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Production of Acrylamide Using Immobilized Cells of *Rhodococcus rhodochrous* M33

Bu-Youn Kim[1] and Hyung-Hwan Hyun[2]*

[1] R&D Center, Tong Suh Petrochemical Corp. Ltd., P.O. BOX 50, Nam-Ulsan, Korea
[2] Department of Bioscience and Biotechnology, Hankuk University of Foreign Studies, Kyunggi-do 449-791, Korea

Abstract The cells *of Rhodococcus rhodochrous* M33, which produce a nitrile hydratase enzyme, were immobilized in acrylamide-based polymer gels. The optimum pH and temperature for the activity of nitrile hydratase in both the free and immobilized cells were 7.4 and 45°C, respectively, yet the optimum temperature for acrylamide production by the immobilized cells was 20°C. The nitrile hydratase of the immobilized cells was more stable with acrylamide than that of the free cells. Under optimal conditions, the final acrylamide concentration reached about 400 g/L with a conversion yield of almost 100% after 8 h of reaction when using 150 g/L of immobilized cells corresponding to a 1.91 g-dry cell weight/L. The enzyme activity of the immobilized cells rapidly decreased with repeated use. However, the quality of the acrylamide produced by the immobilized cells was much better than that produced by the free cells in terms of color, salt content, turbidity, and foam formation. The quality of the aqueous acrylamide solution obtained was found to be of commercial use without further purification.

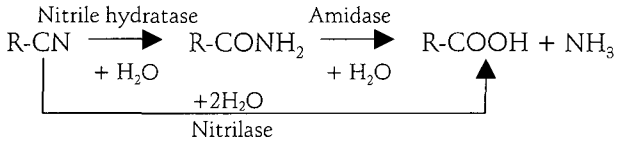
Keywords: nitrile hydratase, immobilization, *Rhodococcus rhodochrous* M33, acrylamide.

INTRODUCTION

Acrylamide is a commodity chemical that is used as a starting material in the production of various polymers used as flocculants or stock additives for petroleum recovery or waste-water treatment, and so forth. Acrylamide was originally produced by the chemical hydration of acrylonitrile with sulfuric acid in the presence of reduced copper as a catalyst [1]. However, chemical hydration reactions have several problems, including the complexity involved in preparing the catalyst, difficulties in the purification and recovery of the acrylamide formed, the formation of by-products, a low conversion yield, and severe reaction conditions [2].

Recently, biotransformation processes which produce acrylamide from acrylonitrile using microorganisms that exhibit nitrile hydratase activity have been developed [3]. During the microbial hydration of nitrile, more than 99% of the acrylonitrile can be converted into acrylamide without the formation of any by-products, plus the enzyme reaction can be carried out under moderate conditions. Several genera of bacteria, such as *Rhodococcus, Pseudomonas, Bacillus, Corynebacterium, Brevibacterium,* and *Arthrobacter* are known to be able to convert nitriles into the corresponding amides [4-9].

Many microorganisms can use nitriles as a source of carbon and/or nitrogen for their growth, and microbial nitrile metabolism is now well understood [10,11]. Aromatic, heterocyclic, and certain unsaturated aliphatic nitriles are converted directly into the corresponding acids and ammonia by nitrilase (EC 3.5.5.1, nitrile aminohydrolase) with little formation of free amides. In contrast, saturated aliphatic nitriles are catabolized in two stages; first, they are converted into the corresponding amides by nitrile hydratase and then into acids and ammonia by amidase [12], as shown below.



Immobilized cells are frequently used for the biotransformation due to advantages such as the prevention of the elution of impurities from the cells, easy separation of the cells from a reaction mixture, repeated use of the immobilized cells, and enhanced stability of the enzymes [13-17]. Wadanabe [8] and Bui *et al.* [18] studied an enzymatic process for the production of acrylamide using cells immobilized in an acrylamide-based polymer gel in a packed bed reactor. Lee and Chang produced acrylamide using cells immobilized in a Ba-alginate and polyacrylamide gel in a recycle fed-batch reactor [19-21].

