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Intracellular pH-dependent efflux of the fluorescent probe pyranine in the yeast *Yarrowia lipolytica*

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Abstract

8-Hydroxypyrene-1,3,6-trisulfonic acid (pyranine) can be used as a vital intracellular pH (pH_i) indicator. In the yeast *Yarrowia lipolytica*, a partial efflux of the probe was detected by using the pH-independent wavelength of 415 nm. A simplified correction of the fluorescent signals was applied, enabling to show for this species a good near-neutral pH_i maintenance capacity in a pH 3.9 medium. Octanoic acid, which is known to have toxic effects on yeast, decreased the pH_i and increased the 260-nm-absorbing compounds leakage. However, this acid inhibited the fluorescent probe efflux linearly with its concentration suggesting a pH_i-dependent efflux of pyranine from cells. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Microbiological Societies.

Keywords: Yeast Intracellular pH; Pyranine; Probe leakage; Octanoic acid; *Yarrowia lipolytica*

1. Introduction

The determination of the intracellular pH (pH_i) in micro-organisms is useful to understand many vital processes like transport mechanisms [1] or cell adaptation in complex media, and more generally, cell energetics. It can also be useful to determine the mechanisms of the action of toxic compounds, since many of them commonly bring about a modification of pH_i, together with an alteration of cell membranes [2].

The use of fluorescent probes for pH_i measurements has been extensively developed during the last decade because of the simplicity of these methods, compared with some other techniques, i.e., the equilibrium distribution of radiolabeled weak acids or ^{31}P nuclear magnetic resonance spectroscopy. The cell permeant probe fluorescein and some of its derivatives are among the most used compounds [3], but many authors have reported their trapping inside yeast vacuoles [4]. The probe leakage can also be a limitation to the fluorescence use in pH_i determination as reported in yeast, for example with the dyes C.SNARF-1 and fluorescein [5]. The probe efflux has also been favorably used to assess yeast membrane integrity [2,6] and cell viability [7].

Yarrowia lipolytica is a non-conventional dimorphic yeast that is able to use fatty acids, alkanes or paraffins as carbon sources and also to excrete large amounts of organic acids [8] or some flavor compounds [9], both potentially toxic for the yeast, affecting thus bioprocesses [10].

The use of the impermeant probe pyranine arises as an interesting alternative method for pH_i measurements since it is introduced in living cells by forming transient pores in plasma membrane, allowing a cytoplasmic preferential location and then a limited efflux [11]. This fluorescent compound was used here to determine the pH_i of *Y. lipolytica* and its decrease induced by octanoic acid. Moreover, measurements at a pH-independent wavelength permitted to monitor the probe efflux.

2. Materials and methods

2.1. Strain and culture conditions

Y. lipolytica W29 (ATCC20460; CLIB89) was grown in a glucose medium in the conditions previously described [9].

2.2. pH_i determination

The method was adapted from that described by Peña et

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al. [11]. Yeast cells from 100 ml of culture broth were harvested by centrifugation (6000×*g*, 5 min), washed twice with physiologic water, then with milliQ® water and resuspended in 3 ml of milliQ® water. 700 μl of this suspension were mixed with 10 μl of a 100 mM pyranine (Molecular probes, Eugene, OR, USA) solution in water, in a cell with a 4-mm gap. Electroporation was as follows: a 1400-V pulse was applied, with a capacitance of 25 μF, a resistance of 200 Ω and a duration of around 3.5 ms. Samples were then divided into 150-μl aliquots and washed three times (10 000×g, 10 s and 4°C) with distilled water. Each cell sample was then used for one pH_i measurement in a 4-ml cuvette. Fluorescence intensities were monitored in a spectrofluorometer (Hitachi Instrument co., F4500, Japan) equipped with a stirred and thermostated (27°C) cuvette holder which was connected to an acquisition and processing system. Emission wavelength was 520 nm (slit width of 5 nm) and excitation was detected at 460 and 405 nm (slit width of 10 nm). After a few minutes, when the fluorescence signal was stable, values at 460 and 405 nm were noted and the content of the cuvette was rapidly taken with a syringe and filtered through a 0.2-μm pore filter. The fluorescence values at 460 and 405 nm of the supernatant were used for correction of the signal value and the ratio (r) was calculated as follows: $r = (460_{total} - 460_{filtrate})/(405_{total} - 405_{filtrate})$. Log ($r$) against pH was then used for pH_i calculations.

