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Citrate Efflux in *Penicillium simplicissimum*: Fundamental Methods for the *in vivo* Study of Efflux Kinetics

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Summary

Several fundamental methods were developed in order to allow a reliable interpretation of in vivo kinetics of citrate efflux in Penicillium simplicissimum. Filamentous growth was achieved by growing P. simplicissimum at pH = 7.3 in a medium containing 1 M HEPES. The volume of intracellular water was determined by thermogravimetry as 1.3 mL/g dry wt. A comparison of different methods for extracting citrate from the hyphae showed that boiling the hyphae in 0.1 M HCl for ten minutes was the most suitable method. Cooling was not necessary to maintain the intracellular citrate concentration during harvesting. An optimized method was developed for measuring the cytoplasmic pH. From the calculation of the chemical gradient of undissociated citric acid it was concluded that simple diffusion of undissociated citric acid is of little importance concerning citrate excretion by P. simplicissimum.

Keywords: *Penicillium*, citrate efflux, intracellular pH, intracellular water, disintegration of hyphae, simple diffusion of undissociated citric acid

Introduction

The interpretation of *in vivo* kinetics of citrate efflux from filamentous fungi is not straightforward. Three obvious difficulties are: *(i)* the hyphae frequently aggregate to pellets resulting in different physiological states of hyphae within the pellet; *(ii)* the pool of intracellular citrate consists of four species and the concentration of each species is pH-dependent; *(iii)* intracellular citrate may be distributed between the cytoplasm and subcellular compartments, for instance mitochondria and vacuoles. Reliable kinetic data from *in vivo* experiments are, however, important, because transport through the plasma membrane is – at least in *Aspergillus niger* – one of the main regulatory points for citrate production *(1)*. Despite of this, even in *A. niger* only preliminary results on the citrate export system have been obtained *(2)*.

Citrate production by *Penicillium simplicissimum* was used for the leaching of zinc from a certain industrial filter dust *(3)*. *P. simplicissimum* tolerated much higher concentrations of zinc and manganese than *A. niger*, and excreted citrate only at a pH higher than 6. This article presents result of investigations which were undertaken in order to arrive at a reliable interpretation of the *in vivo* kinetics of citrate efflux in *P. simplicissimum*. The methods, which are required to reach this goal, are: a method for producing a filamentous growth form of *P. simplicissimum*; a method for determining the volume of the intracellular water; the determination of the effect of harvesting and resuspension on the intracellular citrate concentration; a method for the extraction of citrate, which allows a high sample throughput; and, as the most important, a method for measuring the intracellular, or, more precisely, the cytoplasmic pH. These methods will also turn out to be of value for investigating citrate excretion in *A. niger*. Knowing the intracellular citrate concentration and the intracellular pH we calculated the chemical gradient of undissociated citric acid during citrate excretion. The result supports the hypothesis that simple diffusion of undissociated citric acid does not play a role in citrate excretion by *P. simplicissimum*.

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Materials and Methods

Media

The basic constituents of the medium, which induced citrate excretion in *P. simplicissimum*, were (concentrations given in mM): glucose, 400; $(NH_4)_2SO_4$, 6.25; NH_4Cl, 12.5; KH_2PO_4, 5.8; $MgSO_4 \cdot 7\ H_2O$, 1.6 and 20 mL of a solution of trace elements. Trace element solution were (concentrations given in mM): $Fe(II)SO_4 \cdot 7\ H_2O$, 1.8; $Mn(II)SO_4 \cdot 1\ H_2O$, 1.36; $ZnCl_2$, 1.47; $Cu(II)SO_4 \cdot 5\ H_2O$, 0.2 and $CaCl_2 \cdot 2\ H_2O$, 2.04. A high buffering capacity of the medium was achieved by adding 1 M MES (pH = 6 with NaOH), 1 M HEPES (pH = 7.3 with NaOH), 0.2 M zinc oxide or 20 g/L calcium carbonate. Sugar, salts and the buffer were autoclaved separately. The inoculation density was 10^6–10^7 spores per mL of medium and the flasks were shaken with 100, 250 or 400 r.p.m at 30 °C.

