

Production of Acrylic Acid from Acrylonitrile by Immobilization of *Arthrobacter nitroguajacolicus* ZJUTB06-99

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Immobilized cells of *Arthrobacter nitroguajacolicus* ZJUTB06-99 capable of producing nitrilase were used for biotransformation of acrylonitrile to acrylic acid. Six different entrapment matrixes were chosen to search for a suitable support in terms of nitrilase activity. Ca-alginate proved to be more advantageous over other counterparts in improvement of the biocatalyst activity and bead mechanical strength. The effects of sodium alginate concentration, CaCl₂ concentration, bead diameter, and ratio by weight of cells to alginate, on biosynthesis of acrylic acid by immobilized cells were investigated. Maximum activity was obtained under the conditions of 1.5% sodium alginate concentration, 3.0% CaCl₂ concentration, and 2-mm bead size. The beads coated with 0.10% polyethylenimine (PEI) and 0.75% glutaraldehyde (GA) could tolerate more phosphate and decrease leakage amounts of cells from the gel. The beads treated with PEI/GA could be reused up to 20 batches without obvious decrease in activities, which increased about 100% compared with the untreated beads with a longevity of 11 batches.

Keywords: Acrylic acid, acrylonitrile, *Arthrobacter nitroguajacolicus*, nitrilase, calcium alginate, polyethylenimine

Acrylic acid has been widely applied to the production of paints, coatings, polymeric flocculants, and paper as well as other commercial products. Currently, commercial acrylic acid is produced through the oxidation of propene, which generates several unwanted by-products and a large amount of inorganic waste [16]. An alternative method for the production of acrylic acid from acrylonitrile using biocatalysis should be studied and developed, whose great possibility is offered with development in the biotransformation of nitriles for the synthesis of valuable carboxylic acid and amide as a green and environmentally friendly technology [2, 9, 11, 13, 20]. However, there are few reports about the

biotransformation of acrylonitrile for production of acrylic acid.

Arthrobacter nitroguajacolicus ZJUTB06-99, capable of converting acrylonitrile to acrylic acid, was recently isolated in our laboratory. To date, application of *A. nitroguajacolicus* in enzymatic production of acrylic acid has not been reported. Moreover, no by-product, acrylamide, was observed in the process of biotransformation using *A. nitroguajacolicus* cells as the biocatalyst. Because cell immobilization could offer numerous technical and economical advantages, such as feasibility of continuous processing, lower costs of recovery, recycling, and downstream process, biotransformation reactions with immobilized microbial cells have become a subject of increasing interest and are used in the production of some valuable chemicals [5, 14, 15, 18]. Entrapment is one of the simplest and more important methods currently employed to cell immobilization [2, 4]. Encapsulated cells can offer higher operational stability due to the protection from direct exposure to toxic compounds in the reaction environment; in particular, it is rather beneficial in the case of nitriles as the substrate, with extremely high toxicity to microorganism cells [12]. Moreover, immobilization of whole cells with expensive nitrilase is an inexpensive alternative to increase its reusability and stability [9]. It is reported that nitrilase-producing bacteria have been immobilized in matrixes such as polyacrylamide [6], carrageenan gel [3], chitosan [11], and alginate [2]. However, to date, entrapment of *Arthrobacter* sp. to covert acrylonitrile to acrylic acid has been little reported. In this study, the biotransformation of acrylonitrile to acrylic acid by immobilized *A. nitroguajacolicus* ZJUTB06-99 cells was described to provide a new method with high efficiency for production of acrylic acid.

MATERIALS AND METHODS

Chemicals

Acrylonitrile, acrylic acid, and other chemicals used in this work were of analytical grade and commercially available.

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Strain and Cultivation Conditions

A. nitroguajacolicus ZJUTB06-99, isolated from soil using acrylonitrile as a nitrogen source after enrichments and identified based on morphology, physiological tests, and its 16S rDNA sequence in our laboratory, was used in this study. The strain was incubated aerobically at 30°C for 72 h in the media with the following composition (in g/l): glucose 20, yeast extract 5, K₂HPO₄ 0.5, KH₂PO₄ 0.5, MgSO₄·7H₂O 0.5, ε-caprolactam 4, monosodium glutamate 0.75. Cells were collected by centrifugation at 4°C, 10,000 rpm for 10 min and were washed with 0.9% sodium chloride solution. Then, the cells were harvested by centrifugation and stored at 4°C for further use.

