H. Strasser, unpublished results). Manganese is able to reduce buffer-stimulated citric acid excretion by *P. simplicissimum* (Franz *et al.*, 1991) but not as strongly as in *A. niger* (Kisser *et al.*, 1980). A further difference is that *A. niger* produces citric acid in synthetic media only if the extracellular pH is low (Kubicek & Röhr, 1986), whereas *P. simplicissimum* excretes citric acid in a pH range between 4 and 7. Similarities between the two systems are the requirement for a high concentration of a carbon source and a high oxygen demand. However, if aeration is interrupted, citric acid production by *A. niger* is irreversibly repressed (Kubicek *et al.*, 1980), whereas *P. simplicissimum* produces citric acid at an unaltered rate when oxygen supply is resumed (Burgstaller *et al.*, 1992).

In this study, factors which are known to influence the *Aspergillus* system (carbon and nitrogen sources, presence or absence of metals, inhibition of cytoplasmic protein synthesis) were investigated in the *Penicillium* system. The transport of citric acid across the plasma membrane of *P. simplicissimum*, dependence on metabolic energy and relationship to the activity of one of the most important enzymes of fungal plasma membrane, the H⁺-ATPase (Sanders, 1988), were also studied. The latter was investigated because the activity of the H⁺-ATPase has been connected with the excretion of organic acids in *Neurospora crassa* (Slayman *et al.*, 1990) and *Penicillium cyclopium* (Roos & Slavik, 1987). It had also been suggested that the H⁺-ATPase of *Saccharomyces cerevisiae* may be stimulated by an extracellular buffer (Sigler & Höfer, 1991).

Methods

Strains. *P. simplicissimum* (= *P. janthinellum*) (Oudem.) Thom., was isolated from a soil contaminated with heavy metals and identified by the Centraalbureau voor Schimmelcultures. Two strains of *A. niger* were from the Centraalbureau voor Schimmelcultures, The Netherlands (CBS 420.64, CBS 120.49).

Spore suspension. Rye grains (50 g) washed with distilled water and wetted with 50 ml distilled water were autoclaved at 121 °C, inoculated with 1 ml of a spore suspension and incubated at 30 °C. After 4–6 d spores were washed off with 0.05% Tween 80. Spore suspensions were stored in 50% (v/v) glycerol at –20 °C (short-term storage) or in liquid nitrogen (long-term storage).

Submerged cultivation. Flasks (500 ml) containing 70 ml medium or 100 ml flasks containing 20 ml medium were inoculated with a spore suspension to provide a final concentration of 5×10^7 spores ml⁻¹ and incubated at 30 °C and 150 r.p.m. The standard medium contained (mm): sucrose, 439; NH₄NO₃, 25; MgSO₄·7H₂O, 1.8; KH₂PO₄, 6.5. Alternative carbon sources used included (mm): glucose, 300; lactose, 200; acetic acid, 200; citric acid, 438; α-ketoglutarate, 200; molasses 2.9% (w/v). Alternative nitrogen sources included: (NH₄)₂CO₃, 12.5 or 25 mm; NaNO₃, 55 mm; Casamino acids, 0.77% (w/v); molasses, 2.7% (w/v). The pH in media without an extracellular buffer was adjusted to 4 or 7 by addition of HCl and NaOH, respectively. The standard medium was used with and without a solution of trace

elements (Franz *et al.*, 1991). Further nutrient media used were Czapek Dox medium, a glucose-tartrate medium (275 mm-glucose, 35 mm-diammonium tartrate, 1.6 mm-KCl, 1.3 mm-MgSO₄·7H₂O, 0.88 mm-KH₂PO₄, 0.32 mm-FeSO₄·7H₂O, 0.1 mm-ZnSO₄, pH 5.6; modified from Roos & Slavik, 1987) and a molasses medium (250 g molasses l⁻¹, 6.5 mm-KH₂PO₄).

