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Production of Alkaline Protease by Entrapped *Bacillus licheniformis* Cells in Repeated Batch Process

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In this study, *Bacillus licheniformis* cells were immobilized by entrapment in calcium alginate beads and were used for production of alkaline protease by repeated batch process. In order to increase the stability of the beads, the immobilization procedure was optimized by statistical full factorial method, by which three factors including alginate type, calcium chloride concentration, and agitation speed were studied. Optimization of the enzyme production medium, by the Taguchi method, was also studied. The obtained results showed that optimization of the cell immobilization procedure and medium constituents significantly enhanced the production of alkaline protease. In comparison with the free-cell culture in pre-optimized medium, about 7.3-fold higher productivity was resulted after optimization of the overall procedure. Repeated batch mode of operation, using optimized conditions, resulted in continuous production of the alkaline protease for 13 batches in 19 days.

Keywords: Alkaline protease, *Bacillus licheniformis*, cell immobilization, full factorial, optimization, Taguchi method

Proteases are an important group of industrial enzymes, especially alkaline proteases, which are widely used in the detergent industry. They comprise more than 30% of the global enzyme market. Applications of alkaline proteases have significantly increased in various industrial sectors such as detergent, leather tanning, pharmaceutical, and food industries [2, 6, 17, 19]. Because of their lower production cost and utilization of renewable supplies, microbial proteases, which are used for degrading peptides, are more favorable than their chemical counterparts. These enzymes have been produced by various processes such as solid-state and submerged fermentations, using bacteria, fungi, and yeasts [4, 7, 12, 23]. Bacteria of the genus *Bacillus*, such as *Bacillus licheniformis*, owing to their ability to produce large amount of proteases, with remarkable activity and stability at high pH and temperature, are favored producers of extracellular proteases and share a large portion of commercially produced alkaline proteases [1, 5, 11, 12, 14, 24]. Microbial proteases are usually produced by either free or immobilized cells. Use of immobilized cell processes has some advantages over the free-cell types, such as facilitating the product separation, reusability of the biocatalysts in repeated batch fermentation processes, prevention of washout of the cells, reduction of contamination risk, operational stability, and improvement of productivity [9, 13, 27, 28, 30]. Immobilized cells have been used in many processes such as biotransformation, phenol degradation of toxic substances, preparation of biosensors, and production of extracellular enzyme, antibiotics, and ethanol [10, 18, 20, 22,]. Immobilization encompasses many approaches, but among them, entrapment in calcium alginate is more advantageous because of its simplicity and using non-toxic reagents in the production procedure [10]. Ca-alginate-biocatalyst preparation from microbial cells is a stepwise procedure, in which the stability of the biocatalysts is dependent on various factors, which have to be optimized [26]. Designing a cost-effective medium for production of alkaline protease by immobilized cells is also a necessity [8].

In this research, for preparing stable beads with high reusability, some effective parameters were studied and optimized by statistical full factorial method. Furthermore, alkaline protease production medium was optimized by the Taguchi method [29].

Materials and Methods

Microorganism

The alkaline protease-producing *Bacillus licheniformis* PTCC1525 [21] was obtained from Persian Type Culture Collection (PTCC,

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Iranian Research Organization for Science and Technology, Tehran, Iran). It was maintained on BHI Agar (Merck Co.) slants and subcultured every month.

Inoculum Preparation

The preculture was prepared by transferring a loop-full of a 24-h-old slant of *B. licheniformis* to a 250 ml Erlenmeyer flask, containing 50 ml of sterile Brain Heart Infusion Broth Medium (Merck Co.), pH 7.4. The flasks were incubated on a shaker incubator for 16 h, at 150 rev/min, 30°C. At the end of the incubation time, a defined amount (as described below) of inoculum medium was centrifuged at 4,000 ×*g* for 20 min. The cell pellet was resuspended in sterile sodium chloride solution, 9.0 g/l, and was used as inoculum for both immobilized and free cell fermentations.

