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Effect of Various Factors on the Operational Stability of Immobilized Cells for Acrylamide Production in a Packed Bed Reactor

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The effect of concentrations of phosphate buffer and acrylonitrile, pH, and various salts on the operational stability of the immobilized cells of *Brevibacterium* CH2 in a packed bed reactor were investigated. The effects of salts and carriers on the swelling of the immobilized beads during hydrolysis in a column reactor were also investigated. Immobilization of the cells in Ba-alginate was more desirable than those in polyacrylamide and Ca-alinate for the swelling of the immobilized beads and the desired quality of the acrylamide produced. High quality acrylamide was produced using the Ba-alginate beads in a recycle fed-batch reactor without using an isotonic substrate. The conversion yield was nearly 100%, including a trace amount of acrylic acid produced as a by-product.

Acrylamide is one of the most important chemical commodities, being in great demand as a starting material for the production of various polymers used as flocculants, stock additives, and polymers for petroleum recovery.

Galzy and co-workers, and Yamada and his associates, proposed an enzymatic process for the production of acrylamide, which was quite different from the chemical method using copper catalyst (1, 5, 6, 18). Several groups of bacteria such as *Nocardia, Brevibacterium, Arthrobacter, Rhodococcus, Corynebacterium*, and *Pseudomonas* are known to be able to convert nitriles to corresponding amides (2, 3, 7-9, 12-15, 22, 23). Hwang and Chang reported an acrylamide production using *Brevibacterium* sp. CH1 in a recycle fed-batch reactor and in a dual hollow fiber bioreactor (16, 17). The strain possessed high nitrile hydratase activity for acrylonitrile, but the amidase activity toward acrylamide was negligible. Therefore, the conversion yield was nearly 100% with a trace amount of acrylic acid produced. The two-step degradation pathway of acrylonitrile, which involves hydratase and amidase with acrylamide as an intermediate is expressed as follows (4).

$$CH_2CHCN + H_2O \xrightarrow{\text{nitrile hydratase}} CH_2CHCONH_2 + H_2O$$
$$\xrightarrow{\text{amidase}} CH_2CHCOOH + NH_3$$

Recently, we isolated *Brevibacterium* sp. CH2 which was tolerant to higher acrylonitrile concentration, by repeated cultivation of *Brevibacterium* sp. CH1 in the broth with gradually increasing the acrylonitrile concentration. The specific nitrile hydratase activity of CH2 strain was 3.2 times higher than that of CH1 strain and the enzyme of the former had a higher acrylonitrile concentration tolerance than that of the latter (19-21). The formation of nitrile hydratase in CH2 strain was greatly enhanced by the addition of ferrous and ferric ions to the medium (11).

In the production of acrylamide using microorganisms, acrylonitrile is brought into contact with the microorganisms or immobilized cells in an aqueous substrate such as physiological saline solution and phosphate buffer solution. The use of physiological saline solution, phosphate buffer solution, or the like, as an aqueous substrate results in the contamination of acrylamide product by large amounts of sodium chloride, phosphates, or other salts. This is not desirable in the desired product quality. In particular, in the production of acrylamide-based polymers having a high molecular weight, the presence of

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phosphates in acrylamide is liable to cause water insolubilization of polymers formed. It, therefore, becomes necessary to apply post-treatments such as ion exchange treatment. This leads to the loss of the advantage since a high quality acrylamide aqueous solution can be produced by microorganisms without any special purification step.

On the other hand, if the saline solution, the buffer solution, or the like are not used as the aqueous substrate, the immobilized cells swell in the course of hydrolysis reaction, and the enzymatic activity of the cells is rapidly lost. Furthermore, in the case of the column reaction, when an aqueous solution of acrylonitrile passes through a column that is packed with cells conventionally immobilized with polyacrylamide, the immobilized cells in the column swell shortly after the start of the hydrolysis reaction, and this phenomenon becomes more severe as acrylamide concentration increases. As a result, efficient operation using this method becomes impossible (19, 24).

In this paper we report the effects of various factors on the operational stability of the immobilized cells of *Brevibacterium* sp. CH2 in a packed bed column reactor. In addition, this paper also describes the bench scale production of a high quality acrylamide, by the immobilized cells in a recycle fed-batch reactor without using an isotonic substrate.

