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AN INVESTIGATION INTO THE FEASIBILITY OF USING YEAST PROTOPLASTS TO STUDY THE ION TRANSPORT PROPERTIES OF THE PLASMA MEMBRANE

A.P.R. THEUVENET and R.J.M. BINDELS

Department of Chemical Cytology, Faculty of Science, University of Nijmegen, Toernooiveld, Nijmegen (The Netherlands)

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Summary

A method for studying ion uptake in enzymatically isolated protoplasts from the yeast, *Saccharomyces cerevisiae*, is described. The kinetics of K^+ and Rb^+ uptake, metabolic proton extrusion and cell electrophoretic mobility have been determined. Enzymic removal of the cell wall does not significantly alter the above-mentioned properties of the yeast cells. It is concluded that studies of these properties can be performed equally well with intact yeast cells or protoplasts. However, in studies aimed at determining effects of complex organic substances, e.g., antibiotics, on plasma membrane function the use of protoplasts is recommended. The effectiveness of the antibiotic, Dio-9, for example, in reversing the metabolic proton extrusion into a net proton influx is at least 50 times higher after enzymic removal of the yeast cell wall.

Introduction

Yeast cells are surrounded by a rigid cell wall. This wall represents a physical barrier which prevents direct experimental access to the plasma membrane. For example, chemical inhibitors of specific enzymes isolated from the plasma membrane might show no effect at all when applied to intact cells, due to diffusional limitations exerted by the rigid cell wall. The yeast cell wall as a diffusional barrier or a trap for substrates can, however, be easily eliminated. In the past two decades several reports appeared on the enzymic removal of the yeast cell wall [1—6]. Whether or not this enzymic treatment affects the transport

Abbreviation: Mes, 4-morpholineethanesulfonic acid.

properties of the plasma membrane and the metabolic energization of transport is examined in this report. We will show that isolated yeast protoplasts provide a model system of the yeast cell without the interference by the cell wall for studies on effects of complex organic substances, e.g., antibiotics on the kinetics of ion uptake.

Materials and Methods

Cell culture. A non-flocculent brewing yeast (strain *Saccharomyces cerevisiae* A 294, Whitbread and Co., Ltd., Luton, U.K.) was grown on minimal medium (30 g glucose, 5.9 g succinic acid and 6.7 g Yeast Nitrogen Base (Difco) per l). The pH of the medium was adjusted to 4.5 with K_2HPO_4. The cells were grown overnight at room temperature and harvested in the early exponential phase of growth by centrifugation for 5 min at 2000 × *g*. The cells were washed three times with distilled water.

Protoplast formation. Protoplasts were prepared according to a slightly modified procedure described by Kotahda et al. [6]. The washed yeast cells (10^8 cells/ml) were incubated with gentle shaking at 30°C for 30 min in a medium containing 22.5 mM Tris/Mes buffer, pH 7.5, 12 mM 2-mercaptoethanol, 0.7 mg/ml Zymolyase-5000, 2.1 mg/ml Cellulase Onozuka R-10 and 0.82 M sorbitol as an osmotic stabilizer. By this treatment the yeast cells were completely converted into protoplasts (see Results). The protoplasts were isolated from the medium by centrifugation for 5 min at 2000 × *g* and washed by repeated centrifugation in 0.82 M sorbitol containing 1 mM $CaCl_2$ (osmotic stabilizer solution). All experiments described in this article were performed with freshly prepared protoplasts.

Metabolic proton extrusion. Net proton fluxes were measured by means of a pH-stat (Radiometer TTT2/PHA 942, Copenhagen). Proton fluxes were measured at pH 4.5 and at 25°C under anaerobic conditions. Depending on the direction of the net transmembrane proton fluxes, 30 mM triethanolamine or 0.1 N HCl was automatically titrated into the suspension and the pH was kept constant at pH 4.5.

K^+ uptake. Uptake of K^+ by anaerobic cells was followed by measuring the decrease in K^+ activity in the medium by means of a K^+-sensitive electrode (Philips IS 560-K) connected to a Pye Unicam Model 292 pH meter, in 45 mM Tris/succinate buffer, pH 4.5, provided with 3% (w/v) glucose, 0.82 M sorbitol and 1 mM $CaCl_2$. The KCl was added after a 5 min preincubation period with glucose.