Entrapment of Cells in Calcium Alginate Beads

In order to compare the free-cell culture with immobilized ones, the same volume of preculture, which was used as inoculum for the former, was centrifuged and the cell pellet was resuspended in sterile sodium chloride solution, with a volume equal to the related supernatant, and well mixed for 10 min with sodium alginate, as shown in Table 1, to inoculate the latter. The cell's density in the preculture was about 8.5×10^8 CFU/ml, as measured by the plate counting method. The sodium alginate solution was prepared by dissolving the Na-alginate (Medium and High viscosity types; Sigma-Aldrich) in boiling water and autoclaving it at 121°C for 15 min. Using a 20 ml syringe, the cell–alginate mixture was added dropwise, from a 10 cm distance, into a stirring solution of 2.15% (w/v) $CaCl_2$ to form the beads [25]. Stirring was continued for further 30 min. The bead-containing solution was kept unstirred for 1 h at 4°C for curing. Then the $CaCl_2$ solution was decanted and the beads were washed two times with sterile 9.0 g/l sodium chloride solution. All the steps were performed aseptically under a laminar airflow unit.

In this manner, both the free and immobilized cell cultures were inoculated by the same volume of inoculum and cell density, and precise comparison between these two types of cultures was possible.

Alkaline Protease Production Using Free and Immobilized Cells in Pre-Optimized Production Medium

The initial production medium composition used for alkaline protease production by *B. licheniformis* PTCC 1525 was (g/l) soy bean meal, 30; glucose, 30; beef extract, 15; K_2HPO_4, 1; $MgSO_4 \cdot 7H_2O$, 0.5; $CaCl_2$, 0.5; Na_2CO_3, 10; NaCl, 1.5; and L-cysteine, 0.5; pH 9.5 [25]. To each 250 ml Erlenmeyer flask, containing 50 ml of the above medium, approximately 250 beads (equivalent to 5 ml) were added. Three flasks were also inoculated with 5 ml of preculture, as free-cell culture, for comparison. The flasks were incubated at 140 rev/min, 30°C, for 24 h, before assaying their proteolytic activity.

Table 2. Selected factors and their levels for optimization of production medium.

Factors	Level 1	Level 2	Level 3	Level 4
Soybean meal (g/l)	25	30	35	40
Glucose (g/l)	0	5	15	30
Beef extract (g/l)	0	5	10	15
L-Cysteine (g/l)	0	0.15	0.3	0.5

Optimization of Entrapment Conditions by Statistical Full Factorial Method

To optimize the immobilization process, three factors of (i) type/concentration of Na-alginate, (ii) concentration of $CaCl_2$, and (iii) agitation speed of cultures were selected, as shown in Table 1. Regarding the number of factors and their levels, 18 trials, each in duplicate, were run. The flasks were incubated at 30°C for 15 days and the beads were checked every day regarding their physical integrity. The spent production medium was replaced by fresh medium every 48 h and flasks were discarded if more than 50% of their beads were disintegrated. Design of the experiment and analysis of the results were done by Minitab Ver. 15 software (Minitab Inc., PA, USA).

Effect of Protein Source on Alkaline Protease Production

Three protein sources [soy flour and soy protein meal (both prepared from local manufacturers, sieved by 1 mm sieve) and casein (Merck, Co.)] were tested individually for preparation of production medium. Each experiment was carried out in triplicate. The rest of the constituents were kept constant for all the experiments. All the flasks were similarly inoculated with 250 beads, and incubated at 30°C, 150 rev/min, for 48 h, after which, samples were assayed for alkaline protease activity.