Previously, the current authors reported on fed-batch fermentation for the enhanced production of nitrile hydratase by *Rhodococcus rhodochrous* strain M33 [17]. In this study, the effects of various factors on the produc-

* **Corresponding author**
Tel: +82-31-330-4279 Fax: +82-31-333-1696
e-mail: hhhyun@san.hufs.ac.kr

tion of acrylamide using the immobilized cells of *R. rhodochrous* strain M33 in a stirred fed-batch reactor were investigated.

MATERIALS AND METHODS

Microorganism

Rhodococcus rhodochrous M33 was obtained from the Institute for Genetics for Microorganisms, VNII Genetika (Russia).

Media and Culture Conditions

For the preparation of a frozen stock culture, a culture broth of *R. rhodochrous* M33 was mixed with 30% glycerol at a ratio of 1:1, frozen, and stored in a deep freezer (-80°C). To prepare the seed culture, cells taken from a frozen stock were transferred to a 500-mL Erlenmeyer flask containing 50 mL of a seed medium consisting of 0.5 g of KH_2PO_4, 0.4 g of K_2HPO_4, 0.01 g of $CoCl_2 \cdot 6H_2O$, 0.005 g of $FeSO_4 \cdot 7H_2O$, 0.01 g of EDTA-2Na, 20 g of glucose, 0.5 g of $MgSO_4 \cdot 7H_2O$, and 3 g of urea per liter of demineralized water, and cultivated at 30°C for 48 h with shaking at 250 rpm. The seed culture was then transferred to a 5-L jar fermenter (Korea Fermenter Co., Incheon, Korea) containing 3 L of the same medium as used in the seed cultures with the supplement of 0.01 g/L of NaCl, 7.5 g/L of urea, 1.2 g/L of $MgSO_4 \cdot 7H_2O$, and 0.018 g/L $CoCl_2 \cdot 6H_2O$. The batch fermentations were carried out for 50 h in the same jar fermenter, which was controlled at 30°C, agitated at 500 rpm, and aerated at 0.4 vvm. The pH in the fermenter was controlled at 7.2 with a 10% KOH solution. Antifoam (polyoxyethylene-oxypropylene glycol ether) was added when necessary. The cells were harvested by centrifugation at 8,000 rpm at 4°C and washed twice with demineralized water.

Immobilization of Cells

To immobilize the cells, 0.7 g of dry cells was mixed with 4.5 g of acrylamide and 0.5 g of *N,N'*-methylene-(bis)acrylamide in an appropriate amount of demineralized water, uniformly suspended, and then further diluted with demineralized water to make 40 g. The polymerization was initiated by the addition of 5 mL of 5% (v/v) 3-(dimethylamino)propionitrile and 10 mL of 2.5% (w/v) potassium persulfate and continued for 60 min in a cold chamber (10°C) to prevent the inactivation of the enzyme. The cell-containing gel was pulverized into cubes and washed with a 25 mM phohphate buffer (pH 7.5) to remove any non-polymerized monomers and residues.

Reactions in Stirred Reactor

The acrylamide production reaction was carried out in a 1.5-L jacketed stirred reactor that contained 1 L of the reaction mixture consisting of acrylonitrile, immobilized cells, and demineralized water, was controlled at 20°C by the circulation of cooling water, and agitated at 500 rpm. Acrylonitrile was continuously fed into the reactor by a peristaltic pump to maintain an acrylonitrile concentration below 0.5% during the reaction.

