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Immobilization of Yeast Cells with Polymeric Carrier Cross-Linked Using Radiation Technique

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Various compositions of 2-hydroxyethacrylate (HEA) and methoxy polyethylene glycol methacrylate (M23G) monomers were irradiated by γ-rays at low temperature (−78 °C) to synthesize polymer carriers for effectively immobilizing yeast cells. The yeast cells were immobilized by cell adhesion onto/in these polymers. The ethanol productivity of immobilized yeast cells with the polymer carriers was higher than that of free cells, increasing by 1−3 times. However, the ethanol productivity of immobilized yeast with the polymer carrier resulting from 7%/7% (HEA/M23G) monomer was low, comparatively. The effect of adding cross-linking reagent (4G) to the low concentration of HEA/M23G monomers on the activity of yeast cells immobilized with the cross-linked carriers by radiation polymerization was investigated. The ethanol productivity of immobilized cells with the carriers, which were cross-linked by adding 3−6% 4G to the low concentration of HEA/M23G monomer, was increased by 20−30%, because the pore size, network structure, and mechanical strength of the polymer carriers was well adjusted and cell leakage from the polymer carriers decreased. The relationship between the ethanol productivity of immobilized yeast cells and the interior structure of polymer carriers is discussed and indicated that the interior structure of polymer carriers is crucial for effective immobilization of yeast cells.

KEYWORDS: Polymer carrier; yeast cell; immobilization; ethanol productivity; cross-linking

INTRODUCTION

Immobilization of yeast cells through various techniques, for example, surface adhesion, covalent bonding, encapsulation, and entrapment in a gel matrix, has been reported (*1*−*6*). Agar, polyacrylamide, alginate, or κ-carrageenan have been used for the entrapment of yeast cells. However, the mechanical strength of agar is rather weak and the oxygen permeability is low. Polymerization reaction of polyacrylamide is toxic for yeast cells. Alginate gels decompose when there are excessive amounts of phosphoric ion in the nutrient medium. Radiation polymerization can produce a large amount of artificial, nontoxic polymer carriers for immobilized cells and can easily and continuously change the properties and the structure of the carrier to effectively immobilize cells (*7*, *8*); therefore, this method of immobilized cells has potential application in the food and medical industries. Fujimura (*9*, *10*) and Lu (*11*−*13*) have studied several methods to immobilize yeast cells with artificial polymer carriers produced by radiation polymerization at low temperature, and the immobilized cells thus obtained exhibited high ethanol productivity. However, the mechanical strength of the polymer carriers becomes weak, and low ethanol productivity is observed with increasing water content of the polymer carriers or with decreasing monomer concentration. To improve the efficiency of immobilized yeast cells in low-concentration monomer, we studied the effects of adding cross-linking reagent in low-concentration monomers on the activity of immobilized yeast cells, water content, and structure of the polymer carriers in this work.

MATERIALS AND METHODS

Microorganism. *Saccharomyces formesensis* was used in this work. The yeast cells were precultured under aerobic conditions at 28 °C for 48 h in a nutrient medium consisting of 1% glucose, 0.1% molasses. 0.5% peptone, 0.3% yeast extract, and 0.3% malt extract (pH 4.8).

Preparation of Carriers and Immobilization of Yeast Cells. Three kinds of monomers, 2-hydroxyethacrylate (HEA), methoxy polyethylene glycol methacrylate (M23G), and a cross-linking reagent having a bifunctional group, tetraethylene glycol dimethacrylate (4G), which were obtained from Shin-Nakamura Chemical, were employed in this work. They were mixed with water to various concentrations, and the mixtures were irradiated by γ-rays from a ^{60}Co radiation source for 60 min at a dose rate of 10 kGy/h at −78 °C. The resultant polymer carriers were cut into small pieces, ∼5−10 mm in diameter, and then shaken with an excess amount of water for 3 days in order to become fully swollen. The swollen carriers were sterilized by autoclaving at 120 °C for 40 min and then immersed into the nutrient medium for 2 days to be filled with the nutrient medium. The swollen and sterilized carriers (10 cm^3) were added to the mixture of the nutrient medium (20 mL) with precultured yeast cells (1 mL). The resultant suspension was incubated