The integrity of physiological functions of electroporated cells was tested by comparing the protons efflux with that of reference cells, when transferred to a glucose (100 mM) solution.

For pH_i measurement in presence of octanoic acid (Aldrich, Germany), a volume from a 70-mM stock solution in ethanol was added in the cuvette.

2.3. Octanoic acid effects on cell membrane permeability and on probe leakage

The method was adapted from Sá-Correia et al. [12]. Cells grown for 19 h were recovered by centrifugation (5000×*g*, 5 min, 4°C) and washed with distilled water until the absorbance at 260 nm of the supernatant was inferior to 0.1. The cells were then resuspended in 20 ml of MilliQ® water, containing 0.8% ethanol for the reference or octanoic acid, and incubated on a shaking table (27°C, 140 rpm). At convenient intervals, 1.4-ml samples were removed and centrifuged (12 000×*g*, 5 min, 4°C). The supernatant was removed and transferred to a 1-ml cuvette, the absorbance at 260 nm was determined.

Pyranine efflux was determined by using the pH-independent excitation wavelength of 415 nm. After 5 min, the fluorescence of the medium with the cells then in the filtrate was measured and a percentage of leaked probe was calculated.

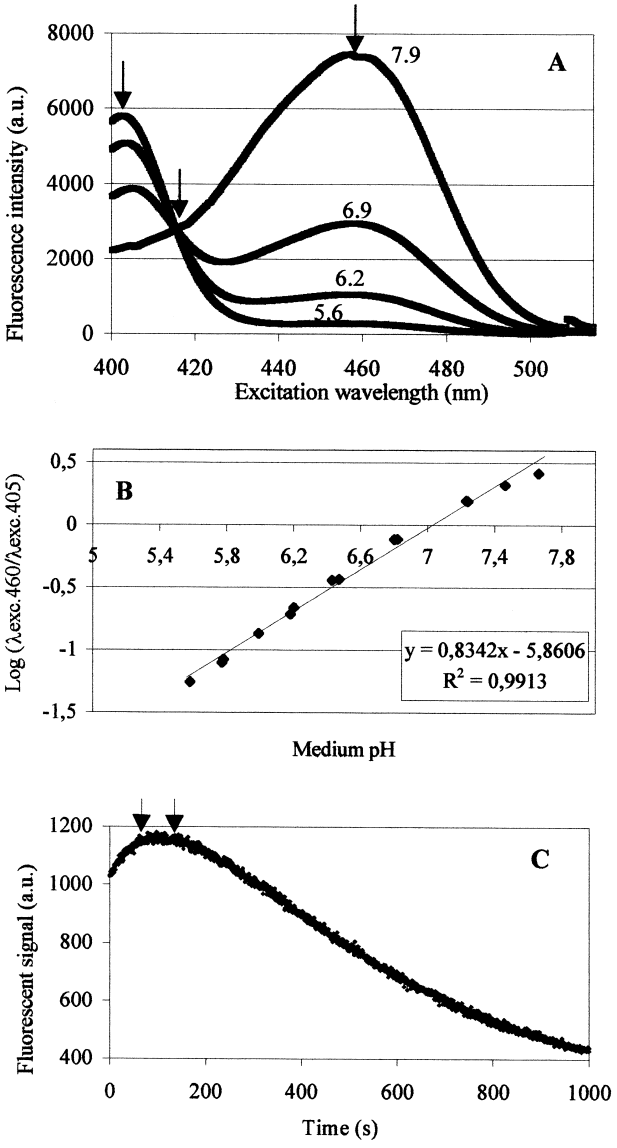

Fig. 1. pH_i measurement with pyranine. A: Excitation spectra of a pyranine solution in phosphate buffers at different pH (indicated on the curves). The arrows indicate the excitation wavelengths used in this study. B: Calibration curve obtained in buffered media in presence of the cells. The equation in the figure was used to calculate pH_i. C: Pyranine efflux monitoring at the excitation wavelength of 460 nm. The time lag between the arrows indicates the suitable time for pH_i measurements.

3. Results and discussion

3.1. pH_i determination

Pyranine excitation spectra in different pH media are presented in Fig. 1A: the signal values for 460- and 405-nm excitation wavelengths were pH-dependent and the isobestic point (pH-independent wavelength) was at 415 nm. In presence of heat-deactivated cells, Log (460/405) was linear between pH 5.4 and 7.8 (Fig. 1B), which corresponds to a physiological pH range.

