Initiation of citric acid accumulation in the early stages of *Aspergillus niger* growth

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**Summary.** Glycerol, which functions as an osmoregulator in the early stages of *Aspergillus niger* growth, slowly diffuses out of the cells and possibly into the mitochondria. Since mitochondrial nicotinic adenine dinucleotide phosphate (NADP⁺)-specific isocitrate dehydrogenase is inhibited by glycerol, citrate starts to accumulate in the cells. At physiological pH values citric acid dissociates and affects the intracellular and extracellular pH. By the phosphorus-31-nuclear magnetic resonance technique a drop in intracellular pH from 7.1 to about 6.5 has been detected, which might significantly influence metabolic rates.

**Introduction**

Much has been reported on the mechanism of citric acid accumulation by *Aspergillus niger* (Kubíček and Rohr 1986), but the studies were mostly performed on the acid-excreting mycelium while the initiation of excretion was not examined. In studies on the germination of *A. niger* under conditions leading to high yields of citric acid, a characteristic series of morphological changes has been observed (Legiša et al. 1981). After spore germination, enlarged bulbous cells form from which a hypha-like filamentous outgrowth spontaneously follows. Concomitant with the change in morphology, citric acid first becomes detectable in the medium (Legiša and Mattey 1986a), followed by a change in carbohydrate metabolism from the pentose phosphate pathway to a mainly glycolytic flux (Legiša and Mattey 1988).

In vitro tests showed that the highest concentration of glycerol was found in the medium at about the same time as the change in morphology. This could account for the inhibition of mitochondrial nicotinic adenine dinucleotide phosphate (NADP⁺)-specific isocitrate dehydrogenase (ICDH) and cause the initiation of citric acid accumulation (Legiša and Mattey 1986a). The present paper attempts to elucidate the initiation of this accumulation.

**Materials and methods**

*Aspergillus niger* (strain NRRL 2270) spores were harvested from a 7-day-old wort agar slant, suspended in 25 ml of 0.1% Tween solution and filtered through Whatman 54 filter paper to remove the clumped spores. The whole suspension was used to inoculate 1 l medium (Legiša et al. 1981) in a 2-l all-glass stirred tank reactor. For manganese-sufficient conditions MnSO₄ was added to the medium to a final concentration of 10 μM. Fermentation was conducted at 30°C and at high dissolved oxygen tension.

For the intracellular determination of different substances the mycelium was collected by suction filtration, washed briefly with distilled water to remove the medium, and finally kept in 50 v/w ratio of ice-cold distilled water for 5 min to remove all traces of medium from the pellets. After washing off the substrate the mycelium was collected by suction filtration, again rinsed with cold distilled water, and extracted with 5% trichloroacetic acid.

Intracellular volume was estimated according to the distribution of glucose and sorbitol between the medium and the mycelium (Newsholm and Start 1981). The concentration of both substances was determined enzymatically. The intracellular volume of the 28-h-old mycelium grown in manganese-sufficient and -deficient medium was 23.3 and 17.5 ml/100 g wet weight respectively.

Glycerol was determined enzymatically (Eggstein and Kuhlmann 1974) using the Boehringer kit (Boehringer, Mannheim, FRG). Before the assays samples were neutralized using 6 M KOH.
Citric acid was determined according to Saffran and Denstedt (1948) modified according to Spencer and Lowenstein (1967).

The changes in the pH value of the medium were recorded by an electrode (Ingold, Frankfurt/Main, FRG) connected to a pH meter (Knick, Berlin (West)). Intracellular pH values were determined from the chemical shifts of P resonances by using a calibration procedure described by Roberts et al. (1980). Phosphorus-31 nuclear magnetic resonance (P31-NMR) spectra were measured on a VXR 300 spectrometer (Varian, Palo Alto, Calif, USA) at 121 MHz. Accumulation of about 2000 scans was carried out employing 60° pulses and a 0.34 s repetition time. Shifts were referred to 85% orthophosphoric acid as a standard located in a capillary tube. All spectra were recorded at 21°C.

Results and discussion

When *A. niger* was grown at different sucrose concentrations different levels of glycerol could be detected in the cells (Table 1), which undoubtedly demonstrates the role of glycerol as an osmotic regulator. In 15% sucrose medium, up to 175 mM glycerol was present from the very beginning of growth onwards and it did not change during the initial phase until 40 h of fermentation. Later on the amount of glycerol decreased slowly until the fifth day of fermentation (Fig. 1). Virtually the same concentration of glycerol has been found in mycelium grown in the manganese-sufficient and -deficient medium (Fig. 1).

Glycerol is mostly formed in the cytosol from intermediates of the pentose phosphate pathway by NADP⁺-specific glycerol dehydrogenase (Legiša and Mattey 1986b). In the early stages of *A. niger* growth a change in the carbohydrate metabolism from pentose phosphate pathway to glycolysis takes place (Legiša and Mattey 1988), which affects glycerol formation and consequently causes a decrease in intracellular glycerol level.

With mammalian cells glycerol diffuses fairly readily through the membranes (Moore 1968) but this does not seem to happen with *A. niger*. The mould appears to conserve glycerol in the cells to function as an osmoregulator, yet the membranes are not completely impermeable to this molecule so the amount of glycerol in the medium slowly increases during the growth of the enlarged bulbous cells. Different levels of glycerol were found at different sucrose concentrations in the media (Fig. 2).

Unfortunately, it was not possible to measure the amount of glycerol in the mitochondria but some diffusion through the mitochondrial membranes may be assumed to cause a similar increase in glycerol in the mitochondrial matrix as that in the extracellular space.

Since the significance of glycerol in the inhibition of mitochondrial NADP⁺-specific ICDH was found in *A. niger* (Legiša and Mattey 1986a) and some other fungi (Brown and Simpson 1972)

Table 1. The intracellular amount of citric acid and glycerol measured at 28 h of fermentation during the growth of *Aspergillus niger* at different sucrose concentrations

<table>
<thead>
<tr>
<th>Sucrose (%)</th>
<th>Glycerol (mM)</th>
<th>Citric acid (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>20.1</td>
<td>3.4</td>
</tr>
<tr>
<td>5</td>
<td>57.8</td>
<td>7.5</td>
</tr>
<tr>
<td>10</td>
<td>121.8</td>
<td>7.3</td>
</tr>
<tr>
<td>15</td>
<td>173.0</td>
<td>9.6</td>
</tr>
<tr>
<td>20</td>
<td>242.5</td>
<td>8.0</td>
</tr>
</tbody>
</table>

Fig. 1. Intracellular glycerol levels in manganese-sufficient (×) and-deficient (●) mycelium

Fig. 2. Glycerol levels found in the media with different sucrose concentrations