Optimization of Production Medium by Taguchi Method

In order to find a cheaper and more effective production medium, the amount of production medium components were optimized by the Taguchi method [29]. The concentration of four factors (soy bean meal, glucose, beef extract, and L-cysteine) each in four levels, were checked for this purpose (Table 2). For designing of the experiments, an L-16 orthogonal array was selected, by which 16 treatments were designed and run. The media were inoculated with immobilized cells and incubated at 150 rev/min, 30°C for 48 h before assaying the enzyme activity. Each treatment was performed in triplicate. Analysis of the results was performed by Qualitek-4 software (Nutek, Inc., Detroit, USA).

Verification Test

In order to verify the optimized amount of ingredients of the production medium, calculated by analysis of the results, the optimum

Table 1. Factors and their levels selected for optimization of immobilization process.

Factors	Level 1	Level 2	Level 3
Type/concentration of sodium alginate	Medium viscosity 3%	Medium viscosity 4%	High viscosity 3%
$CaCl_2$ concentration (M)	0.2	0.3	0.4
Agitation speed of the culture (rev/min)	120	150	-

condition was tested again in triplicate and the actual responses were compared with the expected ones.

Effect of Beads' Cell Loading on the Protease Production

The beads were prepared in four different cell-loading sizes of (v/v) 1%, 5%, 15%, and 30%, as described above. For making a 5% (v/v) loading size, for example, 2.5 ml of inoculum medium was centrifuged; cell pellet was resuspended in 9% (w/v) sodium chloride to a total volume of 2.5 ml and mixed with Na-alginate, as described above. The beads were transferred to the flasks containing 50 ml of optimized production medium. Each experiment was carried out in triplicate. After 48 h of incubation at 150 rev/min, 30°C, samples were assayed for their alkaline protease activity.

Time Course of Enzyme Production

Several 250 ml Erlenmeyer flasks, containing 50 ml of optimized production medium, pH 7.4, were inoculated with immobilized live cells, in triplicate, for evaluating the appropriate time for changing the enzyme-containing spent medium with fresh medium. Some samples were also inoculated with free cells to compare the result. The flasks were incubated at 150 rev/min, 30°C, and sampled at different intervals for measuring their alkaline protease activity.

Alkaline Protease Production in Repeated Batch

The optimized beads were prepared in three different cell loading sizes (v/v) of 5%, 15%, and 30%, transferred to 250 ml Erlenmeyer flasks containing 50 ml of optimized production medium (approximately 250 beads for each flask), pH 7.4, and incubated at 150 rev/min, 30°C, for 35 h (each cell loading size experiment was carried out in triplicate). After incubation and enzyme assay, the spent medium was removed, and after washing the beads twice (with sterile saline solution), fresh medium was added and the flasks were re-incubated.

Alkaline Protease Assay

Assay of alkaline protease activity was performed by the Kunitz method [16]. The samples were centrifuged at 4,000 ×*g* for 20 min and the supernatant, containing alkaline protease, was separated. Then 100 µl of the supernatant was added to 1 ml of 1% (w/v) Hammersten Casein (Merck Co.) in 50 mM Tris-HCl buffer, pH 8.5. The mixture was incubated at 65°C for 10 min. The reaction was terminated by adding 1.5 ml of 10% (w/v) trichloroacetic acid (TCA) solution to the mixture. The mixture was shacked well and incubated at room temperature for 30 min without any movement. For the preparation of the blank, the TCA solution was first added to the supernatant of the production medium in order to inactivate the enzyme. The tubes were centrifuged at 4,000 ×*g* for 20 min and the absorbance of the samples was measured at 275 nm. One unit of enzyme activity was defined as the amount of enzyme that was able to release 1 µg/ml/min of tyrosine under the assay conditions. The amount of tyrosine was calculated by using the tyrosine standard curve.

RESULTS

Optimization of the Entrapment Procedure

The beads were not stable in the alkaline pH (higher than 7.5) for more than 24 h. Even at a pH of 7.4, the beads disintegrated after 5–6 days of inoculation (data not shown). Regarding the low stability of the initially prepared beads, the entrapment condition was optimized by a full factorial method (see Table 3 for both the design and results). The response, recorded as the durability of the beads (as days), was analyzed by Minitab Ver. 15 software, by comparing the average performance of each factor at a

Table 3. Treatments and obtained results of optimization of cell entrapment by full factorial method.

