Development of a Novel Microorganism Immobilization Method Using Anionic Polyurethane

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ABSTRACT: A new microorganism immobilization method involving gelation of anionic polyurethane has been developed. Nontoxic synthetic anionic polyurethane was used, which provides greater mechanical strength than do natural polymers. Similar to the formation of Ca-alginate gel beads, this method has the advantage of easy preparation. Gel beads are formed only by dropping the anionic polyurethane emulsion into a calcium chloride solution. The feasibility of the proposed immobilization method was tested by an ethanol production process with baker's yeast (*Saccharomyces cerevisiae*). The result verified that the anionic polyurethane entrapped–cell method developed in this study has great potential for a variety of applications such as biological processes and biological wastewater treatment. © 2005 Wiley Periodicals, Inc. J Appl Polym Sci 99: 738–743, 2006

Key words: anionic polymerization; polyurethane; gelation

INTRODUCTION

Immobilization of biocatalyst (cells or enzymes) offers a promising potential for improving the efficiency of biological processes. Operational simplicity and efficient cell containment have made entrapment of cells in a proper support matrix a popular means for cell immobilization. Mild conditions are required for gelation of polymers in the presence of cells, so natural algal polysaccharides such as alginate, carrageenan, and agar are the materials most widely used for cell immobilization.[1] However, natural polymers have limited mechanical stability. To overcome such a drawback, synthetic polymers with strong mechanical stability, such as polyacrylamide, polyurethane (PU), and polyvinyl alcohol, have been developed and employed for the immobilization of living microorganisms. Nevertheless, the toxic effect of the chemicals, the high temperature and extremely high pH required for gel formation, as well as the complicated procedures involved have all limited the application of living microorganism immobilization. Therefore, a novel cell entrapment method that involves benign cell gelation and can achieve strong gel structure is much desired and needed.

Among all the synthetic polymers used for cell immobilization, PU is distinct for its excellent properties, such as mechanical, physical, and chemical stability as well as for possibly enabling its mass production at low cost on an industrial scale. However, the isocyanate groups in the raw materials are toxic to cells. To avoid such an unfavorable monomer effect, a method for synthesizing PU prepolymers [Scheme 1(a)] has been developed.[2] Although their toxicity has been reduced to a certain extent, these PU prepolymers still contain some extremely reactive isocyanate groups, which are toxic to living microorganisms.[3] Furthermore, the short handling time required during immobilization makes it almost impossible to prepare a large number of spherical biocatalysts. Therefore, a less toxic poly(carbamoylsulfonate) gel [PCS; Scheme 1(b)] was derived from the blocking of the functional ends of isocyanate groups during polymerization.[4] The PCS group is substantially less toxic to living cells than is the conventional isocyanate group, and the length of time for its gelation can be adjusted over a wide range of pHs. However, immobilization in PCS is laborious. During incubation the mixture becomes so viscous that the production of PCS beads makes it very difficult to handle, although it can still be done successfully.[5] Thus, polyurethane for cell immobilization is almost formed as rigid integral plate foam, which is then chopped into pieces for cell adsorption.

The aim of this study was to develop a fast, simple, and benign procedure for cell entrapment into a gel bead with high mechanical strength that could be done at low cost. The procedure was designed according to the principle of polymerization of PU ionomers. The family of PU ionomers that incorporate the ionic

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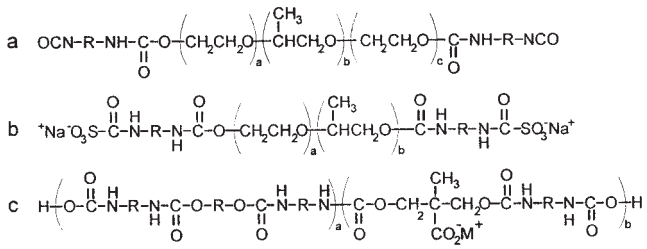

