Repeated-Batch Operation of Immobilized β-Galactosidase Inclusion Bodies-Containing Escherichia coli Cell Reactor for Lactose Hydrolysis

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In this study, we investigated the performance of an immobilized β-galactosidase inclusion bodies-containing Escherichia coli cell reactor, where the cells were immobilized in alginate beads, which were then used in repeated-batch operations for the hydrolysis of o-nitrophenyl-β-D-galactoside or lactose over the long-term. In particular, in the Tris buffer system, disintegration of the alginate beads was not observed during the operation, which was observed for the phosphate buffer system. The o-nitrophenyl-β-D-galactoside hydrolysis was operated successfully up to about 80 h, and the runs were successfully repeated at least eight times. In addition, hydrolysis of lactose was successfully carried out up to 240 h. Using Western blotting analyses, it was verified that the β-galactosidase inclusion bodies were sustained in the alginate beads during the repeated-batch operations. Consequently, we experimentally verified that β-galactosidase inclusion bodies-containing Escherichia coli cells could be used in a repeated-batch reactor as a biocatalyst for the hydrolysis of o-nitrophenyl-β-D-galactoside or lactose. It is probable that this approach can be applied to enzymatic synthesis reactions for other biotechnology applications, particularly reactions that require long-term and stable operation.

Keywords: Repeated-batch, active inclusion body, β-galactosidase, cell immobilization, alginate bead, Escherichia coli

It has been reported that bacterial inclusion bodies (IBs) in Escherichia coli (E. coli) were expressed as biologically active protein aggregates [9–11, 14, 15, 24, 26], even though IBs are generally known to consist of inactive insoluble protein aggregates. In addition, our previous reports also demonstrated that E. coli β-galactosidase (β-gal) IBs were active and their activities were enhanced through the addition of a repressor (α-methyl D-glucopyranoside, α-MG) or an inducer analog (o-fucose) after induction of the araBAD promoter system in E. coli [14, 15]. In addition, the structural properties of IBs have been investigated based on the solubilization of IBs in guanidine hydrochloride solution [6, 22] or the degradation of IBs using a protease [2, 6]. de Groot and Ventura [6] observed that GFP-fused protein IBs formed at high temperature were clearly more resistant to protease digestion and denaturation by guanidine hydrochloride than those formed at lower temperatures. It was reported that this increased resistance was due to the more densely packed structure of IBs, and this structure was attributed to a more rapid formation of IBs at high temperatures [6]. Similar results were also observed for our E. coli β-gal IBs [27]; β-gal IBs expressed in the presence of o-fucose or α-MG were more quickly solubilized in guanidine hydrochloride or degraded by trypsin treatment than those produced when these compounds were not present. Moreover, the β-gal IBs expressed in the presence of o-fucose or α-MG were less stable at various temperatures. Based on these findings, we deduced that the looser structure of β-gal IBs resulted in enhanced enzymatic activity of β-gal IBs upon addition of o-fucose or α-MG after induction.

Although García-Fruitós et al. [9, 11] showed that only the β-gal-fused protein performed very efficiently as catalysts in enzymatic reactions, only a limited number of studies have examined the use of active IBs in enzymatic reactions. Interestingly, a cross-linked IB was previously used in the sialic acid synthesis process [19], which was mimicked by crosslinked enzyme aggregates technology [1]. In particular, in our previous studies, active E. coli β-gal IBs were produced by the addition of a repressor (α-MG) or an inducer analog (o-fucose) after induction of the araBAD promoter system in E. coli [14, 15]. Using these β-gal IBs, a packed-bed reactor was successfully operated for the hydrolysis of o-nitrophenyl-β-D-galactoside (ONPG) [28].

However, because the immobilized cell-containing alginate beads were shown to disintegrate during the longer periods
of operation [28], this approach could not be utilized for long-term operation of the packed-bed immobilized cell reactor in the continuous operational mode. Thus, to achieve stable operation using the immobilized *E. coli* β-gal IBs-containing cells, it is necessary to develop methods to mitigate the disintegration of alginate. This disintegration property was not surprising, since it has been previously shown that alginate beads are easily formed and disintegrated in *in vitro* and *in vivo* systems, and because of this property, alginate beads have been used as a carrier for drug delivery applications [7, 17, 21, 25].

In this study, we first developed methods to prevent the disintegration of alginate beads by changing the buffer system during the operation of the packed-bed reactor. Once a buffering system that was able to prevent disintegration of alginate beads was identified, long-term operation of the *E. coli* β-gal IBs-containing immobilized cell reactor system was investigated. The aim of this study was to demonstrate the long-term operation of an immobilized cell reactor, in which the *E. coli* cells contained active β-gal IBs and the cells were immobilized in alginate beads. In addition, we investigated whether β-gal IBs were stable and active during the long-term operation. The results of this study demonstrate that the use of enzyme IBs in reactor systems may be expanded to include a wide range of applications.

