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NOTES

Yeast Spheroplasts Formed by Cell Wall-Degrading Enzymes from *Oerskovia* sp.[1]

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Oerskovia sp. produces inducible extracellular enzymes which degrade the walls of various yeasts. Yeast spheroplasts are formed from both log- and stationary-phase cells.

Considerable success has been obtained in degrading isolated yeast cell walls with microbial enzymes (8), but the formation of spheroplasts or protoplasts from viable yeasts is more difficult. For successful spheroplast formation, most of the effective microbial enzymes require a long incubation period (7) or the addition of reducing agents such as 2-mercaptoethanol (2). In addition, preincubation in ethylenediaminetetraacetate (12) sometimes is necessary, or the yeast cells have to be in a certain growth state (2, 12). Digestive enzymes from the stomach of *Helix pomatia* (snail enzyme) are useful for producing spheroplasts from yeast cells in the exponential phase of growth (1), but older (stationary-phase) cells are not susceptible (3). Additionally, microbial and snail enzyme preparations frequently are active on the walls of only a limited number of different yeast species. In contrast, we have isolated a soil bacterium which produces inducible enzymes that degrade the walls of a variety of different kinds of yeasts. In a suitable osmotic support, spheroplasts are formed rapidly in the absence of reducing or chelating agents.

The bacterium which produces these degradative enzymes is a gram-positive, branching actinomycete which fragments into motile rod-like elements in older cultures. This nocardoid actinomycete belongs to the proposed genus *Oerskovia* (9), of which *O. turbata* (Statens Seruminstitut of Copenhagen strain 891 and ATCC 25835) is the type species. The chemical constitutions of cell wall preparations (13) are identical between our strain and the type culture; however, physiological tests and temperature requirements indicate differences. Based on these differences a new species will be described in the future.

When 10^8 cells of *Oerskovia* sp. per ml and 10^8 cells of *Saccharomyces cerevisiae* per ml were incubated together in yeast nitrogen base (YNB) containing no carbon source, less than 0.001% of the yeast cells remained viable after 36 hr (Fig. 1). Plate and direct counts for yeast cells were about equal, suggesting that loss of viability was caused by osmotic instability. A control containing only yeast cells in YNB did not decrease in viability. Cell-free samples prepared from the suspension of the two organisms after 18 and 48 hr were capable of producing spheroplasts from log-phase yeast cells within 20 min.

Spheroplasting enzymes also are induced when *Oerskovia* sp. is grown in a mineral salts medium containing purified yeast walls, pressed yeast cake, or autolyzed yeast (Difco). The mineral salts medium consisted of the following (grams per liter): $MgSO_4 \cdot 7\ H_2O$, 0.5; NaCl, 0.1; $CaCl_2 \cdot 2\ H_2O$, 0.1; and $(NH_4)_2SO_4$, 5.0, in 0.05 M potassium phosphate buffer, *p*H 7.0. Autolyzed yeast (Difco; a product which includes yeast cell walls) was used as a carbon source for growth and enzyme production in the following studies. Dialyzed cell-free broth was prepared and used as the source of crude enzyme without additional treatment.

Phase-contrast micrographs show the appearance of yeast cells (*Hansenula anomala*) before (Fig. 2A) and after (Fig. 2B) exposure to the crude enzyme preparation for 20 min.

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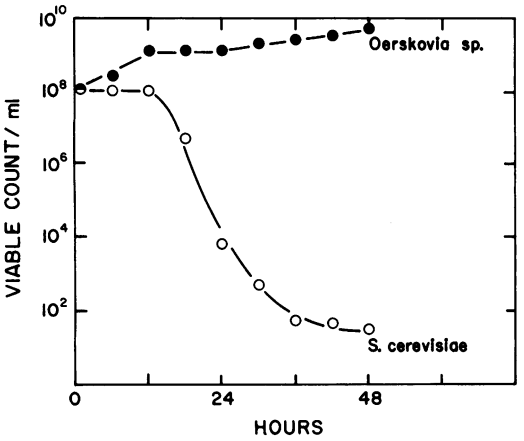

FIG. 1. *Viable counts of S. cerevisiae and Oerskovia sp. in mixed culture. Cells from 24-hr cultures of both organisms were centrifuged, washed two times with distilled water and suspended together in liquid yeast nitrogen base adjusted to pH 7.0. The mixed culture was incubated on a shaker at 28 C. Viable cells were counted by plating duplicate samples periodically on acidified wort agar and nutrient agar.*