Scheme 1 General formula of different kinds of polyurethane: (a) isocyanate-containing PU prepolymer, (b) isocyanate-blocked PU prepolymer (PCS), (c) isocyanat -free anionic polyurethane (APU).

groups either into the molecular backbone or in the side chains to improve the physical properties and water dispersibility of the hydrophobic organic polymers is one of the most active branches in PU chemistry.[6] PU ionomers are well known for their ability to disperse or dissolve in water. However, anionic polyurethane [APU; Scheme 1(c)] dispersion is naturally unstable in the presence of polyvalent cations, and the APU is rapidly flocculated when it comes into contact with such cations. Thus, a large number of porous cell beads can be prepared easily in a very mild aqueous condition without using organic solvent or reactive chemical regents.

To demonstrate that the unstable APU dispersion phenomenon could be applied to the cell entrapment process, we first assessed the toxic effect of APU on microorganisms and the general effect of the immobilization conditions on microorganism activity. The mechanism of gel bead formation also was investigated. The feasibility of using this method was finally confirmed by the finding that production of ethanol using APU-immobilized *Saccharomyces cerevisiae* was efficient.

EXPERIMENTAL

Chemicals

Polycaprolactone (PCL), neopenty glycerol (NPG), trimethylol propane (TMP), dimethylolpropinic acid (DMPA), isophoronediisocyanate (IPDI), ethylene diamine (EDA), and triethylamine (TEA) were purchased from Aldrich (St. Louis, MO). All other chemicals used were of reagent grade.

Synthesis of anionic polyurethane

PCL (125 g), NPG (13 g), TMP (2.25 g), and DMPA (13.4 g) were mixed in a 500-mL flask and dehydrated under vacuum at 130°C for 1 h. The temperature was then adjusted to 70°C–80°C before the dropwise addition of IPDI (111.1 g). After 1 h of reacting, dried acetone was added to reduce the viscosity of the mixture. On completion of the reaction, which took another 6 h, the mixture was dripped into a vessel containing H_2O (500 g), EDA (7.5 g), and TEA (10.1 g). The final solution was stirred vigorously for 1 h, and a stable APU emulsion was obtained.

Microorganism

Baker's yeast (*S. cerevisiae*) was isolated from commercialized active yeast. The yeast cells were cultured at 28°C for 24 h on an orbital shaker at 150 rev/min.

Immobilization

First 10 mL of the APU emulsion (solid content 50%) was mixed with 3 g of centrifuged cells to produce an APU–cell mixture. The resulting cell suspension was mixed thoroughly in order to achieve homogeneous distribution of the cells. The cell suspension was extruded as discrete droplets into a 5% (w/v) $CaCl_2$ solution for 1–2 h in order to allow formation of gel beads, which were collected by filtration or decantation. It was found that the beads had an average diameter of 2 mm. During incubation, the entrapped cells in the gel beads were propagated in the complete medium on a rotary shaker at 30°C.

Viability of free cells

Respiration rate was determined by measuring the rate of O_2 uptake of the free cells and of the cell mixture with APU emulsion. Baker's yeast was dispersed as free cells. The cell mixture with APU emulsion was stirred for 1 h before testing. The culture medium saturated with atmospheric O_2 was stirred in a full 150-mL incubation flask with 10 mL of free cells or the cell mixture (with the same biomass). The respiration rate was calculated by measuring the decrease in dissolved oxygen (DO). Calculation of the viability of free cells in contact with the APU emulsion was as follows[7]:

$$P = (R_{r1}/R_{r0}) \times 100\%$$

where P is the viability (%); R_{r1} is the respiration rate after mixing with the APU emulsion (mg O_2/min); and R_{r0} is the respiration rate of free cells with the same weight (mg O_2/min).