**MATERIALS AND METHODS**

**Chemicals**

α-Fucose, o-nitrophenyl-β-o-galactoside (ONPG), ampicillin, α-naphthol, and DNase I were purchased from Sigma-Aldrich (St. Louis, MO, USA). Sodium alginate was from Yakuri Chemical Co. (Kyoto, Japan). B-PER II reagent, SuperSignal West Pico chemiluminescent substrate, and Hyperfilm ECL were from Pierce (Rockford, IL, USA), and nitrocellulose membrane (Hybond-C, Extra) was from Amersham Biosciences (Uppsala, Sweden). Rabbit anti-β-galactosidase was from Molecular Probes (Carlsbad, CA, USA), and horseradish peroxidase conjugate was from Abcam (Cambridge, UK). Whey (Samik Dairy Products, Jeonbuk), lactose (Daejung Chemical Co., Gyeonggi-Do), chloroform (Daejung Chemical Co.), and acetonitrile (Daejung Chemical Co.) were from Korean companies. The other reagents were reagent-grade chemicals.

**Recombinant β-Gal-Expressing E. coli**

In this study, we used a recombinant *E. coli* in which the expression of β-gal was controlled by the araBAD promoter. The β-gal gene was cloned into the pBAD/Myb-His lucZ vector (7.2 kb) using the pBAD/Myb-His expression kit (V440-01, Invitrogen) [14, 15]. *E. coli* MC1061 was used as an expression host and induction was carried out by the addition of L-araabinose.

**Preparation of β-Gal IB-Containing Cells**

Fermentor cultures were conducted in a 2.5 l jar fermentor (Applikon Biotechnology, The Netherlands) with a working volume of 1.0 l, under conditions previously described [14, 15]. The composition of the medium was as follows: glycerol, 20 g/l; yeast extract, 20 g/l; KH₂PO₄, 2.31 g/l; and Na₂HPO₄. 10.22 g/l. Ampicillin was added at a concentration of 100 µg/ml. L-araabinose induction was performed at 0.05%, at an elapsed time of 3 h. If necessary, 0.01% α-fucose was added at an elapsed time of 3.5 h.

**Preparation of Alginate Beads**

An equal volume of 6% sodium alginate and *E. coli* culture broth, which was harvested at an elapsed time of 5.5 h, were mixed, and then the mixture was added drop-wise to a 0.05 M CaCl₂ solution, using a 5.0 ml syringe with needle (number 23). Alginate beads in the 0.05 M CaCl₂ solution were fully hardened after 1–2 h at 4°C.

**Packed-Bed Immobilized Cell Reactor**

Alginate beads, which were prepared using a 10 ml mixture of the alginate solution and *E. coli* culture broth, were poured into a 10 ml syringe (1.7 cm ID × 7.5 cm H). The silicone tubing, which was attached to the needle hub, was connected to a Masterflex C/L pump (Model 77120-62, Cole-Parmer), and the ONPG feed solution was supplied upward at a rate of 0.24 ml/h. Details of the operation conditions were described previously [28]. ONP (o-nitrophenol) production was measured in the effluent from the top of this reactor. The temperature of the packed-bed immobilized cell reactor was maintained at 28°C by immersion in a water bath.

**Repeated-Batch Immobilized Cell Reactor**

After 500 alginate beads prepared using the above method were washed by distilled water, the beads were placed into a 250 ml Erlenmeyer flask. Then, after 40 ml of ONPG or whey solution was added into the Erlenmeyer flask, the hydrolysis reaction was started. The reaction was carried out at 60 rpm and 28°C in a shaking incubator, and 200 µl was collected intermittently. In the repeated-batch operation, the reaction supernatant and alginate beads were separated by allowing the beads to settle down. The supernatant was then removed by decanting. Next, 40 ml of the substrate solution was added, and the new batch operation was started. This procedure was carried out repeatedly throughout the entire operation. When the hydrolysis reaction was carried out overnight, the flask containing the beads was stored in the refrigerator (4°C).

**ONP Production**

The production of ONP was monitored by measuring the color development of the effluent at 415 nm [optical density (OD) at 415 nm] using a microplate reader (Bio-Rad), in which 200 µl of effluent was added to each well of a 96-well microplate, and then 91 µl of 1 M Na₂CO₃ was added to stop the reaction.