Assay of Nitrile Hydratase Activity

To measure the nitrile hydratase activity in the free and immobilized cells, the free cell samples were diluted with a 25 mM phosphate buffer (pH 7.5) based on a 0.04 mg-dry cell weight (DCW) per mL, whereas the immobilized cell samples were fully ground in a mortar and diluted with the above buffer to a 0.2 optical density at 540 nm. The reactions were carried out by incubating the reaction mixtures consisting of 1 mL of a 1% (w/w) acrylonitrile solution in a 25 mM phosphate buffer (pH 7.5) and 1 mL of the cell suspension at 20°C for 10 min with moderate shaking, and then stopped by adding 40 μL of conc. HCl. The amount of acrylamide produced in the reaction mixture was determined using a gas chromatography (AutoSystem GC, Perkin Elmer, USA).

One unit of nitrile hydratase activity was defined as the amount of cell enzyme that catalyzed the formation of 1 μmol of acrylamide per min under the above reaction conditions. The specific activity of the nitrile hydratase was expressed as units per mg of dry cells.

Analytical Methods

To determine the culture turbidities, the culture broths were appropriately diluted with demineralized water and the optical densities were measured measured at 540 nm using a UV-visible spectrophotometer (Cary, Varian Co., Australia). The dry cell weight was estimated using a calibration curve based on the relationship between the optical density at 540 nm and the dry cell weight, and 1.0 OD_{540} was equivalent to a 0.2 g dry cell weight/L.

The amounts of nitrile consumed and amide formed in the reaction mixture were determined using a gas chromatograph (AutoSystem GC, Perkin Elmer, USA) equipped with a frame ionized detector. The column used was a stainless steel column packed with an 8% free fatty acid phase for the amide analysis and Porapak Type Q (80-100 mesh) for the nitrile analysis. The detector and injector temperatures were both 230°C and the column temperature was 190°C. The carrier gas was N_2 and its flow rate was 80 mL/min. The integration and calibration of the peak areas were carried out using a PE-NELSON model 1020.

The turbidity of the acrylamide solution was measured by a laboratory nephelometer (Model TA1, Monitek Inc., USA), while the conductivity was measured by a conductometer (Model 644, Metrohm Inc., Switzerland). The color of the acrylamide solution was determined by visual comparison with known concentrations of platinum-cobalt solutions.

Chemicals

The medium components were normal commercial products and used without further purification. All other chemicals were reagent grade and purchased from Aldrich Chemical Company, Inc., USA.

RESULTS AND DISCUSSION

Immobilization of Whole Cells

Since polyacrylamide is known to be the best carrier for the immobilization of whole cells as regards the stability of nitrile hydratase [21-23], it was used as the carrier to immobilize the whole cells of *R. rhodochrous* M33 in the current study. Experiments were then conducted to determine the optimum ratio between the amount of whole cells and the amount of polyacrylamide support. As shown in Fig. 1, the maximum specific activity was obtained when 0.7 g of dry cells was loaded in 60 mL of the polymer solution. When more than 0.7 g-DCW was loaded, this caused the aggregation and precipitation of the cells during the immobilization and resulted in a lack of homogeneity and no further increase in the specific enzyme activity.

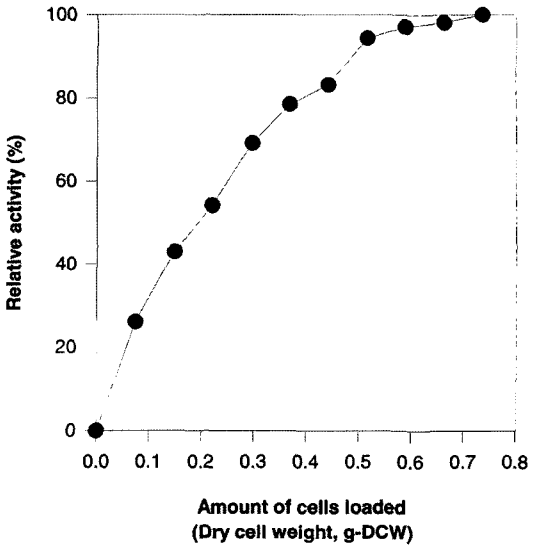


Fig. 1. Determination of optimum cell concentration for immobilization of whole cells. Different amounts of cells (g-DCW) were loaded in the reaction mixture for immobilization consisting of 40 mL of water, 4.5 g of acrylamide, 0.5 g of *N,N'*-methylenebisacrylamide, 5 mL of 5% 3-(dimethylamino)-propionitrile, and 10 mL of 2.5% potassium persulfate. 100% relative activity corresponds to 107 units/mg-DCW.