Electron micrographs of enzyme-treated cells of *S. cerevisiae* are shown in Fig. 2C to F. These cells (10^8/ml) were incubated in a 1:10 dilution of crude enzyme in a 0.6 M KCl osmotic support. Samples taken between 0 and 60 min were fixed by the double-aldehyde procedure of Karnovsky (4), stained in 0.5% (w/v) aqueous uranyl acetate, dehydrated, then embedded in Araldite 6005 (11). Sections were stained 10 min with lead citrate (10) then 2 min with 2% (w/v) aqueous barium permanganate. The electron micrographs show that stainable portions of cell wall were progressively removed (Fig. 2D–F) and that peripheral cytoplasmic bodies (Fig. 2D, E) were released as spheroplasts (Fig. 2F) were formed. Control cells had intact walls and peripheral cytoplasmic bodies (Fig. 2C). Intact cells, spheroplasts, and cells with partially degraded walls were found in all samples treated 5 min or longer with the dilute enzyme preparation. The number of spheroplasts and cells with partially degraded walls increased progressively with longer periods of exposure to the enzyme.

In another experiment, 2×10^8 cells of various yeasts (Table 1) representing six different genera were incubated in reaction mixtures containing 1 ml of crude enzyme and 1 ml of 1.2 M KCl in 0.1 M potassium phosphate buffer, pH 6.5. Dilution with water causes lysis of osmotically sensitive cells. The number of intact cells in the dilutions was determined microscopically in a counting chamber. Intact cells were easy to count because the cell wall material from osmotically disrupted cells generally was completely degraded. The values in Table 1 were calculated from the differences in the cell counts in water dilutions compared to those diluted with the osmotic support, KCl. With the exception of *Rhodotorula rubra*, spheroplasts were produced by all strains after 20 min. Evidently, walls of stationary-phase (24 hr) cells of *S. cerevisiae* were slightly more resistant to degradation than those of log-phase (4 hr) cells. Within 45 min, however, all of the 24-hr cells of *S. cerevisiae* were osmotically sensitive, and only spheroplasts could be detected with a microscope. The resistance of *R. rubra* walls to lysis by enzymes from *Oerskovia* sp. may be characteristic of basidiomycetous yeasts. In another experiment, the enzyme preparation failed to form spheroplasts from cells of *Sporobolomyces salmonicolor*.

Crude preparations contained the following enzymes: α-mannanase (5), chitinase (agar plate assay), and endo-laminarinase which has been implicated in spheroplast formation in other systems (14). Preliminary separations on Sephadex G-75 show that our enzyme preparation contained two endo-laminarinases, only one of which is essential for spheroplasting activity on live yeast cells. Phosphomannanase, an enzyme which is required for yeast protoplast formation in other systems (6), evidently was not present since *Oerskovia* sp. broth failed to depolymerize phosphomannan Y-2448 from *H. holstii*.

The ecological significance of yeast lysis by *Oerskovia* sp. in soil has not been determined. Degradation of yeast cell walls catalyzed by enzymes produced by this bacterium may be more useful for preparing spheroplasts or isolating organelles than previously described enzyme systems. Conceivably, these lytic enzymes can provide an economic method for disrupting yeast cells in the production of single-cell protein for human and animal consumption.