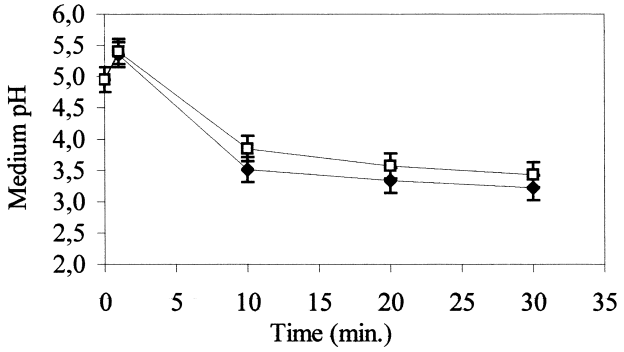

Fig. 2. Acidification of a medium containing glucose (100 mM), by electroporated (□) cells and control cells (♦).

The insertion of the fluorescent probe in the cytoplasm of the cells was verified by fluorescence microscopy (not shown). The treatment did not affect neither yeast viability (a methylene blue staining showed the same viability as reference cells (not shown)) nor physiology as is ascertained in Fig. 2: the electroporated cells acidified a glucose-containing medium in the same extent as reference cells. Once in the measurement cuvette, the fluorescence signal stabilized after a few minutes and it was maintained for several minutes, the cell suspension was rapidly filtrated, enabling the determination of fluorescent signals in the filtrate and then calculation of pH_i. If the cells were left longer, a decrease in fluorescence intensity at 460 nm could be observed (Fig. 1C), corresponding to a probe efflux as could be verified by detecting pyranine concentration in the supernatant at the pH-independent wavelength of 415 nm.

The pH_i of *Y. lipolytica* in a pH 3.9 medium was 7.1 (Fig. 3A), demonstrating a good ability of the yeast to maintain a high transmembrane pH gradient (3.2 pH units), even if no carbon source was added. This is, to our knowledge, the first determination of pH_i for *Y. lipolytica* and the observed value was in agreement with reports of some authors using a non-fluorescent method in *Saccharomyces cerevisiae* [13] and was slightly higher than values reported by Kotyk and Georghiou [3] in three yeast species using the fluorescent probe fluorescein diacetate, or by Peña et al. [11] using pyranine in *S. cerevisiae*.

3.2. pH_i decrease induced by octanoic acid

The presence of octanoic acid in the medium decreased pH_i almost linearly with its concentration (Fig. 3A), reaching a pH_i of 5.4 for 80 mg l^{-1} acid. This decrease was in agreement with that reported in *S. cerevisiae* by using [2-^{14}C]propionic acid distribution [14].

When octanoic acid was added to the cell suspension, a drop in the 460-nm signal was observed and it stabilized after a few seconds (Fig. 4). The acid concentrations we used did not modify the extracellular pH and the rapid decrease was thus not due to an effect on the probe present in the supernatant: the pH_i variation induced by octanoic acid appears to be a rapid process.