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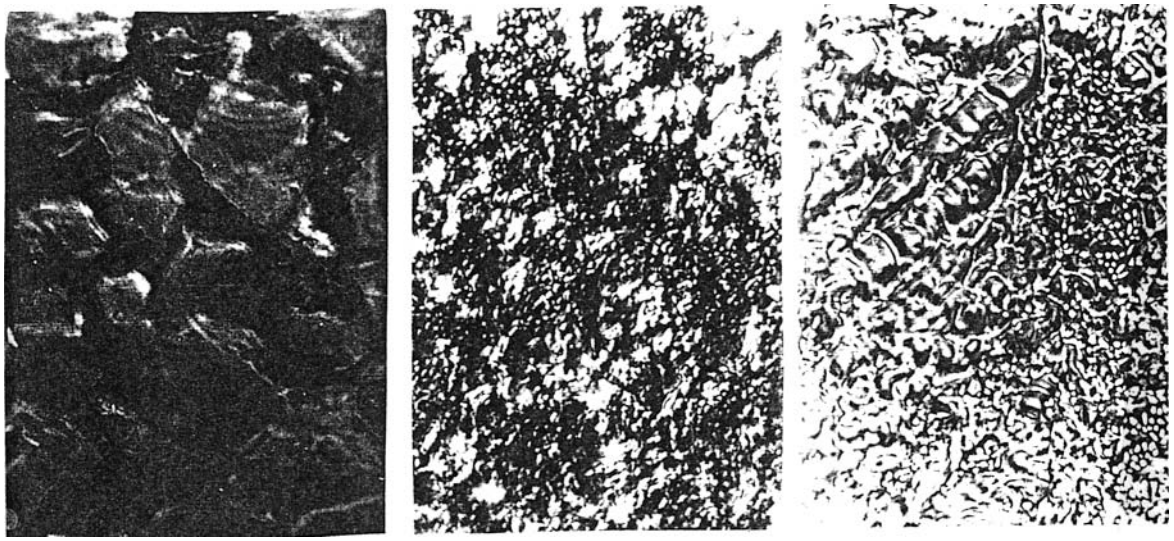

Figure 1. Structure of poly(HEA/M23G) carrier and yeast cells of immobilization with the carriers: (A, left) 40%/40%/20% (HEA/M23G/water); (B, middle) 20%/10%/70% (HEA/M23G/water); (C, right) 7%/7%/86% (HEA/M23G/water).

at 30 °C under an aerobic condition in a rotary shaker at 130 rpm for 72 h. The nutrient medium was changed every 24 h. The composition of the nutrient medium used in this work was 12% glucose, 1% molasses, 0.15% yeast extract, 0.25% NH_4Cl, 0.1% NaCl, 0.001% $CaCl_2$, and 0.3% lactic acid (pH 4.8).

Evaluation of Activity in Immobilized Yeast Cells. After aerobic incubation for 72 h, the polymer carrier immobilized yeast cells were washed well with the nutrient medium. The washed immobilized cells were put into 10 mL of the nutrient medium and fermented by incubation at 30 °C in a rotary shaker. After fermentation for 60 min, the concentration of ethanol was determined by using alcohol dehydrogenase (*14*).

Evaluation of Water Content of Polymer Carriers. Fully swollen polymer carriers were decanted and lyophilized for 72 h until the weight of carrier remained constant. The water content was calculated with following equation:

$$\text{water content (\%)} = \frac{\text{wt of fully swollen polymer} - \text{wt of dried polymer}}{\text{wt of fully swollen polymer}} \times 100$$

Observation of Structure of Polymer Carrier. The fully swollen polymers and polymers with immobilized yeast cells were sliced by knife as thinly as possible, and then the structure of the polymers and features of immobilized cells with the polymers were observed by means of an optic microscope (Nikon DIAPHOT-TMDP and pictures taken.

RESULTS AND DISCUSSION

Relationship between Ethanol Productivity and Water Content of Polymer Carriers. The dependence of ethanol productivity on the water content of the polymer carriers was studied without the cross-linking reagent through the change of the monomer composition in HEA/M23G copolymers. The results are shown in **Table 1**. The ethanol productivity of the immobilized yeast cells increased with the increase of water content of the carriers and reached a maximum at 94.5% water content, where it was 4 times higher than that of free cells, and then it sharply decreased when the water content of the polymer carriers increased further. The results indicated that the water content of the polymer carrier had a critical point for immobilized yeast cells, and there was an optimum water content for effective immobilization and multiplication of cells in the carrier. Excessive water content of polymer carrier was not favorable to the immobilization and multiplication of the cells.