Trial number	Factors and their levels			Obtained result shown as Stability[a] (Days)
	Type and concentration of sodium alginate	$CaCl_2$ concentration (M)	Agitation (rpm)	
1	Medium viscosity 3%	0.2	120	5.5±0.71
2	Medium viscosity 3%	0.2	150	5±0
3	Medium viscosity 3%	0.3	120	15±0
4	Medium viscosity 3%	0.3	150	15±0
5	Medium viscosity 3%	0.4	120	15±0
6	Medium viscosity 3%	0.4	150	12±0
7	Medium viscosity 4%	0.2	120	7±0
8	Medium viscosity 4%	0.2	150	4.5±0.71
9	Medium viscosity 4%	0.3	120	6.5±0.71
10	Medium viscosity 4%	0.3	150	5±0
11	Medium viscosity 4%	0.4	120	6±1.41
12	Medium viscosity 4%	0.4	150	8±1.41
13	High viscosity 3%	0.2	120	2±0
14	High viscosity 3%	0.2	150	2±0
15	High viscosity 3%	0.3	120	2±0
16	High viscosity 3%	0.3	150	2±0
17	High viscosity 3%	0.4	120	2±0
18	High viscosity 3%	0.4	150	2±0

[a]Average of 2 replications ± standard deviation.

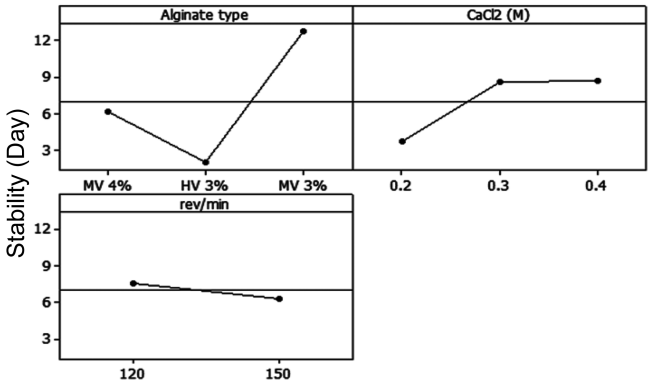

Fig. 1. Main effects plot for optimization of the stability of cell entrapment, which shows the average performance of each factor at a given level.

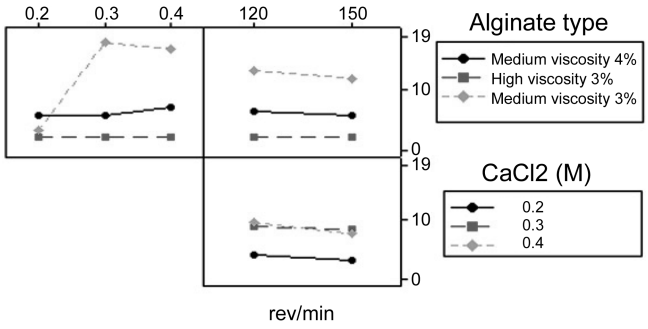

Fig. 2. Interaction plot for optimization of stability of cell entrapment.

given level. As shown in Fig. 1, the highest stability of the beads was observed at level 3 of sodium alginate concentration/type, level 3 of calcium chloride concentration, and level 1 of the agitation speed. The prepared beads, by these conditions, were stable for 15 days. The results showed that increase of agitation speed, from 120 to 150 rev/min, did not have significant effect on beads stability. Thus, regarding the better aeration in 150 than 120 rev/min, needed for better activity of *Bacillus* cells, the former value was selected. Since the difference between 0.4 and 0.3 M concentrations of $CaCl_2$ was not significant, the latter was chosen.

The stability of optimized beads in alkaline pH was also improved, since the beads were stable at pH 8, 8.5, and 9 for 8, 5, and 4 days, respectively.