MATERIALS AND METHODS

Microorganism

The microorganism used was *Brevibacterium* sp. CH2 which had a high nitrile hydratase activity and a high acrylonitrile concentration tolerance (10, 19).

Medium and Culture Conditions

The culture medium contained (per liter): glucose, 15 g; yeast extract, 3 g; malt extract, 3 g; bacto peptone, 5 g; KH_2PO_4, 1 g; K_2HPO_4, 1 g; NaCl, 1 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; $FeSO \cdot 7H_2O$, 0.02 g. The initial pH of the medium was adjusted to 7.1 with a 2N NaOH solution. Batch culture was carried out in a 5-liter jar fermenter (KFC, Korea Fermenter Co., Korea) for 23 h at 30℃, pH 7.1, and 500 rpm agitation. The culture broth was inoculated with 100 m*l* of a preculture grown in an Erlenmeyer flask containing the same medium. Aeration was achieved by bubbling filtered air into the vessel at 1 vvm.

Preparation of Resting Cells

Cells were harvested from culture broth by centrifugation at 18,000×g for 5 min at 5℃. The harvested cells were washed with 0.1 M NaH_2PO_4/Na_2HPO_4 buffer (pH 7.1) containing 20 mM n-butyric acid, and then suspended in the same buffer. This cell suspension was used for the cell immobilization.

Cell Immobilization

Acrylamide: The resting cells (12 g wet wt) were suspended in 40 m*l* of 0.1 M phosphate buffer (pH 7.1), and then 4.5 g of acrylamide as a monomer and 0.5 g of N,N'-methylenebisacrylamide as a crosslinking agent were added to the cell suspension. The polymerization was initiated by the addition of 5 m*l* of 5% (v/v) β-dimethylaminopropionitrile and 10 m*l* of 2.5% (w/v) potassium persulfate. Polymerization was carried out in a cold chamber (5℃) to protect the deactivation of enzyme activity. The cell-containing gel was pulverized into cubes. Then these cubes were mixed with 200 m*l* of a 0.1 M phosphate buffer and 0.8 m*l* of 25% (v/v) aqueous solution of glutaraldehyde, and were subjected to a glutaraldehyde treatment at 5℃ for 50 min with mild stirring. Prepared immobilized cells were washed with a 0.1 M phosphate buffer in order to remove nonpolymerized monomers and residues.

Calcium and Barium Alginate: 20 g of wet cells were mixed with 100 m*l* of 1.5% (w/v) Na-alginate solution. The solution was dropped into a 3% (w/v) $CaCl_2$ or $BaCl_2$ solution to form beads. The beads were mixed with 200 m*l* of distilled water and 0.8 m*l* of 25% (v/v) aqueous solution of glutaraldehyde, and the mixture was subjected to a glutaraldehyde treatment at 5℃ for 50 min with mild stirring.

Analytical Methods

The nitrile hydratase activity of whole cells was assayed in a reaction mixture containing 1 m*l* of 6% (v/v) acrylonitrile solution and a centrifuged whole cells of 1 m*l* of culture broth. Acrylonitrile solution was prepared by adding 60 m*l* of acrylonitrile per 1 liter of 0.1M phosphate buffer (pH 7.0). Reaction was carried out at pH 7.0 and 5℃ for 2 min with moderate shaking and then terminated by adding 0.1 m*l* of conc. HCl. One unit of nitrile hydratase activity was defined as the amount of the whole cells that catalyzed the formation of 1 μmole of acrylamide per min under these reaction conditions. The specific activity was expressed as units per mg of dry cells.

The concentration of acrylonitrile and acrylamide was determined by gas chromatography (Varian model 3300, Varian Inst. Co., U.S.A.) equipped with a flame ionization detector using a column packed with chromosorb W 80~100 mesh as solid phase and carbowax 20 M 10% as stationary phase. Operating conditions were: detector and injection port temperature, 210℃; column temperature, 160℃. Helium was used as a carrier gas at the flow rate of 30 m*l*/min. The integration and calibration of the peak areas were carried out with an integrator (SP 4290, Spectra-Physics, U.S.A.).

