Effect of ethanol on cell growth, metabolism and cation fluxes in the yeast *Metschnikowia reukaufii*

HEINZ-ULRICH GLÄSER and MILAN HÖFER*

Botanisches Institut der Universität Bonn, Kirschallee 1, 5300 Bonn, FRG

(Received 4 May 1989; revised 16 January 1990; accepted 13 March 1990)

The yeast *Metschnikowia reukaufii* could not utilize ethanol as a growth substrate, although ethanol stimulated cellular O₂ consumption and the reduction of intracellular nicotinamide adenine nucleotides. Also, ethanol inhibited cell growth in glucose-containing medium. The effect was stronger when the K⁺ concentration in the growth medium was lowered from 5.8 to 0.6 mM. Addition of glucose to an aerated cell suspension caused an initial H⁺ efflux and K⁺ influx in a ratio of approximately 1:1. This was followed by a phase of continuing extracellular acidification without any measurable uptake of K⁺. In contrast, in cells energized by glucose, ethanol stimulated K⁺ efflux; concomitantly, H⁺ extrusion was markedly lowered by ethanol. The rates of H⁺ extrusion correlated with the intracellular level of glucose 6-phosphate and not of ATP. It is concluded that there is a regulatory interaction, though not by a direct effect, between glucose 6-phosphate and the plasma-membrane ATPase. Ethanol appears to activate electron transfer from cytosolic NADH to O₂ by a pathway independent of the mitochondrial respiratory chain.

Introduction

Ethanol is produced industrially by anaerobic fermentation of carbohydrates by yeasts or bacteria (Righelato, 1980). The problem in biotechnology is that high concentrations of ethanol kill the cells producing it. Therefore, the inhibitory effect of ethanol on cellular metabolism and growth, especially of the yeast *Saccharomyces cerevisiae*, has been the subject of considerable research (Leão & van Uden, 1982; Millar *et al.*, 1982; Ingram & Buttkle, 1984). Under aerobic conditions *S. cerevisiae* can also utilize ethanol as a carbon and energy source (Wasungu & Simard, 1982) and thus overcome its inhibitory effect on metabolism. This is in contrast to *Metschnikowia reukaufii* as shown in the present work.

Metabolism supplies the cell with energy to build up an electrochemical proton gradient ($\Delta\mu_{H^+}$) across the plasma membrane (Eddy, 1978) which, in turn, can be used for active sugar uptake (Aldermann & Höfer, 1981). As shown in reconstituted plasma-membrane vesicles the plasma-membrane-bound ATPase is the main generator of $\Delta\mu_{H^+}$ (Gläser & Höfer, 1987). Moreover, this H⁺-pump is stimulated strikingly in the presence of

K⁺ (Gläser & Höfer, 1986, 1987). Since addition of glucose to yeast suspensions usually causes an increase of active H⁺ extrusion *in vivo*, it seems very likely that the ATPase can be stimulated also by some metabolic signals. On the other hand, ethanol inhibited the H⁺ extrusion induced by glucose. Hence, in the present work the effect of ethanol on the energy-supplying system was investigated.

Methods

Growth conditions. The facultative anaerobic yeast *Metschnikowia reukaufii* (CBS 5834) was grown in a glucose-containing standard medium as described by Aldermann & Höfer (1981). After harvesting in the late exponential phase (15–17 h) and washing in double-distilled water, the cells were aerated for 2 h before use for the experiments. In growth media with reduced K⁺ concentrations, K₂HPO₄ was replaced as appropriate by Na₂HPO₄.

Measurements of pH and of K⁺ concentration. The extracellular pH was measured by a glass electrode and recorded by an analog digital transformer (Plischke & Buhr) connected to a Copam PC-401 Turbo personal computer. The intracellular pH was determined by the distribution of [¹⁴C]propionic acid (1 mM, 18.5 GBq mol⁻¹) across the plasma membrane as described by Cartwright *et al.* (1986), except that the buffer pH was 4.3. Potassium was measured by atomic absorption spectrophotometry (AAS type 360, Perkin-Elmer) as described by Hauer *et al.* (1981).