Table 1. Relationship between Monomer Composition and Water Content of Polymer Carriers or Ethanol Productivity of Immobilized Yeast Cells with the Polymer Carriers

monomer composition %			water content of polymer carrier (%)	ethanol productivity of immobilized yeast cells (mg/mL·h)
HEA	M23G	water		
40	40	20	86.05	13.0
15	15	70	94.91	22.0
10	20	70	94.84	26.0
20	10	70	94.75	29.0
7	7	86	97.85	22.0
	free cells			7.50

Moreover, we investigated the relationship between monomer composition of the polymer carriers and water content of the polymer carriers after they had been fully swollen. From **Table 1** it can be seen that as total monomer volume (percent) decreased or water (percent) in monomer composition increased, the water content of the polymer carriers after swelling also increased. The reason is that, after polymer synthesis by radiation-induced polymerization at low temperature (−78 °C), the mixture of the monomers with water was cooled with dry ice and the ice precipitated from the homogeneous monomer solution. After radiation polymerization, monomer was polymerized and became solid polymer. When the polymer was kept at room temperature, the ice in the polymer melted, forming pores, so the polymer carrier became a porous structure. Obviously, the porous structure of the polymer carrier depended on the ice volume and ice size that resulted from the water volume in the monomer solution.

Then, the structures of the polymer carriers were observed by microscope. It was found that the structure of the carrier obtained from 40%/40%/20 HEA/M23G/water was dense and very high in mechanical strength but very low in pore number (**Figure 1A**). In this case, the ethanol productivity of immobilized cells with the carrier was lower because there was less space for the multiplication of yeast cells and because of the strong diffusion limit of substrate and O_2.

The structure of carrier yielded from 20%/10%/70% HEA/M23G/water was a network, porous structure, like a honeycomb, and had a larger pore size and optimum mechanical strength. The ethanol productivity with this carrier is the maximum, 29 mg/mL·h, indicating the carrier is an ideal one for the im-

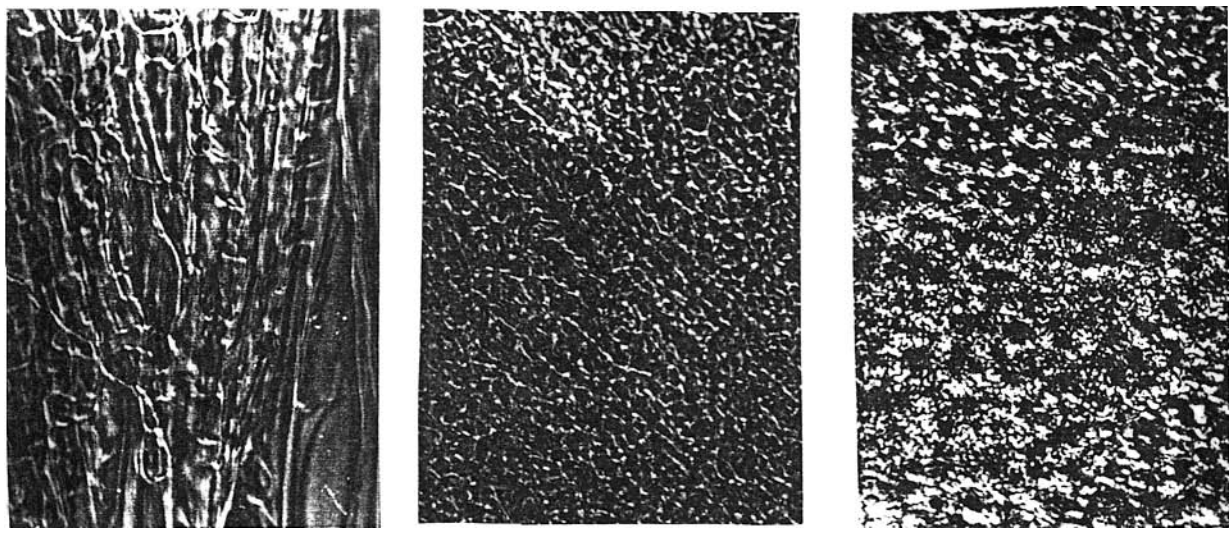

Figure 2. Effect of adding cross-linking reagent to low-concentration (HEA/M23G) monomer on structure of cross-linked carrier and immobilization of yeast cells: (A, left) 5%/5%/90% (HEA/M23G/water); (B, middle) 5%/5%/90% (HEA/M23G/water) with added 0.6% 4G; (C, right) state of cells immobilized with the carrier, resulting from (B) by radiation polymerization at −78 °C.

