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Separation of yeast protoplasts from membrane ghosts using an aqueous two-phase system

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Protoplasts, prepared from *Saccharomyces cerevisiae*, were separated from membrane ghosts using an aqueous two-phase system of Dextran T 500, 5% (w/w) and polyethylene glycol (PEG) 8000, 5% (w/w). The protoplast preparation was prewashed in the top phase. After resuspension in fresh top phase an equal volume of bottom phase was added. A protoplast preparation almost free from membrane ghosts was obtained as a precipitate in the bottom phase. The membrane ghosts partitioned to the interphase.

Preparation of protoplast from yeast can be achieved by commercially available enzymes [1], gut juice of a snail, *Helix pomatia* [2], or an enzyme preparation derived from *Arthrobacter luteus*, zymolyase [3]. The yeast culture, being asynchronous, is composed of cells at different stages of the cell life-cycle, which will be reflected in the properties of the protoplasts derived from the cells.

The yield of protoplast is determined by the physiological state of the cells as well as by the cell population. The age of the culture markedly influences the protoplast yield, so that young cells of *Saccharomyces cerevisiae* from exponentially growing cultures are readily converted to protoplast [4–6], while cells from stationary phase cultures are resistant to lysis. The resistance to lytic enzymes has been reported to develop rapidly in the transitional period from the exponential to the stationary phase [7].

Though literature on medium effect on protoplast release is not extensive, protoplast yield from *Schizosaccharomyces pombe* was found to be enhanced from cells grown in the presence of 2-deoxy-D-glucose [8,9].

Both effects, culture age and culture medium, suggest that modification or change in the cell wall might be involved in altering its susceptibility to lysis. Thiol compounds have been used extensively to enhance protoplast release. This has been ascribed to the reduction of disulfide bonds in cell wall proteins, which will allow the penetration of lytic enzymes [10].

Microscopic observations generally show that protoplast preparations are greatly contaminated with membrane ghosts. However, it has been found necessary to prepare protoplasts without contamination of membrane ghost for characterization of protoplast membrane surface properties (Hosono, K. and Hahn-Hägerdal, B., unpublished data). Similarly, it is important to prepare non-contaminated protoplasts for electric field fusion, which has recently attracted great interest as an alternative to polyethylene glycol (PEG) fusion [11–14].

Usually different cell constituents are fractionated by means of centrifugation, especially

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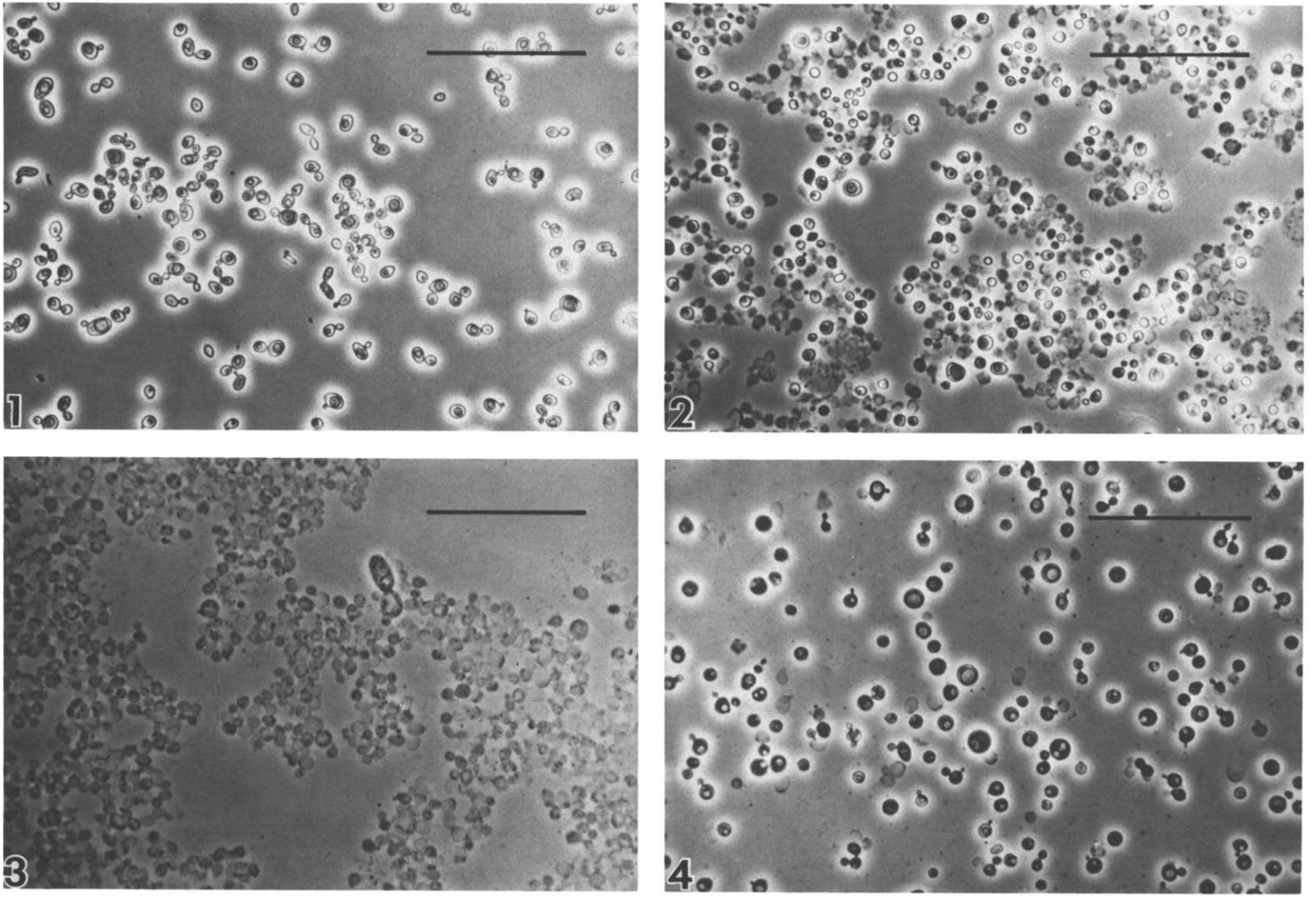