Abbreviations: FCCP, *p*-trifluoromethoxyphenylhydrazone; RQ, respiratory quotient.

Determination of intracellular ATP and glucose 6-phosphate concentrations. Cell suspension (1 ml) was mixed with 0.1 ml perchloric acid (6 M), frozen, thawed, and neutralized by a buffer containing 1.5 M-triethanolamine, 0.1 M-acetic acid and 4 M-KOH. The sample was centrifuged and the supernatant (0.1–0.4 ml) was added to 2 ml of buffer containing 50 mM-triethanolamine, pH 7.5, 1 mM-MgCl₂, 20 mM-KCl, 0.6 mM-NAD⁺ and 20 mM-glucose. The reaction was started at 20 °C by the addition of glucose-6-phosphate dehydrogenase (5 µl, 1100 U ml⁻¹) to measure the glucose 6-phosphate concentration. At the end of the reaction hexokinase (5 µl, 560 U ml⁻¹) was added for the determination of ATP. The stoichiometrically coupled reduction of NAD⁺ was measured as the increase of fluorescence by using a Hitachi fluorescence spectrometer type 650-10 M. The excitation and emission wavelengths were 342 and 460 nm, respectively. The increase of fluorescence was calibrated by defined amounts of both metabolites added successively to the assay.

For the determination of the internal cell volume, which was necessary for the calculation of intracellular pH and metabolite concentrations, the cell dry weight was measured: 1 mg dry wt corresponded to 2 µl intracellular volume as determined by the methods of Rottenberg (1979).

Changes in NADH content. Reduction of nicotinamide adenine nucleotides can be followed *in vivo* by measuring the increase in cellular fluorescence (Duysens & Ames, 1957). The cell suspension (0.16 mg dry wt ml⁻¹) was stirred at 20 °C in a cuvette of the fluorimeter (see above) to maintain aerobic conditions. The increase in relative fluorescence after addition of a substrate was recorded. In control experiments cells were uncoupled by 0.2 mM FCCP or boiled for 30 min; no fluorescence change by added substrates was observed.

Measurements of cellular O₂ consumption and CO₂ production. The decrease in O₂ concentration in cell suspensions (1.5–2 mg dry wt ml⁻¹) immediately after substrate addition was measured by an oxygen-sensing electrode (Beckmann). For longer observations and for the determination of CO₂ production a conventional Warburg manometer (B. Braun) was used as described by Kleinzeller (1965). For these experiments cells were resuspended (4–6 mg dry wt ml⁻¹) in 0.15 M-KH₂PO₄ buffer, pH 4.3, at 22 °C.

Measurements of enzyme activity. The isolation of plasma membranes from *M. reukaufii* and the enzyme assay to determine the plasma-membrane ATPase activity were described by Gläser & Höfer (1986, 1987). Alcohol dehydrogenase activity was determined in crude extracts after disruption of cells with glass beads (Ballotini). Extract (0.2 ml) was added to 2.2 ml buffer (pH 8.7) containing 0.4 mg glutathione ml⁻¹ and 0.2 mg NAD⁺ ml⁻¹. The reaction was started at 20 °C by addition of 1.5 M-ethanol and the increase in absorbance at 366 nm was measured in an Eppendorf photometer type 1101 M.

Chemicals. Diethylstilboestrol, semicarbazide and sodium pyrophosphate were from Sigma; ATP, hexokinase, glucose-6-phosphate dehydrogenase and NAD⁺ were from Boehringer. Orthovanadate was from ICN Pharmaceuticals and FCCP from Fluka. All other chemicals were from Merck. Solutions were made in double-distilled water.