Rb^+ uptake. Washed cells (protoplasts or intact yeast cells, 1.3—2.0 mg protein/ml) were preincubated in 45 mM Tris/succinate buffer, pH 4.5, provided with 3% (w/v) glucose, 0.82 M sorbitol and 1 mM $CaCl_2$ at 25°C. After a 5 min preincubation period, ^{86}RbCl with or without non-radioactive RbCl (1—10 mM final concentration) was added to the cell suspension. The radioactive cocktail also contained carrier-free [^{14}C]mannitol for determination of the mannitol-permeable space in order to correct the ^{86}Rb counts for contribution by extracellular ^{86}Rb counts. Nine successive 0.5 ml samples of the suspension were taken during a 5—10 min incubation period. Uptake of Rb^+ was stopped by diluting the 0.5 ml sample with 1 ml of ice-cold, pH 4.5

buffered, osmotic stabilizer solution also containing 50 mM KCl in an Eppendorf tube. The tube was centrifuged for 15 s in a microcentrifuge at maximal speed (10 000 × *g*). The supernatant was removed by aspiration and the centrifuge tube was carefully blotted dry. After resuspension of the pellet in 0.6 ml osmotic stabilizer solution, 0.5 ml was dissolved in 4.5 ml Packard scintillation fluid. Radioactivity (^{14}C and ^{86}Rb) was measured in a Philips liquid scintillation analyser.

Cell electrophoresis. Electrophoretic mobilities were measured at 25°C using a rectangular cuvette and apparatus as described by Fuhrmann et al. [7]. The composition of the buffer solutions was 45 mM Tris adjusted to the desired pH with succinic acid. From electrophoretic mobility, the zeta potential was calculated by using the Helmholtz-Smoluchowski equation [8]. The S.E. was in the order of 2—4%, never exceeding 10%.

Microscopy. Light micrographs were taken of freshly prepared protoplasts and yeast cells in osmotic stabilizer solution with a Carl Zeiss photomicroscope with phase contrast optics.

For freeze-etching, glutaraldehyde-fixed cells, placed on a copper disc, were frozen in liquid Freon 22. Freeze-etching was performed in a Balzers BA 360 apparatus. A platinum-carbon replica of the etched object surface was investigated in a Philips EM 300 electron microscope.

Protein determination. Prior to the protein determination according to the method of Lowry et al. [9], cell lipids were extracted in an ethanol/ether (3 : 1) mixture, according to the method of Wanka [10].

Osmotic lysis. The formation of protoplasts was followed during the enzymic digestion by taking samples at intervals and diluting them in either osmotic stabilizer solution or in water and counting the cells under a Wild phase-contrast microscope. If in water no intact cells were observed, the enzymic digestion of the cell wall was considered to be completed.

Chemicals. Zymolyase-5000 was purchased from Kirin Brewery Co., Ltd. and Cellulase-Onozuka R-10 from Kinki Yakult Co., Ltd. Dio-9 was from Gist-Brocades at Delft. Yeast Nitrogen Base was from Difco. ^{86}Rb and [^{14}C]mannitol were from the Radiochemical Centre, Amersham. Packard liquid scintillation fluid (Emulsifier Scintillator-299) was from Packard Becker B.V. Chemical Operations, Groningen. All other reagents were A.R. grade.

Results

Protoplast formation

Three criteria were used to evaluate the formation of protoplasts.

(a) Their osmotic fragility, measured by microscopic counting of the cells before and after dilution in water, which caused bursting of protoplasts. Fig. 1 shows that after 30 min of enzymic treatment all yeast cells became osmofragile.

(b) Their spherical shape, very different from the ovoid shape of ordinary cells of *S. cerevisiae* (Figs. 2 and 3).

(c) The presence of characteristic invaginations in the plasma membrane [11, 12], which can be seen in a freeze-etch replica, provided the cell wall is completely removed. These invaginations were clearly seen in the preparation of 30-min enzyme-treated yeast cells (Fig. 4).

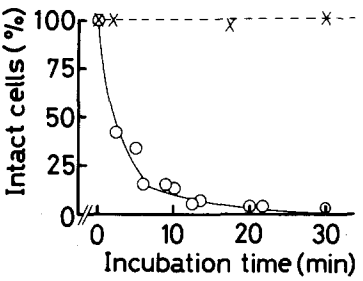

Fig. 1. Osmotic lysis resistance of yeast cells as a function of the time of enzymic treatment. The cells were lysed by osmotic shock in bi-distilled water. Results were estimated by microscopic counting. (X) Control, no osmotic shock; (○) after osmotic shock. Number of cells at zero time was taken as 100%. Points represent means of triplicate determinations.

