Immobilization of Yeast Cells with Ionic Hydrogel Carriers by Adhesion-Multiplication

Lu Zhaoxin*,† and T. Fujimura‡

College of Food Science & Technology, Nanjing Agriculture University, 1 Weigang, Nanjing 210095, China, and Takasaki Radiation Chemistry Establishment, Japan Atomic Energy Research Institute, Takasaki, Gunma 370-12, Japan

The mixture of an ionic monomer, 2-acrylamido 2-methylpropanesulfonic acid (TBAS), and a series of poly(ethylene glycol) dimethacrylate (nG) monomers were copolymerized with ^{60}Co γ-rays, and the produced ionic hydrogel polymers were used for immobilization of yeast cells. The cells were adhered onto the surface of the hydrogel polymers and intruded into the interior of the polymers with growing. The immobilized yeast cells with these hydrogel polymers had higher ethanol productivity than that of free cells. The yield of ethanol with poly(TBAS-14G) carrier was the highest and increased by 3.5 times compared to the free cells. It was found that the ethanol yield increased with the increase of glycol number in poly(ethylene glycol) dimethacrylate. The state of the immobilized cells was observed with microscope, and it was also found that the difference in the ethanol productivity is mainly due to the difference in the internal structure and properties of polymer carrier, such as surface charge, hydrophilicity, and swelling ability of polymer carrier.

Keywords: *Immobilization; yeast cells; ionic polymer; adhesion-multiplication; ethanol productivity*

INTRODUCTION

In methods of immobilized cells, adhesion is the simplest and the oldest method. The cell adhesion, however, has several advantages: simplicity, low cost, and easy sterilization, reuse of carrier, and good initial activity retention. The adhered cells, however, are desorbed with the change of pH and ionic strength.

Immobilization of yeast cells with various carriers (e.g. acrylamide gel, carrageenan, sodium alginate, glass bead and polymer by radiation polymerization) has been reported (Aykut et al., 1988; Misura et al., 1980; Simon 1989; Haecht et al., 1985; Iersel et al., 1999; Pilkington et al., 1998). The success of immobilization lies on the quantity of survival cells on/in the carrier, the efficient intake of substrate, and removal of products from a simple diffusion. Immobilized method, carrier structure, culture condition, and others determine the quantity of immobilized growing cells. The diffusion depends on the internal structure, such as porosity and pore diameter of the carrier. Therefore it is important to prepare the proper carrier. Radiation polymerization can produce artificial polymer carriers for immobilized cells and change easily and continuously the property and the structure of the carrier (Lu et al., 1992a, 1992b, 1994, 1995).

In this work, immobilization of yeast cells by the adhesion-multiplication method with the carriers, which was produced from hydrophilic, ionic monomer 2-acrylamido-2−2-methylpropanesulfonic acid (TBAS) (Japan patent 1976) with poly(ethylene glycol) dimethacrylate monomers (nG) by radiation polymerization, was studied. The relation among the properties, the structure of polymer carriers, and the activity of immobilized yeast cells was discussed.

Table 1. Composition and Property of Carriers and Ethanol Productivity of Immobilized Cells

monomer composition	glycol no.	property		ethanol	
		swelling	strength	productvty (mg/L·h)	conversn (%)
TBAS-2G 15:15	2	worse	hardness	21.9	33.7
TBAS-4G 15:15	4	bad	hardness	23.0	35.4
TBAS-9G 15:15	9	good	softness	26.6	40.3
TBAS-14G 15:15	14	better	softness	29.0	44.6
free cells				6.6	10.2

MATERIALS AND METHODS

Microorganism. *Sacchamyces fermosensis* was used in this work. The yeast cells were precultured under aerobic conditions for 48 h at 28 °C in a 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. A hydrophilic, ionic monomer TBAS was mixed with diethylene glycol dimethacrylate (2G), tetraethylene glycol dimethacrylate (4G), nonaeethylene glycol dimethacrylate (9G), and tetradecaethylene glycol dimethacrylate (14G), respectively (see Table 1). The mixture was irradiated at −78 °C with 10 kGy/h of γ-rays from ^{60}Co for 1 h. The resultant polymer carriers were cut to small pieces, approximately 3−5 mm in diameter, and shaken with an excess amount of water for 3 days in order to be fully swollen. The swollen hydrogels were sterilized by autoclaving at 120 °C for 40 min and then immersed into the aseptic nutrient medium for 2 days to be filled with the nutrient medium. The precultured yeast cells (1 mL) were inoculated into 20 mL of the nutrient medium with 10 cm^3 of aseptic carrier. The resultant suspension was incubated at 30 °C under aerobic conditions in a rotary shaker in 130 rpm for 72 h. For every 24 h, the nutrient medium was changed. The composition of the nutrient medium used in this

† Nanjing Agriculture University.