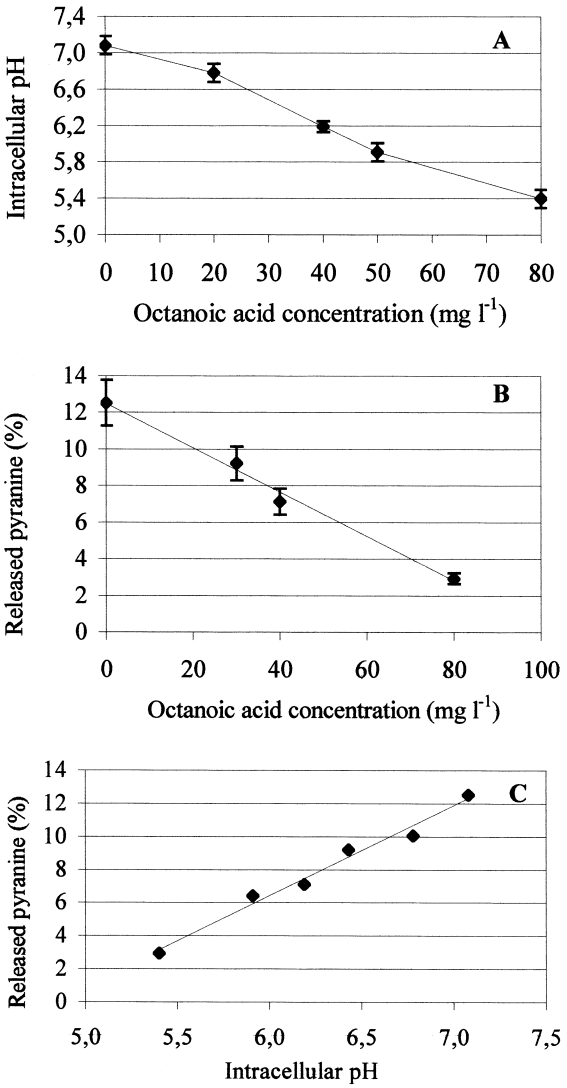

Fig. 3. Effect of octanoic acid concentration on, (A) pH_i in a pH 3.9 medium. (B) Release of pyranine from yeast cells. (C) Relation between release of pyranine and pH_i.

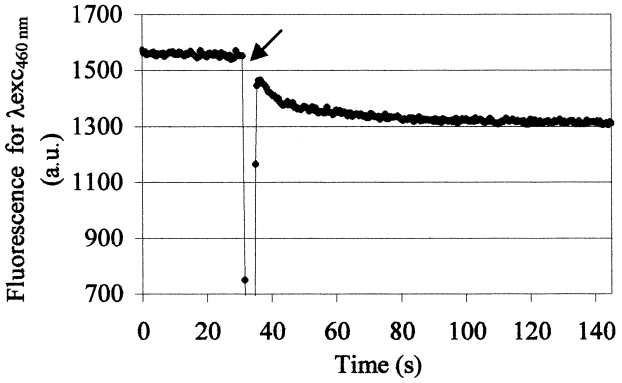

Fig. 4. Effect of octanoic acid (50 mg l^{-1}) on the fluorescence signal obtained for an excitation wavelength of 460 nm. The arrow indicates the acid addition to the cell suspension.

Octanoic acid is known to act as many lipophilic weak acids by entering yeast cells as undissociated molecules that are soluble in membrane lipids. Once inside the cell, the acid dissociates, releasing anions and protons, resulting in an acidification of the cytosol. Thus, this action is increased for low extracellular pH [13].

3.3. Octanoic acid effects on cell membrane permeability

When cells were maintained several hours in water, an increase in absorbance at 260 nm was observed in the supernatant. This absorbance is due to a leakage from an intracellular free pool of amino acids and 260-nm-absorbing compounds, that were shown to diffuse from cells by a passive mechanism [15]. The presence in the medium of agents enhancing membrane permeability, stimulates the leakage of these compounds [12]. Octanoic acid at concentrations of 40 mg l^{-1} and 80 mg l^{-1} increased the absorbance at 260 nm (Fig. 5), demonstrating an effect of this compound on cell membrane integrity.

3.4. Pyranine release by cells with acidified cytoplasm

After 5 min in the reference medium with or without ethanol 0.8%, around 13% of the probe was released (Table 1). If the medium contained glucose (50 mM), the percentage was slightly higher (15.7%). In the presence of octanoic acid, the percentage of released pyranine decreased linearly with the acid concentration in the medium, reaching 3% for 80 mg l^{-1} acid (Fig. 3B). The percentage of released probe was linearly correlated with the above-determined pH_i (Fig. 3A,B): the efflux of pyranine diminished for lowered pH_i. This observation is not consistent with a passive efflux of pyranine from the cells: one of the p*K*a values for pyranine-ionizable groups, in the conditions we used, was around 7.2, for increasing octanoic acid concentrations, the pH_i became lower, increasing the concentration of the undissociated form of pyranine inside the cell. An efflux occurring by a passive mechanism should be increased with a higher concentration of the undissociated acid, since the ion cannot pass through the phospholipid membrane.

The presence of glucose in the medium weakly increased the leakage, significantly compared to what was observed in the medium without carbon source but not to the medium containing ethanol (0.8%), which can be metabolized by *Y. lipolytica* [8]. Yeast cells in a medium without carbon source can still maintain a transmembrane pH gradient (Fig. 3A), however the functioning of cellular energy-dependent mechanisms might be improved with the availability of a carbon source such as glucose or in a lesser extent ethanol.