Immobilization of ZJUTB06-99 Cells

For entrapment in alginate, cells were mixed with an equivalent mass of 0.9% (w/v) sodium chloride and added to 1.5% (w/v) sodium alginate solution. The mixture was dropped to the solution composed of 3.0% (w/v) calcium chloride by a syringe. After being entrapped overnight, beads were washed with 0.9% sodium chloride and stored in the same composition of sodium chloride until use. For cells immobilized in chitosan, 2.0% (w/v) chitosan was firstly dissolved in 1.0% (v/v) acetic acid solution. The mixture of cells and chitosan was dropped to the solution of 1.5% (w/v) sodium triphosphate. After immobilization for 1 h, the beads were washed according to the methods mentioned above. When agar was used for entrapment, the harvested cells were resuspended in the 0.9% sodium chloride, and then mixed with 4% (w/v) agar above gelling temperature. The obtained gel was cut into cubes (approximately 3 mm×3 mm×3 mm) for use. For entrapment in glutin matrix, the cells were mixed with 10% (w/v) glutin. After gelation, the gel was treated with 0.5% (w/v) glutaraldehyde and cut into cubes (approximately 3 mm×3 mm×3 mm). The beads were washed twice and stored for later use. For polyacrylamide immobilization, the gel consisted of acrylamide, cross-linking agent N,N'-methylene-bisacrylamide, dimethylaminopropionitrile, and ammonium persulfate. The final concentration of polyacrylamide gel was 15%. The polymerization of the gel was finished in 10 min. The gels were sliced into particles of average size 3 mm×3 mm×3 mm. The beads were washed twice and stored for later use. When the cells were immobilized in egg white, the cells, egg white, and final concentration of 2% (v/v) glutaraldehyde were mixed. The gels were sliced into average 3 mm×3 mm×3 mm particles after polymerization at 25°C for 2.5 h subsequently with physiological saline washing.

Enzyme Activity Assay Methods

Biotransformation using the immobilized cells was performed in 50-ml conical flasks containing 3.0 g of wet beads and 10 ml of physiological saline when the beads were untreated with PEI-GA. Reactions were performed in the phosphate buffer (pH 7.0) with the treated beads. Biotransformation was carried out on a shaker at 150 rpm in a 30°C water bath for 3 h after substrate acrylonitrile was added. Samples were withdrawn and analyzed by gas chromatography (Agilent 6890) equipped with a flame ionization detector and a flap column (30 m×0.25 mm×0.33 μm). The carrier gas was nitrogen. The column, injection, and detector temperatures were 150, 220, and 220°C, respectively. One unit of nitrilase activity was defined as the amount of enzyme that catalyzed the formation of 1 μmol acrylic acid per minute under standard conditions. All assays were performed in triplicate.

Determination of the Bead Mechanical Strength

The mechanical strength of the Ca-alginate beads was indirectly expressed with the amount of the bead fragment in the samples. The process was carried out with 3.0 g of wet beads and 10 ml of reaction buffer contained in the 50-ml conical flasks on an orbital shaker at 150 rpm at 30°C for 24 h. Samples were withdrawn and centrifuged at 12,000 rpm for 10 min at 4°C. The amount of precipitation was tested.

Optimization of Bead Immobilization Parameters

For the preparation of beads with optimal nitrilase activity and good bead mechanical strength, various concentrations of sodium alginate (1.0–3.0%, w/v), CaCl₂ (1.0–4.0% w/v), different amounts of cells immobilized (2–14%, w/v) and different bead sizes (2.0–5.0 mm) were tested.

Chemical Cross-Linking of Alginate Beads

The alginate beads were treated with polyethylenimine (PEI) and glutaraldehyde (GA). Firstly, the beads were suspended in PEI-HCl (pH 7.0) solution adding Ca²⁺ and stirred for 24 h in a 30°C water bath. Then, the beads were washed twice and later treated with GA, stirring for 30 s. After cross-linking, the beads were washed twice and stored in physiological saline until further use [5, 10].