Results

Growth of *M. reukaufii* on media containing glucose and/or ethanol under aerobic conditions

When cultures were transferred to glucose-containing standard medium, exponential growth continued and the stationary phase was reached after 18–21 h (Fig. 1). However, the cells could not grow on ethanol as sole

Fig. 1. Effect of ethanol on growth of *M. reukaufii* under aerobic conditions. Growth media: no carbon source (■); 0.13 M-glucose (○); 0.5 M-ethanol (▲); 0.13 M-glucose + 0.5 M-ethanol (●).

carbon source (Fig. 1). The generation time of *M. reukaufii* cells in glucose-containing standard medium was increased by ethanol (0.5 M) from 3.3 to 4.5 h (Fig. 1). Moreover, during growth in the presence of glucose plus ethanol the cell shape changed from elongated to ovoidal (not shown). Although the K⁺ concentration in glucose-containing standard media was 5.8 mM, a concentration of 0.6 mM was sufficient for maximal growth of *M. reukaufii*. The generation time in medium with a low K⁺ concentration (0.6 mM) was increased more by ethanol (to 5.7 h) than in medium with the standard K⁺ concentration (see above). This is very likely due to the induction by ethanol of K⁺ (and H⁺) fluxes across the plasma membrane (see Fig. 2b).

Effect of glucose and ethanol on H⁺ and K⁺ fluxes in aerated cell suspensions

As observed in several other yeasts, e.g. *S. cerevisiae* (Eddy, 1978; Sigler *et al.*, 1981), *Schizosaccharomyces pombe* (Foury *et al.*, 1977) and *Rhodotorula glutinis* (Höfer & Misra, 1978), glucose induced a strong acidification of *M. reukaufii* cell suspensions (Fig. 2a). CO₂ production (see Fig. 3a) contributed to the acidification only to a minor extent, since bubbling CO₂ into yeast suspensions increased the H⁺ concentration to only 80 µM. Diethylstilboestrol (0.2 mM) and orthovanadate (0.2 mM), specific inhibitors of the plasma-membrane ATPase (Goffeau & Slayman, 1981), lowered the initial velocity of glucose-induced acidification by 79% and 58%, respectively, following 15 min preincubation (not shown). This inhibition of acidification correlated with the reduction of the ATPase activity of purified plasma membranes, which was inhibited half-maximally by 8 µM-orthovanadate and 74 µM-diethylstilboestrol. These results indicate that a stimulation of the plasma-membrane ATPase is mainly responsible for the glucose-induced acidification.

Fig. 2. H^+ ($\circ$) and K^+ ($\square$) concentrations measured in unbuffered suspensions of *M. reukaufii* following addition (open symbols) at zero time of (a) glucose, (b) ethanol and (c) glucose + ethanol as compared to cell suspensions without added substrate (filled symbols in b). In addition, the intracellular glucose 6-phosphate concentration ($\triangle$) was determined. The inset in (a) shows extracellular H^+ ($\circ$) and K^+ ($\square$) concentration at the start of experiments plotted on an extended time-scale. Yeast cells were suspended in double-distilled water ($7.3 \text{ mg dry wt ml}^{-1}$ for ion measurements and $10.8 \text{ mg dry wt ml}^{-1}$ for determination of intracellular metabolites). Concentration of added substrates: glucose (0.02 M); ethanol (0.5 M). The results are means of three measurements.

Ethanol (0.5 M) did not induce acidification of *M. reukaufii* suspensions (Fig. 2b) but strongly inhibited glucose-induced acidification (Fig. 2c), possibly because the plasma-membrane ATPase is inhibited by ethanol: ATPase activity of purified plasma membranes was inhibited 30% by 0.5 M -ethanol. Another reason for reduced acidification may be the reduced (by up to 40%) rate of glucose uptake in the presence of 0.5 M -ethanol. However, at the same time respiration was stimulated (see Fig. 3) with virtually no change in cellular ATP level (data not shown).