Comparison of activity of free and immobilized cells

The activity of the free and the immobilized cells was determined by measuring ethanol production efficiency in a pH range of 3.0–7.0 at 30°C and in a temperature range of 20°C–50°C at pH 5. The experiments were carried out in a 50-mL Erlenmeyer flask

containing 30 mL of ethanol production medium (1 L of the medium contained 30 g of glucose, 8 g of KH_2PO_4, 0.5 g of $MgSO_4$, 2 of $(NH_4)_2SO_4$, and 8 g of yeast extract). Either 3 mL of the cell suspension (20 mg dry cell weight/mL) or 5 g of the cell beads (12 mg dry cell weight/g of gel) were added to the flask.

Ethanol production

The batch conversion of glucose to ethanol was carried out in a 500-mL Erlenmeyer flask containing 200 mL of ethanol production medium. Ten grams of cell beads were added to the flask and incubated at 30°C on an orbital shaker at 150 rev/min. During the process, the concentrations of glucose, ethanol, and cells in the gel beads were measured.

Gelation mechanism

Infrared (IR) spectra were utilized to determine the gel bead formation mechanism. Films for IR spectrum testing were prepared in a Teflon mold, the inner dimensions of which were $10 \times 10 \times 0.5$ cm. The APU emulsion was diluted with tetrahydrofuran (THF) solvent and then poured into the mold. Because it was difficult for gel beads to dissolve, the gel beads were swelled by THF for 4 h and then cast into the mold. After standing at room temperature overnight to allow evaporation of the solvent, the films were vacuum-dried at 50°C for 24 h to remove the residual solvent completely.

Analytical methods

The biomass concentration was determined by dry cell weight after 24 h of drying at 105°C. The protein content of the cells as well as the calculated biomass was determined according to the bicinchoninic acid assay method as modified in our previous study.[8] The IR spectra were recorded using an FTIR spectrophotometer (Bomem DA 8.3 with attenuated total reflectance) at a resolution of 2 cm^{-1}. Ethanol production was quantified by a gas chromatographer (G3000, Hitachi, Japan) equipped with an FID detector. Glucose concentration was analyzed by HPLC using a Jasco 880 PU (Jasco, Japan) equipped with an RI detector.

RESULTS AND DISCUSSION

Toxic effect of anionic polyurethane

The respiration rates of the cells in contact with/without APU emulsion were 0.57 and 0.55 mg $O_2 \cdot min^{-1}$, respectively. This reveals that the viability of the baker's yeast remained almost constant after the cells came into contact with the APU emulsion. Sumino et al. reported that the viability of nitrifying bacteria was completely lost after the cells were mixed with 1%–12% (w/v) toluene diisocyanate (TDI).[7] Vorlop et al. showed that the residual activity of yeast cells (*S. cerevisiae*) was obviously inhibited in the presence of a low concentration of the functional group isocyanate, and the activity of the cells decreased slightly at a higher concentration of blocked TDI.[9] Because the APU emulsion did not contain any isocyanate groups or plasticizer, toxicity was expected to be negligible. The structure of these three kinds of PUs are compared in Scheme 1.

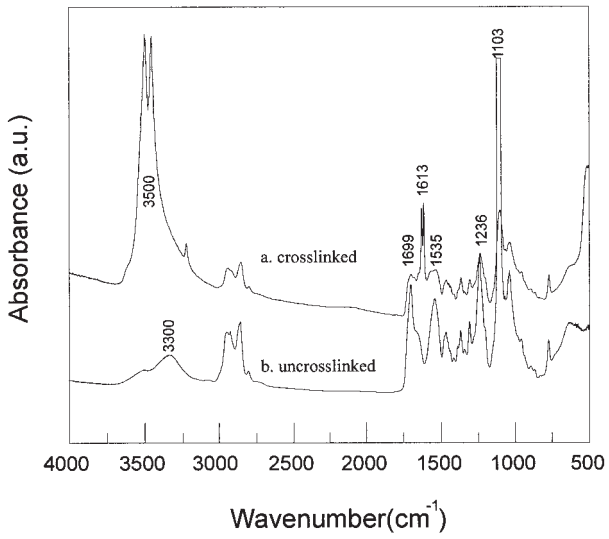

Figure 1 FTIR spectra of APU film before and after being crosslinked.