Metabolic proton extrusion

Under anaerobic conditions, addition of glucose to the cell suspension caused an initial proton uptake by the cells which gradually changed into a net proton extrusion. Fig. 5 shows that enzymic removal of the cell wall did not affect this typical glucose response of the yeast cells. However, the sensitivity of the metabolic proton extrusion for the antibiotic Dio-9 was greatly enhanced after removal of the cell wall. A dose of 3 μg/ml of Dio-9 caused a reversal of the net proton flux when applied to protoplasts, whereas with intact

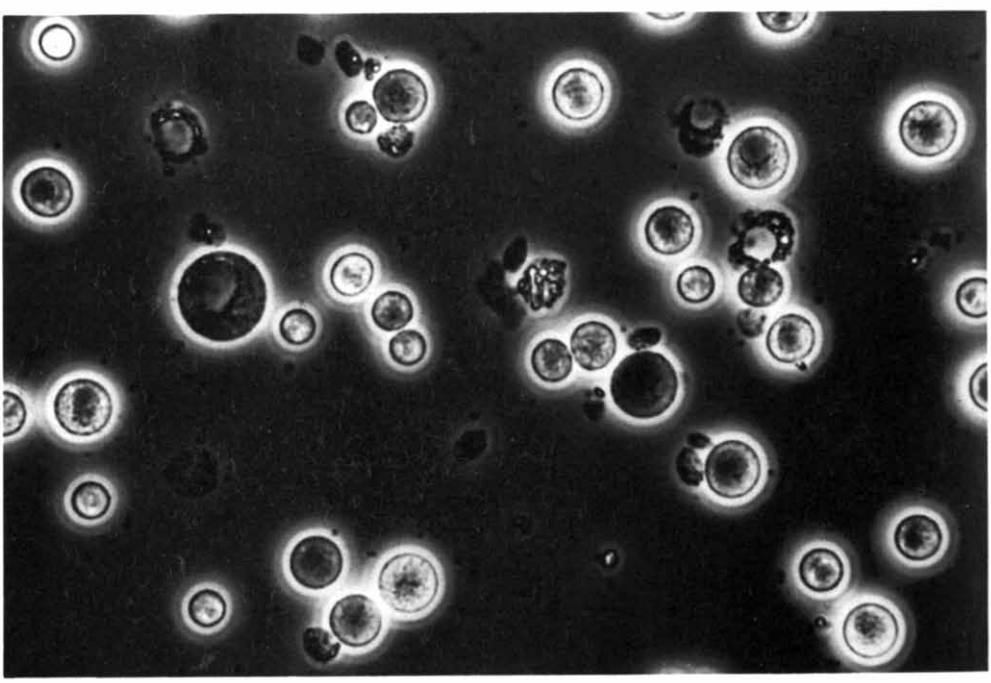

Fig. 2. Protoplasts of *Saccharomyces cerevisiae* A 294 observed in phase-contrast microscopy. Magnification: X1080. The micrograph shows a sample of the cell suspension after 30-min enzymic treatment. The dark spots are residues of cell walls.