The interaction plot shown in Fig. 2 indicates that there were no significant interactions between performances of these three factors. This means that the factors have exerted their effects on the stability of the beads, independent from each other. The only significant interaction was seen between alginate (medium viscosity, 3%) and $CaCl_2$ concentration.

Selected Protein Source for Alkaline Protease Production

The amount of alkaline protease production, using pre-optimized production medium, after 48 h of inoculation by optimized immobilized (5% cell loading) and free cells (5% inoculum size) was shown to be 205 and 170 U/ml, respectively.

Results showed that the highest alkaline protease production, 200 U/ml, was obtained by using soybean meal in the production medium. The amount of produced enzyme, using soy protein meal and casein, was 189 and 102 U/ml, respectively.

Table 4. Treatments and obtained results for optimization of the production medium by the Taguchi method.

Trial number	Soybean meal (g/l)	Glucose (g/l)	Beef extract (g/l)	L-Cysteine (g/l)	Produced enzyme (U/ml)[a]
1	25	0	0	0	993.3±17.5
2	25	5	5	0.15	760.0±30.1
3	25	15	10	0.30	428.7±13.8
4	25	30	15	0.50	190.0±8.9
5	30	0	5	0.30	556.0±19.9
6	30	5	0	0.50	361.7±14.6
7	30	15	15	0	261.7±4.7
8	30	30	10	0.15	209.7±9.5
9	35	0	10	0.50	305.3±16.0
10	35	5	15	0.30	216.0±16.1
11	35	15	0	0.15	647.0±7.6
12	35	30	5	0	261.0±15.7
13	40	0	15	0.15	318.0±16.5
14	40	5	10	0	539.3±16.9
15	40	15	5	0.50	519.0±21.9
16	40	30	0	0.30	547.7±15.9

[a]Figures are the average of three replications ± standard deviation.

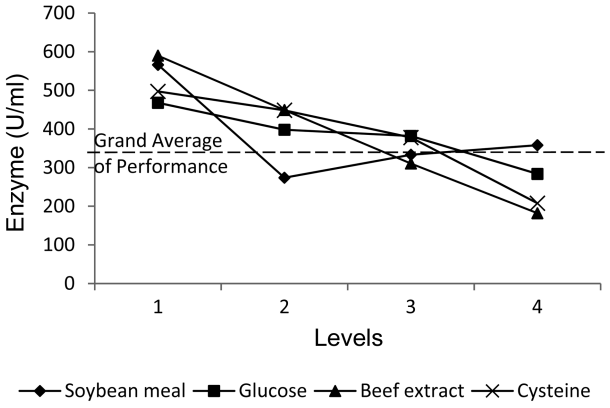

Fig. 3. Main effects plot for optimization of the alkaline protease production medium, which shows the average performance of each factor at a given level.

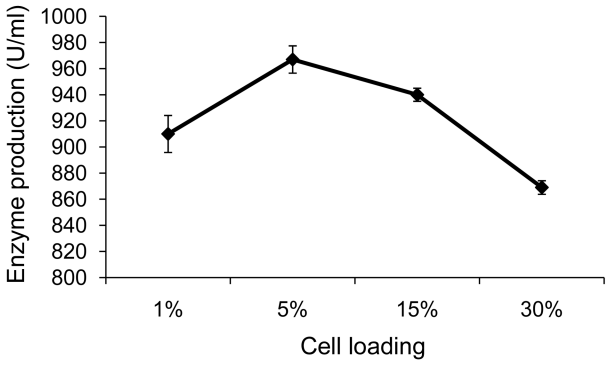

Fig. 4. Effect of cell loading size on the protease production. Error bars represent the standard deviation of 3 replicates.