Comparison of Physicochemical Properties of Free and Immobilized Cells

Effect of pH on activity

The dependence of the nitrile hydratase activity of the free and immobilized cells on pH is compared in Fig. 2. Both the free and immobilized cells displayed a similar pH range for the enzyme activity (pH 5.8-8.4) and the maximum nitrile hydratase activity was observed around pH 7.4.

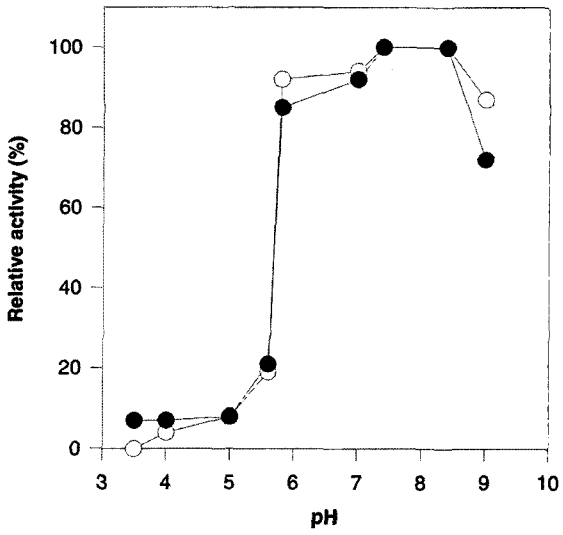


Fig. 2. Relation of pH to nitrile hydratase activity of free and immobilized cells. The enzyme activity was measured at 20°C, as described in Materials and Methods, using 0.2 M of a Na-acetate buffer (pH 3.5-5.6), 0.1 M of a phosphate buffer (pH 5.8-8.0), and 0.2 M of a Tris-HCl buffer (pH 8.4-9.0). 100% relative activity corresponds to 106 units/mg-DCW. ●, immobilized cells; □, free cells.

Effect of temperature on activity

The relation of temperature to the nitrile hydratase activity of the free and immobilized cells was also examined. Fig. 3 illustrates that in both the free and immobilized cells the enzyme was active within a broad temperature range (*i.e.*, 5-45°C) and displayed a temperature optimum for activity at 45°C. When an Arrhenius plot of the specific enzyme activity versus temperature was determined (data not shown), a Q_{10} value of 2.8 was calculated for both the free and immobilized cells within a temperature range of 25°C to 45°C when the enzyme activity sharply increased.

Effect of acrylamide concentration

Acrylonitrile and acrylamide, the substrate and product of nitrile hydratase, respectively, are both known to be strong inactivators of the enzyme [21]. Therefore, to test whether the stability of the nitrile hydratase was improved by immobilization, the effect of the acrylamide concentration on the enzyme activity of the free and immobilized cells was examined under the following-

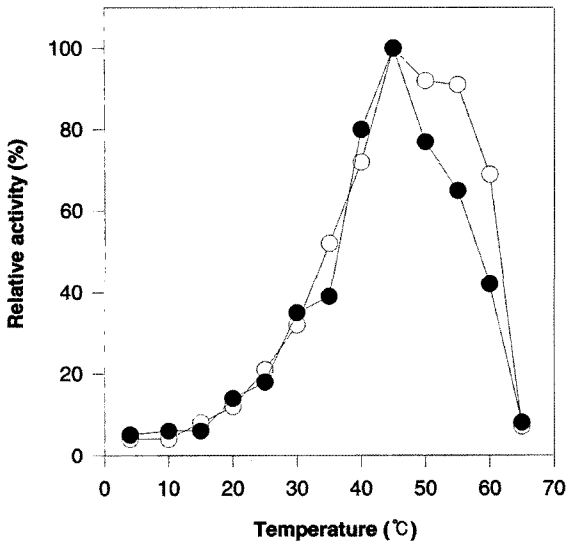


Fig. 3. Dependence of nitrile hydratase activity of free and immobilized cells on temperature. 100% relative activity corresponds to 931 units/mg-DCW. ●, immobilized cells; □, free cells.