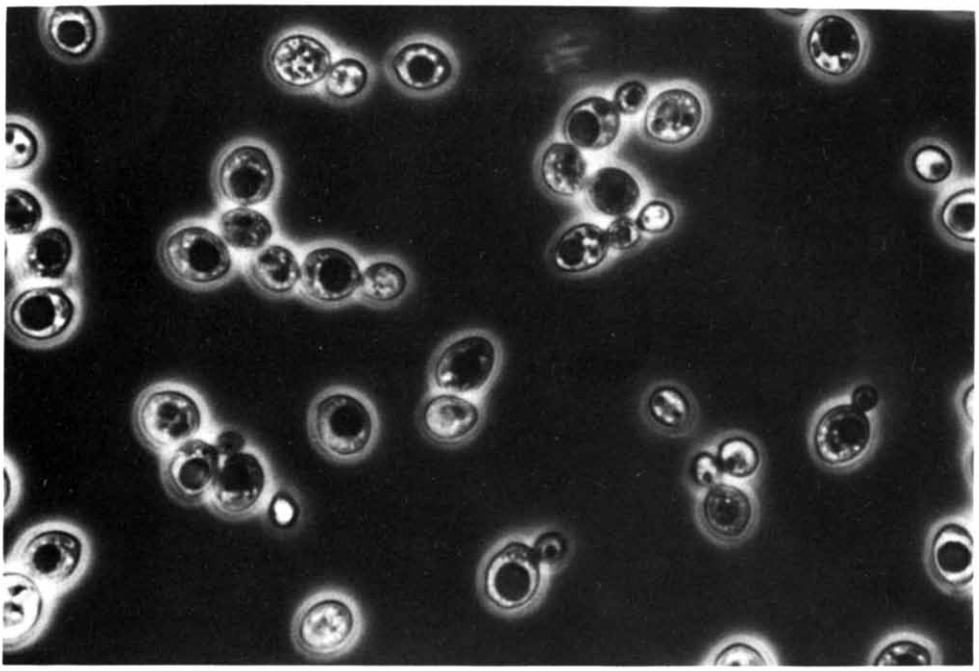

Fig. 3. Cells of *Saccharomyces cerevisiae* A 294 in the early exponential growth phase in osmotic stabilizer solution. Observation in phase contrast. Magnification: ×1080. Observe the ovoid shape of the cells in contrast to the spherical shape of the protoplasts.

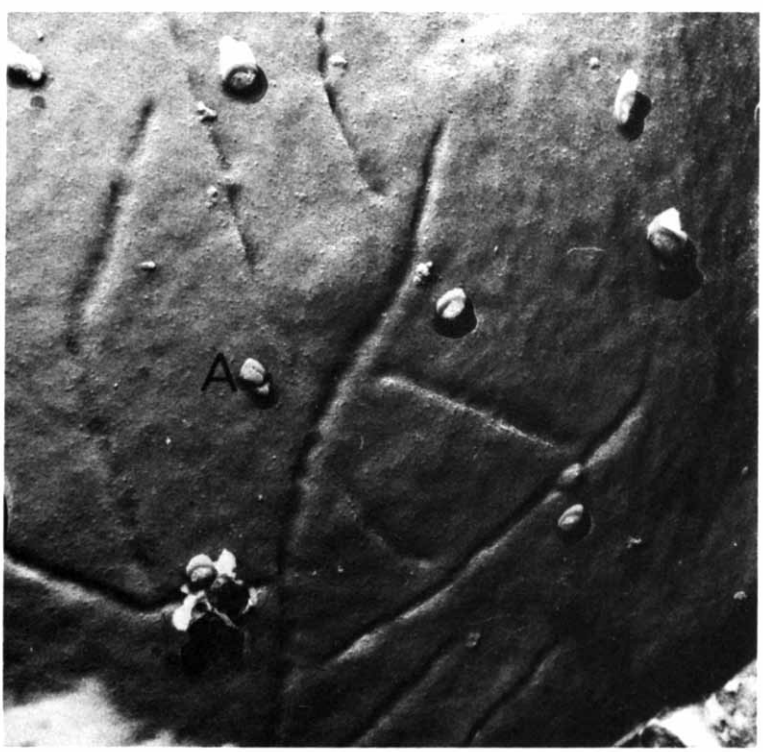

Fig. 4. Electron micrograph of a platinum-carbon replica of the surface of *Saccharomyces cerevisiae* A 294 after 30-min enzymic treatment, showing the characteristic invaginations of the plasma membrane. A, artifacts introduced by the freeze-drying technique applied. Magnification: ×40 480.

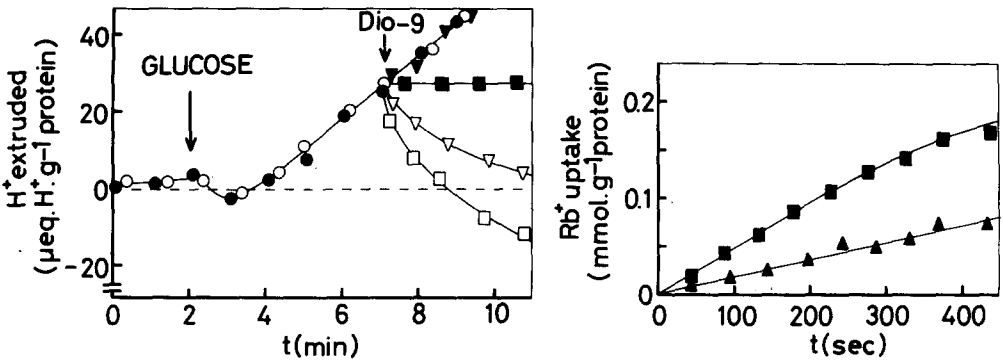

Fig. 5. Effect of Dio-9 on metabolic proton extrusion by protoplasts (○, no addition; ▽, 3 μg/ml; □, 150 μg/ml) and by intact yeast cells (closed symbols). See also legend to Fig. 1.