Operational Stability Test and Bench Scale Reactor Operation

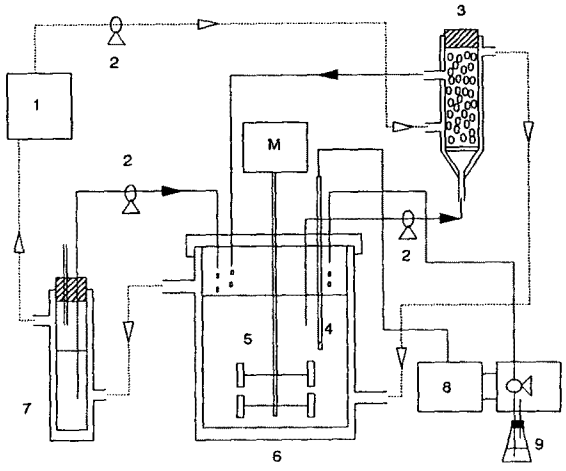

Fig. 1. A schematic diagram of the experimental equipment of the recycle fed-batch reactor.
(1) water bath; (2) peristaltic pump; (3) packed bed reactor with the immobilized cells; (4) pH electrode; (5) stirrer; (6) substrate and product reservoir; (7) pure acrylonitrile; (8) pH controller; (9) 0.5 N Na_2CO_3.

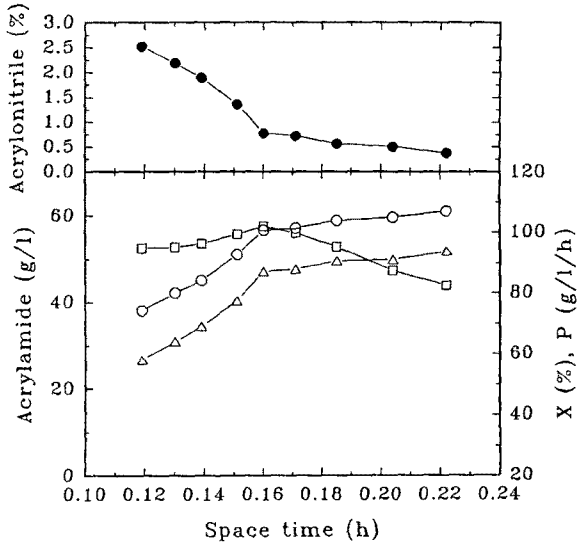

Fig. 2. The conversion, acrylamide concentration and productivity as a function of space time in a packed bed reactor.
(●) acrylonitrile concentration, (○) acrylamide concentration, (□) productivity (P), (△) conversion (X).

Operational stability of the immobilized cells was measured in a jacketed glass column equipped with sintered glass bottom to support the immobilized cells. Occurrence of the swelling of immobilized beads was measured by volume increase in the column reactor during the hydrolysis for 24 h at 4℃. The substrate reservoir was placed in a water bath, and the packed bed was maintained at the same temperature (4℃) as that of the reservoir by circulating water through the jacket from the constant temperature water bath (Cole-parmer model 1258-00, Cole-parmer Inst. Co., Chicago, U.S.A.). The reactor operation was carried out in a continuous mode by continuously feeding a 5% (v/v) acrylonitrile solution into the packed bed reactor (2.5 cm bed diameter and 12 cm height).

A schematic diagram of the bench scale reaction system is shown in Fig. 1. Temperature of the substrate reservoir and the packed bed reactor was maintained at 10℃ by circulating water from the water bath. The reactor was operated in a recycle fed-batch manner by recirculating the substrate solution into the substrate-product reservoir (1 liter solution of 5% (v/v) acrylonitrile initially) and the packed bed (8.0 cm bed diameter and 30 cm height). At the same time pure acrylonitrile (99.9 %) was continuously fed into the substrate-product reservoir by a peristaltic pump.

RESULTS AND DISCUSSION

In the acrylamide production by enzymatic method, only low acrylonitrile concentration, less than 2%, was used because of the severe substrate inhibition. Recently, *Brevibacterium* CH2 with nitrile hydratase functioning even at 6% (v/v) acrylonitrile was isolated (10, 20, 21). This makes it possible to feed higher concentration of acrylonitrile to the reactor.

