Immobilization of *Mortierella vinacea* Cells by Radiation Polymerization

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Synopsis

Immobilization of *Mortierella vinacea* cells, which contain active α-galactosidase, by radiation polymerization at low temperatures was studied. The durability of the enzymatic activity of the immobilized cells was examined by repeating the batch enzyme reaction. The enzymatic activities of the immobilized cells obtained with hydrophilic monomers was affected by the concentrations of the cells and monomer in which optimum conditions were observed. The enzymatic activity of the immobilized cells obtained with hydrophilic monomers was compared to that of hydrophobic monomers. Michaelis constants of the immobilized cells varied with monomer concentration. The effect of addition of porous solid substances on the immobilization of the cells was studied.

INTRODUCTION

To produce useful substances by enzymatic or fermentation method, microbial cells have been effectively used in the food, medical, and chemical industries. Furthermore, the study of immobilized cells as well as immobilized enzyme has been the subject of increased interest.[1–3]

In the beet sugar industry, raffinose is known as an obstacle substance for the crystallization of beet, as it is gradually increased during storage to 0.1–0.2%. Therefore, the hydrolysis of raffinose in beet sugar is important in the beets sugar industry. *Mortierella vinacea* cells, which contain active α-galactosidase, easily hydrolyze raffinose to galactose and sucrose.[4,5] Thus, the immobilization of this cell is important to the beet sugar industry.

The authors have studied the radiation polymerization of glass-forming monomers having remarkable polymerizability at low temperatures and have shown that microbial cells and enzymes could be immobilized effectively by this method.[6,7] In this work, the immobilization of *Mortierella vinacea* cells by radiation polymerization at low temperatures was studied.

EXPERIMENTAL

Martierella vinacea cells were obtained from Hokkaido Togyo Co., Ltd. Various monomers with glass states at low temperatures, such as hydroxyethyl methacrylate (HEMA), hydroxyethyl acrylate (HEA), trimethyrolpropane triacrylate (A-TMPT), and polyethylene glycol diacrylate (A-nG), used in this work were obtained from Mitsubish Gas Chemical Co., Ltd., and were used without further purification. The purity of these monomers, measured by gas chromatography, was above 99.5%. Impurities were inhibitors and other monomers that do not affect to the enzymatic activity of the cells.

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The immobilization of the cells was carried out as follows. A mass of dried cells was suspended in 0.05 *M* acetate buffer solution (pH 4.5) containing the monomer, to a total volume of 2.0 ml. This mixture was charged in a glass tube (2.0 cm in length and 0.8 cm in diameter), rapidly shaken, and frozen at −78°C. The γ-ray irradiation (1 MR) was carried out using a ^{60}Co source of 5×10^5 Ci for 1 h at a dose rate of 1 MR/h, at a distance of 25 cm from the vessel. The irradiation temperature was kept at −78°C by immersing the vessel in a Dewar flask filled with Dry Ice–methanol. The immobilized cell composite piece obtained using hydrophilic monomers was cut into thin pellets after irradiation.

The durability of the enzymatic activity of the immobilized cells was examined by repeating the batch enzyme reaction (1.0 h at 40°C). The enzymatic activity (%) was obtained from the D-glucose formation ratio in the immobilized and intact cells with each batch of enzyme reaction. From the initial reaction rate, the enzymatic activity of the cells was obtained, the reaction rate being linear during the reaction time course of the assay. A 1.0% melibiose solution containing 0.05 *M* acetate buffer solution was used as the substrate throughout. The D-glucose formed was measured with glucose-specific reagent (GOD-PODLK, Nagase Sangyo Co., Ltd.).

RESULTS AND DISCUSSION

Variation of Enzymatic Activity with Repeated Batch Reaction

The variation of the enzymatic activity of the immobilized cells with repeated batch enzyme reaction is shown in Figure 1. The enzymatic activity increased as the number of batch enzyme reactions increased at the early stages and then reached a constant value. The phenomenon of this initial increase appeared to be a characteristic of the immobilized cells obtained by radiation polymerization of hydrophilic monomer. The increase can be attributed to the swelling effect of the polymer matrix by water, which increases the diffusivity of the substrate into cells entrapped in the inner part of the matrix. Swelling of the