Table 1
Released pyranine from yeast cells in various pH 3.9 substrate-containing media

	None	Ethanol 0.8%	Glucose (0.05 M)
Released pyranine after 5 min (%)	12.5 ± 1.4	13.4 ± 1.2	15.7 ± 1.1

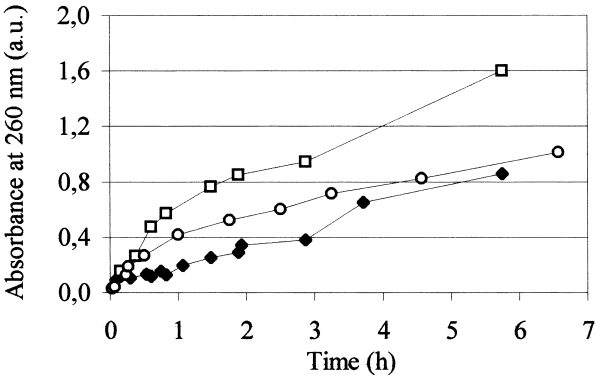

Fig. 5. Effect of octanoic acid on 260-nm-absorbing compounds release by reference yeast cells (♦) and in presence of octanoic acid 40 mg l^{-1} (○) or 80 mg l^{-1} (□).

These elements could thus indicate that pyranine extrusion from cells occurs via an energy-dependent carrier-mediated mechanism. Indeed, octanoic acid increased passive efflux of neutral compounds (Fig. 5), but did not raise the efflux of pyranine, which was correlated with the pH_i. One hypothesis for this could be that the activity of the membrane carrier could be altered by a cytoplasmic pH withdrawing from the optimal pH value: many metabolic processes in yeast are submitted to a fine regulation of the pH_i [16,17]. Holyoak et al. [18] reported in *S. cerevisiae*, the extrusion of the anionic fluorescent probe fluorescein via an ATP-binding cassette (ABC) transporter, also known to catalyze mono-anionic compounds extrusion. The fluorescein efflux was competitively inhibited by sorbic or benzoic acids and the ABC transporter appeared also to be specific for monocarboxylic acids with chain lengths from C1 to C7. Sorbic and benzoic acid are known to acidify the pH_i in yeast [17,19], but in the study of Holyoak et al. [18], the cells grown in the presence of these acids, adapted their pH_i which was then not directly linked to the fluorescein efflux. The occurrence in *Y. lipolytica* of a pyranine excretion via an ABC transporter-like carrier, competitively inhibited by octanoic acid, can be hypothesized too, since these transporters are present in all types of cells [20]. However, pyranine and octanoic acid are very different molecules with a different number of charged groups, turning difficult the consideration of a competition between both compounds towards a specific carrier, that is moreover, inducible.

The type of carrier implicated here could be, most likely, a transport system for the extrusion of more or less toxic anionic compounds from yeast cells, as hypothesized by Breeuwer et al. [7] for the efflux of fluorescein in *S. cerevisiae*.

The use of pyranine introduced in yeast cells by electroporation appears to be a convenient method for pH_i de-

termination. Our simple adaptation enabled a rapid signal correction, that was essential since part of the probe leaked from the cell interior. Moreover, the use of the pH-independent wavelength 415 nm can be useful to monitor the probe efflux. The method applied to the yeast *Y. lipolytica*, gave results in agreement with previously reported pH_i values in other yeast species. The cell-acidifying agent octanoic acid, proved to increase membrane permeability to neutral freely diffusing compounds, but inhibited pyranine release: the efflux of this compound was rather correlated with the pH_i of cells. These results constitute the first data dealing with the mechanisms of pyranine efflux: this pH-dependent phenomenon, could constitute a model system for the carrier-mediated efflux of compounds in living cells and could also help to better understand the excretion of secondary metabolites by yeast.

Acknowledgements

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References

[1] Von Recklinghausen, I.R., Molenaar, D., Konings, W.N. and Visser, J. (1993) Application of pH-sensitive fluorescent dyes to fungi. In: Biotechnology Applications of Microinjection, Microscopic Imaging, and Fluorescence (Bach, P.H. et al., Eds.), pp. 207–219. Plenum, New York.

[2] Ziegelbauer, K., Grusdat, G., Schade, A. and Paffhausen, W. (1999) High throughput assay to detect compounds that enhance the proton permeability of *Candida albicans* membranes. Biosci. Biotechnol. Biochem. 63, 1246–1252.

[3] Kotyk, A. and Georghiou, G. (1991) Protomotive force in yeasts – pH, buffer and species dependence. Biochem. Int. 24, 641–648.