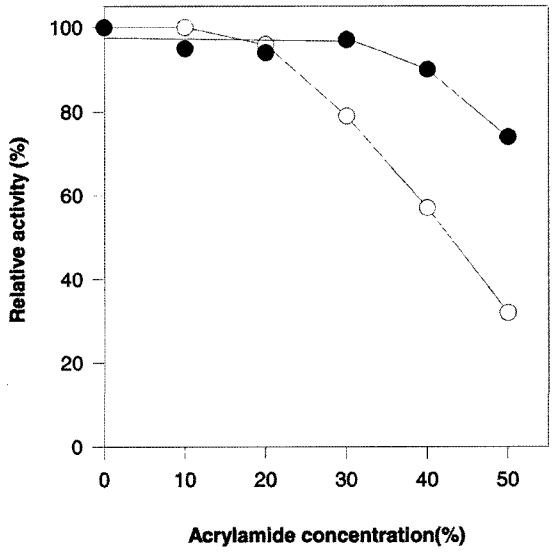


Fig. 4. Effect of acrylamide concentration on enzyme stability of free and immobilized cells. 100% relative activity corresponds to 177 units/mg-DCW. ●, immobilized cells; □, free cells.

conditions. The free and immobilized cells were treated with varying concentrations of acrylamide for 1 h, then the residual activity was measured. As shown in Fig. 4, the enzyme activity of the free cells decreased rapidly in the presence of more than 20% (w/v) acrylamide, whereas the enzyme of the immobilized cells remained stable in the presence of 30% (w/v) acrylamide, yet gradually became inactivated at a higher concentration. The fact that the nitrile hydratase of the immobilized cells was more stable with acrylamide than that of the

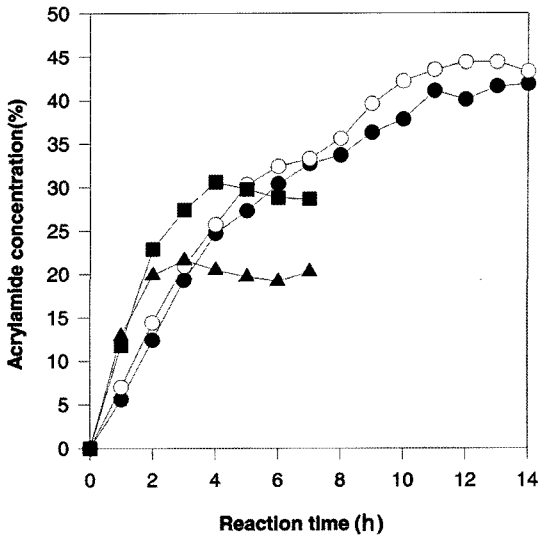


Fig. 5. Effect of temperature on acrylamide production by immobilized cells in stirred reactor. ●, 10°C; ○, 20°C; ▲, 30°C; ■, 40°C.

free cells suggests that a higher product concentration can be obtained when using immobilized cells with the same amount of enzyme.

Optimization of Reaction Conditions in Stirred Reactor

To establish the optimal reaction conditions for the bioconversion of acrylonitrile into acrylamide in a stirred reactor using immobilized cells of *R. rhodochrous* M33, a fed-batch type of reactor into which the substrate is continuously fed was employed.