In Fig. 2, the conversion, acrylamide concentration, and productivity of the reactor as a function of space time in a column reactor, which were packed with the cells immobilized in polyacrylamide are shown. Acrylonitrile substrate (6% (v/v), pH 7.1) was continuously fed into the column reactor. The acrylamide concentration and conversion increased with the space time. However, the peak productivity of 100 g/l/h was obtained at a space time of 0.16 h with the acrylamide concentration of 55 g/l and the conversion of 80%.

Fig. 3 shows the effect of phosphate buffer concentration on the operational stability of the cells immobilized in polyacrylamide. The reaction mixture contained 5% (v/v) of acrylonitrile and an indicated concentration of phosphate buffer (pH 7.2). The substrate was fed into the column reactor by a peristaltic pump at the space time of 0.16 h. When 0.2 M phosphate buffer was used as an aqueous substrate, about 80% of initial activity remained after 260 h of the hydrolysis reaction and the pH of the outlet decreased to 7.1. The pH decrease resulted from the production of acrylic acid by amidase. However, when 0.05 M phosphate buffer was used as an aqueous substrate, the half-life of the immobilized

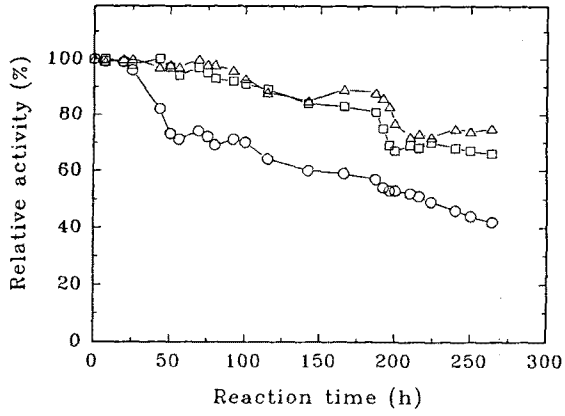

Figure 3. Effect of phosphate buffer concentration on the operational stability of the immobilized cells.
(○) 0.05 M, (□) 0.1 M, (△) 0.2 M.

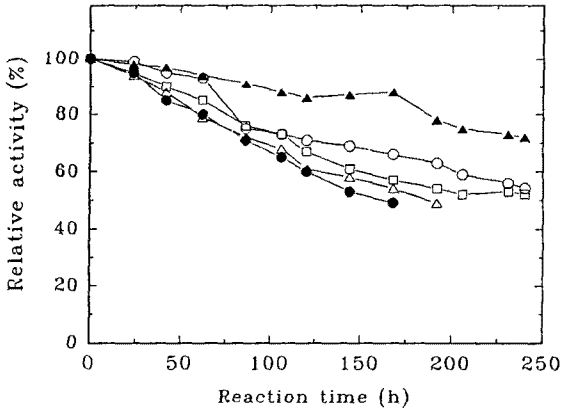

Fig. 4. Effects of various salts on the operational stability of the immobilized cells.
(▲) $FeSO_4 \cdot 7H_2O$, (○) $CaCl_2 \cdot 2H_2O$, (□) $MgSO_4 \cdot 7H_2O$, (△) $CuSO_4 \cdot 5H_2O$, (●) $(NH_4)_2SO_4$.

cells was 240 h and the pH of the outlet decreased to 6.8. The lower operational stability at the lower buffer concentration is due to the deactivation by the pH change which increased with decrease of the buffer concentration in the column reactor (8).

Fig. 4 shows the effects of various salts on the operational stability of the cells immobilized in polyacrylamide. The reaction mixture contained 5% (v/v) of acrylonitrile, 0.1 M phosphate buffer (pH 7.2), and 0.1 g/*l* of salts indicated. Operational stability was slightly enhanced by the addition of 0.1 g/*l* of $FeSO_4 \cdot 7H_2O$ then that of the case of using a phosphate buffer only (Fig. 3). It was due to the fact that Fe^{3+} ion functioned as a cofactor in nitrile hydratase of *Brevibacterium* sp. CH2 (11).