‡ Japan Atomic Energy Research Institute.

* Corresponding author. (telephone, 86-25-4396431; fax, 86-25-4395155; e-mail, fmb@lib.njau.edu.cn).

10.1021/jf000520d CCC: $19.00 © 2000 American Chemical Society
Published on Web 11/22/2000

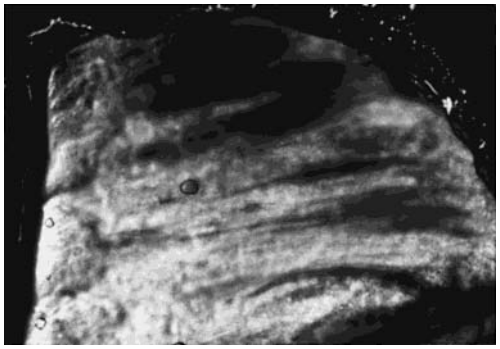

Figure 1. Process of immobilization of yeast cells with poly(TBAS-14G). The black part show the cells because of transparent poly(TBAS-14G).

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 Ethanol Productivity of Immobilized Yeast Cells. After aerobic incubation for 72 h, the polymer carriers immobilized yeast cells were washed well with the nutrient medium and then put into 10 mL of a new nutrient medium and fermented by an incubator at 30 °C under anaerobic conditions in a rotary shaker. After fermentation for 60 min, the concentration of ethanol was determined by using alcohol dehydrogenase (Bonichsen, 1971).

Observation of Structure of Polymer Carrier. The observation of the structure of the polymer carrier was carried out by means of optical microscope (NIKON DIAPHOT-TMD).

RESULTS AND DISCUSSION

Process of Immobilization of Yeast Cells. The poly(TBAS-nG) carriers, porous hydrogels, were immersed in the mixture of precultured yeast cells and the nutrient medium. The resultant suspension was incubated at 30 °C under aerobic conditions on a rotary shaker for 24 h, and the many colonies of yeast cells were observed on the surface and in the inside of the carriers, suggesting that the yeast cells are immobilized by adhesion of the cells onto the surface of the polymer carrier and subsequent multiplication of the cells, which was named as the adhesion-multiplication method. Figure 1 shows the feature of yeast cells entering the poly(TBAS-14G), indicating the immobilizing process of yeast cells.

Ethanol Productivity of Yeast Cells Immobilized with Poly(TBAS-nG). To measure activity of the immobilized yeast cells, the immobilized cells were incubated under anaerobic conditions for 1 h. The result is shown in Table 1. The ethanol productivity of immobilized cells was higher than that of the free cells, increasing by 2−3.5 times. The ethanol conversion ratio was the highest, nearly 50% in 1 h, for poly(TBAS-14G) carrier. The result indicted that the yeast cells immobilized with ionic hydrogels by the adhesion-multiplication method were of higher activity. However, the ethanol productivity varied with the carriers; the possible reason may be due to a different structure and surface property of the polymer.

Adhesion-multiplication method for cell immobilization has significant advantages when compared to other immobilization methods. One would be able to avoid cell damage or injury due to the chemical reactions in the carrier preparation in entrapment immobilization. Another may be able to increase the surface area of the carrier and to decrease the leakage of immobilized cells due to the network structure in absorption immobilization. As a result, the immobilized cells grew and multiplied well, and the quantity of the immobilized cells per unit volume was much greater than the free cells.

In this work, we used the adhesion-multiplication method for immobilization of yeast cells. Therefore, the effect of immobilization was relative to the initial adhesion of cells and polymer structure. Initial adhesion of cells depends on the surface properties, such as hydrophobicity, bearing-charge, and roughness, of the carrier. Several research investigations have demonstrated that the application of a cationic species, such as gelatin, aluminum ion, and cationic starch, improves the cells adhesion to the carriers. To enhance the initial adhered cells quantity, the TBAS monomer, which bears positive and negative charge groups, was introduced for the immobilization of yeast cells. When the pH changed, TBAS had the following changes:

$$CH_2{=}CH{-}CO{-}NH^{+}{-}C(CH_3)_2{-}CH_2{-}SO_3H \rightleftharpoons CH_2{=}CH{-}CO{-}NH{-}C(CH_3)_2{-}CH_2{-}SO_3H \rightleftharpoons CH_2{=}CH{-}CO{-}NH{-}C(CH_3)_2{-}CH_2{-}SO_3^{-}$$