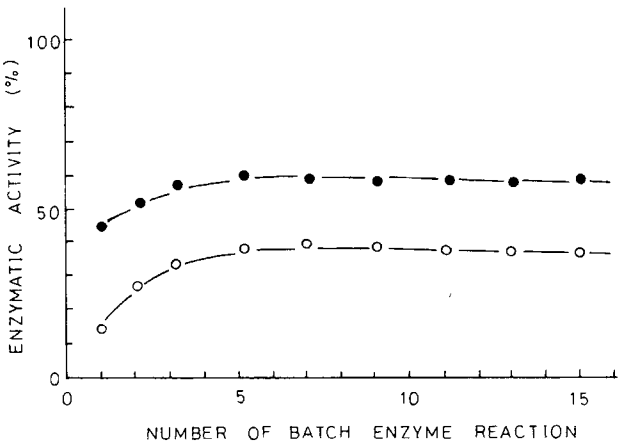

Fig. 1. Variation of enzymatic activity of immobilized cells with repeated batch reaction. HEMA monomer concentration: (●) 15%; (○) 50%. Cell concentration, 0.5%.

polymer matrix by water occurred at the initial stage of repeated batch reaction and then reached an equilibrium after three to five batch reactions, as does the polymer matrix in the immobilized *Streptomyces phaeochromogenes* cells.[6] Swelling of the polymer matrix has the apparent effect of increasing enzymatic activity of the cells. It is assumed that the diffusion of the substrate to the position of trapped cells is promoted by the swelling of the polymer matrix. In addition, the mobility of the trapped cells might increase by the swelling, taking the more favored configuration for contact and reaction with substrate in the polymer matrix.

Relationship Between Monomer Concentration and Enzymatic Activity

The relationship between HEMA monomer concentration and enzymatic activity is shown in Figure 2, in which the enzymatic activities are the values after 15 batch enzyme reactions. The enzymatic activity of the immobilized cells had a maximum value at 20% monomer concentration, decreasing at lower and higher monomer concentrations. In the immobilized cells prepared with lower monomer concentrations, below 10%, small amounts of the cells leaked in the solution from the polymer matrix after the enzyme reactions were detected with the optical microscope. The polymer matrix of the immobilized cells prepared with higher monomer concentrations, of 70%, had a low porosity of 15%.

From these results, it appears that the decrease of the enzymatic activity at lower monomer concentrations is due to the leakage of the cells from the polymer matrix, and that at higher monomer concentrations it is due to the increase of the cells trapped in the polymer matrix having a small porosity. The polymer matrix obtained by radiation polymerization at low temperatures has a porous structure which is formed by the melting of ice. The formation of a porous structure is one of the most characteristic features, of the polymer matrix obtained by radiation polymerization of glass-forming monomers at low temperatures, and is the key factor in determining enzymatic activity.[8]

The monomer concentration has a direct effect on enzymatic activity. The

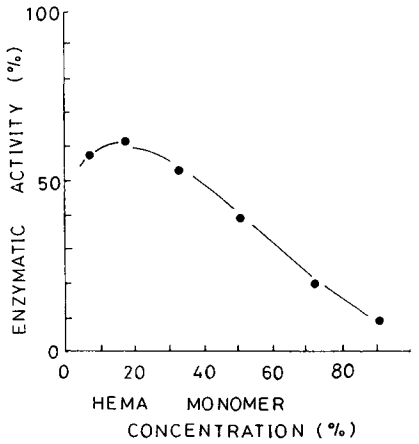

Fig. 2. Effect of HEMA monomer concentration on enzymatic activity. Cell concentration, 0.5%.

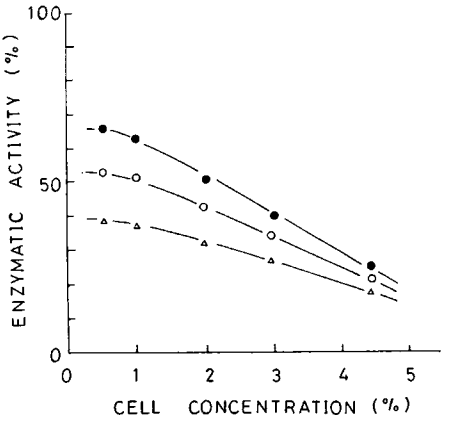

Fig. 3. Effect of cell concentration on enzymatic activity. HEMA monomer concentration: (●) 20%; (○) 30%; (△) 50%.

optimum monomer concentration giving maximum enzymatic activity in the immobilized cells was ca. 20%, as shown in Figure 2. The optimum monomer concentration in immobilized enzymes was 50–60%, as previously reported.[8] This could be attributed to the difference in leakage behavior due to difference in size and/or shape of the cell and the enzyme molecule.