[4] Slayman, C.L., Moussatos, V.V. and Webb, W.W. (1994) Endosomal localization of fluorescent indicators: vacuolar sequestration of –am dyes in *Neurospora* and in cultured mammalian fibroblasts. Biophys. J. 66, A275.

[5] Haworth, R.S. and Fliegel, L. (1993) Intracellular pH in *Schizosaccharomyces pombe* – comparison with *Saccharomyces cerevisiae*. Mol. Cell. Biochem. 124, 131–140.

[6] Liao, R.S., Rennie, R.P. and Talbot, J.A. (1999) Assessment of the effects of amphoterecin B on the vitality of *Candida albicans*. Antimicrob. Agents Chemother. 43, 1034–1041.

[7] Breeuwer, P., Drocourt, J.-L., Rombouts, F.M. and Abee, T. (1994) Energy-dependent, carrier-mediated extrusion of carboxyfluorescein from *Saccharomyces cerevisiae* allows rapid assessment of cell viability by flow cytometry. Appl. Environ. Microbiol. 60, 1467–1472.

[8] Barth, G. and Gaillardin, C. (1996) The dimorphic fungus *Yarrowia lipolytica*. In: Non-conventional yeasts in biotechnology (Wolf, K., Ed.), pp. 314–388. Springer-Verlag, Berlin.

[9] Waché, Y., Laroche, C., Bergmark, K., Møller-Andersen, C., Aguedo, M., Le Dall, M.-T., Wang, H., Nicaud, J.-M. and Belin, J.-M. (2000) Involvement of acyl coenzyme A oxidase isozymes in biotransformation of methyl ricinoleate into γ-decalactone by *Yarrowia lipolytica*. Appl. Environ. Microbiol. 66, 1233–1236.

[10] Endrizzi, A., Pagot, Y., Le Clainche, A., Nicaud, J.-M. and Belin, J.-M. (1996) Production of lactones and peroxisomal beta-oxidation in yeasts. Crit. Rev. Biotechnol. 16, 301–329.

[11] Peña, A., Ramírez, J., Rosas, G. and Calahorra, M. (1995) Proton pumping and the internal pH of yeast cells, measured with pyranine introduced by electroporation. J. Bacteriol. 177, 1017–1022.

[12] Sá-Correia, I., Salgueiro, S.P., Viegas, C.A. and Novais, J.M. (1989) Leakage induced by ethanol, octanoic and decanoic acids in *Saccharomyces cerevisiae*. Yeast 5, S123–S127.

[13] Pampulha, M.E. and Loureiro-Dias, M.C. (1989) Combined effect of acetic acid, pH and ethanol on intracellular pH of fermenting yeast. Appl. Microbiol. Biotechnol. 31, 547–550.

[14] Viegas, C.A. and Sá-Correia, I. (1995) Toxicity of octanoic acid in *Saccharomyces cerevisiae* at temperatures between 8.5 and 30°C. Enzyme Microb. Technol. 17, 826–831.

[15] Lee, T.C. and Lewis, M.J. (1968) Mechanism of release of nucleotidic material by fermenting brewer's yeast. J. Food Sci. 33, 124–128.

[16] Imai, T. and Ohno, T. (1995) The relationship between viability and intracellular pH in the yeast *Saccharomyces cerevisiae*. Appl. Environ. Microbiol. 61, 3604–3608.

[17] Krebs, H.A., Wiggins, D., Sols, S. and Bedoya, F. (1983) Studies on the mechanism of the antifungal action of benzoate. Biochem. J. 214, 657–663.

[18] Holyoak, C.D., Bracey, D., Piper, P.W., Kuchler, K. and Coote, P.J. (1999) The *Saccharomyces cerevisiae* weak-acid-inducible ABC transporter pdr12 transports fluorescein and preservative anions from the cytosol by an energy-dependent mechanism. J. Bacteriol. 181, 4644–4652.

[19] Bracey, D., Holyoak, C.D. and Coote, P.J. (1998) Comparison of the inhibitory effect of sorbic acid and amphotericin B on *Saccharomyces cerevisiae*: is growth inhibition dependent on reduced intracellular pH? J. Appl. Microbiol. 85, 1056–1066.

[20] Higgins, C.F. (1992) ABC transporters: from microorganisms to man. Annu. Rev. Cell Biol. 8, 67–113.