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Development of a continuous L-lysine bioconversion system for cadaverine production
Continuous bioconversion of L-lysine into cadaverine using barium alginate packed reactor

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ABSTRACT

Cadaverine, a five carbon diamine (1,5-diaminopentane), plays a role as a building block of polyamides and it can be made by fermentation or direct bioconversion. To improve its production by increasing reusability of immobilized enzyme and avoid separation of enzyme in bioconversion, a continuous L-lysine bioconversion process for cadaverine production has been developed. Various divalent cations, alginate concentrations, cell density with alginate and flow rate of feed were examined to maximize the lysine decarboxylase activity of the whole-cell immobilized beads. Under the selected conditions, 123 h of continuous cadaverine production has been performed and 5.5 L of 819 mM cadaverine were produced with 14 mL reactor resulting in 466.5 g of cadaverine. Cadaverine production was possible with small volume reactor maintaining relatively high concentration of substrate.

Introduction

Q4 Increasing energy demands along with environmental problems have stimulated development of sustainable industry. Therefore, as an alternative to petrochemical production, biochemical production has received attentions [1–5]. However, economic feasibility has prevented its successful industrialization. Compared with petrochemical production, biochemical production has several drawbacks, such as relatively low production titer, purity, and productivity [6]. Therefore, to overcome the economic barrier, developing a cost-efficient bioprocess is an important step in the industrialization of biochemical production.

Cadaverine, a five carbon diamine (1,5-diaminopentane), plays a role as a building block of polyamides such as PA 5.4, 5.6, 5.10, and 5.12, which can be alternatives to petroleum based polyamides [7]. In particular, bio-based PA 5.10 has mechanical properties comparable to those of the well-established petrochemical polyamides PA 6 and PA 6.6, suggesting it to be a possible replacement for conventional petrochemical nylons. As the global market for petroleum-based polyamides has increased, and is currently ~6.6 million tons per year, interest in biological cadaverine production has also increased [7]. Cadaverine is produced by decarboxylation of L-lysine, which is an essential amino acid synthesized by bacteria and plants, by lysine decarboxylase (E.C. 4. 1. 1. 18). Metabolic engineering has enabled *Escherichia coli* and *Corynebacterium glutamicum* to overproduce cadaverine with various substrates [8–12]. Particularly, using a L-

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lysine over-producing *C. glutamicum* as a host strain, high titers (103 g/L) and molar yields (50%) were achieved [13].

Direct bioconversion of concentrated L-lysine into cadaverine using whole-cell biocatalysts is another possible cadaverine production process [14,15]. Because lysine production has been successfully commercialized, biotransformation using commercial lysine as a substrate could be considered. *E. coli* whole-cell biocatalysts overexpressing lysine decarboxylase achieved a 92–99% conversion ratio with L-lysine over 1 M (mol/L) [14–16]. In addition, immobilized whole-cell biocatalyst enabled repetitive reactions by increasing the stability of the *E. coli* whole-cell catalyst [17]. Accordingly, it is important to develop and evaluate processes for industrialization of cadaverine production [14].

In this study, we designed a continuous cadaverine production system based on an immobilized *E. coli* whole-cell biocatalyst. A bead-packed column reactor was designed for continuous L-lysine bioconversion into cadaverine by optimizing the reaction efficiency through adjusting immobilization and operating conditions. The simple and small reactor (14 mL working volume) was operated for 123 h with a constant flow rate and achieved continuous cadaverine production. This report suggests the feasibility of cadaverine production using immobilized whole-cell biocatalysts and provides insight into bulk cadaverine production using a continuous bioconversion reactor.

Immobilized cells have long been applied to continuous production systems [18–24]. These systems have potential for industrial production using various types of packed-bed reactors. Whole-cell bioconversion of L-lysine into cadaverine is a rapid reaction that converts over 90% of 1 M L-lysine into cadaverine within 2 h [15]. We reported previously that immobilization of the whole-cell biocatalyst using barium alginate was stable and effective for repetitive reactions [17]. Therefore, as a proof of concept, we designed a continuous process using a packed-bed column reactor and investigated its potential for industrial production of cadaverine (Fig. 1). The designed glass reactor had a working volume of 14 mL and height of 14 cm, and was able to be packed with about 1500 2-mm-diameter beads. The beads encapsulate *E. coli* whole-cells overexpressing lysine decarboxylase (CadA). Feed would pass through the bead packed reactor with constant flow rate. As it undergoes reaction, carbon dioxide (CO_2) gas is emitted to top of the reactor and product would be collected to the effluent storage.