A high extracellular buffering capacity was achieved by adding a filter dust from the converter of a copper works (Montanwerke Brixlegg, Brixlegg, Austria) or 1 M-phthalic acid/triethanolamine pH 7.5 to the standard medium. The filter dust consisted of the following metals in the form of oxides (w/w): 59% Zn, 11% Pb, 6% Sn, 0.5% Cu, 0.1% As, 0.07% Fe, 0.03% Sb, 0.02% Ni, 0.004% Ag. Filter dust (25 g l⁻¹) or 17 g zinc oxide l⁻¹ was added to the standard medium. Filter dust and zinc oxide were sterilized separately. A solution of trace elements was added to media supplemented with zinc oxide or phthalic acid/triethanolamine (Franz *et al.*, 1991).

Investigation of physiological parameters. Stock solutions of chloramphenicol and valinomycin were made in methanol, and of cycloheximide, 2,4-dinitrophenol and sodium orthovanadate in double-distilled water. Inhibitors were added on day 3 for non-replacement experiments and when the pellets were suspended in fresh medium for replacement experiments.

The influence of individual metals was tested by adding a single metal (50 µmol l⁻¹, final concentration) to the standard medium supplemented with zinc oxide and the solution of trace elements (Franz *et al.*, 1991).

Intracellular citric acid was determined by extracting mycelium frozen in liquid nitrogen with perchloric acid according to Smith & Ng (1972) and Kubicek & Röhr (1978).

The short-term influence of sodium orthovanadate on the respiration rate was examined using a Twin Flow respirometer (Cyclobios). A suspension of pellets (0.5 ml, 6 d old) diluted with 5 ml fresh standard medium was used. Oxygen concentrations were measured at a stirring rate of 500 r.p.m. and a temperature of 25 °C.

Replacement technique. Three-day-old pellets were separated from the medium (70 ml) by filtration (pore size 25 µm) and washed in 100 ml distilled water and 150 ml fresh medium and incubated for 3 d in fresh standard medium.

Analytical methods. Zinc was measured by atomic absorption spectrophotometry using standard conditions. Organic acids and sugars were determined by HPLC (column: Aminex HPX 87H cation exchanger with micro guard cation H precolumn; mobile phase, 5 mM-H₂SO₄; flow rate, 0.5 ml min⁻¹; temperature, 30 °C; detection, refractive index detector and UV detector at 213 nm). After ultra-filtration (10 kDa) samples were diluted 1:50 with distilled water.

Biomass concentrations of cultures grown in the presence of filter dust or zinc oxide were calculated from the difference between the weight after drying at 80 °C for 24 h and ashing at 500 °C for 4 h. In the most unfavourable case, the contribution of the biomass ash to the total ash weight was not more than 5%.

Sampling was carried out daily and all values are the mean values of three or four replicates. Standard deviation was normally lower than 5% and in a few cases between 5 and 10%.

Results and Discussion

Growth in standard medium

Generally speaking, most of the total citric acid produced by *A. niger* during a fermentation period is excreted not during the exponential growth phase but during a secondary 'production' phase which is characterized by a low extracellular pH and a reduced growth rate

Fig. 1. Course of biomass formation (▲, ▽) and extracellular pH (◆, ◇) in cultures of *P. simplicissimum* in standard medium with trace elements with (closed symbols) and without (open symbols) filter dust, as well as production of citric acid (●) in the presence of filter dust. Without filter dust no citric acid was produced. Cultivation was carried out in 100 ml flasks containing 20 ml standard medium. Three flasks were sampled each day.

(Chmiel, 1975; Röhr *et al.*, 1983; Steinböck *et al.*, 1991; Wallrath *et al.*, 1992). In contrast, citric acid production by *P. simplicissimum* occurred only in the presence of an industrial filter dust exhibiting a high buffering capacity (Franz *et al.*, 1991), in a pH range between 4 and 7 (Fig. 1; Schinner & Burgstaller, 1989) and paralleled the formation of biomass throughout cultivation (Fig. 1). A closer investigation of the mechanism of citric acid excretion in *P. simplicissimum* provided evidence that different imbalances of metabolism were responsible for the excretion of large amounts of citric acid in *A. niger* and *P. simplicissimum*.