Homogenous suspension of hyphae

We carried out a series of tests to produce a homogenous suspension of hyphae by applying the following treatments: *(i)* oxygen limitation and low pH *(4)*; *(ii)* addition of polyacrylamide *(5)*; *(iii)* formation of spherocytes by adding ethylene glycol to spores *(6)*; *(iv)* high inoculation density, low shaking speed (100 r.p.m.) combined with a subsequent disintegration of aggregates by mechanical stirring; *(v)* treatment with a mechanical disintegrator (Ultra Turrax) and subsequent regeneration *(7)*; *(vi)* growth in 1 M HEPES at pH = 7.3 *(8)*.

Disintegration of hyphae

Before disintegration the hyphae were washed thoroughly: after five washing steps all extracellular citrate was removed from the hyphae. Hyphae of *P. simplicissimum* were disintegrated by three methods: grinding in a glass bead mill at high speed (»Bead Beater«, Biospec); boilling for ten minutes (in 0.02 M HEPES pH = 7.8, 0.1 M HCl, 1 M HCl or 65%) HNO_3; treatment with 1 M $HClO_4$ in an ultrasonic bath *(9)*.

Intracellular water

Intracellular water was determined by thermogravimetry *(10)*. In short, aliquots of the wet biomass were dried on an infrared balance. At the transition from the evaporation of extracellular water to the evaporation of intracellular water the curve showed a distinct change of slope. Because of this the intracellular water could be calculated from the kinetics of the evaporation of water from the wet biomass. A value of 1.3 ± 0.34 mL/g dry wt (n = 20) was determined for the intracellular water of *P. simplicissimum*.

Intracellular pH

Three fluorescent probes were tested for their suitability to measure the intracellular pH in *P. simplicissimum*: Fluorescein, BCECF and SNARF (Molecular Probes Europe, The Netherlands). The efficiency by which the esterified dyes were hydrolyzed was tested using a crude cell-free extract *(11)*. The standard procedure for loading of SNARF-AM into the hyphae was as follows: 0.1 g of fresh weight was suspended in 5 mL of 0.05 M MES (pH = 6) and 50 μg of SNARF-AM (dissolved in 50 μl DMSO) were added. The suspension was incubated at 30 °C and 400 rpm for 15 minutes. The hyphae were then washed with 2 x 10 mL of buffer, suspended in buffer at a mycelial density of 10 mg wet weight per mL, and stored on ice. This suspension could be used for at least six hours. For fluorescence measurements this suspension was diluted 10-fold (final dry weight concentration: 0.2 mg dry weight/mL). This mycelial density quenched less than 5% of the fluorescence of SNARF added to a mycelial suspension. The oxygen supply for the stirred mycelial suspension in the cuvette was sufficient (this was checked by means of an oxygen microelectrode).

Fluorescence was measured in a Hitachi F 4500 fluorimeter equipped with a thermostated, stirred cuvette and appropriate filters on both the excitation and the emission side. SNARF was excited at 540 nm and emission was measured simultaneously at 585 and 624 nm. The ratio of the fluorescence at the two wavelengths (585/624) was then related to the pH of the solution. The following substances did not influence the fluorescence of SNARF: ethanol (1%; volume fraction), DMSO (1%; volume fraction), CCCP (0.05 mM), nystatin (0.05 mM), valinomycin (0.05 mM), azide (10 mM), glucose (50 mM), butyrate (50 mM); and vanadate (1 mM).

The efflux of SNARF from loaded hyphae was not small enough to be neglected. Therefore, after measuring the total fluorescence of the mycelial suspension, the suspension was filtered and the fluorescence of the filtrate measured. The fluorescence of the filtrate at both emission wavelengths was then substracted from the total fluorescence before the ratio was calculated.

