Succinate synthesis and excretion by *Penicillium simplicissimum* under aerobic and anaerobic conditions

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Abstract

Succinate is an interesting chemical for industries producing food and pharmaceutical products, surfactants, detergents and biodegradable plastics. Succinate is produced mainly by a mixed-acid fermentation process using anaerobically growing bacteria. However, succinate excretion is also widespread among fungi. In this article we report results on the intracellular concentration and the excretion of succinate by *Penicillium simplicissimum* under aerobic and anaerobic conditions. The intracellular concentration of succinate increased slightly with the specific growth rate and strongly if the respiratory chain was inhibited by sodium azide or anaerobic conditions (N₂). A strong increase of succinate excretion was observed if the respiratory chain was inhibited. It is suggested that succinate synthesis under functional (sodium azide) or environmental (N₂) anaerobic conditions occurs via the reductive part of the tricarboxylic acid cycle. Succinate is then excreted because the oxidative part of the tricarboxylic acid cycle is inactive. A possible role of succinate synthesis in the regeneration of NAD (‘fumarate respiration’) is discussed. © 2002 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

Keywords: Tricarboxylic acid cycle; Oxidative and reductive part; Continuous cultivation

1. Introduction

Succinate is an interesting chemical for industries producing food and pharmaceutical products, surfactants, detergents and biodegradable plastics [1,2]. Succinate is produced mainly by a mixed-acid fermentation process using anaerobically growing bacteria [1]. However, succinate excretion is also widespread among fungi. Succinate is a by-product of ethanol fermentation by yeasts [3–5]. Succinate excretion is characteristic of the genus *Fusarium* as a whole and is also excreted by other filamentous fungi [6]. We came about succinate excretion by the filamentous fungus *Penicillium simplicissimum* during the investigation of citrate excretion [7]. We observed that succinate was the only tricarboxylic acid (TCA) cycle intermediate whose excretion was strongly stimulated if respiration was inhibited by anaerobic conditions or by addition of sodium azide. Additionally, succinate was the major intracellular organic acid in growing [8] and non-growing [7] hyphae of *P. simplicissimum*. For these reasons we summarize in this article the results which we have obtained on intracellular and extracellular succinate in *P. simplicissimum* under aerobic and anaerobic conditions. These results suggest that (i) the intracellular level of succinate responds to both the specific growth rate and the activity of the respiratory chain, and (ii) under anaerobic conditions succinate may be formed via the reductive part of the TCA cycle and excreted with high yield. This could be a starting point for further investigations into succinate excretion by filamentous fungi with their high metabolic capacity.

2. Materials and methods

2.1. Organism, inoculum and medium

*P. simplicissimum*, isolated from soil contaminated with heavy metals and identified by the Centraalbureau voor Schimmelcultures [9], was grown in a minimal medium (mM): NH₄Cl 12, KCl 6, NaH₂PO₄·H₂O 6, Na₂SO₄ 1.6, MgCl₂·6H₂O 1.6, glucose 200; 20 ml per litre medium of a solution of trace elements; pH 4, 6 or 7 (with NaOH or HCl). Trace element solution (mM): Fe(II)SO₄·7H₂O...
1.8, Mn(II)SO₄·3H₂O 1.4, ZnCl₂ 1.47, Cu(II)SO₄·5H₂O 0.2, CaCl₂·2H₂O 2.0. The glucose was sterilized separately, the solution of trace elements was sterilized by filtration. A filamentous inoculum to start the chemostat culture was produced by growing P. simplicissimum in the minimal medium to which 1 M HEPES pH 7.3 was added. A 100-ml aliquot of medium was inoculated with 10⁶–10⁷ spores ml⁻¹ and grown at 400 rpm and 30°C for 2 days.

2.2. Chemostat experiments

All chemostat experiments were performed in a Biostat M bioreactor (Braun, Germany) at 30°C, 700–1000 rpm and 1–2 vvm (volume air per volume of fermentation liquid and minute), with a working volume of 1700 ml. The conditions for bioreactor runs were as described previously [10].

The chemostat was inoculated with 100 ml of a filamentous pre-culture and processed in the batch-mode overnight. Then the chemostat-mode was started by switching on the feed and alkali pump. Between four and six generation times were allowed to pass for the culture to reach the steady state. Samples were taken every 1–4 generation times (four samples per steady state). Twenty millilitres from the chemostat culture were filtered through a cellulose-acetate filter (pore size 0.45 μm). The filtrate was stored at −20°C for further analysis.