The basic features of growing *P. simplicissimum* in the standard medium without trace elements (the standard medium was a typical citric acid production medium modified after Rehm, 1979; for composition see Methods) were as follows: *P. simplicissimum* strongly acidified this medium during the first 2–3 d of growth (pH 2 or below). This acidification was stoichiometric (1:1) with the uptake of ammonium. A similar acidification behaviour has been described for *P. cyclopium* in a non-buffered medium with ammonium chloride as nitrogen source (Roos & Luckner, 1984). *P. simplicissimum* formed about 2 g dry wt l⁻¹ during these 3 d.

If the standard medium was supplemented with trace elements, *P. simplicissimum* resumed growth after a short phase of zero growth (Fig. 1). The resumed growth was accompanied by an increase in pH (Fig. 1).

No organic acids were excreted into the standard medium (with or without trace elements) by *P. simplicissimum* (Table 1). In contrast, two different strains of

Table 1. Stimulation of citric acid excretion in *P. simplicissimum* in a strongly buffered medium

P. simplicissimum was cultivated in the standard medium (see Methods) with ammonium nitrate as nitrogen source. The buffers filter dust ($\text{ZnO} + 2\text{H}^+ \rightarrow \text{Zn}^{2+} + \text{H}_2\text{O}$) or zinc oxide plus trace elements were added as indicated. Flasks (500 ml) with 70 ml of medium were inoculated with 5×10^7 spores ml⁻¹ and incubated at 150 r.p.m. and 30 °C for 9 d.

Culture condition	pH		Biomass (g dry wt l ⁻¹)	Citric acid [mmol (g dry wt ⁻¹) ⁻¹]
	Start	End		
Without buffer, without trace elements	4.0	2.1	2.2	0
Without buffer, plus trace elements	4.0	6.0	25.0	0
With 2.5% (w/v) filter dust, without trace elements	6.5	4.5	35.1	2.9
With 1.7% (w/v) zinc oxide, plus trace elements	6.5	4.7	39.7	1.8

Table 2. Influence of nitrogen sources on citric acid excretion in non-buffered cultures of *P. simplicissimum*

P. simplicissimum was cultivated in the standard medium (see Methods) with different nitrogen sources, different concentrations of nitrogen sources and different starting pH values. No filter dust and no trace elements were added. Flasks (500 ml) with 70 ml of medium were inoculated with 5×10^7 spores ml⁻¹ and incubated at 150 r.p.m. and 30 °C for 9 d.

Nitrogen source	pH		Biomass (g dry wt l ⁻¹)	Citric acid [mmol (g dry wt ⁻¹) ⁻¹]
	Start	End		
Urea (12.5 mM)	4.0	4.0	3.0	2.1
	7.0	6.7	3.0	1.3
Urea (25 mM)	4.0	4.1	1.7	0
	7.0	6.3	5.4	0
Casamino acids	4.0	2.6	12.1	2.2
	7.0	4.3	12.0	1.2
Molasses [2.7% (w/v)]	4.0	4.2	26.6	0.3
	7.0	5.1	18.6	0.5
NaNO ₃ (55 mM)	4.0	6.6	14.5	0.2
Other media:				
Czapek Dox medium	7.0	5.9	22.2	0.4
Molasses medium	7.0	5.9	23.0	1.2

A. niger (CBS 420.64 and CBS 120.49) produced up to 100 mM-citric acid after 6 d when cultivated in this medium (without trace elements; data not shown).

Supplementation of the standard medium with 2.5% (w/v) filter dust stimulated the excretion of considerable amounts of citric acid in *P. simplicissimum* cultures (Fig. 1; Table 1). *A. niger* was not able to grow in the presence of 2.5% (w/v) filter dust. It was concluded that the buffering effect of the filter dust ($\text{ZnO} + 2\text{H}^+ \rightarrow$

Fig. 2. Influence of the presence of a buffer (1 M-phthalic acid/triethanolamine, pH 7.5) on specific citric acid production by *A. niger* (□) and *P. simplicissimum* (■). Cultivation was carried out for 3 d in 100 ml flasks containing 20 ml standard medium plus trace elements. Four flasks were sampled each day.

