Note

Preparation of Protoplasts Capable of Strongly Oxidizing Salicylaldehyde from Aureobasidium pullulans

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In the previous work, Aureobasidium pullulans No. 14 was isolated as one of fungi capable of assimilating phenol, cresols and monohydroxybenzoates, and subsequent studies suggested the feasibility of its practical application for treatment of phenolic waste water. Since recent studies have suggested the hopeful application of the protoplast-fusion technique to improvement of antibiotic productivity, we prepared protoplasts of strain No. 14 for the purpose of increasing the phenol-utilizing activity.

Jeffries et al., Dominguez et al. and Finkelman et al. reported protoplast formation from A. pullulans cells. In our present study satisfactory results were obtained using a yeast cell-wall lytic enzyme, Zymolyase. This paper describes the preparation of protoplasts capable of metabolizing phenols from cells of strain No. 14 and also describes procedures for maintaining their high activity. Salicylaldehyde (SAD)-oxidizing activity, induced when the cells were cultured in o-cresol-containing medium, was adopted as a criterion of physiologically strong phenol-utilizing activity.

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The difficulties encountered in such studies were: 1) In harvesting large quantities of protoplasts induced to oxidize SAD, and 2) in preservation of such enzyme activity for a long time. In the synthetic culture medium with o-cresol as a sole carbon source the growth of this Aureobasidium was very scanty. Moreover, when incubated too long in the o-cresol medium, its cell-wall became non-lysable by Zymolyase.

Preparation of protoplasts was carried out by the following procedure. Inoculum cells (approximately 10^7/ml) cultured exponentially in 10 ml of M.P.Y. medium were washed twice with sterilized distilled water and transferred to 100 ml of o-cresol-I-medium. Young cells cultured for 14~15 hr in the I-medium were harvested and washed three times with distilled water. Fundamental Zymolyase treatment was as follows. Approximately 3 x 10^7 of the cells were treated in a 10 ml reaction mixture (0.1 m Na_2SO_3, 0.8 m KCl, final 5 units/ml of Zymolyase and 25 mm Tris-HCl buffer, pH 7.5) at 25°C for 60 min. The protoplasts thus obtained were collected by centrifugation (3000 rpm, 5 min) and washed three times with hypertonic protoplast washing solution (0.1 m Na_2SO_4, 0.5 m KCl or 0.5 m MgSO_4-7H_2O and 25 mm Tris-HCl buffer, pH 7.5). The efficiency of protoplast formation was confirmed by phase-contrast microscopy and by calculating the burst ratio as follows. Centrifuged protoplasts (3000 rpm, 5 min) were burst by osmotic shock by resuspending them in the same volume of distilled water as that of the Zymolyase reaction mixture. The burst ratio was calculated as (a-b)/ax 100 (%), where a = OD_800 immediately after incubating the reaction mix-

\[ \text{Assay Method} \]

- Cell Growth, SAD-Oxidizing Activity and Protoplast Formation.

The initial cell size inoculated into o-cresol-I-medium from a M.P.Y. medium culture was varied in several series of experiments. Cell growth in o-cresol-I-medium was determined spectrophotometrically according to the procedure in the previous paper. SAD-oxidizing activity of intact cells and their efficiency of protoplast formation were determined after appropriate incubation periods in o-cresol-I-medium. SAD-oxidizing activity was determined using a Bioxygraph at 30°C. The reaction mixture consisted of 2.7 ml of 0.1 m K-phosphate buffer, pH 7.0, 0.4 ml of cell suspension containing 1.2 mg cells as dry weight and 0.2 ml of 20 mm SAD.

O-O, cell growth in several series of experiments; ■■, SAD-oxidizing activity.
ture and \( b = \text{OD}_{900} \) after bursting the protoplasts. A successful protoplast forming experiment usually resulted in a burst ratio of over 80%.

Preparation of protoplasts showing SAD-oxidizing activity were yielded as indicated in Fig. 1. An incubation period in o-cresol-I-medium of 10~15 hr was the most favorable regardless of the initial cell size. An incubation period shorter than this resulted in insufficient induction of the oxidizing activity: On the contrary, if longer, the efficiency of protoplast formation decreased presumably owing to the increased resistance of the cell-wall against the lytic enzyme.

It seemed of interest that Aureobasidium cells, a filamentous mold, could be converted to protoplasts by a yeast cell-wall lytic enzyme, Zymolyase, like a typical yeast e.g. Saccharomyces.\(^9\)

The stability of SAD-oxidizing activity was manometrically examined with the resultant protoplasts (Fig. 2). Protoplasts treated with a hypertonic solution with MgSO\(_4\) maintained their SAD-oxidizing activity stably, while the activity of protoplasts treated with KC\(_1\) solution decreased as time passed. MgSO\(_4\) was much more favorable than KC\(_1\) as an osmotic stabilizer for the protoplast washing and suspending solution, like for the respiratory activity of protoplasts from a hydrocarbon-utilizing yeast toward n-alkane.\(^{10}\)

Figure 3 compares phase-contrast micrographs of intact cells (A), and MgSO\(_4\)-washed (B) and KCl-washed protoplasts (C). Both protoplasts were similarly round-shaped in contrast to the rod-shaped intact cells. However, while MgSO\(_4\)-washed protoplasts assumed a dark blue color like intact cells, KCl-washed ones were a much lighter blue. The difference in color may reflect the stability of SAD-oxidizing activity. These phenomena suggested that SAD-oxidizing activity is located in the cell-membrane region. The color of protoplasts was reversibly changed depending on the osmotic stabilizer contained in the washing solution.

![Fig. 2. Effect of Osmotic Stabilizers on Preservation of SAD-Oxidizing Activity of Protoplasts.](image)

Protoplasts were washed, resuspended in MgSO\(_4\)- or KCl-containing washing solution and stored at 4°C. Their SAD-oxidizing activities were assayed using a Warburg manometer at 30°C. The reaction mixture consisted of 1.6 ml of MgSO\(_4\)- or KCl-containing protoplast washing solution, 0.2 ml of protoplast suspension containing ca. 4 mg cells as dry weight and 0.2 ml of 20 mm SAD.

- •••, MgSO\(_4\)-containing washing solution; ○○○, KCl-containing washing solution.

![Fig. 3. Micrographs of Intact Cells and Protoplasts of Strain No. 14.](image)

(A) Intact cells grown in o-cresol-I-medium for 14 hr.
(B) Protoplasts washed with MgSO\(_4\)-containing washing solution.
(C) Protoplasts washed with KCl-containing washing solution. The length of the bars represents 10 \( \mu \)m.
REFERENCES