Optimization of Production Medium Using Taguchi Method

The medium composition in each trial and the obtained results are shown in Table 4. The data were analyzed by Qualitek-4 software. The "standard analysis," using average of results, and the state of "bigger is better," as the QC condition for analysis of results, were selected for data analysis. As the main effects plot (Fig. 3) shows, the optimum level for all the four factors (soybean meal, glucose, beef extract, and L-cysteine) was their first level.

As Fig. 4 shows, the highest alkaline protease production was obtained at cell loading size of 5%. At cell loading size of 30%, the amount of protease production was reduced significantly.

In free-cell fermentation, the highest level of protease production, which was equal to 623 U/ml, was reached after 35 h. Compared with this, in the immobilized cell process, the highest amount of enzyme, 1,083 U/ml, was produced after 48 h, which was 74% higher than the former (Fig. 5). The highest enzyme productivities in both processes resulted after 35 h, which were 17.8 and 27.4 U/ml/h for free and immobilized processes, respectively. The optimum pH and temperature of protease activity were equal to 8–9 and 65°C, respectively.

Alkaline Protease Production in Repeated Batch Process

As mentioned previously, for running the repeated batch fermentation, beads were prepared in three cell loading sizes of 5%, 15%, and 30%. As depicted in Fig. 6, during repeated batch process, in all of the three cell loading sizes, the production of the enzyme was almost in the same level, although a slight decrease of production was observed in the higher loading sizes. Each batch was continued for 35 h, before changing the spent medium with the fresh one. An unwanted bacterial contamination, which was detected in the twelfth batch in the majority of the flasks, caused a significant reduction of protease production after this point and eventually stopped the process. At the end of the thirteenth batch, the number of whole beads was counted and it was found that approximately 50% of the

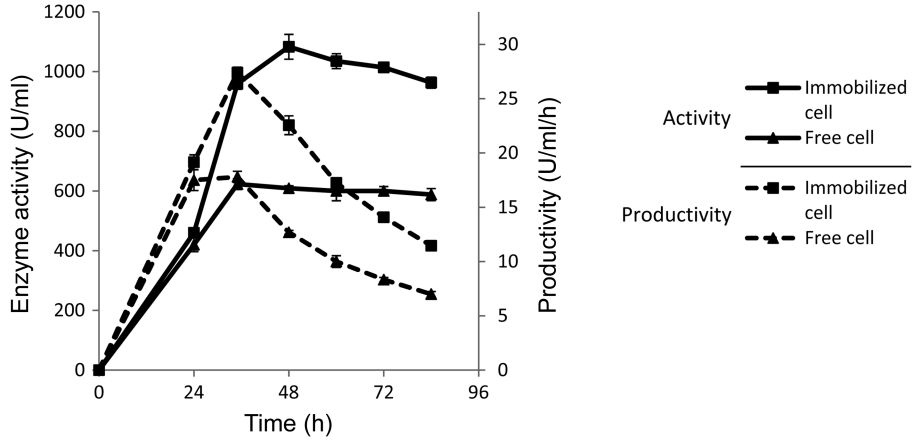

Fig. 5. Comparison of production of protease and productivities of the processes by free and immobilized cell cultures. Error bars represent the standard deviation of 3 replicates.

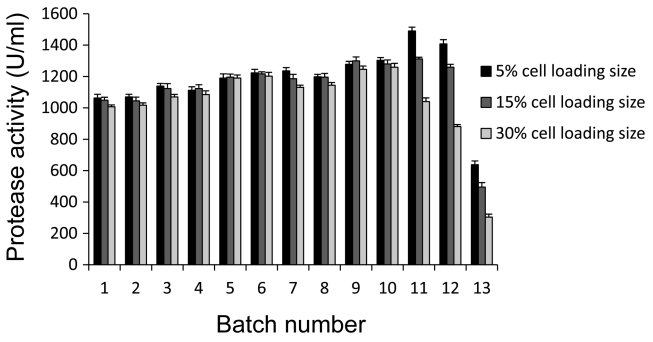

Fig. 6. Production of protease in repeated batch process in different cell loading sizes.
Error bars represent the standard deviation of 3 replicates.

beads were unimpaired in all the three cell loading sizes, which was evidence of the satisfactory stability of the beads. Table 5 shows the overall result obtained at this stage.