Various methods were applied to find the best way to calibrate intracellular SNARF. Nigericin/KCl, Triton X 100, Saponin and nystatin were applied in order to permeabilize the plasma membrane. Incubation with nystatin for five minutes turned out to be the most suitable procedure (50 μg of nystatin per mg of fresh weight). Nystatin equilibrated extra- and intracellular pH without greatly increasing the efflux of SNARF. A further advantage of nystatin is, that it permeabilizes only the plasma membrane and not the vacuolar membranes.

Investigation of the kinetics of citrate excretion

For the investigation of the kinetics of citrate efflux five gram of fresh weight of *P. simplicissimum* biomass were suspended in 200 mL of a solution of 0.1 M glucose, 1 mM NaCl and 10 μM KCl. The suspension was stirred and aerated in order to give a constant oxygen saturation (between 50 and 80%). The pH and the oxygen concentration were measured continuously during the experiment. Samples (12 mL) were taken at different times and filtered rapidly (47 mm filter holder, nylon net 20 μm). The mycelium was then washed by pressing 6 x 12 mL of cold 0.1 M glucose through the filter holder. Measuring citrate in the washing solutions revealed that this washing procedure was sufficient to remove all extracellular citrate from the hyphae. The biomass was divided into three parts and put into polypropylene tubes which were immediately immersed into liquid nitrogen. Both the filtrate and the frozen myce-

lium were stored at –20 °C until further treatment. The whole procedure from the taking of the sample until the mycelium was frozen took about one minute.

Analytical methods

Intra- and extracellular citrate was determined by HPLC using an Aminex HPX 87 H column (Bio-Rad; eluent: 2 mM H_2SO_4; flow rate: 0.6 mL/min; temperature: 41 °C)*(8)*. The total citrate was determined by UV absorption at 210 nm. Potassium was measured by atomic absorption spectrometry using a standard addition method.

Results and Discussion

Growth form

P. simplicissimum excreted citrate only in strongly buffered media, *i.e.* when the medium contained either 0.2 M zinc oxide, 20 g/L calcium carbonate, 1 M MES, pH = 6, or 1 M HEPES, pH = 7.3. Growth curves of *P. simplicissimum* in these four media are shown in Fig. 1. Similar extracellular citrate concentrations per gram of biomass were achieved in all four media: between 0.5 and 1.0 mmol/g of dry weight after two days of exponential growth.

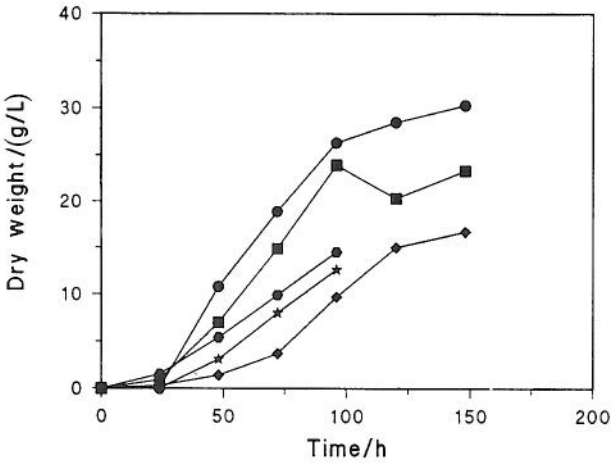

Fig. 1 Growth of *Penicillium simplicissimum* in media which induced an excretion of citrate. ● – medium buffered with 0.2 M ZnO (250 r.p.m.); ■ – medium buffered with 1 M MES (pH = 6; 250 r.p.m.); ⬣ – medium buffered with 20 g/L calcium carbonate (400 r.p.m.); ★ – medium buffered with 1 M HEPES (pH = 7.3; 400 r.p.m.; ◆ – medium buffered with 1 M HEPES (pH = 7.3; 250 r.p.m.).