Operational Stability

The batch reusability of the immobilized cells was investigated to determine the number of cycles that it could be used without significant loss of activity. Each reaction cycle was carried out under the same condition. The enzyme activity was assayed and the particles were washed with distilled water before the next cycle.

The operational stability of the immobilized beads was determined by the following equation: Operational efficiency (%) = 100 × (C_x/C₁), where C_x is the activity of beads in the x cycle of batch, and C₁ is the activity of the beads used for the first cycle.

Bead Morphology

The Ca-alginate beads prepared as mentioned above were cut into hemispheres. Then, the hemispheres were vacuum-dried overnight. The outside and inside surface morphology of freeze-dried beads was investigated using environment scanning electron microscopy (ESEM XL-30, Japan). All samples were sputter-coated with platinum before observation [9].

RESULTS AND DISCUSSION

Choice of Entrapment Material

To select the suitable immobilization matrix of cells, various entrapment carriers were screened based on the activity and the stabilization of the biocatalyst. As seen from Fig. 1, the calcium alginate carrier showed the highest immobilization efficiency of the materials tested. However, other materials such as polyacrylamide exhibited lower activity. Cells entrapped in agar could easily leak out of the matrix. Ca-alginate as a low-cost and low-toxicity support is considered to be a desirable material for cell immobilization in view of the enzyme activity and the reusability of the beads. The data obtained also revealed the difference

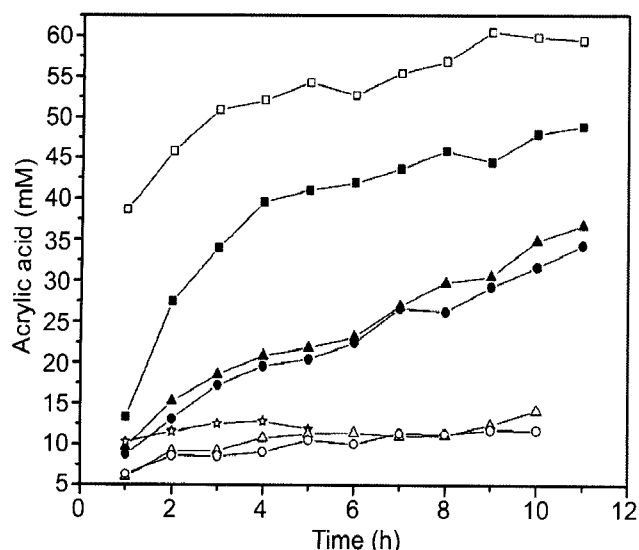


Fig. 1. Biotransformation of acrylonitrile by immobilized cells in different matrixes.

Symbols: (□) resting cells; (■) Ca-alginate; (▲) chitosan; (●) agar; (☆) gelatin; (△) polyacrylamide; (○) egg white.

between free cells and immobilized cells in the bioconversion of acrylonitrile to acrylic acid. After 1 h, the acrylic acid concentration reached 38.61 mM in the freely suspended cells system, but only 13.30 mM in the system of Ca-alginate beads. This could be ascribed to the mass transfer restriction during the initial reaction stage. The conversion of acrylonitrile reached 80% by alginate bead in the prolonged period. Immobilization slightly decreased the ZJUTB06-99 activity, which was consistent with previous reports [4, 17]. However, considering the reusability, the approach of cells immobilized in Ca-alginate was advisable in this study.

Effects of Alginate and CaCl_2 Concentrations

Alginate and CaCl_2 concentrations are the important parameters for enzyme gel entrapment. In Fig. 2, different alginate concentrations ranging from 1.0% (w/v) to 3.0% (w/v) were tested. Alginate concentration greatly affected the bead mechanical strength, especially when the concentration shifted from 1.0 to 1.5% (w/v). Too low sodium alginate concentration resulted in soft beads with low mechanical strength, which would be broken in the process of biotransformation. The rate of acrylic acid formation was the most rapid at 1.5% (w/v) concentration of alginate. Sodium alginate above 1.5% (w/v) hardened the beads but might cause a diffusion problem [18]. This is presumably due to the fact that the increased concentration of alginate resulted in a more densely cross-linked gel and substrate was restricted to diffuse easily to the cells [7]. The CaCl_2 concentration is another parameter for bead preparation. Fig. 3 showed that as the CaCl_2 concentration ranged from 1.0% (w/v) to 4.0% (w/v), the relative activities were all situated above 80% of the maximum activity. This implied that the CaCl_2 concentration had a smaller effect on the