Table 2. Effect of Adding Cross-Linking Reagent on the Ethanol Productivity of Immobilized Cells

monomer composition (%)			cross-linking agent (%) 4G (of total monomer vol mL/100 mL)	ethanol productivity of immobilized cells (mg/L·h)
HEA	M23G	water		
10	10	80	0 (0)	24.8
10	10	80	3 (0.6)	28.8
10	10	80	6 (1.2)	26.4
10	10	80	9 (1.8)	21.65
7	7	86	0 (0)	25.2
7	7	86	3 (0.4)	24.2
7	7	86	6 (0.8)	31.7
7	7	86	9 (1.3)	26.6
5	5	90	0 (0)	28.9
5	5	90	3 (0.3)	30.75
5	5	90	6 (0.6)	35.75
5	5	90	9 (0.9)	28.8
	free cells			7.50

mobilization of yeast cells because there is enough space for multiplication of yeast cells and because of the lower diffusion limit of substrate and O_2 due to the porous network structure (**Figure 1B**).

In the carriers made from 7%/7%/86% HEA/M23G/water, the structure of the polymer was a network structure partly and the pore was very large and tubelike (**Figure 1C**). Although this structure contained enough space for cells to enter and multiply, the cells easily leached out and the mechanical strength of the carrier was too weak and was broken by intensive shaking owing to the large pore size, so the ethanol productivity was decreased. From the above discussion, we came to the conclusion that the porosity, pore size, network structure, and mechanical strength in polymer carrier is very important for effective immobilization of yeast cells, whereas the water content of the polymer synthetically reflects the effects of these factors and can be used as an indictor to evaluate quantitatively properties of the polymer for immobilization of cells.

Immobilization of Yeast Cells with Cross-Linking Carrier. The compositions of HEA/M23G monomer to cross-link were 10%/10%, 7%/7%, and 5%/5%, respectively. The concentrations of cross-linking agent, 4G, added were 3%, 6%, and 9% of total monomer volume in each composition as shown in **Table 2**. A relationship can be seen between the cross-linking agent concentration and the ethanol productivity of yeast cells immobilized with the cross-linked carriers. The ethanol productivity of yeast cells immobilized with the cross-linked carriers also increased, and it increased with increasing concentration of 4G, reached a maximum, and then decreased. The same trend was found for all three kinds of monomer compositions. However, the added 4G concentrations reaching the highest ethanol productivity were different in the three kinds of monomer compositions, that is, 3% in 10%/10%, 6% in 7%/7%, and 6% in 5%/5% monomer compositions, respectively. The ethanol productivities of immobilized cells with the three carriers were 28.8, 31.7, and 35.75 mg/L·h, respectively, ∼4−5 times free cells. This result indicated that adding cross-linking agent is favorable for effective entrapment of yeast cells.

It was found that the effect of adding 4G to a relatively low monomer concentration, such as 5%/5%, on the ethanol productivity of immobilized cells was better than that in a relatively high one. The lower the concentration of monomer was, the larger the pore size was. To obtain a suitable carrier with an appropriate pore size for effective entrapment and multiplication of yeast cells, the concentration of added 4G in 5%/5% monomer solution must be higher than that in 10%/10% so that the cross-linking molecular number in the polymer carrier was increased. However, when the concentration of 4G was excessively high, the cross-linking molecular number in the polymer carrier was further increased, resulting in an excessively small pore size and less space for multiplication of immobilized yeast cells. As a result, the ethanol productivity decreased.

To confirm the above discussion, the structure of polymer carriers to which cross-linking agent had been added was observed by optical microscope. **Figure 2A** shows that the structure of the polymer resulting from 5%/5% monomer solution has a tubelike structure and large pore size. In this structure, yeast cells leached out easily and carrier was also broken easily by shaking in a rotary shaker. The carrier resulting from 5%/5% monomer solution to which 6% 4G had been added was of network structure like a honeycomb and had certain elasticity and mechanical strength. The pore size of the carrier became smaller than that in 5%/5%, ∼20−30 μm (**Figure 2B**). The state of immobilized yeast cells with the carrier after incubation for 72 h is shown in **Figure 2C**. As can be seen, the

vision field of the microscope was filled with yeast cells. The results indicated that the immobilized yeast cells multiplied actively and had filled all parts of the carrier after incubation for 72 h. Thus, the ethanol productivity reached the maximum, 35.75 mg/L·h.

By adding less cross-linking reagent into a low concentration of HEA/M23G, the water content, cross-linking density, porosity, and mechanical strength of the polymer can be well adjusted owing to the hydrophobic property and cross-linking function of 4G, so polymer carriers that have proper pore size, network-like structure, and suitable mechanical strength can be obtained. Therefore, it is a very useful method not only for enhancing the efficiency of immobilized yeast cells but also for reducing the cost of polymer carriers because less monomer solution is used.

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