The highest productivity, 33.7 U/ml/h, was obtained by the immobilized cells with 5% loading size, when optimized production medium was used. The next ranks belonged to the 15% and 30% cell loading sizes with the productivities of 32.5 U/ml/h and 29.8 U/ml/h, respectively.

DISCUSSION

The purpose of the present study was to find the behavior of the alginate beads regarding its stability and optimization of production of the alkaline protease by alginate-entrapped *Bacillus licheniformis* PTCC 1525, a native isolate, in such a way that it can be used in repeated batch fermentation. As the first step, the condition for entrapment of *B. licheniformis* cells in Ca-alginate was optimized by full factorial method, where using 3% (w/v) concentration of medium viscosity sodium alginate, as the supporting material, 0.3 M of calcium chloride, as the geling agent, and agitation speed of 150 rev/min, were determined as the best condition for highest stability of the beads. In continuation, the composition of production medium was optimized by the Taguchi method. As the result, a medium containing (g/l) soybean meal, 25; KH_2PO_4, 1; $CaCl_2$, 0.5; $MgSO_4 \cdot 7H_2O$, 0.5; and NaCl, 1.5 was determined as the optimum production medium for *B. licheniformis* PTCC 1525. Using this medium, after 48 h, 1,010 U/ml of the enzyme was produced by immobilized cells, which was 4.9 times more than the enzyme produced by pre-optimized medium.

According to the previous studies, the amount of yield and productivity, by using immobilized cell cultures for production of alkaline protease, is remarkably higher than free-cell fermentation [25]. Reusing the immobilized cells in repeated batch fermentation is possible, and preparation of inoculum is not required for starting new batches. The optimized beads were used for 13 cycles. In spite of some reports indicating that the use of immobilized cells in pH 9 and above can be continued for 7 batches and more [3, 15, 25], the results of the present study showed that calcium alginate beads stability reduces remarkably at pH 8 and above.

Protease productivity resulted from the immobilized cells in repeated batch fermentation was 89% more than that of free-cell cultures.

In repeated batch fermentation, using optimized immobilized cells with 5% cell loading size, a productivity of 33.7 U/ml/h resulted, which was 7.3 times higher than the free-cell fermentation using pre-optimized medium (4.6 U/ml/h).

Optimization of production medium composition not only reduced the cost of production medium, but also significantly increased the production. By using immobilized cells in repeated batch fermentation for 13 cycles, owing to re-using the inoculum, the cost of production may be further reduced. As a result, use of *B. licheniformis* cells immobilized in calcium alginate beads as biocatalysts for alkaline protease production in repeated batch fermentation can be a suitable alternative for ordinary fermentation using free cells.

Table 5. Comparison of alkaline protease production in different states.

Culture condition[a]	Fermentation time of each batch (h)	Batch numbers	Total fermentation time (h)	Total alkaline protease production (U/ml)	Average of produced alkaline protease (U/ml)	Productivity (U/ml/h)
A	35	1	35	160	160	4.6
B	35	1	35	625	625	17.8
C	35	1	35	185	185	5.3
D	35	13	455	15,348	1,181	33.7
E	35	13	455	14,789	1,137	32.5
F	35	13	455	13,576	1,044	29.8

[a]Culture conditions: (A) Free cell with 5% inoculum size using pre-optimized production medium; (B) Free cell with 5% inoculum size using optimized production medium; (C) Immobilized cell with 5% cell loading size using pre-optimized production medium; (D) Immobilized cell with 5% cell loading size using optimized production medium (repeated batch); (E) Immobilized cell with 15% cell loading size using optimized production medium (repeated batch); (F) Immobilized cell with 30% cell loading size using optimized production medium (repeated batch).

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