We tested fifteen different media in the course of our work and in all but one (the medium containing 1 M HEPES at pH = 7.3), *P. simplicissimum* grew in the form of pellets. Pellets could not be used for the fluorimetric measurement of the intracellular pH, because they led to unreproducible fluorescence values. Several treatments for producing a homogenous suspension of hyphae were tested (see Materials and Methods). Cultivation of *P. simplicissimum* in 1 M HEPES at pH = 7.3 was chosen as standard method because no further treatment of the mycelium was necessary subsequent to growth.

Extraction of citrate

Citrate has a high metabolic turnover rate. It is therefore important to measure its intracellular concentration during the whole transport experiment. This means that in many samples the hyphae must be disintegrated. Boiling the hyphae for ten minutes in 0.1 M HCl proved to be the method of choice, because it combined a high efficiency of citrate extraction with a high sample throughput. Concerning the extraction of potassium, the boiling of the hyphae in 0.1 M HCl was as effective as the boiling in nitric acid or disruption in a glass bead mill. The respective values for intracellular potassium were: 234 ± 11 mM (n = 8) with the boiling of mycelium in 0.1 M HCl; 287 ± 26 mM (n = 10) with disruption in the glass bead mill; 288 ± 34 mM (n = 6) with the boiling in nitric acid. The intracellular citrate concentrations were: 23.8 ± 3.3 mM (n = 4) with the boiling in 0.1 M HCl and 25.7 (n = 1) with the glass bead mill. Increasing the concentration of hydrochloric acid to 1 M did not increase citrate extraction (22.8 ± 2.2 mM; n = 4).

Intracellular citrate during harvesting and aeration in a glucose solution

Cooling was not necessary during harvesting to maintain the intracellular citrate concentration: intracellular citrate was 119 mM (n = 2) in a sample taken directly from the culture and disintegrated immediately, 115 ± 6.7 mM (n = 6) in mycelium harvested by filtration at room temperature, and 81 ± 5.9 mM (n = 6) in mycelium harvested by filtration at 4 °C.

In *Corynebacterium glutamicum* lysine efflux could be started by rapidly increasing the temperature of a cooled suspension of cells *(12)*. This is a convenient method for starting an efflux experiment. In *P. simplicissimum*, however, cooling did not completely stop citrate efflux (Fig. 2). Harvesting, washing and suspending of the mycelium in a fresh glucose solution was therefore carried out at room temperature.

After the suspension of the harvested mycelium in a fresh glucose solution (without nitrogen) the intracellu-

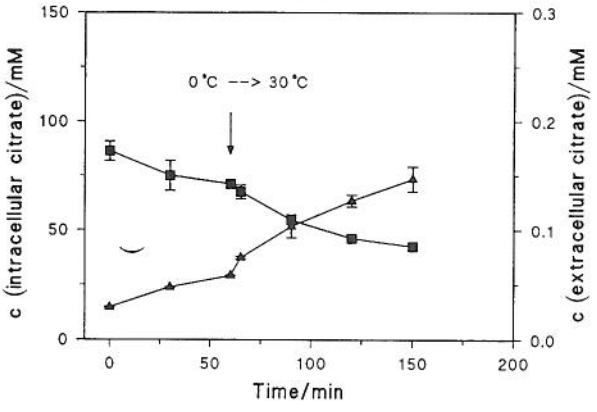

Fig. 2. Effect of cooling on intracellular citrate and citrate efflux in *Penicillium simplicissimum* (growth medium: buffered with 1 M MES pH = 6; conditions: see Material and Methods); ■ – intracellular citrate; ▲ – extracellular citrate.