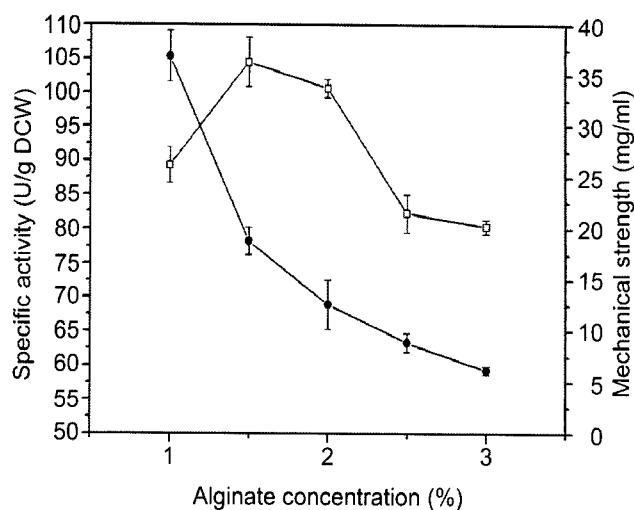


Fig. 2. Effects of sodium alginate concentrations on biosynthesis of acrylic acid by the immobilized ZJUTB06-99 cells.

Symbols: (□) specific activity; (●) bead mechanical strength.

enzyme activity than the alginate concentration in the range tested. The leakage of the bead was decreased as the Ca^{2+} concentration increased. Above 3.0%, the gel network formed a tight structure, which resulted in mass transfer resistance. Considering the activity and the bead mechanical strength, the alginate and CaCl_2 concentrations were 1.5% and 3.0%, respectively, in this study.

Effects of Ratio of Cells to Alginate

The activity depended on the amount of cell used in a certain way. Usually, the more cells entrapped in per unit volume of the matrix, the higher beads activity was attained. As shown in Fig. 4, the relative activity was increased as the ratio of

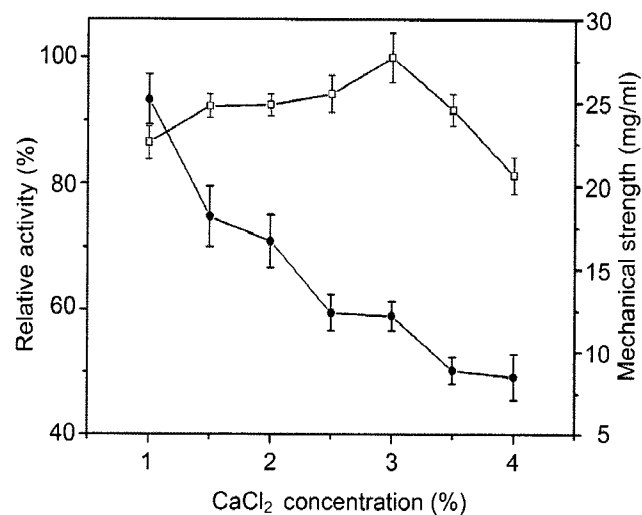


Fig. 3. Effects of CaCl_2 concentrations on biosynthesis of acrylic acid by the immobilized ZJUTB06-99 cells.

The relative activity was expressed at the percentage of the reaction when CaCl_2 was 3% (w/v) (specific activity, 110.73 U/g DCW). Symbols: (□) relative activity; (●) bead mechanical strength.

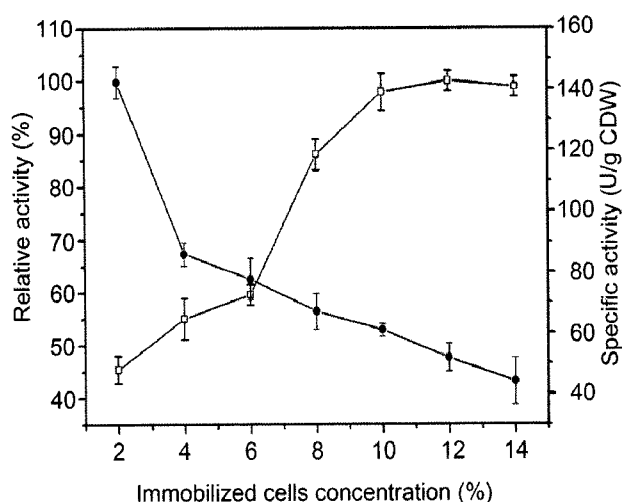


Fig. 4. Effects of immobilized cells concentrations on biosynthesis of acrylic acid.