Dependence of Enzymatic Activity on Cell Concentration at Various Monomer Concentrations

The enzymatic activity of the immobilized cells is plotted against cell concentration in Figure 3. The enzymatic activity had a maximum value at 0.5% cell concentration, decreasing with higher cell concentration. The decrease in enzymatic activity with increasing cell concentration may be due to the leakage of the cells from the polymer matrix.

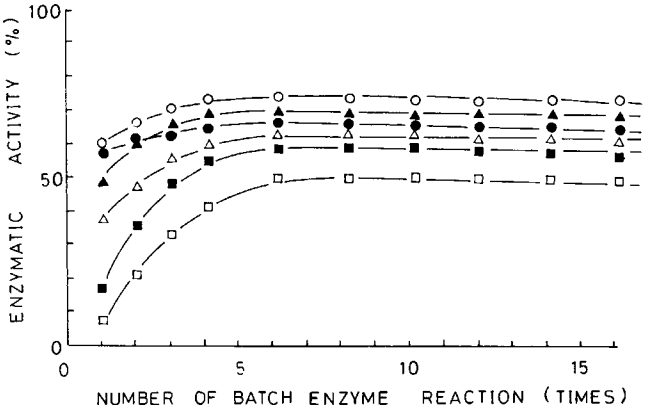

Fig. 4. Variation of enzymatic activity of immobilized cells obtained using HEA monomer with repeated batch reaction. Monomer concentration: (●) 5%; (○) 10%; (▲) 20%; (△) 30%; (■) 50%; (□) 70%. Cell concentration, 0.5%.

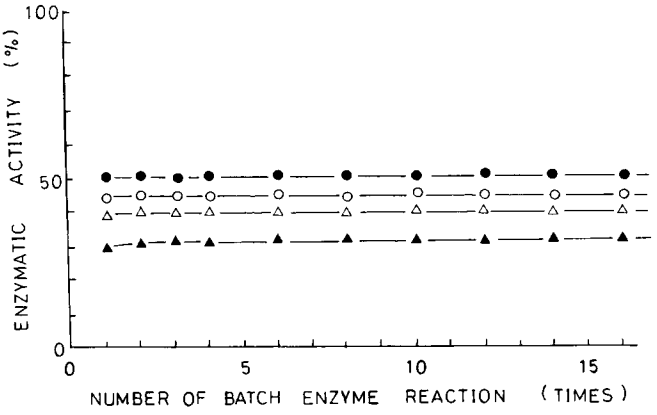

Fig. 5. Variation of enzymatic activity of immobilized cells obtained using A-TMPT monomer with repeated batch reaction. Monomer concentration: (●) 15%; (○) 30%; (△) 50%; (▲) 70%. Cell concentration, 0.5%.

Immobilization of the Cells Using Various Monomers

The immobilization of the cells using various hydrophilic and hydrophobic monomers was studied. The variation of the enzymatic activity with repeated batch reaction in the case of HEA of a higher hydrophilic monomer than HEMA is shown in Figure 4. The increase in enzymatic activity at the initial stages of the batch reaction in HEA was similar to that in HEMA, but the enzymatic activity at later stages was higher. The water content of the HEA polymer matrix corresponding to the porosity was larger than that of HEMA; that is, these values were 46 and 26%, respectively. This fact is a desirable feature for increasing the enzymatic activity.

The variation of the enzymatic activity with repeated batch reaction in the case of hydrophobic A-TMPT monomer is shown in Figure 5. The enzymatic activity did not vary with repeated batch reactions. This result was different

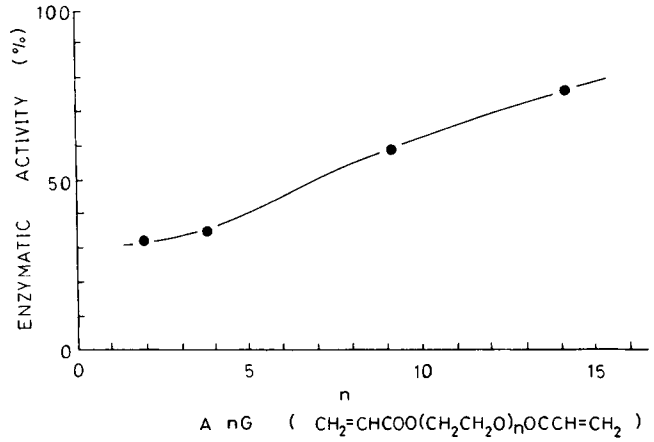

Fig. 6. Enzymatic activity of immobilized cells obtained using A-nG monomer. Monomer concentration, 30%; cell concentration, 0.5%.