Effect of temperature

Fig. 5 shows the acrylamide production profiles when the reactor was controlled at different temperatures. The data illustrate that the higher the reaction temperature, the higher the reaction rate, whereas the final product concentrations were higher at low temperatures (0°C and 20°C) than at high temperatures (30°C and 40°C). The maximum acrylamide concentration was obtained at 20°C, indicating that the immobilized enzyme was still unstable at a high temperature and high product concentration.

Effect of immobilized-cell concentration

As shown in Fig. 6, the production rate as well as the final concentration of acrylamide increased with an increase in the concentration of immobilized cells. The concentration of acrylamide reached about 42% (w/v) within 8 h when the reaction was carried out with 150 g/L of immobilized cells, corresponding to a 1.91 g/L dry cell weight, and only a negligible amount of acrylic

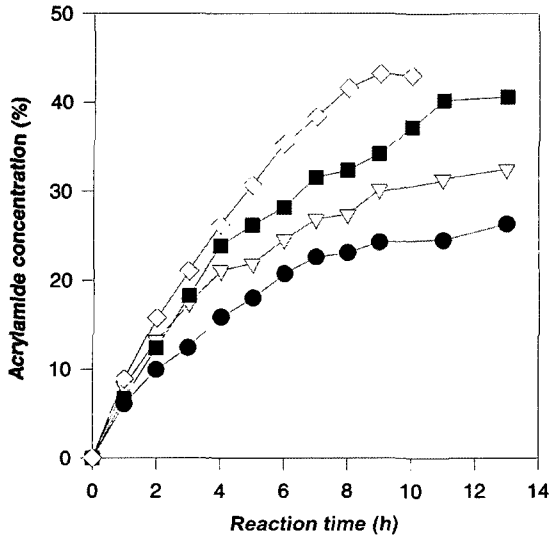


Fig. 6. Effect of immobilized cell concentration on acrylamide production in stirred reactor. ●, 50 g/L; ▽, 75 g/L; ■, 100 g/L; ◇, 150 g/L.

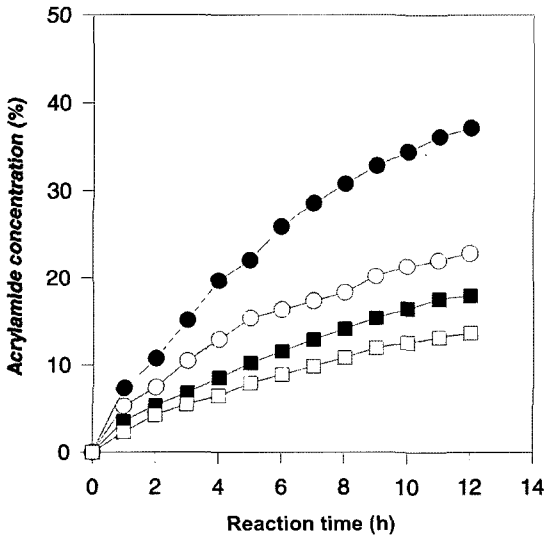


Fig. 7. Change in acrylamide production profiles with repeated use of immobilized cells. ●, 1st batch; ○, 2nd batch; ■, 3rd batch; □, 4th batch.

acid was detected.