The relative activity was expressed at the percentage of the reaction when cell concentration was 12% (w/v) (specific activity, 120.61 U/g DCW). Symbols: (□) relative activity; (●) specific activity.

cells to alginate increased below 12%. Above 12% of cell ratio to alginate, the activity was slightly decreased. However, the activity of per unit weight of cells was decreased as the ratio increased. The specific activities were 142.32 and 44.11 U/g DCW for ratio of 2% and 14%, respectively. This behavior could be caused by the diffusional restrictions of the substrate in the capsule membrane so that not all the immobilized cells could participate effectively in the reaction as free cells did.

Effects of Bead Size

As immobilized cells generally experience mass transfer resistance, the size of beads in which cells were entrapped may be one of the most important parameters [9]. The sizes of beads were controlled by changing the sizes of the needles. Ten beads were used to give an average bead size. Fig. 5 showed the effect of five different bead sizes on nitrilase activity. As mass transfer barrier increased as the bead diameter increased, the activity of immobilized beads was reduced accordingly. Maximum specific activity was attained for the minimum bead diameter of 2 mm with a 126.04 U/g DCW. A smaller bead size could produce higher activity because of an increase in the surface volume ratio or a lower mass transfer resistance. In addition, the larger bead size was more prone to swell and crack during the reaction process, and the bead mechanical strength decreased as the bead size increased.

Reinforcement of the Intensity of Ca-Alginate Beads

The immobilization of microbial cells by entrapment in calcium alginate is not very efficient, for the cells leak out during the course of converting time. Moreover, alginate matrixes were susceptible to cation-chelating agents such as phosphate, which caused the beads to dissolve. To overcome

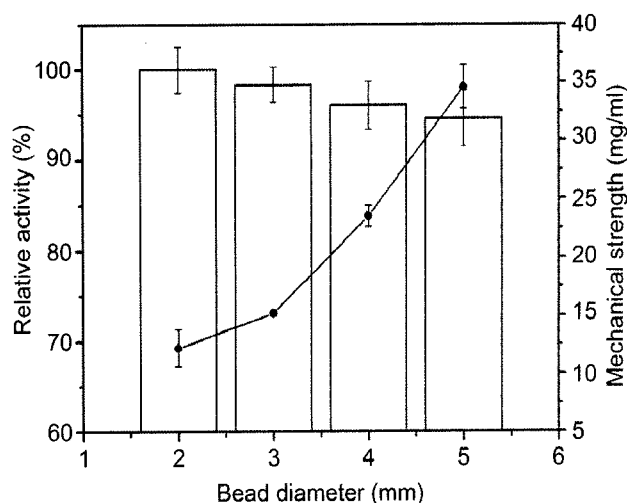


Fig. 5. Effects of bead diameters on biosynthesis of acrylic acid by the immobilized ZJUTB06-99 cells.

The relative activity was expressed at the percentage of the reaction when bead diameter was 2 mm (specific activity, 137.24 U/g DCW). Bar: (□) relative activity; Symbols: (●) bead mechanical strength.

these problems, PEI and GA were used to coat the beads. The conditions of the cross-linking were investigated [10]. Figs. 6 and 7 exhibited the single factor experiment results of the concentration of PEI and GA optimization. The mechanical strength of the beads was enhanced with the treatment of PEI and subsequently GA. Fig. 6 showed that the lower the concentration of PEI, the higher the bead activity. Fig. 7 revealed that the ideal concentration for GA was 0.75% (v/v). It was found that cross-linking resulted in greater bead stability of the biocatalyst at the cost of further increase of mass transfer resistance. Another cause of the reduced activity could be ascribed to the excessive GA