In aerated suspensions of *M. reukaufii* a steady K^+ efflux over a period of 5 h was observed; this efflux was

virtually unchanged in the presence of ethanol (Fig. 2b). However, release of K^+ from cells was maximal when glucose was applied together with ethanol (Fig. 2c). In contrast, glucose alone induced a transient uptake of K^+ into the cells which was initially stoichiometric with H^+ extrusion; the net flux of K^+ subsequently ceased and the external K^+ concentration changed only slightly over the next 4 h (Fig. 2a). It is evident that ethanol enhanced the permeability of the plasma membrane to K^+ , particularly in energized cells. A similar stimulation of K^+ efflux (but without any delay) was observed when glucose was added to cell suspensions preincubated with diethylstilboestrol (results not shown).

Comparison of the effect of glucose and of ethanol on cell metabolism

The stimulation of H^+ extrusion by added glucose could not be caused by the enhanced (5 mM) ATP concentration; the intracellular ATP concentration in resting cells was 4 mM and hence sufficiently high to permit maximal ATPase activity. The K_m for ATP of the plasma-membrane ATPase is 0.6 mM (Gläser & Höfer, 1986).

In contrast, the intracellular concentration of glucose 6-phosphate in *M. reukaufii* first increased after addition of glucose and then decreased after 8 min (Fig. 2a). The increase was delayed and considerably lessened in the presence of ethanol (Fig. 2c) as expected from the observed decrease in glucose uptake. The rate of H^+ extrusion was also delayed and reduced by ethanol ($0.8 \text{ nmol ml}^{-1} \text{ min}^{-1}$ after 6 min as compared with $17 \text{ nmol ml}^{-1} \text{ min}^{-1}$ in the presence of glucose alone), and correlated well with the increase in glucose 6-phosphate concentration. However, the ATPase activity of purified plasma membranes was stimulated by neither glucose nor glucose 6-phosphate. Addition of ethanol alone did not change (not shown) the endogenous glucose 6-phosphate concentration ($<0.3 \text{ mM}$).

The intracellular pH of *M. reukaufii* cells also decreased (from 6.64 to 6.40) following addition of glucose. Ethanol did not cause intracellular acidification and did not affect that induced by glucose. The intracellular acidification in the presence of glucose, in spite of the increased H^+ extrusion, may be explained by glucose catabolism (phosphorylation of glucose, stimulated CO_2 production; cf. Figs 2 and 3).

Effect of ethanol on cell respiration and on the redox state of nicotinamide adenine nucleotides

CO_2 production and O_2 consumption by *M. reukaufii* are shown in Fig. 3(a, b). O_2 consumption was stimulated by both ethanol (0.5 M) and by glucose (0.02 M) to the same

extent when measured shortly after their addition (Fig. 3b). When glucose and ethanol were added together the effect was additive (not shown). Prolonged measurements over a period of 2 h revealed that the stimulation in O_2 consumption induced by ethanol decreased with time, whereas that stimulated by glucose persisted (Fig. 3b). The non-metabolizable monosaccharide xylose did not stimulate O_2 uptake (not shown).

No increase in CO_2 production was observed following addition of either ethanol (Fig. 3a) or xylose (the latter not shown). This again indicates that *M. reukaufii* cannot utilize ethanol for growth. However, the intracellular NADH content was increased more by ethanol than by glucose (cf. Fig. 4b and Fig. 4a, respectively). Correspondingly, the crude extract exhibited a NAD^+ -

dependent alcohol dehydrogenase activity of $0.058\text{ U (mg protein)}^{-1}$. When glucose and ethanol were added together (Fig. 4c) the intracellular NADH content reflected both the characteristic rapid reduction of NAD^+ by ethanol (cf. Fig. 4b) and the initial drop followed by a steady increase in NADH content characteristic of glucose catabolism (cf. Fig. 4a). In addition, since respiration and CO_2 production from glucose metabolism were not inhibited by ethanol (Fig. 3a, b) interference by ethanol with glucose metabolism can be excluded.