TABLE I
Michaelis Constants of the Immobilized Cells

HEA monomer concentration, %	K_m, mM
10	0.49
30	0.57
50	0.66
70	0.82
Intact cells	0.40

from those in hydrophilic monomers such as HEA and HEMA. The polymer composite piece obtained by radiation suspension polymerization of hydrophobic monomers at low temperatures was of particle shape (100–200 μm) in which the cells were trapped on the surface of the particle. Therefore, the diffusion of the substrate inside the polymer matrix with swelling was not necessary, by which the enzymatic activities at the initial and later stages of the batch reaction became constant. The immobilization of the cells by radiation polymerization of A-nG monomers was studied, and the variation of the enzymatic activity with n numbers of —CH_2CH_2O— units in A-nG monomers is shown in Figure 6. The enzymatic activity increased with increasing n, i.e., as the hydrophilic property of the polymer matrix increased. The water content of A-14G and HEA polymer was 50 and 46%, respectively; thus, both values were of the same order, indicating that the enzymatic activities in both monomers are comparable.

Michaelis Constants of the Immobilized Cells

Michaelis constants of the immobilized cells were obtained with Lineweaver–Burk plots with various HEA concentrations, and the values are listed in Table I. The K_m value of the immobilized cells decreased with decreasing monomer concentration and then reached a value near that of intact cells. This result showed that the situation of the immobilized cells prepared with a mod-

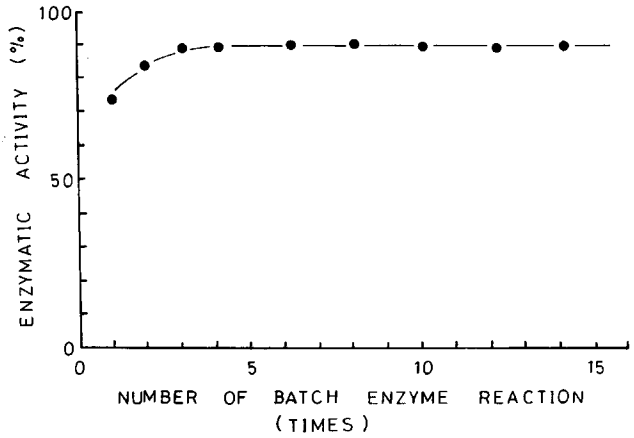

Fig. 7. Effect of Kanuma earth additive on immobilization of cells. HEMA monomer concentration, 30%; additive concentration, 60%; cell concentration, 0.5%.

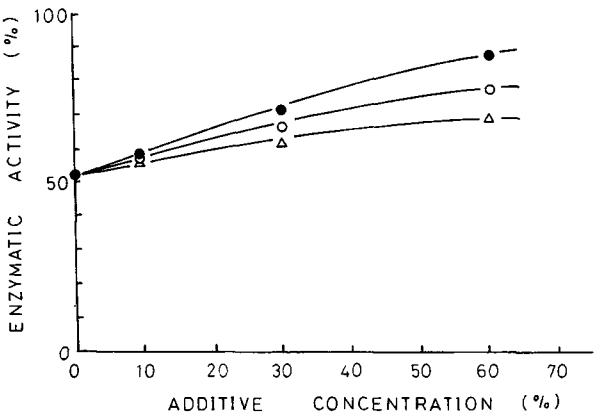

Fig. 8. Effect of various porous solid additives on immobilization of cells. HEMA monomer concentration, 30%; cell concentration, 0.5%. Additives (●) Kanuma earth; (○) activated carbon; (△) unglazed pottery.

erate low monomer concentration is similar to that of the intact cells, though the leakage of the cells is taking place in lower monomer concentrations.

Effect of Addition of Porous Solid Additives

A polymer matrix having a large diffusibility for the substrate is recommended in the immobilization of the cells. To increase further the porous structure of the polymer matrix, the effect of addition of various solid porous substances was studied. The variation of the enzymatic activity of the immobilized cells in Kanuma earth additive with repeated batch reaction is shown in Figure 7. The enzymatic activities at initial and later stages were increased considerably by the addition of Kanuma earth additive.

The effect of various additives on the enzymatic activity of the immobilized cells is shown in Figure 8. The enzymatic activity increased with increasing additive concentration and reached a saturation value. The effect of the additives could be attributed to the enlarging of the porous structure, by which the diffusibility of the substrate is increased. Such an explanation for the increased enzymatic activity with additives would be reasonable.

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