**Thin-Layer Chromatography**

The hydrolysis of ONPG or lactose was analyzed via thin-layer chromatography (TLC) with a TLC plate (20 × 10 cm, Partisil K5F (Whatman, Kent, UK)) using acetonitrile solution [acetonitrile:water = 85:15 (v/v)] as the mobile phase. The sample loading volume was 1.0 µl. For visualization of the bands, TLC plates were soaked in 0.5% α-naphthol and 5% H₂SO₄ in ethanol and then dried in an oven at 80°C for 15 min. The percent hydrolysis of ONPG or lactose was quantitatively determined via TLC. The ONPG or lactose bands in the scanned images of the TLC plate were transformed to peaks using the AlphaEase FC software (Alpha Innotech, USA), and the percent hydrolysis of ONPG or lactose was calculated as follows:
Percent hydrolysis of ONPG or lactose (%) = \frac{A_t - A_0}{A_0} \times 100

where \(A_0\) and \(A_t\) are the amount of ONPG or lactose in the sample obtained from the \(\beta\)-gal IB-containing immobilized cell reactor at time zero and time \(t\), respectively.

Western Blotting
Ten alginate beads containing immobilized \(\beta\)-gal IBs-containing \(E.\ coli\) cells were disrupted by pipetting up and down with 3 ml of PBS (Phosphate-buffered saline). The disrupted bead suspension was then microcentrifuged, and, after removal of the supernatant, the debris were resuspended in a mixed solution of 5 ml of B-PER II reagent and 50 \(\mu\)l of DNase I solution (1 mg/ml). Soluble and insoluble fractions were then fractionated according to a previously described method [14, 15, 27, 28]. Those fractions were separated by 12.5% SDS–polyacrylamide gel electrophoresis (SDS–PAGE). The proteins were transferred to nitrocellulose membranes, which were subsequently probed with 1:5,000 rabbit anti-\(\beta\)-gal and 1:10,000 goat anti-rabbit IgG-HRP conjugate. Immunoreactive bands were visualized using the SuperSignal West Pico chemiluminescent substrate followed by exposure on Hyperfilm ECL. The details were described in previous reports [14, 15, 27, 28].

RESULTS AND DISCUSSION

Buffer System for Preventing the Disintegration of Alginate Beads
Stable operation of the immobilized cell reactor without disintegration of the immobilization system is crucial for practical operation for industrial applications. In particular, disintegration of alginate beads was frequently observed during the operation of the reactor system when a phosphate ion-containing buffer system was used [3–5]. We previously observed the disintegration of alginate beads during the operation of a packed-bed reactor in a phosphate buffer system, and this disintegration occurred within 24 h [28]. Generally, alginate beads are known to easily disintegrate in citrate or phosphate solutions [23]. Therefore, as shown in Fig. 1, we investigated the effect of the buffer system on the disintegration of alginate beads. Since we previously showed that the addition of chloroform, SDS (sodium dodecyl sulfate), and \(\beta\)-mercaptoethanol to the buffer system was beneficial to the \(\beta\)-gal activity [28], these chemicals were added to the Tris and phosphate buffer systems. When the reactor was operated in the phosphate buffer system, the alginate beads were disintegrated at 6 h (Fig. 2A). However, when the Tris buffer system was used, disintegration of the beads was not observed until 26 h (Fig. 2B). Therefore, the Tris buffer system containing chloroform, SDS, and \(\beta\)-mercaptoethanol was used for repeated-batch operation.

Repeated-Batch Operation for ONPG Hydrolysis
To verify the feasibility of long-term operation and to examine the durability of alginate beads in the Tris buffer system, we carried out repeated-batch operation using the immobilized \(\beta\)-gal IBs-containing \(E.\ coli\) cell reactor. After 500 alginate beads were prepared, the operation was carried out in 40 ml of ONPG solution in a 250 ml Erlenmeyer flask (Fig. 3). As shown in Fig. 3, the repeated-batch operations were successfully carried out for more than 15 h, and the runs were successfully repeated four or five times. Moreover, the alginate beads were not totally disintegrated because the Tris buffer system was used (data not shown), and ONPG production reached a maximum value when the ONPG concentration was more than 19.2 mM. Using an ONPG concentration of 19.2 mM, we examined the potential of long-term operation using the immobilized \(\beta\)-gal IBs-containing \(E.\ coli\) cell reactor (Fig. 4A). ONPG hydrolysis

Fig. 2. Sodium alginate beads during operation of the packed-bed immobilized cell reactor. A. When the reactor was operated in the phosphate buffer system, samples were collected at 6 h in Fig. 1 (arrow #1). B. When the reactor was operated in the Tris buffer system, samples were collected at 26 h in Fig. 1 (arrow #2).
was observed up to about 80 h; therefore, the runs were successfully repeated at least eight times. However, ONP production gradually decreased at longer operation times. However, we did not also observe disintegration of the alginate beads (data not shown). Fortunately, the performance of this repeated-batch operation was superior to that of the packed-bed operation using the immobilized β-gal IBs-containing E. coli cell reactor (Fig. 4). The disintegration of beads was not observed during the packed-bed operation; however, a loss of β-gal IBs-containing E. coli cell was observed, which was consistent with a previous study [28]. In addition, because the total amounts of β-gal IBs in the two reactor systems were quite different, it is difficult to directly compare the results obtained using the two systems. However, these results demonstrate that β-gal IBs activity was lost more easily in the packed-bed reactor system. In other words, the flow used in the packed-bed reactor was detrimental to maintaining β-gal activity.