$\text{Zn}^{2+} + \text{H}_2\text{O}$) rather than a better supply of trace elements in the presence of filter dust was responsible for this enhanced citric acid excretion: the specific citric acid excretion rate in the presence of filter dust was considerably higher than that obtained in Czapek Dox medium which supported similar biomass formation in the absence of a high buffering capacity (Table 2). This conclusion was confirmed by a stimulated citric acid excretion if the standard medium was supplemented with 1 M-phthalic acid/triethanolamine, pH 7.5 (Fig. 2) or other buffers (data not shown) instead of filter dust. For *A. niger* the opposite is true: less citric acid is excreted if the medium is buffered compared to the non-buffered low pH medium (Fig. 2; see also Steinböck *et al.*, 1991). The different responses of *A. niger* and *P. simplicissimum* to the extracellular pH imply different mechanisms for citric acid excretion.

The physiological events leading to the excretion of large amounts of citric acid in *A. niger* – most important a deficiency of manganese – have been well defined (Kubicek & Röhr, 1986). For buffer-stimulated citric acid excretion in *P. simplicissimum* only preliminary indications about the effect of an extracellular buffer on the physiology of the cells have been gained. If *P. simplicissimum* was grown in the presence of filter dust, all the filter dust was adsorbed onto the hyphae. Preventing the adsorption of filter dust by packing it into a dialysis bag resulted in lower specific citric acid production and lowered growth rate: 1.7 mmol (g dry wt)⁻¹ with adsorbed filter dust and 0.8 mmol (g dry wt)⁻¹ with the filter dust in dialysis bag (day 6; biomass: 43 and 16 g dry wt l⁻¹, respectively). The adsorbed filter dust presumably acted as a pH stabilizer ($\text{ZnO} + 2\text{H}^+ \rightarrow \text{Zn}^{2+} + \text{H}_2\text{O}$) in the vicinity of the cell surface. Thus the local pH was increased in comparison to

normal conditions (Sentenac *et al.*, 1989). In *S. cerevisiae* a higher extracellular pH also resulted in a higher growth rate which was closely correlated to an increased activity of the plasma membrane H⁺-ATPase (Portillo & Serrano, 1989). That such a causal chain of events is also probable for increased growth rate of *P. simplicissimum* cultures with adsorbed filter dust is supported by the effect of the plasma membrane H⁺-ATPase inhibitor vanadate (see below). The filter dust in the dialysis bag could obviously not act as a pH stabilizer in the vicinity of the cell surface and this resulted in a lower growth rate.

Influence of the nitrogen source

Ammonium is the most widely used nitrogen source for citric acid production in *A. niger* (Röhr *et al.*, 1983; Kubicek & Röhr, 1986), although Xu *et al.* (1989a) found that urea was the best nitrogen source, followed by yeast extract, peptones, ammonium sulphate, malt extract and sodium or ammonium nitrate. In cultures of *P. simplicissimum* without filter dust and with ammonium nitrate as nitrogen source no citric acid was excreted (Table 1). If ammonium nitrate was replaced by urea or Casamino acids, citric acid excretion was observed (Table 2). Both the specific and absolute citric acid excretion were significantly lower compared to that in media with added filter dust. Absolute amounts of excreted citric acid were lower due to lower biomass formation in these media (presumably due to the lack of trace elements). At least for urea the mechanism underlying citric acid excretion seemed to be different from the buffer-stimulated citric acid efflux: whereas the latter was proportional to the amount of buffer present in the medium (Schinner & Burgstaller, 1989), a decrease in the concentration of urea increased citric acid excretion (Table 2).

With *A. niger* sodium nitrate was as effective as ammonium nitrate for citric acid production (Xu *et al.*, 1989a). With *P. simplicissimum* sodium nitrate as nitrogen source in the absence of filter dust led to a small citric acid efflux whereas ammonium nitrate did not (Tables 1 and 2). Nitrate is taken up by fungi via a proton symport (Sanders, 1988). Thus a pH stabilizing effect through the uptake of nitrate similar to the one assumed with filter dust can be anticipated. However, *P. simplicissimum* could not grow on sodium nitrate in the presence of filter dust. A reduced amount of protons available for proton symports in this case could explain this finding.