Mechanisms of gel bead formation

APU with carboxylate groups can be dispersed in water. The dispersed emulsion is generally unstable and flocculated when it comes in contact with multivalent cations—that is, APU will be crosslinked by multivalent cations and then will precipitate. Therefore, this concept from the above flocculation phenomenon could be developed as a method of producing immobilized-cell gel beads. The results of IR spectra for the films of crosslinked and uncrosslinked PUs cast from THF solutions are shown in Figure 1. The peak of the carboxylate groups, originally at 1699 cm^{-1} for the uncrosslinked PU, shifted downward by TEA ionization. The extent of the shift increased with the degree of ionization, and the peak centered at 1613 cm^{-1} when the carboxylate group was fully ionized by calcium ions was 86 cm^{-1} away. In the region of the NH band, a peak at 1535 cm^{-1} occurred only for the uncrosslinked PU because of the NH group of TEA. After APU was crosslinked with calcium ions, TEA was replaced by calcium ions, and the peak at 1535 cm^{-1} disappeared. The broad peak at 3300 cm^{-1} was attrib-

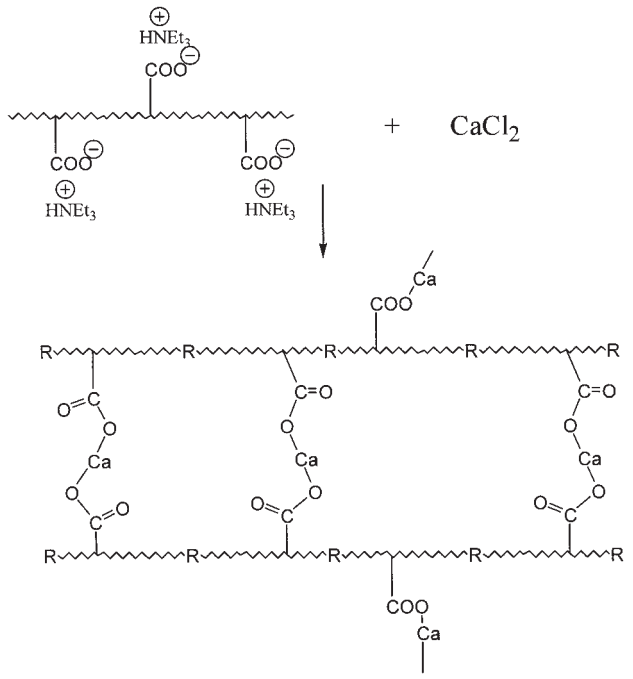

Figure 2 Chemical structure of APU crosslinked with calcium ions.

uted to the hydrogen-bonded NH group of TEA, and the peak from free NH was almost absent for uncrosslinked APU. After APU was crosslinked with calcium, the peak of the hydrogen-bonded NH group of TEA disappeared, and a split peak at 3500 cm^{-1} was observed because of the strong hydrogen bond between the calcium-ionized carboxylate groups and the hydrogen in the main chain of APU. These results showed that the crosslinking reaction was conducted by the carboxylate group and calcium ions. The proposed chemical structure is shown in Figure 2. It is similar to that of Ca-alginate beads. However, this process is irreversible and APU gel beads cannot be counteracted by chelating agents such as phosphate. These results revealed that the immobilization of cells could conveniently be carried out under very mild conditions. Furthermore, we could not find the peak of the isocyanate group at around 2200 cm^{-1} in Figure 1. This indicates the absence of toxic isocyanate groups in the APU emulsion, confirming that the viability of cells was nearly 100% as described above.