In addition, the reactor performance was better in the repeated-batch reactor (Fig. 4). Under these conditions, a loss in β-gal activity was more clearly observed by the estimation of percent hydrolysis of ONPG in the repeated-batch reactor during the entire operation (Fig. 5). The reason for the loss of β-gal activity during the latter period of the operation time was examined by Western blot analysis (Fig. 5B) and TLC analyses (Fig. 6). As shown in Fig. 5B, the β-gal IBs were maintained in the immobilized cells until 54 h; however, no β-gal IBs were observed in the immobilized cells at longer time periods. Therefore, it was deduced that the loss of β-gal activity in the latter period of operation resulted from a loss of β-gal IBs-containing cells in the alginate beads. The loss of enzymes and cells in the alginate beads has also been frequently
observed in other reports [12, 13]. To overcome this problem, the cell viability in the alginate beads needs to be improved and new immobilization methods need to be developed.

**Repeated-Batch Operation for Lactose Hydrolysis**

For practical applications, we attempted to hydrolyze lactose in whey using the repeated-batch operation with the immobilized β-gal IBs-containing *E. coli* cell reactor. As shown in Fig. 7A and 7B, hydrolysis of lactose was observed for up to about 250 h, although the time intervals for the repeated batches were retarded compared with the hydrolysis of ONPG. In this repeated-batch operation, the β-gal IBs were maintained up to 240 h; however, IBs were no longer observed at 312 h (Fig. 7C). The retardation of

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**Fig. 5.** Elucidating the cause for loss of β-gal activity during the latter period of the operation time.

**A.** Percent hydrolysis of ONPG in the repeated-batch reactor during the entire operation. Samples were collected from the experiment described in Fig. 4A. To estimate the percent hydrolysis of ONPG, the amounts of residual ONPG were analyzed via TLC (Fig. 6). The asterisk (*) indicates the sample that was analyzed by Western blotting (B). **B.** Western blot analysis of active β-gal IBs-containing recombinant *E. coli* cells, which were immobilized in alginate beads. Sampling times are described on the top of the figure, and the samples were collected from the periods indicated in Fig. 4A (Fig. 5A). M indicates β-gal standard (G-5635, Sigma). S and I indicate soluble fractions and insoluble fractions from *E. coli* cells in alginate bead.

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**Fig. 6.** TLCs of the repeated-batch reactor in Fig. 5A.
The hydrolysis of ONPG was analyzed via TLC. O and G indicate ONPG and galactose as the standards, respectively. Numbers below the elapsed times indicate the consecutive run numbers in the repeated-batch reactor.
hydrolysis of lactose was not beneficial for the performance of the operation, and fortunately the β-gal IBs were maintained for longer periods than observed for the hydrolysis of ONPG. These results were probably due to the fact that whey consists of protein, lipid, lactic acid, citric acid, mineral, etc, as well as lactose [18]. Therefore, these ingredients might resist against the mass transfer phenomena around the alginate beads. In addition, owing to the limitation of mass transfer, inhibition of β-gal activity occurred as a result of the accumulation of galactose in the alginate beads [8, 16, 20, 28]. Consequently, limiting the mass transfer around the alginate beads might result in quite different results as shown in Fig. 7A and 7B, when compared with those in Fig. 4A and 5A.

In this study, alginate beads during the operation of an immobilized β-gal IBs-containing E. coli cell reactor were not disintegrated when a Tris buffer system was used. Throughout the repeated-batch operation of this immobilized cell reactor, ONPG hydrolysis was successfully observed up to about 80 h; thus, the runs could be successfully repeated at least eight times. In addition, disintegration of alginate beads was not observed during the repeated operation. When we attempted to hydrolyze lactose in whey using this immobilized β-gal IBs-containing E. coli cell reactor system, the reactor could be operated for up to 240 h. The presence of β-gal IBs in the alginate beads after hydrolysis of ONPG and lactose was confirmed by Western blotting analyses. However, the length of operation time for the hydrolysis of lactose in whey was remarkably different from that of ONPG. It was deduced that, owing to some ingredients in whey, there was a diffusional limitation around the alginate beads. In this study, we verified experimentally that β-gal IBs-containing E. coli cells could be used in a repeated-batch reactor as a biocatalyst for the hydrolysis of ONPG or lactose. Finally, it is probable that this approach can be applied to enzymatic synthesis reaction in the other fields of biotechnology, particularly in the transgalactosylation by β-gal.

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REFERENCES