From the above results, if the buffer solution was used as an aqueous substrate, the immobilized cells in the

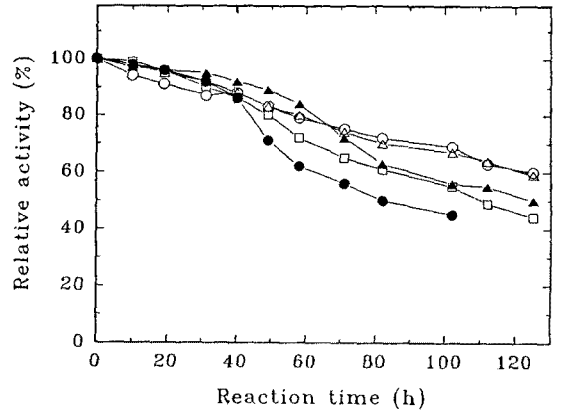

Fig. 5. Effect of the acrylonitrile concentration and sodium acrylate as an aqueous substrate on the operational stability of the immobilized cells.
(○) 2% (v/v), (△) 3%, (▲) 4%, (□) 5%, (●) 6%.

column reactor would not swell, and then the enzymatic activity would be maintained for a long period of time. Furthermore, the hydration reaction could be efficiently carried out for a long period of time. However, the use of phosphate buffer as an aqueous substrate results in the contamination of acrylamide product by large amounts of phosphates, which is not desirable in the desired acrylamide quality. When phosphates, sodium chloride, or the like are present in such small amounts, they exert no substantial adverse influences on the polymerization of acrylamide. However, when they are present in large amounts, the purity of the product may be reduced, therefore, they are preferably added in amounts of 1% or less, based on the acrylamide (24).

Fig. 5 shows the effects of acrylonitrile concentration and sodium acrylate as an aqueous substrate on the operational stability of the cells immobilized in polyacrylamide. The reaction mixture contained an indicated concentration of acrylonitrile and 0.1% sodium acrylate (neutralized with Na_2CO_3 to pH 7.2). The concentration of acrylamide produced in the outlet increased when a concentration of acrylonitrile was added. As shown in Fig. 5, the operational stability decreased when a concentration of acrylonitrile was more added. This was due to the enzyme inactivation by higher concentration of acrylonitrile and acrylamide (11). When 0.1% sodium acrylate as an aqueous substrate was used, this compound exerted no substantial adverse influence on the quality of acrylamide produced, but the operational stability decreased as compared with the case of using the phosphate buffer. It resulted from the enzyme inactivation by the pH change which increased when a concentration of acrylonitrile was added and was observed to be more severe than the case in which phosphate buffer

was used. The pH change between the inlet and the outlet solution increased from 0.8 to 1.5 with increasing the acrylonitrile concentration of 2 to 6%.

Although the reason why the immobilized cells swell during the hydrolysis reaction is not completely clear, it is believed to be due to the following two reasons, (1) the binding force of bead is weakened by the continuous flow of substrate and product stream and (2) the repulsion force generated among negatively charged cells when passing a substrate solution, and the difference in the osmotic pressure between the outside and the inside of the immobilized cells. These phenomena are believed to be due to the difference between the concentrations of acrylonitrile and acrylamide between the inside and the outside of the immobilized cells when the acrylonitrile enters into the immobilized cells and converts into acrylamide, and the formed acrylamide migrates out of the immobilized cells. Furthermore, it is also believed that the deterioration of the enzymatic activity as the result of the swelling phenomenon is due to the facts that the enzyme was liable to leak out of the immobilized cells due to the swelling, and that the stable conformation in normal cells in which the enzyme was not swollen cannot be maintained.

In the case of polyacrylamide bead, as shown in Table 1, when the reaction was carried out in an isotonic substrate such as a physiological saline solution, a phosphate buffer solution, and a sodium acrylate solution, the swelling of the immobilized cells could not occur. In these cases no great difference in the osmotic pressure between the outside and the inside of the immobilized cells was created, and therefore the swelling of the immobilized cells could be prevented while at the same time the enzyme could be maintained in a stable condition.