Discussion

Cells of the yeast *M. reukaufii* could not use ethanol as substrate for growth or for oxidative metabolism producing CO_2 . In contrast, cells of *S. cerevisiae* can metabolize ethanol either added to the medium (Brown *et al.*, 1981; Wasungu & Simard, 1982) or produced by the cells under aerobic conditions (Haarasilta & Oura, 1975; Käppli, 1986). The minimal K^+ concentration for uninhibited growth was 0.4 mM (Camacho *et al.*, 1981). Mutants of *S. cerevisiae* have been selected which display similar physiological behaviour to *M. reukaufii*: they cannot grow in media containing ethanol as sole carbon source despite their unchanged glucose catabolism (Aguilera & Benitez, 1986). It was therefore of interest to investigate *in vivo* the specific effect of ethanol on cell metabolism and on ion fluxes across the plasma membrane which are driven by metabolic energy.

The extracellular acidification induced by glucose metabolism results from the functioning of the plasma-

Fig. 3. CO_2 production (a) and O_2 consumption (b) by *M. reukaufii* cell suspensions after addition (arrowed) of 0.02 M-glucose (○, ●), 0.02 M-glucose + 0.5 M-ethanol (▲, △) or 0.5 M ethanol (□, ■). The results are means of three to six measurements by conventional manometry.

Fig. 4. Changes in intracellular NADH content induced by addition (arrowed) of 0.02 M-glucose (a), 0.5 M-ethanol (b) or both (c). Cells were suspended in double-distilled water and stirred continuously. The change in fluorescence of NADH was measured at 342 nm (excitation) and 460 nm (emission).

membrane ATPase. This conclusion is based on the correlation of inhibition of the two processes by diethylstilboestrol and by orthovanadate. Moreover, the contribution of CO₂ released from glucose metabolism to the decrease in extracellular pH was negligible in the pH region below 4.5. The extrusion of H⁺ through the ATPase was initially accompanied by an approximately stoichiometric uptake of K⁺, which subsequently decreased and then ceased after 5–10 min. The electrical compensation for H⁺ extruded under these conditions is not clear. In *S. cerevisiae* an efflux of organic anions occurs (Pulver & Verzar, 1940; Rothstein & Bruce, 1958; Sigler *et al.*, 1981) which could compensate for the charge translocation by the ATPase.

The rate of H⁺ extrusion in *M. reukaufii* was not regulated by intracellular ATP concentration which remained fairly constant (4–5 mM) both in the presence and absence of glucose or ethanol. By contrast, in *S. cerevisiae* the intracellular ATP concentration was, although only transiently (5–15 min), greatly increased in the presence of glucose or ethanol (Rothman & Cabib, 1969; Maitra & Estabrook, 1967). However, in *M. reukaufii* the intracellular glucose 6-phosphate concentration appeared to be correlated with the rate of H⁺ extrusion induced by added glucose, and both were lowered in the presence of ethanol. Hence, glucose 6-phosphate appears to be involved in the regulation of H⁺ extrusion by the plasma-membrane ATPase. However, since neither glucose nor glucose 6-phosphate stimulated the ATPase activity of isolated plasma membranes their intracellular concentrations may act as signals for as-yet-unidentified metabolic intermediate(s) involved in regulation of the plasma-membrane ATPase activity. Sychrova & Kotyk (1985), however, reported an increased ATPase activity in the isolated plasma-membrane fraction from *S. cerevisiae* cells incubated with glucose. A similar observation was made with *M. reukaufii*: the ATPase activity of the plasma-membrane fraction from starved cells (aerated for 2 h at 30 °C) was one-third lower than that of freshly harvested cells grown on glucose (results not shown).