Feasibility for ethanol production using APU-immobilized cell beads

For an immobilization method to be successful, the cells must be able to grow in the gel beads as well as function normally without much interference from environmental factors. Therefore, the feasibility of using the newly developed immobilization method was evaluated by the ability of cells to grow, the flexibility of cells in response to changes in pH and temperature, and the efficiency of ethanol production expressed as dry-cell weight calculated from the protein content of cells in the APU-immobilized gel beads. Because APU gel is nontoxic and the reaction conditions were friendly or benign to cells, the cells would grow rapidly when the cell beads were transferred into the growth medium. After 27 h of incubation, the culture reached maximum cell density (17 mg dry cell/g gel), comparable to that of yeast grown in κ-carrageenan gel[10] and alginate gel.[11] This result showed that the network in the interstices of the APU gel also provided a good environment for cell growth like natural polysaccharide.

The extended capability of APU-immobilized cell beads was shown by experimenting with the influence of pH and temperature on the cell system. Figure 3 shows the effect of pH on ethanol production by free and immobilized yeast cells in the above-mentioned medium at 30°C. The maximum activity of immobilized and free cells was 1.1 and 0.8 g h^{-1} g^{-1} dry cell, respectively. The optimum pH for ethanol production by immobilized cells was similar to that of free cells. In contrast, the immobilized system showed a wider operating range of optimal pHs. In other words, the activity of immobilized cells became less sensitive to pH, both at higher and at lower pH values. Residual activity of the immobilized cells was maintained at more than 80% of the maximum activity at a pH as high as 8.0 or as low as 3.0, whereas the free cells lost about 60% of their activity at a pH of 8.0 or 3.0. When yeast grows, the production of carbon dioxide acidifies the local environment. This could explain why there was no obvious decrease in activity at an alka-

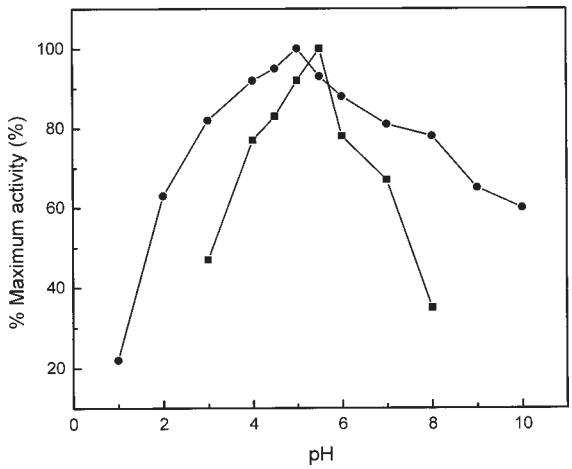

Figure 3 Effect of pH on activity of immobilized yeast cells, determined from ethanol concentrations after 120 min of fermentation. Relative activity was defined as a percentage of maximum activity: (■) free cell (maximum activity: 0.8 g h^{-1} g^{-1} dry cell), (●) immobilized cell (maximum activity: 1.1 g h^{-1} g^{-1} dry cell).

line pH. Furthermore, the carboxylate groups of the APU could have provided a buffering capacity, and therefore activity can have been maintained at a low pH. The temperature activity profile of the free and immobilized cells is shown in Figure 4. The optimal temperature was 40°C for both free and immobilized cells. The maximum activity of the immobilized and free cells was 1.3 and 1.4 g h^{-1} g^{-1} dry cell, respectively. The optimum temperature range, which showed as a relatively broad concave curve, was found to be between 30°C and 45°C for the cells entrapped in APU gel beads. This implies that the stability of cells against pH and temperature was increased after immobilization in the APU.

The ethanol production experiment was carried out in a batch mode in order to determine the fermentation capacity of yeast cells entrapped in APU gel beads. The time course of ethanol production of sucrose is shown in Figure 5. The cell concentration of the immobilized-cell system was calculated according to the cell concentration in gel beads (10 g of gel beads were added in this experiment). Compared with a free cell, an immobilized cell grew slowly in the gel beads (from 0.45 to 0.9 g/L), and most of the glucose was used to produce ethanol. After 20 h of incubation, the concentration of ethanol rose above 3.5% (v/v) for the immobilized-cell system and 1.5% (v/v) for the free-cell system. The initial specific ethanol productivity during fermentation reached 1.1 g h^{-1} g^{-1} dry cell at 30°C. The immobilized cells showed higher specific ethanol productivity than did the suspended cells under similar conditions (about 0.8 g h^{-1}•g^{-1} dry cell).