Table 1. Effects of salts and carriers on the swelling of the immobilized cells during the hydrolysis

Carrier	Salt in 5% (v/v) acrylonitrile	Swelling
Ba-alginate	distilled water	not occurred
Ca-alginate	distilled water	occurred
	0.1 M phosphate buffer	occurred
	10^{-4} M $CaCl_2 \cdot 2H_2O$	not occurred
Acrylamide	distilled water	occurred
	0.1 M phosphate buffer	not occurred
	saline solution	not occurred
	0.1% sodium acrylate	not occurred
	5×10^{-4} M $CaCl_2 \cdot 2H_2O$	not occurred
	10^{-4} M $CaCl_2 \cdot 2H_2O$	occurred
	10^{-3} M $(NH_4)_2SO_4$	occurred
	10^{-3} M $FeSO_4 \cdot 7H_2O$	occurred
	10^{-3} M $MgSO_4 \cdot 7H_2O$	occurred
	10^{-3} M $ZnSO_4 \cdot 7H_2O$	occurred
	10^{-3} M $MnSO_4 \cdot 7H_2O$	occurred

In the biological conversion of acrylonitrile into acrylamide by the immobilized *Brevibacterium* sp. CH2 in an aqueous substrate with a very small amount of $CaCl_2$, the swelling of the immobilized cells did not occur and the enzymatic activity could be maintained for a long period of time. It is also believed that no great difference in the osmotic pressure between the outside and the inside of the immobilized cells was created. In the case of Ca-alginate bead, the role of Ca^{2+} ion is also believed that the hardening of beads by offering a chance of bind to the alginate during the reaction period. The use of a 5×10^{-4} M $CaCl_2$ solution as substrate introduces a trace amount of salt into the formation of an aqueous acrylamide solution. This is no serious problems in the quality of the produced acrylamide. Fig. 6 shows the operational stability of the resting cells entrapped in polyacrylamide when the hydrolysis was carried out in a 5% (v/v) acrylonitrile solution containing 5×10^{-4} M $CaCl_2$ (pH 7.1) as an aqueous substrate. About 70% of initial activity remained after 200 h of the reaction. The hydrolysis reaction by the continuous column mode using the immobilized cells could be efficiently carried out for a long period of time and permitted the production of acrylamide, of excellent quality. However, when the reactions were carried out in distilled water, 10^{-4} M $CaCl_2$, 5×10^{-3} M $(NH_4)_2SO_4$, etc., the swelling of the immobilized cells occurred. It is also believed that a great difference in the osmotic pressure as mentioned previously.

In the case of hydrolysis of acrylonitrile into acrylamide by the cells immobilized in Ca-alginate, the addition of a 10^{-4} M $CaCl_2$ into a substrate solution introduces a trace amount of salt into the formation of an aqueous acrylamide solution. However, as shown in Fig. 6, when 10^{-4} M $CaCl_2$ was used as an aqueous substrate, the half-life of the Ca-alginate beads was 70 h, so Ca-alginate carrier was not suitable. On the other hand, Ba-alginate beads did not swell in the course of the hydrolysis reaction in which the isotonic substrate was not used. It is believed that the Ba^{2+} ion is more tightly bind to the alginate than the Ca^{2+} ion. Therefore, immobilization of the cells in Ba-alginate was more desirable than that of those in polyacrylamide and Ca-alginate for the swelling of the immobilized cells and the desired quality of the acrylamide produced.

Fig. 7 shows the effect of pH on the operational stability of the cells immobilized in Ba-alginate. Temperature of the reactor was maintained at 10℃. When the pH of the reaction mixture (contained 5% (v/v) of acrylonitrile only) was adjusted to 7.4, the operational stability was at maximum, and about 70% of initial activity remai-

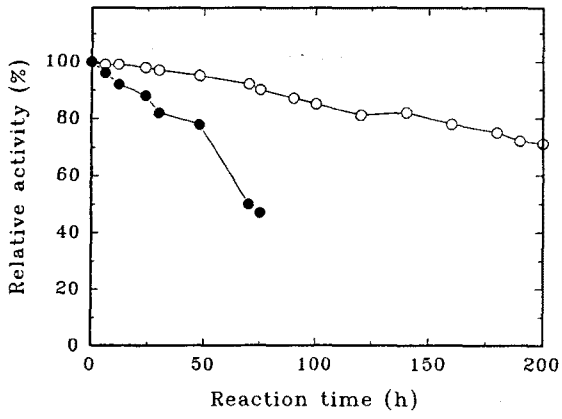

Fig. 6. Effects of the carriers and $CaCl_2$ solutions on the operational stability of the immobilized cells.
(○) polyacrylamide bead in 5×10^{-4} M $CaCl_2$, (●) Ca-alginate bead in 10^{-4} M $CaCl_2$.