Repeated use of immobilized cells

Bioconversion processes that produce acrylamide using immobilized cells have several advantages from an economical viewpoint, as previously described. One of the important advantages is that immobilized cells can be used repeatedly without a severe loss of enzyme activity. Fig. 7 compares the acrylamide production time courses when the immobilized cells with a polyacrylamide gel were repeatedly used in a stirred reactor. The data indicate that the more immobilized cells used, the greater the loss of enzyme activity. In general, when a non-isotonic solution is used to prepare a reaction mixture containing immobilized cells, a significant loss of enzyme activity occurs with repeated use of the immobilized cells. This phenomenon is known to be due to the swelling and lysis of the immobilized cells. However, if isotonic solutions, such as physiological saline, a phosphate buffer, or sodium acrylate solution, are used to prevent the swelling of the cells, then a large amount of sodium chloride, phosphate, or sodium acrylate are left in the aqueous acrylamide solution formed. The removal of the salts, which lower the quality of the product, requires post-treatments, such as an ion exchange treatment. To overcome the swelling problem of immobilized cells, Wadanabe [8] developed an acrylamide production method using cells immobilized with a cationic acrylamide-based polymer gel. Therefore, the effect of the addition of a cationic monomer to the polyacrylamide gel on the acrylamide production was examined. The acrylamide production time courses for the cells immobilized in polyacrylamide gels with and without 2-(dimethylamino)ethylmethacrylate, a cationic monomer, are compared in Fig. 8. The data show that the acrylamide production rate by cells immobilized using a cationic acrylamide-based polymer gel decreased more rapidly with repeated use, when compared to that by cells immobilized without a cationic monomer. This fact indicates that, although the swelling of the immobilized cells was prevented by employing a cationic acrylamide-based polymer with an enhanced affinity for the cells, the enzyme was seemingly more

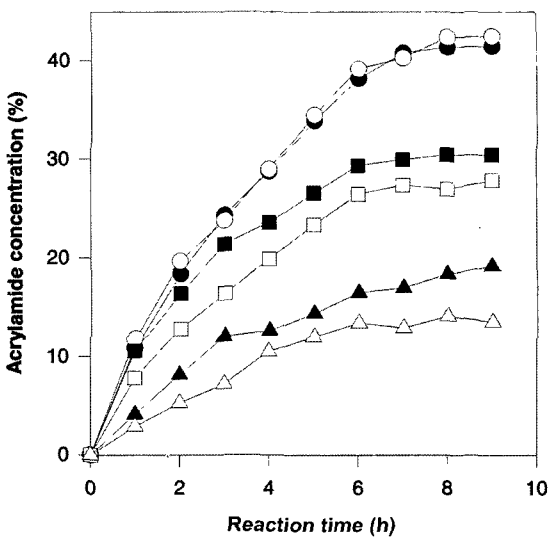


Fig. 8. Comparison of acrylamide production profiles by immobilized cells prepared with (open symbols) and without (closed symbols) addition of 2-(dimethylamino) ethylmethacrylate and used repeatedly. ●, ○, 1st batch; ■, □, 2nd batch; ▲, △, 3rd batch.

Table 1. Comparison of quality of acrylamide produced by free and immobilized cells

Item	Unit	Acrylamide produced by	
		Free cells	Immobilized cells
Concentration	%(w/w)	40	40
Color	APHA	40-50	10
Turbidity	NTU	3.1	1.5
Conductivity	μs/cm	180	30
Foam test	mL	Overflow	50-100

rapidly inactivated by the higher concentration of acrylamide that remained within the immobilized cells due to the cationic monomers. Therefore, it was concluded that it is very difficult to prevent the swelling phenomenon using a cationic acrylamide-based polymer when the final concentration of acrylamide is over 40% (w/v), as in the current study, as opposed to the 20% (w/v) used in Wadanabe's work.

For the industrial production of high quality acrylamide, it is very important to prevent the elution of impurities from the cells, especially in the repeated use of immobilized cells. Table 1 compares the quality of the acrylamide produced by the free and immobilized cells. The acrylamide product produced by the immobilized cells exhibited much better qualities than that produced by the free cells in terms of color, turbidity, salt content, and foam formation, thereby indicating that the acrylamide produced by the immobilized cells contained a lower amount of proteins, salts, and other impurities. Therefore, although the swelling of the immobilized cells could not be prevented, the quality of the aqueous acrylamide solution produced by the immobilized cells was high enough for the commercial preparation of polyacrylamide without any post-treatments, such as ion exchange treatment or ultrafiltration. Accordingly, since the use of immobilized cells for the production of acrylamide does require purification processes, its industrial application shows promise as a cost-effective process.

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