Influence of the carbon source

Both glucose and sucrose led to very similar levels of citric acid excretion by *P. simplicissimum* in the presence of filter dust. For *A. niger* sucrose is a better carbon

Fig. 3. Influence of trace metals and heavy metals ($50 \mu\text{mol l}^{-1}$ final concentration) on buffer-stimulated specific citric acid excretion by *P. simplicissimum*. Cultivation was carried out in 100 ml flasks containing 20 ml standard medium plus trace elements. Four flasks were sampled on day 3 (□) and day 4 (■).

Fig. 4. Intracellular (□) and extracellular (■) citric acid in *P. simplicissimum* cultivated in standard medium with added filter dust. Intracellular citric acid in cultures without filter dust (glucose–tartrate medium) was below the detection limit.

source than glucose (Hossain *et al.*, 1984; Xu *et al.*, 1989b).

Using acetate, lactose, α -ketoglutarate, and citrate as sole carbon sources for *P. simplicissimum* cultures in the presence and absence of filter dust gave similar results to that observed in *A. niger* (Xu *et al.*, 1989b). Acetate could not be used by either fungus. *P. simplicissimum* and *A. niger* (Xu *et al.*, 1989b) are able to utilize lactose but neither *A. niger* nor filter dust supplemented cultures of *P. simplicissimum* produced citric acid with lactose. Growth of *P. simplicissimum* on α -ketoglutarate and citrate in the presence of filter dust indicated an undisturbed function of the tricarboxylic acid cycle in the presence of heavy metals. These results are in accordance with the postulation of Kubicek (1988a, b) that organic acid excretion is due mainly to a cytosolic overflow

rather than to a bottle neck in the tricarboxylic acid cycle.

Influence of metal ions

The metals zinc (Schinner & Burgstaller, 1989; Burgstaller *et al.*, 1990) and iron (Fig. 3) do not decrease buffer-stimulated citric acid excretion in *P. simplicissimum* as is the case with *A. niger* (Röhr *et al.*, 1983; Kubicek & Röhr, 1986). An increase in citric acid excretion in the presence of calcium has also been found for *A. niger* (Röhr *et al.*, 1983), whereas the effect of lead requires further investigation to test if its addition could be used to increase citric acid excretion by *P. simplicissimum* during leaching processes.

Physiological parameters

A first step in the investigation of the metabolic changes occurring in the presence of a buffer (filter dust) in *P. simplicissimum* was the estimation of intracellular citric acid in leaching and non-leaching cultures. The intracellular water was taken as $1.5 \mu\text{l (mg dry wt)}^{-1}$ (*P. cyclopium*; Roos, 1989; other values: $2.5 \mu\text{l}$ for *A. niger*, C. P. Kubicek, personal communication; $3.0 \mu\text{l}$ for *Neocosmospora vasinfecta*, Budd, 1989; $2.0 \mu\text{l}$ for *Penicillium chrysogenum*, Packer *et al.*, 1992). The comparison of intra- and extracellular citric acid at day 6 (Fig. 4) indicated that the excretion of citric acid proceeded against the electrochemical potential gradient of citric acid; this means that metabolic energy must have been used to excrete citric acid. In *A. niger* citric acid excretion is a 'downhill' transport process (Kubicek & Röhr, 1978; Kubicek *et al.*, 1980). This is an additional indication that the mechanism of buffer-stimulated citric acid excretion in *P. simplicissimum* is different from the one in *A. niger*.

Two further results are important: (i) if *P. simplicissimum* was cultivated without filter dust (in a glucose–tartrate medium, which was very similar to the standard medium), intracellular citric acid in *P. simplicissimum* was below the detection limit; this means that the presence of filter dust has the same consequence for *P. simplicissimum* as manganese deficiency for *A. niger*, namely an increase in intracellular citric acid concentration; and (ii) intracellular citric acid in filter dust cultures was ten times lower than found in mycelia of *A. niger* during accumulation of citric acid (Kubicek & Röhr, 1978).