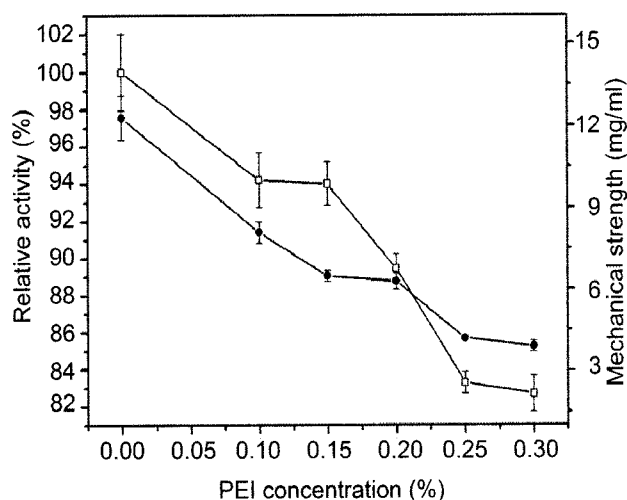


Fig. 6. Effects of PEI concentrations on catalysis reaction and bead mechanical strength.

The relative activity was expressed at the percentage of the reaction without treatment of PEI (specific activity, 136.25 U/g DCW). Symbols: (□) relative activity; (●) bead mechanical strength.

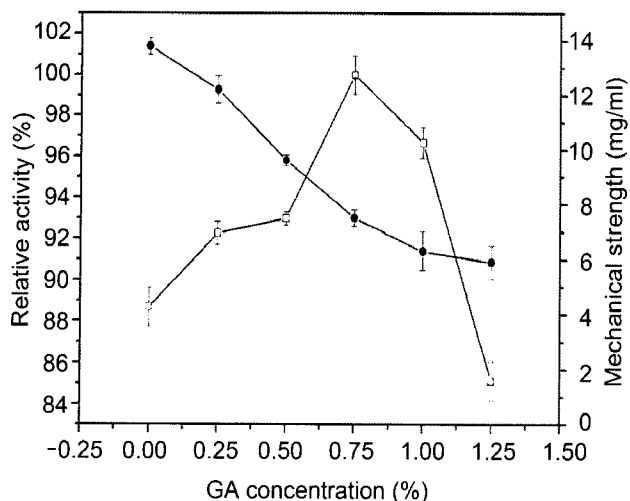


Fig. 7. Effects of GA concentrations on catalysis reaction and bead mechanical strength.

The relative activity was expressed as the percentage of the reaction when GA concentration was 0.75% (v/v) (specific activity, 145.62 U/g DCW). Symbols: (□) relative activity; (●) bead mechanical strength.

concentration that would inactivate the thiol-containing enzyme such as nitrilase [5]. However, the reduced activity was worthy to compare the stabilization of the bead after coating with PEI/GA. The reused batch was obviously increased after being treated. The bead strength was enhanced and cells did not easily leak out, which minimized the downstream processing. Furthermore, the tolerance to phosphate was improved. Beads kept intact in 50 mM phosphate buffer in the reaction process. However, the beads without treatment completely dissolved in the same concentration of phosphate during reaction.

Repeated Use of Nitrilase-Entrapped Beads

With the purpose of testing the stability of nitrilase immobilized in Ca-alginate beads, the number of cycles that it could be used was tested [1, 8, 19]. The reusability of the beads with PEI/GA treatment and without treatment

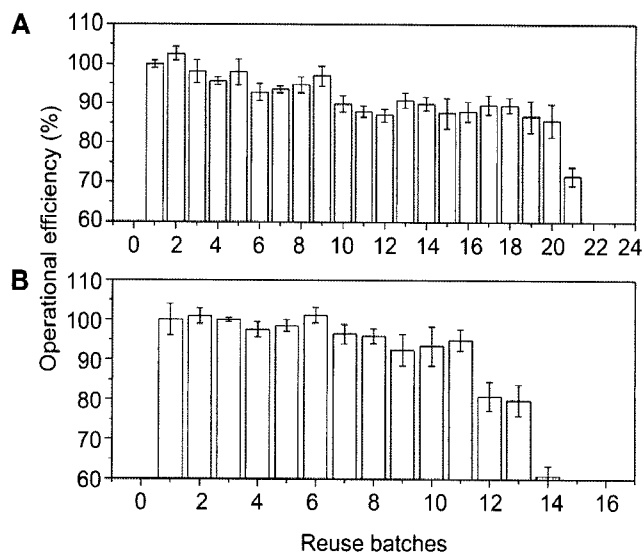


Fig. 8. Reuse batches of immobilized alginate cells with and without treatment by PEI and GA.