Fig. 6. Time course of Rb^+ uptake by protoplasts at pH 4.5. (▲) 1 mM Rb^+; (■) 10 mM Rb^+, using ^{86}Rb as a tracer. Points represent means of triplicate determinations.

yeast cells net proton extrusion was hardly affected. Much higher concentration of the antibiotic were needed to reduce proton extrusion by the intact cells to an appreciable extent.

Rb^+ and K^+ uptake

The applicability of the technique applied to the study of Rb^+ uptake by yeast protoplasts is demonstrated in Fig. 6. At the higher Rb^+ concentrations applied (e.g., 10 mM) uptake of Rb^+ was not linear with time. We therefore determined the initial rates of uptake from the slopes of the tangents to the uptake curves at zero time. Fig. 7 shows the concentration dependence of the Rb^+ uptake rates represented graphically according to Hofstee [13]. The concentration dependence of the Rb^+ uptake by protoplasts was not significantly

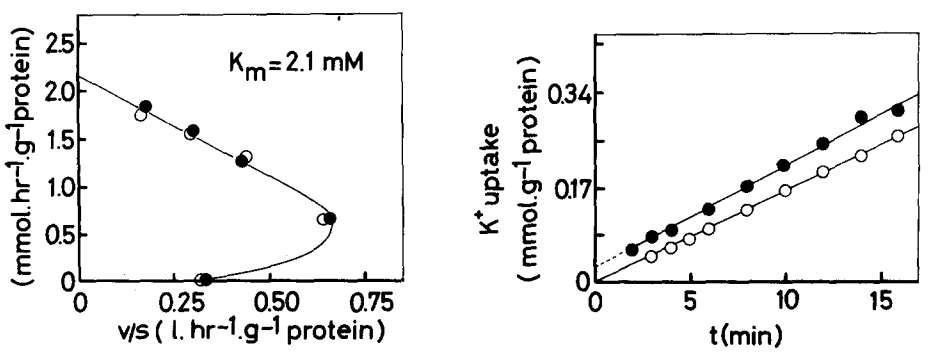

Fig. 7. Hofstee plot of the Rb^+ uptake by protoplasts (○) and by intact yeast cells (●) at pH 4.5. See also legend to Fig. 6.

Fig. 8. Time course of K^+ uptake at pH 4.5 by protoplasts (○) and by intact yeast cells (●) measured with the K^+-sensitive electrode from a 0.6 mM K^+ solution. See also legend to Fig. 6.

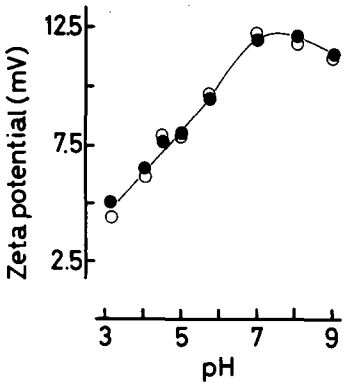

Fig. 9. pH dependence of the zeta potential of protoplasts (○) and of intact yeast cells (●). Each point represents the mean value of at least 20 determinations.

different from that by intact yeast cells. Just as in the case of Rb^+ uptake by *S. cerevisiae*, strain Delft II, a deviation from linearity in the Hofstee plot was found at the lower Rb^+ concentration [14].

The uptake of K^+, at concentrations between 0.5 and 2.5 mM, by protoplasts and also by yeast cells was linear for at least 10 min. The rate of uptake was not affected by enzymic removal of the cell wall, in agreement with the results of the Rb^+ uptake experiments described above. Plots of K^+ uptake by intact yeast cells showed an intercept with the ordinate (Fig. 8 is shown as an example), probably originating from accumulation of K^+ in the cell wall space.