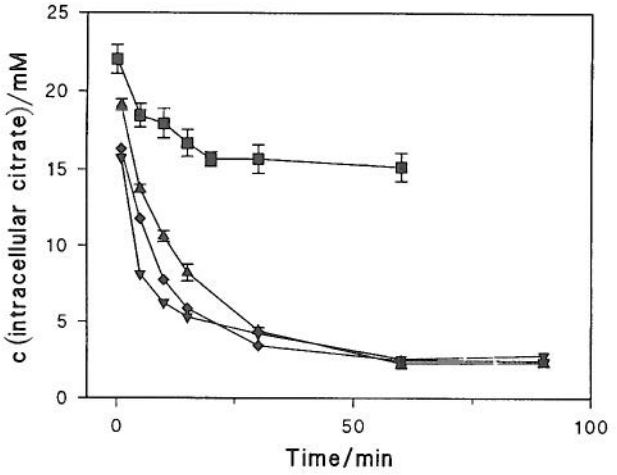

Fig. 3. Intracellular citrate in *Penicillium simplicissimum* during the »transient time« (after suspension of freshly harvested hyphae in an aerated glucose solution; conditions: see Material and Methods); ■ – pellets from medium buffered with 1 M MES (pH = 6; 250 r.p.m.); ▲, ◆, ▼ – filamentous mycelium from medium buffered with 1 M HEPES (pH = 7.3; 400 r.p.m.; values are from three separate experiments).

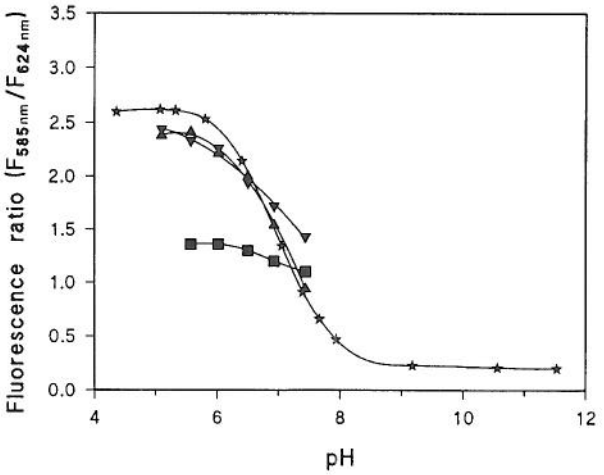

Fig. 4. Calibration of extra- and intracellular SNARF: ★ – SNARF in 0.2 M sodium gluconate + 0.01 M HEPES (pH = 7); ▼ – intracellular SNARF in hyphae, which were permeabilized with nystatin; ▲ – extracellular SNARF in the experiment with permeabilized hyphae; ■ – intracellular SNARF in non-permeabilized hyphae.

lar citrate concentration decreased and reached a new steady-state concentration after about twenty minutes (Fig. 3). This opens up the possibility of measuring citrate efflux either at a set of different intracellular citrate concentrations during the transient time or at a constant steady-state intracellular citrate concentration.

Intracellular pH

The following criteria were used to compare the three fluorescent probes fluorescein diacetate (FDA), BCECF-AM and SNARF-AM: the degree of hydrolysis of the esterified dye by a crude cell extract; the site of intracellular accumulation of the dye: the rate of leakage of the dye from loaded hyphae. Taking hydrolysis of FDA as 100%, SNARF-AM was hydrolyzed to 60%, whereas BCECF-AM was hydrolyzed to less than 5%. A cytoplasmic fraction (won by differential centrifugation of the crude cell extract) hydrolyzed SNARF-AM just as well as the total crude extract. This indicated that hydrolysis of SNARF-AM took place mainly in the cytoplasm. Fluorescence microscopy of loaded hyphae supported this assumption: SNARF was mainly accumulated in the cytoplasm, whereas BCECF was accumulated in subcellular compartments, most probably vacuoles *(13, 14)*. Fluorescein rapidly leaked from loaded hyphae (50% after five minutes), whereas SNARF was retained much better (50% leakage after 45 minutes). According to these results SNARF was the best suited dye for measuring the intracellular pH in *P. simplicissimum*.