While pH is lowered, the reaction may go forward to the left, becoming the positive charge or decreasing negative charge in the molecular. This surface property is favorable to the adhesion of yeast cells, enhancing initial adhesion by electrostatic interaction of cells and polymer due to the negative charge of the microorganism surface. In this work, the polymer carriers may bear a positive charge at pH 4.8, which promoted an initial adhesion of yeast cells with electrostatic interaction, thus an increase of ethanol productivity was observed. As for the 15%:15% ratio of the poly[HEA-M23G; (HEA: 2-hydroxy ethacrylate; M23G: methoxy poly(ethylene glycol) methacrylate)] carrier, which has a similar structure including pore size, porosity, hydrophilicity, and mechanical strength to poly(TBAS-14G), but not bearing a charge group, the ethanol productivity of yeast cells immobilized was 19.8 mg/L·h (Lu and Fujimura, submitted), which indicated that the polymer carrier with a cationic group is superior to the polymer carrier polymerized with two kinds of hydrophilic monomers (HEA and M23G) without a cationic group for immobilization of yeast cells.

Dependence of ethanol productivity of yeast cells immobilized with poly(TBAS-9G) and free yeast cells on incubation time is shown in Figure 2. For immobilized cells, the ethanol productivity was rapidly increased with increasing the incubation time to 120 h and reached 40 mg/mL·h. While the ethanol productivity of free cells was increased with an increase in the incubation time to 72 h, it came to 6.6 mg/mL·h. The result indicated the stability and higher ethanol productivity of immobilized cells with the carrier.

The Structure of Polymer Carriers and the Ethanol Productivity of Immobilized Yeast Cells. To investigate the relationship between the structure of polymer carriers and the state of immobilized cells, the internal structure of carriers was observed with an optical microscope. Figure 3 showed the structures of poly(TBAS-2G) and poly(TBAS-14G). Poly(TBAS-2G) appeared to be porous but smaller and less porous than poly(TBAS-14G). For the former, though yeast cells could be introduced into the interior of the carrier, the

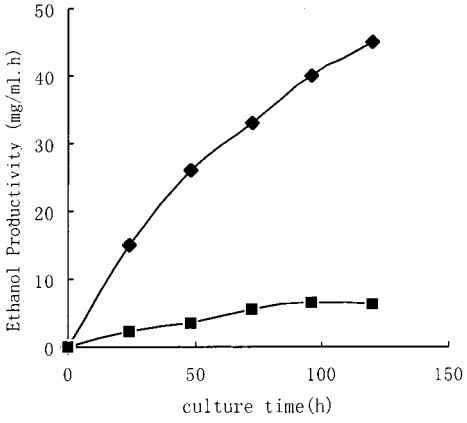

Figure 2. Ethanol productivity of cells immobilized with poly-(TBAS-9G) and free cells: ◆, immobilized cells; ■, free cells.

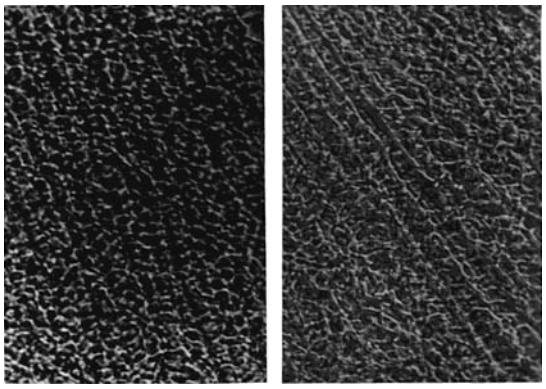

Figure 3. The structure of poly(TBAS-2G) and poly(TBAS-14G); left, Poly(TBAS-2G); right, poly(TBAS-14G).

diffusion of the nutrient, O_2, and the product was affected, and the density of immobilized cells decreased because they were smaller and less porous due to 2G being a hydrophobic monomer. As a result the ethanol productivity of immobilized yeast cells with poly(TBAS-2G) was lower. For the latter, a higher hydrophilic polymer owing to both 14G and TBAS being hydrophilic monomers, yeast cells adhered onto the surface of the carrier and then intruded into the interior and began to multiply. With more pores and larger pore size, the diffusion of the nutrient, O_2, and the product was easier, consequently, yeast cells not only could intake enough nutrient but also could grow and multiply as well. Therefore, the ethanol productivity of immobilized yeast cells with poly(TBAS-14G) was higher, rising by 30% in comparison with that of poly(TBAS-2G). But in previous results, however, indicated that excessively large pores could cause the decrease of polymer strength and linkage of immobilized cells.