The mechanism of the increased K⁺ efflux from cells incubated with both glucose and ethanol is also unclear. It coincided with a reversal of H⁺ efflux and a drop in respiration. Direct inhibition of glucose catabolism by ethanol can be excluded (except for the decrease in glucose 6-phosphate concentration) since CO₂ production in the presence of glucose with and without ethanol was virtually the same. Addition of ethanol alone stimulated cellular respiration without an increase in CO₂ production, thus leading to respiratory quotient (RQ) values around 0.3 (the theoretical RQ for ethanol is 0.66). Correspondingly, the intracellular ATP concentration was not increased by added ethanol. In contrast to

CO₂ production, O₂ consumption appeared to be additive in the presence of glucose plus ethanol. The correlation of early changes in the redox state of cellular nicotinamide adenine nucleotides in the presence of glucose, ethanol or both supports this view. The low RQ values in the presence of ethanol suggest that electrons from ethanol are transferred to O₂ via a pathway outside the mitochondria (Crane *et al.*, 1982). This pathway is obviously activated by added ethanol.

Ethanol affects the physiology of *M. reukaufii* cells by indirectly inhibiting the plasma-membrane ATPase. This effect is correlated with changes in intracellular glucose 6-phosphate concentration and increased plasma-membrane permeability to K⁺ (in the presence of glucose). The intracellular glucose 6-phosphate concentration also correlates with the rate of H⁺ extrusion by the ATPase. Hence, a regulatory interaction between this early phosphorylated intermediate of the glucose catabolic pathway and the ATPase function of the plasma membrane has to be postulated. This regulatory mechanism requires further investigation.

This paper is dedicated to Professor Augustin Betz on the occasion of his 70th birthday.

References

- AGUILERA, A. & BENITEZ, T. (1986). Ethanol-sensitive mutants of *Saccharomyces cerevisiae*. *Archives of Microbiology* **143**, 337–344.
- ALDERMANN, B. & HÖFER, M. (1981). The active transport of monosaccharides by the yeast *Metschnikowia reukaufii*: evidence for an electrochemical gradient of H⁺ across the cell membrane. *Experimental Mycology* **5**, 120–132.
- BROWN, S. W., OLIVER, S. G., HARRISON, D. E. F. & RIGHELATO, R. C. (1981). Ethanol inhibition of yeast growth and fermentation: differences in the magnitude and complexity of the effect. *European Journal of Applied Microbiology and Biotechnology* **11**, 151–155.
- CAMACHO, M., RAMOS, J. & RODRIGUEZ-NAVARRO, A. (1981). Potassium requirements of *Saccharomyces cerevisiae*. *Current Microbiology* **6**, 295–299.
- CARTWRIGHT, C. P., JUROSZEK, J.-R., BEAVEN, M. J., RUBY, F. M. S., DE MORAIS, S. M. F. & ROSE, A. H. (1986). Ethanol dissipates the proton-motive force across the plasma membrane of *Saccharomyces cerevisiae*. *Journal of General Microbiology* **132**, 369–377.
- CRANE, F. L., ROBERTS, H., LINNANE, A. W. & LOW, H. (1982). Transmembrane ferricyanide reduction by cells of the yeast *Saccharomyces cerevisiae*. *Journal of Bioenergetics and Biomembranes* **14**, 191–205.
- DUYSENS, L. N. M. & AMESZ, J. (1957). Fluorescence spectrophotometry of reduced phosphopyridine nucleotide in intact cells in the near-ultraviolet and visible region. *Biochimica et Biophysica Acta* **24**, 19–26.
- EDDY, A. A. (1978). Proton-dependent solution transport in microorganisms. *Current Topics in Membranes and Transport* **10**, 278–360.
- FOURY, F., BOUTRY, M. & GOFFEAU, A. (1977). Efflux of potassium induced by Dio-9, a plasma membrane ATPase inhibitor in the yeast *Schizosaccharomyces pombe*. *Journal of Biological Chemistry* **252**, 4577–4583.
- GLÄSER, H.-U. & HÖFER, M. (1986). Effect of cations on the plasma-membrane-bound ATPase from the yeast *Metschnikowia reukaufii*. *Journal of General Microbiology* **132**, 2615–2620.