The fluorescence intensity of SNARF changed with the ionic strength of the solution. Therefore, *in vitro* calibration curves were prepared at a constant ionic strength, I = 0.25 M (KCl). Additionally, a constant buffering capacity over the whole tested pH range was achieved by using a mixture of three buffers (0.05 M phthalic acid, pK_a = 5.4; 0.05 M dimethylglutaric acid, pK_a = 6.3; 0.05 M imidazole, pK_a = 7). Extracellular and intracellular calibration curves for SNARF are shown in Fig. 4.

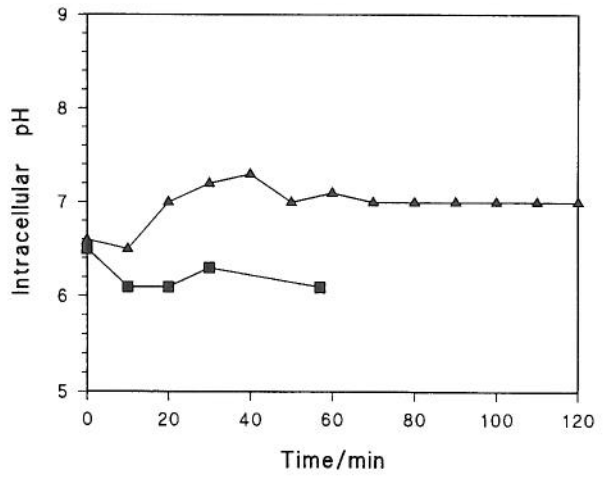

Fig. 5. Intracellular pH in energized and de-energized hyphae of *Penicillium simplicissimum* (growth medium: buffered with 1 M HEPES pH = 7.3; conditions: see Material and Methods). ▲ – energized hyphae (aerated in a glucose solution); ■ – de-energized hyphae (plus 5 mM sodium azide).

The intracellular pH of non-growing hyphae of *P. simplicissimum* in the absence of glucose was 7.1 ± 0.39 (n = 21). The intracellular pH during a transport experiment in non-growing, but energized hyphae (aerated in a glucose solution) is shown in Fig. 5. During the first 60 minutes the intracellular pH varied between 6.8 and 7.2; afterwards the pH remained constant. Sodium azide (5 mM) decreased the cytoplasmic pH by about one unit (Fig. 5). Other metabolic inhibitors (CCCP, NEM) also decreased the cytoplasmic pH (data not shown).

The intracellular concentration of undissociated citric acid was calculated using the measured intracellular citrate concentrations and the measured intracellular pH values (pK_a values at an ionic strength of I = 0.15 M were used: pK_{a1} = 2.9, pK_{a2} = 4.3, pK_{a3} = 5.6 *(15)*. The

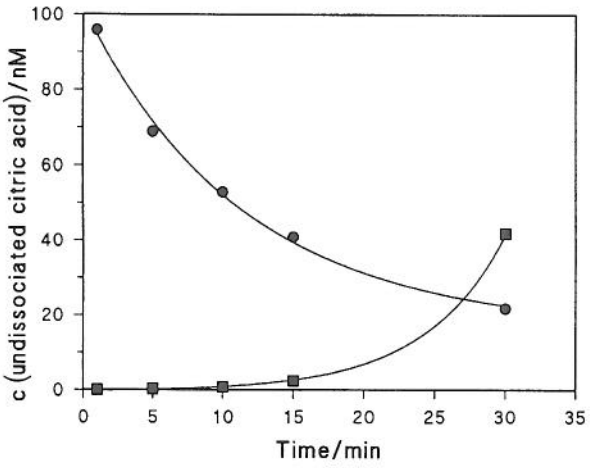

Fig. 6. Intracellular and extracellular concentration of undissociated citric acid during citrate efflux calculated from the total intracellular citrate concentration and the intracellular pH. ● – intracellular undissociated citric acid; ■ – extracellular undissociated citric acid.

same was done for the extracellular citrate using pK_a values at $I = 0$ M *(15)*. The changes in the chemical gradient of undissociated citric acid during a standard transport experiment is shown in Fig. 6. After thirty minutes the extracellular concentration of undissociated citric acid exceeded its intracellular concentration. Because simple diffusion cannot proceed uphill, it is clear the simple diffusion of undissociated citric acid does not play an important role in citrate efflux in *P. simplicissimum*.