Cell electrophoretic mobility

The pH dependence of the electrophoretic mobility of yeast cells and that of protoplasts did not differ significantly (Fig. 9). Qualitatively, a similar pH dependence of the electrophoretic mobility has been found also with other strains of yeast [15]. Typically, the isoelectric points were rather low (less than pH 3).

Discussion

The present results show that under the experimental conditions applied, yeast cells were completely converted into protoplasts by the enzymic treatment. After 30 min of enzymic treatment the cells became completely osmofragile (Fig. 1), the ovoid shape of the yeast cell became spherical (Figs. 2 and 3) and in freeze-etch replicas of cell preparations characteristic invaginations of the plasma membrane could be visualized (Fig. 4), which are not apparent in untreated cells. By repeated washing with osmotic stabilizer solution a protoplast suspension could be obtained, free of cellular debris and fragments of cell wall, suitable for characterization of the ion transport properties of the yeast plasma membrane as will be argued below.

Metabolic proton extrusion was not impaired by enzymic removal of the cell wall (see Fig. 5). Quantitatively, no difference in the proton flux was found between protoplasts and untreated yeast cells. The initial proton influx

observed after the addition of glucose was unexpected. Glucose-proton cotransport has not been found hitherto in strains of *S. cerevisiae* [18]. However, it might be possible that our observation of a net proton influx was due to a transient impairment of the proton pump which may be still operative at a low rate in apparently non-metabolizing yeast cells [17] instead of proton-glucose cotransport.

Foury et al. [19] showed that the antibiotic Dio-9 induces in metabolizing yeast cells of the strain, *Schizosaccharomyces pombe*, uptake of protons. A similar action of this antibiotic on the proton fluxes of *S. cerevisiae* (Fig. 5) was found in the present study. The effectiveness of Dio-9, however, was much greater when applied to protoplasts. Apparently, the interaction of the antibiotic with the yeast plasma membrane was seriously hindered by the presence of the cell wall when applied to untreated yeast cells.

The relatively simple method we have applied for studying ion uptake by means of radioactive tracers produced reproducible results. The double-labeling technique provided a useful means for correcting the data for non-absorbed tracer. These corrections were appreciable at the higher Rb^+ concentrations. For example, a plot of the uncorrected data of the upper curve in Fig. 6 gave an intercept with the ordinate of 0.1 mmol/g protein. The plot of the corrected data showed no intercept at all, illustrating that dilution of the samples in ice-cold osmotic stabilizer solution containing 50 mM KCl effectively stopped the uptake of Rb^+ and, in addition, effectively reduced the amount of adsorbed tracer. The results presented in Figs. 6 and 7 show that protoplasts were able to take up Rb^+. Moreover, they show that the transport properties of the cells were not affected by the enzymic removal of the cell wall. The deviation from a linear Hofstee plot at the lower Rb^+ concentrations (Fig. 7) indicates that uptake of Rb^+ in this yeast is also mediated by a translocator with at least two binding sites (see Ref. 16 for detailed argumentation). Results of experiments performed with the K^+-sensitive electrode support the conclusion that the transport properties of the cells were not affected by the enzymic treatment (see Fig. 8). The rates of K^+ uptake were identical for the protoplasts and for the intact yeast cells.

We argued in a series of earlier publications [20—22] that the K_m of ion translocation across cellular membranes depends on their surface potential. Our observation of a single relationship between the K_m of the Rb^+ uptake and the zeta potential of the yeast cell [23] supports that notion. Fig. 9 shows that the zeta potential of yeast cells, over a large pH range, was not affected significantly by enzymic removal of the cell wall. Similar results were found in a comparative study of the zeta potential of intact yeast cells and yeast plasma membrane vesicles [12]. Also as no difference in K_m of Rb^+ uptake was observed between intact yeast cells and protoplasts it might be hypothesized that the zeta potential of protoplasts and of intact yeast cells, as well, is mainly determined by charges on the plasma membrane, and that charges in the cell wall are of minor importance. The finding of Eddy and Rudin [24] that the zeta potential of isolated cell walls was not much different from that of intact yeast cells does not support this hypothesis. This, however, might be only apparently so. It is possible that the zeta potential of their cell wall preparation was still determined mainly by charges of the plasma membrane. Nurminen et al. [25],

namely, showed that isolated yeast cell walls may be contaminated appreciably by membrane remnants.

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