- GLÄSER, H.-U. & HÖFER, M. (1987). Ion-dependent generation of the electrochemical proton gradient $\Delta\tilde{\mu}_{H^+}$ in reconstituted plasma membrane vesicles from the yeast *Metschnikowia reukaufii*. *Biochimica et Biophysica Acta* **905**, 287–294.
- GOFFEAU, A. & SLAYMAN, C. W. (1981). The proton-translocating ATPase of the fungal plasma membrane. *Biochimica et Biophysica Acta* **639**, 197–223.
- HAARASILTA, S. & OURA, E. (1975). On the activity and regulation of anapleurotic and gluconeogenic enzymes during the growth phase of baker's yeast. *European Journal of Biochemistry* **52**, 1–7.
- HAUER, R., UHLEMANN, G., NEUMANN, J. & HÖFER, M. (1981). Proton pumps of the plasmalemma of the yeast *Rhodotorula gracilis*. Their coupling to fluxes of potassium and other ions. *Biochimica et Biophysica Acta* **649**, 680–690.
- HÖFER, M. & MISRA, P. C. (1978). Evidence for a proton/sugar symport in the yeast *Rhodotorula gracilis* (glutinis). *Biochemical Journal* **172**, 15–22.
- INGRAM, L. O. & BUTTKE, T. M. (1984). Effects of alcohols on microorganisms. *Advances in Microbial Physiology* **25**, 254–300.
- KÄPPELI, O. (1986). Regulation of carbon metabolism in *Saccharomyces cerevisiae* and related yeasts. *Advances in Microbial Physiology* **28**, 181–209.
- KLEINZELLER, A. (1965). *Manometrische Methoden*. Jena: Gustav Fischer.
- LEÃO, C. & VAN UDEN, N. (1982). Effect of ethanol and other alkanols on the glucose transport system of *Saccharomyces cerevisiae*. *Biotechnology and Bioengineering* **24**, 2601–2604.
- MAITRA, P. K. & ESTABROOK, R. W. (1967). Studies of baker's yeast metabolism. II. The role of adenine nucleotides and inorganic phosphate in the control of respiration during alcohol oxidation. *Archives of Biochemistry and Biophysics* **121**, 129–139.
- MILLAR, D. G., GRIFFITH-SMITH, K., ALGAR, E. & SCOPES, R. K. (1982). Activity and stability of glycolytic enzymes in the presence of ethanol. *Biotechnology Letters* **4**, 601–609.
- PULVER, R. & VERZAR, F. (1940). Der Zusammenhang von Kalium und Kohlehydratstoffwechsel bei der Hefe. *Helvetica Chimica Acta* **23**, 1087–1100.
- RIGHELATO, R. C. (1980). Anaerobic fermentation: alcohol production. *Philosophical Transactions of the Royal Society* **B290**, 303–312.
- ROTHMAN, L. B. & CABIB, E. (1969). The efflux of potassium from yeast cells into a potassium free medium. *Biochemistry* **8**, 3332–3341.
- ROTHSTEIN, A. & BRUCE, M. (1958). Regulation of glycogen synthesis in the intact yeast cell. *Journal of Cellular and Comparative Physiology* **51**, 439–455.
- ROTTENBERG, H. (1979). The measurements of membrane potential and ΔpH in cells, organelles, and vesicles. *Methods in Enzymology* **55**, 547–549.
- SIGLER, K., KOTYK, A., KNOTKOVA, A. & OPERAKOVA, M. (1981). Processes involved in the creation of buffering capacity and in substrate-induced proton extrusion in the yeast *Saccharomyces cerevisiae*. *Biochimica et Biophysica Acta* **643**, 583–592.
- SYCHROVA, H. & KOTYK, A. (1985). Conditions of activation of yeast plasma membrane ATPase. *FEBS Letters* **183**, 21–24.
- WASUNGU, K. M. & SIMARD, R. E. (1982). Growth characteristics of baker's yeast in ethanol. *Biotechnology and Bioengineering* **24**, 1125–1134.