Our further aims are to construct pH maps of *P. simplicissimum* hyphae *(16)* and to clarify the subcellular compartmentation of citrate in *P. simplicissimum*.

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References

1. N. V. Torres, C. Regalado, A. Sorribas, M. Cascante, In: *Modern Trends in Biothermokinetics*, S. Schuster *et al.* (Eds.), Plenum Press, New York (1993) pp. 115–124.
2. A. Netik, N. V. Torres, J.-M. Riol, C. P. Kubicek, *Biochim. Biophys. Acta, 1326* (1997) 287.
3. W. Burgstaller, H. Strasser, H. Wöbking, F. Schinner, *Environ. Sci. Technol. 26* (1992) 340.
4. H. W. van Versefeld, M. Metwally, M. el Sayed, M. Osman, J. M. Schrickx, A. H. Stouthamer, *Antonie van Leeuwenhoek, 60* (1991) 313.
5. P. Jones, B. A. Shahab, A. P. J. Trinci, D. Moore, *Trans. Br. Mycol. Soc. 90* (1988) 577.
6. C. L. Slayman, G. N. Zuckier, *Methods Enzymol. 174* (1989) 654.
7. F. Pilz, G. Auling, D. Stephan, U. Rau, F. Wagner, *Exp. Mycol. 15* (1991) 181.
8. C. Womersley, L. Drinkwater, J. H. Crowe, *J. Chromatogr. 318* (1985) 112.
9. C. Prömper, R. Schneider, H. Weiss, *Eur. J. Biochem. 216* (1993) 223.
10. J. L. Uribelarrea, S. Pacaud, G. Goma, *Biotechnol. Lett. 7* (1985) 75.
11. R. S. Haworth, B. D. Lemire, D. Crandall, E. J. Cragoe Jr., F. Fliegel, *Biochim. Biophys. Acta, 1098* (1991) 79.
12. S. Bröer, R. Krämer, *Eur. J. Biochem. 202* (1991) 131.
13. C. L. Slayman, V. V. Moussatos, *J. Exp. Biol. 196* (1994) 419.
14. I. R. von Recklinghausen, D. Molenaar, W. N. Konings, J. Visser, In: *Biotechnology Applications of Microinjection, Microscopic Imaging, and Fluorescence*, P. H. Bach *et al.* (Eds.), Plenum Press, New York (1993) pp. 207–219.
15. L. G. Sillen, A. E. Martell: *Stability Constants of Metal-Ion Complexes*, Spec. Publ. Chem. Soc. No. 17 (1964) pp. 477–480.
16. W. Roos, *Bot. Acta, 105* (1992) 153.

Izdvajanje citrata u *Penicillium simplicissimum*: osnovni postupci za proučavanje kinetike izdvajanja *in vivo*

Sažetak

Razrađeno je nekoliko osnovnih postupaka za ispravnu procjenu kinetike izdvajanja citrata iz P. simplicissimum in vivo. Filamentozni rast postignut je uzgojem P. simplicissimum pri pH = 7,3 u podlozi koja je sadržavala 1M HEPES. Volumen intracelularne vode određen je termogravimetrijom te iznosi 1,3 mL/g suhe tvari. Uspoređivanjem različitih postupaka za ekstrakciju citrata iz hifa, pokazalo se da je najpogodniji postupak kuhanje hifa u 0,1 M HCl tijekom 10 minuta. Hlađenje nije bilo potrebno za održavanje unutarstanične koncentracije citrata tijekom ekstrakcije. Razvijen je najpovoljniji postupak za mjerenje citoplazmatskog pH. Na temelju izračunatog kemijskoga gradijenta nedisocirane limunske kiseline zaključeno je da je jednostavna difuzija nedisocirane limunske kiseline beznačajna u odnosu na izdvajanje citrata iz P. simplicissimum.