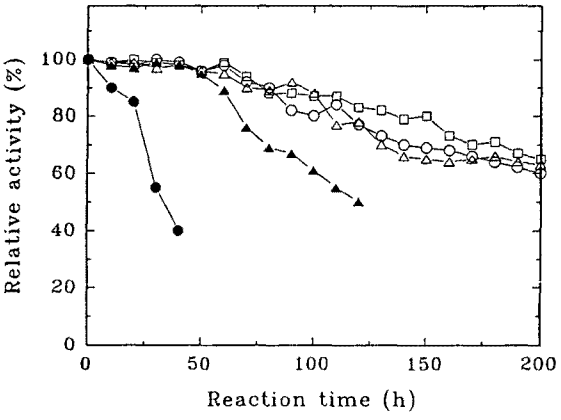

Fig. 7. Operational stability of the cells immobilized in Ba-alginate as a function of pH.
(●) pH 5.9, (▲) 6.4, (△) 6.9, (□) 7.4, (○) 7.9.

ned after 200 h of the reaction and the pH of the outlet decreased to 6.95. In consideration of the operational stability, the optimal pH for acrylamide production by the immobilized cells in the column reactor was nearly 7.4. From the above results, Ba-alginate beads seem to be most suitable when considering the quality of the acrylamide produced and the operational stability of the immobilized cells.

Fig. 8 shows a time course of the acrylamide production with the cells immobilized in Ba-alginate in a bench scale reactor. The reactor was operated in a recycle fed-batch mode in order to obtain a higher product concentration and to avoid a substrate inhibition. In order to obtain a high quality acrylamide, isotonic substrate was not used. The reactor operation was carried out repeatedly by removing all substrates after reaching 18% final

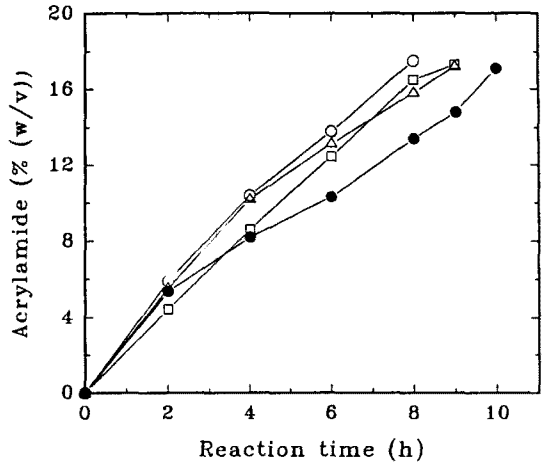

Fig. 8. Time course of repeated acrylamide production in a recycle fed-batch reactor.
(○) first batch, (○) second batch, (△) third batch, (●) fourth batch.

concentration of acrylamide. Temperature of the reservoir and packed bed reactor was controlled at 10℃, and the pH of the reaction mixture was controlled at 7.4± 0.1 with 0.5 N n-butyric acid and 0.5 N Na_2CO_3. As the recirculating rate of the substrate solution shifted from 2 to 3.5 l/h, the acrylamide production rate increased slightly, so the recirculating rate of the substrate was regulated at 3.4 l/h. Acrylonitrile concentration was controlled by the regulation of feeding rate so as not to exceed a concentration of 4% (v/v). The four batch reactions proceeded for nearly 40 h at 10℃ without significant decrease of the enzyme activity. However, after the four batch reactions were stopped, Ba-alginate beads swelled and the enzyme activity significantly decreased. Swelling of the Ba-alginate beads becomes more severe as the acrylamide concentration increases.

As a conclusion, Ba-alginate beads were the best carriers among the three carriers tested in preventing the swelling of the immobilized cells and producing the desired acrylamide quality. Acrylamide, of excellent quality, was produced by the Ba-alginate beads in a recycle fed-batch reactor without using an isotonic substrate. The operational stability of the immobilized cells in a packed bed reactor will decide the economics of the production of acrylamide. For the economical production of high quality acrylamide, it is necessary to develop the immobilized beads which do not swell in the course of the hydration reaction and possess a high operational stability without using an isotonic solution.

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