A. Twenty repeated batches (each of 3 h duration) with total of 60 h bead operation time by immobilized cells treated with PEI and GA. B. Eleven repeated batches (each of 3 h duration) with total of 33 h bead operation time by immobilized cells without treatment.

was compared. As shown in Fig. 8, the entrapped ZJUTB06-99 activity remained unchanged during the first 20 and 11 batches, respectively. After these batches, the activities were progressively decreased. There was a phenomenon of expansion for beads without treatment by PEI and GA in the process of the test of the reusability. The initial bead diameter was 2.0 ± 0.2 mm, based on the mean of 10 Ca-alginate beads. The untreated beads swelled and a significant increase in the mean diameter of beads was observed after 8 runs of reuse. The decline of activity of the beads after 11 batches could be attributed to the beads being cracked, leading to leakage of cells from the gel or the loss of beads during the repeated use. Stabilization with PEI and subsequently GA could avoid the problem of swelling, as it

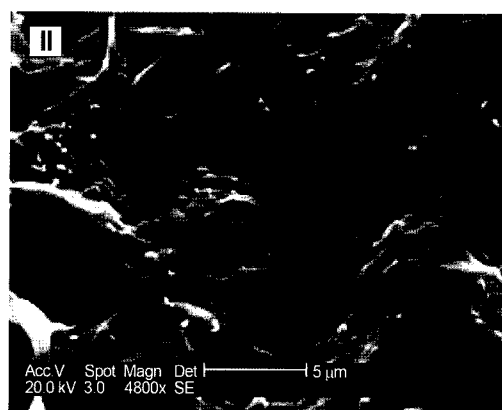
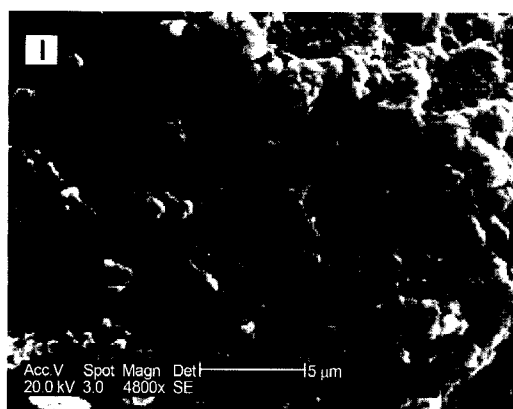


Fig. 9. ESEM pictures of cells immobilized in Ca-alginate matrix.

I. Surface of the bead without treatment by PEI and GA. II. Surface of the bead with treatment by PEI and GA.

formed an alkaline membrane in the surface of the sphere. Repeated-use capability was obviously improved. The reuse reached 20 batches without obvious activity decrease and cell leakage. Therefore, biotransformation of acrylonitrile to acrylic acid using immobilized ZJUTB06-99 cells appeared to be a reasonable route considering the reusability of the cells and the other advantages of immobilization.

Bead Morphology

The surfaces of beads with or without PEI/GA treatment were observed with ESEM. As shown in Fig. 9, cells were randomly distributed in the pores of the matrix. Changes appeared in the surface morphology of the beads with treatment. The treatment of PEI/GA made the bead form an alkaline membrane in the surface in which the gel network structure was tighter than untreated. Furthermore, a comparison of Fig. 9 (I) and Fig. 9 (II) indicated that the coating material affected the surface morphology of the alginate beads.

The results in this study showed that the calcium alginate gel was a feasible cell immobilization matrix for ZJUTB06-99 to biotransform acrylonitrile to acrylic acid. Immobilization operational parameters were investigated. Chemical cross-linking with PEI and GA was tried to reinforce the beads mechanical strength and further extend the reuse batches to 20 times. The applications of entrapped cells of *A. nitroguajacolicus* ZJUTB06-99 in reactors are currently being studied in our laboratory.

Acknowledgments

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