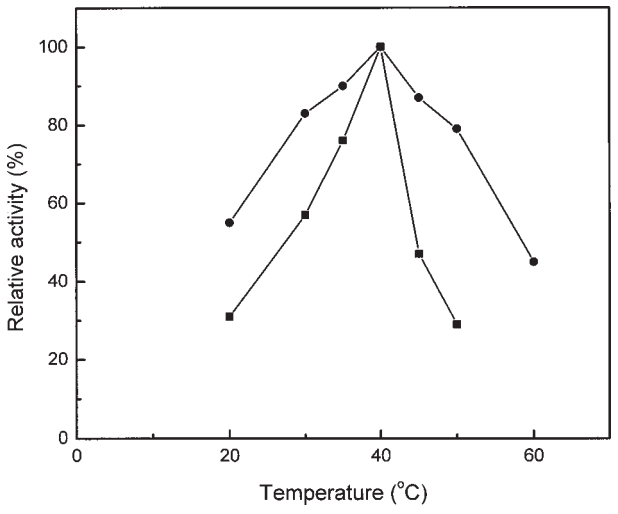

Figure 4 Effect of temperature on activity of immobilized yeast cells, determined from ethanol concentrations after 120 min of fermentation. Relative activity was defined as a percentage of maximum activity: (■) free cell (maximum activity: 1.4 g h^{-1} g^{-1} dry cell), (●) immobilized cell (maximum activity: 1.3 g h^{-1} g^{-1} dry cell).

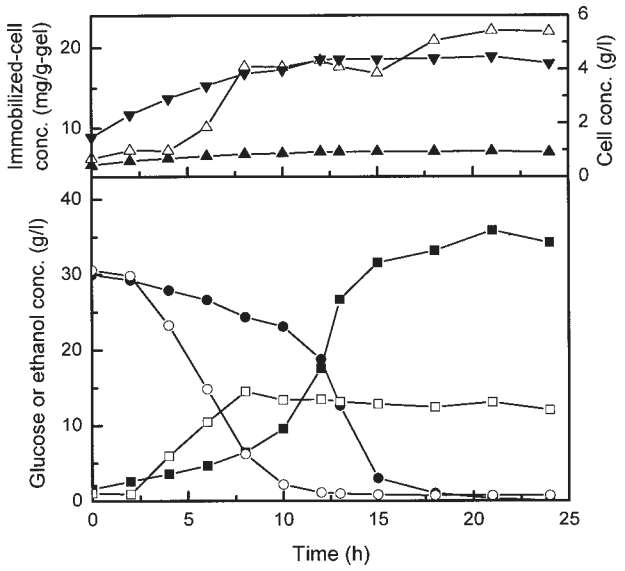

Figure 5 Ethanol production by immobilized cells and free cells: (▼) cell concentration in gel bead, (▲, □) cell concentration (according to volume of culture), (■) ethanol concentration of immobilized cells, (□) ethanol concentration of free cells, (●) glucose concentration of immobilized cells, (○) glucose concentration of free cells.

We suggest that the newly developed carriers in our experiments served to promote ethanol tolerance and production in yeast.

CONCLUSIONS

The newly developed immobilization method detailed here has the advantages of easy operation and the use of synthetic PU that is nontoxic like natural polymers but possesses higher mechanical strength. This method of forming immobilized microorganism gel beads can easily be accomplished by dropping the APU-cell mixed suspension into a solution of $CaCl_2$. Because the chemical reagents it uses during the preparation process have no toxic effects, this method can optimally be used for entrapment of living microorganisms. Furthermore, APU emulsion, which has been widely used in the chemical industry, can be mass-produced at low cost. This convenient technique is promising for the future in the immobilization of various microbial cells. Further applications of this technique are under development.

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