Fig. 1. Yeast cells immediately after harvesting and washing in distilled water. Phase contrast microscopy. Bar, 50 μm.
Fig. 2. Yeast cells after treatment with Zymolyase 20T. Bar, 50 μm.
Fig. 3. Membrane ghosts harvested from the interphase of the aqueous two-phase system. Bar, 50 μm.
Fig. 4. Yeast protoplasts recovered as a precipitate in the bottom phase of the aqueous two-phase system, after washing twice in 0.6 M sorbitol. Bar, 50 μm.

using various gradient techniques, the preparation of which is time-consuming. Aqueous two-phase systems are alternatives and, in comparison, very simple separation techniques, which are especially suitable for preparing various cell components [15]. Thus, whole cells of *Bacillus megaterium* can be separated from protoplast in an aqueous two-phase system [16].

The aim of the present study was to develop a method by which protoplast from yeast could be separated from membrane ghosts as this was found to be necessary for further characterization of the membrane surface properties.

Saccharomyces cerevisiae ATCC 22244 was grown in a liquid medium at 30°C. The medium was composed of 1% yeast extract, 2% peptone and 2% glucose. The cells were harvested after overnight culture, washed twice with distilled water (Fig. 1) and resuspended to a density of approximately 250 mg (wet weight) per ml in 0.6 M sorbitol containing 25 mM EDTA and 2.5% β-mercaptoethanol. The mixture was incubated with slow stirring for 30 min at room temperature, centrifuged, and then the cells were washed twice with 0.6 M sorbitol.

The yeast cells were resuspended to approximately 250 mg (wet weight) per ml in protoplasting buffer, consisting of 0.6 M sorbitol and 10 mM EDTA in 20 mM Tris-HCl (pH 7.5). Depending on the physiological state of the cells approximately 0.5 mg/ml of Zymolyase 20T (Kirin brewery, Japan) was added. The mixture was incubated with stirring at 30°C and the formation of protoplast was followed by phase contrast microscopy. Under these conditions cells were typically converted to protoplasts in less than 15 min. However, the young cells were lyzed to become membrane ghosts. The protoplasts were centrifuged at 2000 rpm, 10 min at room temperature and washed twice with 0.6 M sorbitol. This fraction was sticky, being a mixture of protoplasts and ghosts (Fig. 2).

The protoplasts were then purified in an aqueous two-phase system composed of 5% (w/w) Dextran T500 (Pharmacia Fine Chemicals, Uppsala, Sweden), 5% (w/w) polyethylene glycol (PEG) 8000 (Union Carbide, New York, U.S.A.) and 90% (w/w) 0.6 M sorbitol. The light PEG-rich phase and the dense dextran-rich phase were separated after equilibration and stored separately in a refrigerator. The protoplasts were first resuspended in the light PEG-rich phase. After centrifugation at 5000 rpm for 10 min the supernatant was discharged. It was necessary to pre-wash the membrane ghost containing protoplast preparation in the light PEG-rich phase, because it was found that when the entire phase system was used directly phase separation did not occur. The pellet was then resuspended in fresh PEG-rich phase, the same volume of the dense dextran-rich phase was added and mixed well with the light phase containing protoplasts and ghosts. The mixture was centrifuged at 7000 rpm, 10 min. Membrane ghosts were partitioned to the interphase between the light and the dense phase (Fig. 3), and protoplasts could be recovered as a precipitate at the bottom of dense phase. Both a swing type and a bucket type rotor can be used for this separation. Protoplasts were resuspended in 0.6 M sorbitol and washed three or four times with 0.6 M sorbitol with centrifugation at 1000 rpm, 10 min. After this treatment, purified protoplasts with minor contamination of membrane ghosts were obtained (Fig. 4).

The concentration of sorbitol, the osmotic stabilizer, was only 0.6 M. This was mainly due to the fact that the protoplasts in this study were prepared for contact angle measurements using aqueous two-phase systems (Hosono, K. and Hahn-Hägerdal, B., unpublished data) – a method by which the surface properties of the protoplast membrane can be characterized [17]. This low concentration of sorbitol was chosen in order to reduce the viscosity because the high viscosities make contact angle measurements increasingly difficult. If sorbitol was excluded from the aqueous two-phase system in the separation procedure no protoplasts could be obtained.

All procedures were carried out at room temperature.

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