The Relationship between the Glycol Number of the Monomer and the Ethanol Productivity of Immobilized Yeast Cells. The relationship between the glycol number of the monomer and the ethanol productivity of immobilized yeast cells was investigated and plotted in Figure 4 as a function of the glycol number. The ethanol productivity of immobilized cells was increased with an increase of the glycol number, indicating that it is good to use the monomer of a more glycol group for the immobilization of yeast cells in the work. The molecular formula of nG monomers is $CH_3-CH=CH-CO(O-CH_2-CH_2O)_nCO-CH=CH-CH_3$. As the glycol number is increased, the length of the molecular chain becomes longer, the hydrophobicity of

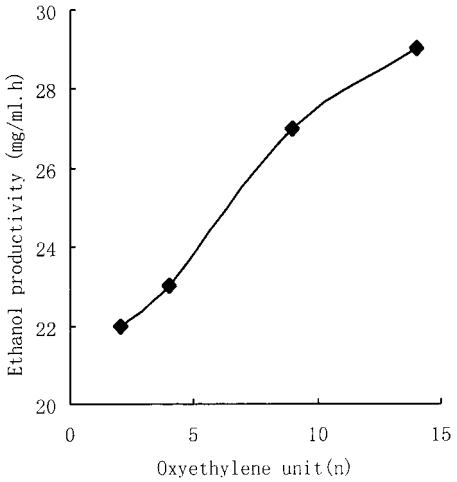

Figure 4. Relation between oxyethylene unit and ethanol productivity of immobilized cells.

the monomer becomes weaker, but the hydrophilicity appears to get stronger. Generally, 2G and 4G are hydrophobic monomers, while the monomers over 9G are hydrophilic monomers. When the solution of TBAS with nG was polymerized by radiation, the hydrophilicity of poly(TBAS-14G) was stronger than that of poly-(TBAS-2G) (Table 1). So poly(TBAS-14G) has higher swelling capacity and elasticity in the property and has larger and more pores in the internal structure than that of poly(TBAS-2G). The result indicated that the effect of the glycol number on ethanol productivity of immobilized yeast cells was attributed to the change of the polymer structure. As above-mentioned, however, excessive hydrophilicity and excessively large pores lead to the decrease of polymer strength and linkage of immobilized cells. Therefore, the use of 9G or 14G is favorable in preparing the carrier with TBAS.

LITERATURE CITED

Aykut, G.; Hasirci, V. N.; Alaeddinoglu, G. Immobilization of yeast cells in acrylamide gel matrix. *Biomaterials* **1988**, *9*, 168−172.

Bonichsen, R. *Method of Enzymatic analysis*; Academy Press: New York, 1971; p 285.

van Iersel, M. F. M.; van Dieren, B.; Rombouts, F. M.; Abee, T. Flavor formation and cell physiology during the production of alcohol-free beer with immobilized *Saccahromyces cerevisiae*. *Enzyme Microb. Technol.* **1999**, *24*, 407−411.

Japan Patent. Hydrophilic acrylamide polymer (JP. 51-19090), 1976; pp 512−522.

Mitsura, W. Kato, J.; Chibata, I. Continuous production of ethanol using immobilized growing yeast cells. *Microbiol. Biotechnol.* **1980**, *10*, 275−278.

Lu, Z.-X.; Kumakura, M. New immobilization method of filamentous cells with thin paper carrier surface modified by radiation. *J. Microbiol. Method* **1994**, *19*, 29−38.

Lu, Z.-X.; Fujimura, T. A study on ethanol production of yeast cells immobilized with polymer carrier produced by radiation polymerization. *Radiat. Phys. Chem*. **1992a**, *42*(4−6), 923−926.

Lu, Z.-X.; Carenza, M.; Kaetsu, I.; Kumakura, M.; Yoshita, M.; Fujimura, T. Immobilization of yeast cell on hydrogel carriers obtained by radiation induced-polymerization. *Radiat. Phys. Chem*. **1992b**, *40*(6), 579−926.

Lu, Z.-X.; Kumakura, M. Production of Gibberellic acid in *Gebberella fujikuroi* adhered onto plymeric fibrous carriers. *Process Biochem*. **1995**, *30*(7), 661−665.

Lu, Z.-X.; Fujimura, T. Immobilization of Yeast Cells with Polymeric Carrier Cross-linked Using Radiation Technique. *Enzyme Microb. Technol*., submitted for publication.

Pilkinton, P. H.; Margaritis, A.; Mensour, N. A.; Russell, I. Fundamentals of immobilized yeast cells for continuous beer fermentation: a review. *J. Inst. Brewing* **1998**, *104*, 19∼31.

Simon, J. P. *Physiology of immobilized cells−an international symposium*; 1998; pp 54−56.

Van Haecht, J. L. M.; Bolipombo, P. G. RouxhetImmobilization of *Saccharomyces cerevisiae* by adhesion. *Bioeng. Biotechnol.* **1985**, *27*(3), 217−224.

Received for review April 26, 2000. Revised manuscript received September 6, 2000. Accepted September 11, 2000.

JF000520D