2.3. Replacement experiments

Efflux of organic acids from non-growing hyphae was investigated with mycelium from the exponential growth phase (medium with 1 M HEPES). The mycelium was harvested and washed with 200 ml of a 100-ml glucose solution. Five grams of this freshly harvested mycelium were suspended in a beaker containing 200 ml of a solution containing 100 mM glucose, 1 mM NaCl and 0.01 mM KCl. The suspension was stirred in order to maintain an oxygen saturation between 50 and 80%. The pH and the oxygen concentration were measured continuously during the experiment. Samples of 12 ml were taken at different times and filtered rapidly (nylon net, 20 μm). The mycelium was washed with 60 ml of cold glucose (100 mM). The biomass was divided into three aliquots and put into polypropylene tubes that were immediately immersed in liquid nitrogen. Both the filtrate and frozen mycelium were stored at −20°C until further treatment.

2.4. Analytical methods

The dry weight of the mycelium was determined after drying at 105°C for 24 h. Intermediates of the tricarboxylic acid cycle were extracted as described before [7]. Extracellular and intracellular organic acids were determined by HPLC [11]. An intracellular volume of 1.3 ml (g dry wt.)⁻¹ was used for the calculation of intracellular concentrations [12]. ATP was extracted and measured lumino metrically as described by Meraner [13].

2.5. Reproducibility

In the experiments with non-growing hyphae (replacement experiments), results from one representative experiment are shown: at each sampling time, one measurement was carried out to determine the extracellular concentration of organic acids, whereas the intracellular concentration was determined from the extraction of three aliquots of biomass. The experiments with non-growing hyphae were repeated three times and showed similar values of intracellular and extracellular concentrations of organic acids. The results from continuous cultivation are from a single chemostat run.

3. Results

Both in batch culture and in glucose- as well as ammonium-limited continuous culture the intracellular concentration of succinate was much higher than the concentration of citrate, oxoglutarate, fumarate and malate. In

<table>
<thead>
<tr>
<th>Condition</th>
<th>Intracellular acids (mM)</th>
<th>Acid efflux (μmol g⁻¹ h⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Succinate</td>
<td>Citrate</td>
</tr>
<tr>
<td>Growing hyphae (NH₄-limited chemostat culture)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+Air</td>
<td>48</td>
<td>30</td>
</tr>
<tr>
<td>+N₂</td>
<td>60</td>
<td>44</td>
</tr>
<tr>
<td>Non-growing hyphae (replacement experiment)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+Air</td>
<td>27.3</td>
<td>2.7</td>
</tr>
<tr>
<td>+N₂</td>
<td>157</td>
<td>6.2</td>
</tr>
<tr>
<td>+Azide</td>
<td>144</td>
<td>24</td>
</tr>
<tr>
<td>Growth as pellets</td>
<td>96</td>
<td>–</td>
</tr>
</tbody>
</table>

The fungus grew in a filamentous form if not indicated otherwise.

Table 1

Intracellular concentration as well as efflux of succinate and citrate in growing (chemostat culture) and non-growing (replacement experiments) hyphae of P. simplicissimum under different conditions
glucose- and ammonium-limited continuous culture intracellular succinate increased with the growth rate (Fig. 1). No intracellular succinate could be detected in hyphae growing in nitrate-limited and phosphate-limited continuous culture (data not shown).

The intracellular succinate concentration in non-growing hyphae was strongly increased if air was replaced with nitrogen or if sodium azide was added (Table 1, Fig. 2). Also in nitrogen-limited chemostat culture replacing air by N2 (which stopped growth) resulted in an increased intracellular succinate concentration (Table 1).

In growing and non-growing hyphae an increase in intracellular succinate was always accompanied by an increase in succinate excretion (Table 1, Fig. 3). If P. simplicissimum grew in the form of pellets, succinate excretion was also increased (Table 1). The highest succinate excretion rate was measured in non-growing hyphae inhibited by azide (562 ± 11 μmol g⁻¹ h⁻¹; Table 1).

4. Discussion

4.1. Intracellular succinate under aerobic conditions

In glucose- and ammonium-limited continuous culture intracellular succinate increased in parallel with the oxygen consumption (for data concerning the oxygen consumption see [10]). In other words, intracellular succinate increased in parallel with the activity of the TCA cycle and of the respiratory chain. This suggests that under aerobic conditions succinate is formed via the oxidative part of the TCA cycle. With a low energy status, e.g. during phosphate limitation (limited ATP synthesis) or nitrate limitation (energy consuming nitrate reduction), no intracellular succinate could be detected. Therefore, the value of the intracellular succinate concentration seems to be an indi-
Succinate excretion by \textit{P. simplicissimum} is different from succinate excretion by \textit{Saccharomyces cerevisiae} in two respects. First, succinate excretion is not coupled to ethanol formation in \textit{P. simplicissimum}. And second, a depolarization of the plasma membrane (which is brought about by azide and nitrogen in filamentous fungi) [19] does not inhibit succinate excretion as is the case with \textit{S. cerevisiae} [20].

Acknowledgements

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References