Chloramphenicol, an inhibitor of mitochondrial protein synthesis, did not inhibit buffer-stimulated citric acid efflux in *P. simplicissimum* to a great extent (Table 3). In contrast, cycloheximide, an inhibitor of eukaryotic translation, caused a strong inhibition of citric acid efflux

Table 3. Influence of metabolic inhibitors on buffer-stimulated citric acid excretion in *P. simplicissimum*

P. simplicissimum was cultivated in the standard medium (see Methods) with ammonium nitrate as nitrogen source in the presence of filter dust. The effect of inhibitors was tested either by direct addition of the inhibitor to a 3-d-old culture or after suspending 3-d-old pellets in fresh medium containing the respective inhibitor. Incubation was continued for 3 d after addition of the inhibitor. Similar results were obtained with both techniques.

Compounds added	Culture age (d)	Final pH	Citric acid (mM)	Citric acid per unit of consumed sugar	Degree of inhibition (%)
Control (no added inhibitor)	3	4.0	121	0.19	
Chloramphenicol (0.77 mM)	3	4.3	108	0.18	5
Cycloheximide (0.89 mM)	3	6.0	24	0.07	63
2,4-Dinitrophenol (5 mM)	4	5.8	26	0.04	79
Sodium orthovanadate (10 mM)	3	6.1	26	0.05	74

(Table 3). Although sugar consumption – and therefore overall metabolism – was also reduced by cycloheximide, inhibition of citric acid excretion was nevertheless a specific effect, because the excreted citric acid per unit of consumed sugar was also decreased by cycloheximide (Table 3). Therefore, in *P. simplicissimum*, as in *A. niger*, *de novo* cytoplasmic protein synthesis is necessary for an increased citric acid efflux. The most important cytoplasmic enzyme for citric acid excretion in *A. niger* is pyruvate carboxylase (Jaklitsch *et al.*, 1991; Steinböck *et al.*, 1991).

The fact that dinitrophenol did not significantly lower sugar consumption, but decreased citric acid excretion, supported the conclusion drawn from the estimation of intracellular citric acid concentration, namely that metabolic energy was needed to excrete citric acid. That metabolic energy is needed to excrete an organic acid already present at high concentration in the cell was also found for *S. cerevisiae* (Borst-Pauwels, 1981).

An involvement of the plasma membrane H⁺-ATPase in buffer-stimulated citric acid excretion was indicated by the observation that vanadate reduced citric acid excretion specifically: sugar consumption was not reduced to a great extent and oxygen consumption during a short-term respiration test was not reduced by up to 10 mM-vanadate in non-leaching cultures nor by up to 20 mM-vanadate in leaching cultures.

Conclusions

A variety of extracellular factors may stimulate a fungal hypha to excrete large amounts of citric acid, for instance: (i) a deficiency of trace elements combined with

a low extracellular pH (Kubicek & Röhr, 1986); (ii) the presence of a specific nitrogen source; and (iii) the presence of a (high) extracellular buffering capacity (Roos & Luckner, 1984). These results show that with *P. simplicissimum* conditions (i) and (ii) did not lead to the excretion of large amounts of citric acid, but condition (iii) did. This and the fact that citric acid excretion was dependent on metabolic energy indicated a different mechanism of citric acid production compared to *A. niger*. Condition (i), which is used to produce citric acid commercially with *A. niger*, cannot be achieved in metal leaching processes (Burgstaller & Schinner, 1993). Therefore, a more detailed investigation of condition (iii) is of interest. The decrease of citric acid excretion when the activity of the H⁺-ATPase was reduced by vanadate indicated that one regulatory point concerning condition (iii) must be looked for in the plasma membrane. Additionally, the mutual relationships between the three different environmental conditions and the question of whether there are species-specific effects or not, are of interest for a better understanding of organic acid excretion by fungi. Organic acids are involved, for instance, in modulating the availability of micronutrients for plants (Msaky & Msanya, 1986), the contamination of disposal sites by solubilized heavy metals (Francis *et al.*, 1992), weathering and the winning of valuable metals from oxide or silicate ores and industrial secondary raw materials (Burgstaller & Schinner, 1993).

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