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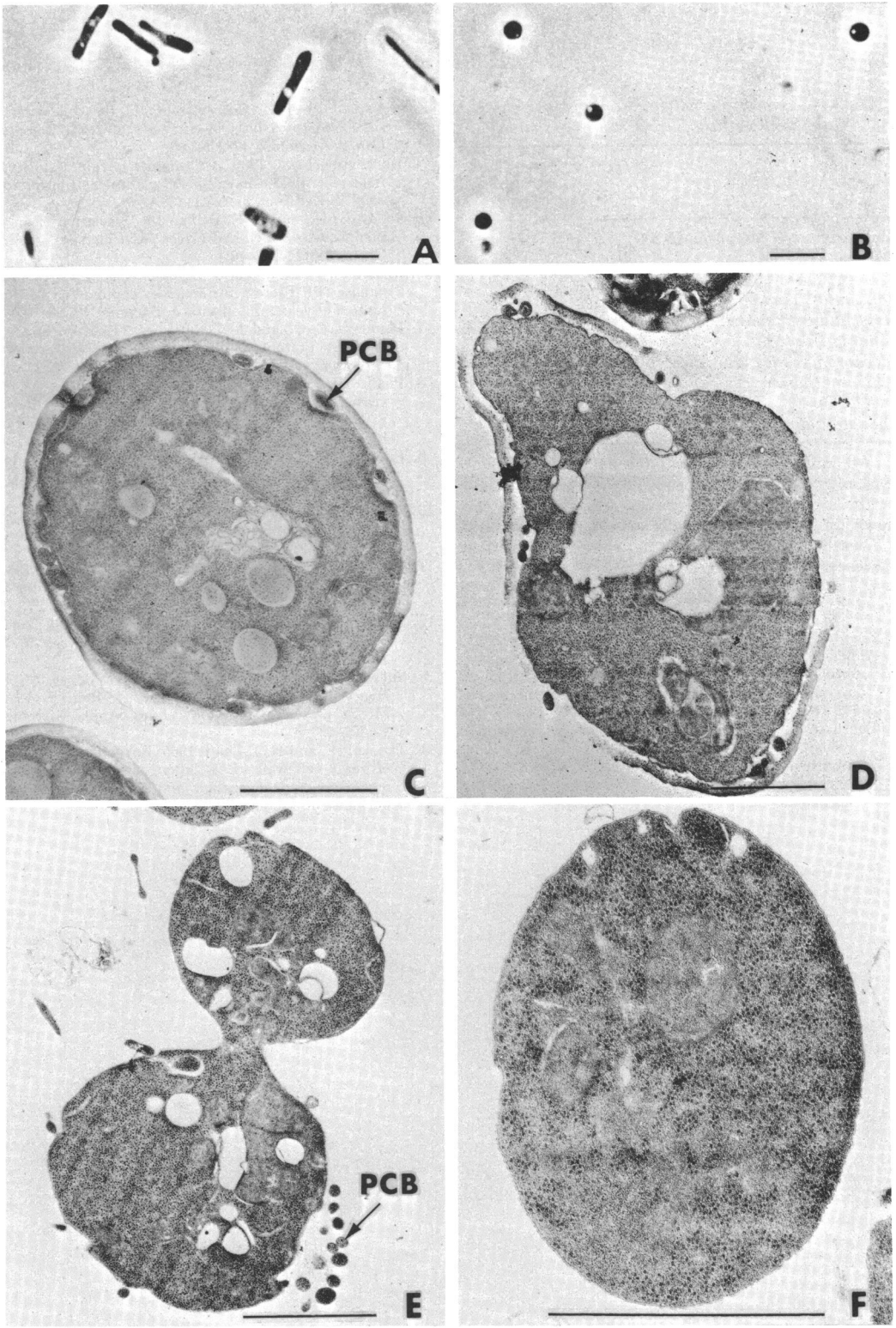

FIG. 2. *Phase-contrast and electron micrographs of osmotically supported yeast cells before (A, C) and after (B, D, E, F) exposure to cell wall-degrading enzymes from Oerskovia sp. (A, B) H. anomala phase contrast. (C–F) Electron micrographs of S. cerevisiae. (C) Control, no enzyme treatment. The progressive removal of stainable wall material (D) and release of peripheral cytoplasmic bodies (E) results in the formation of spheroplasts (F) which have no detectable cell wall material. (E) A budding cell, where stainable wall material has been removed from both the mother cell and the bud. PCB, Peripheral cytoplasmic bodies. Scale lines indicate 10 μm (A, B) and 1 μm (C–F).*

TABLE 1. *Osmotic sensitivity of various yeasts treated with culture filtrate from Oerskovia*

Yeast[a]	Osmotically sensitive cells (%)
Saccharomyces cerevisiae C-299 (4 hr)	100
S. cerevisiae C-299	86
Hansenula anomala C-317	90
Metschnikowia krissii 61-31	78
Candida tropicalis	76
Saccharomycodes ludwigii C-114	32
Schizosaccharomyces pombe 2478 (ATCC)	27
Rhodotorula rubra C-46A	0

[a] All cultures were grown for 24 hr at 28 C on yeast nitrogen base (Difco) plus 0.5% glucose (except for the log-phase culture of *S. cerevisiae* which was grown for 4 hr). After growth, cells were centrifuged and washed with distilled water, and suspended in the reaction mixture for 20 min.

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