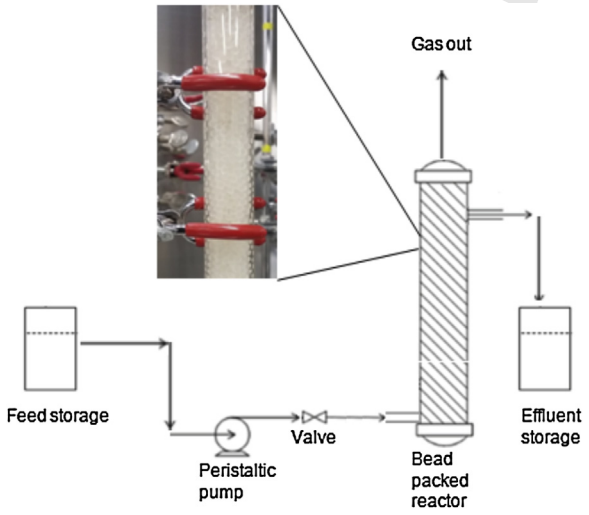

Fig. 1. Schematic diagram of continuous cadaverine production by the L-lysine bioconversion system. The bead packed reactor contains alginate beads encapsulating *E. coli* whole-cell lysine decarboxylase (CadA).

Because barium alginate immobilization reduced the conversion rate by 50% [17], further optimization was required. The interaction between divalent cations and alginate determines the structure and complexation of alginate gels because of the different affinity of alginate towards cations [25,26]. Therefore, we screened out divalent cations to improve activity. The lysine decarboxylase activity of immobilized whole cells using six divalent cations, Mg^{2+}, Cu^{2+}, Zn^{2+}, Sn^{2+}, Co^{2+}, Mn^{2+} and the equal amount of free cell for control was compared to that of a barium alginate whole-cell catalyst (Fig. 2A). Except Mg^{2+}, they formulated sphere beads while their intensity was different from each other. Unfortunately, however, only alginate gels using Sn^{2+} or Ba^{2+} retained their shape after 1 M L-lysine bioconversion. Also, barium alginate exhibited the highest activity among the alginate beads. Because calcium alginate beads have been reported to be unstable [17], use of Ba^{2+} for whole-cell immobilization of the *E. coli* overexpressing lysine decarboxylase would be the most effective cation. For further improvement, the sodium alginate concentration was varied (Fig. 2B). At alginate concentration with less than 1.5%, no significant reduction in whole-cell activity was observed. However, above 1.5% alginate, the whole-cell activity decreased. In general, a low alginate concentration in beads results in higher permeability but lower strength [27,28]. Likewise, an alginate

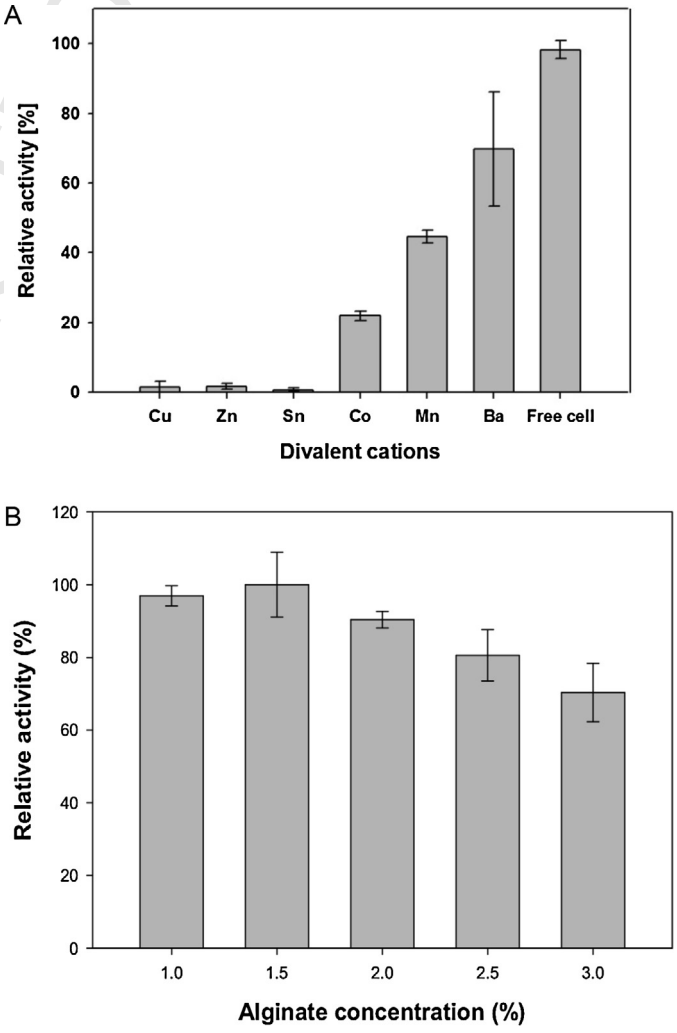

Fig. 2. Optimization of whole-cell immobilization. A: Effect of divalent cations, B: Effect of alginate concentration on lysine decarboxylase activity. The *error bars* represent standard deviations of three independent experiments.

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concentration at more than 1.5% might reduce bead permeability, hindering mass transfer of the substrate and product.

As well as the properties of the alginate beads, the amount of whole-cell biocatalyst is also important. Immobilization of greater amount of cells in a bead would result in greater L-lysine conversion [17]. However, excess cells in a bead would reduce the reaction efficiency because of the reduced diffusion coefficient. In addition, the number of beads should be considered to optimize reaction efficiency. Therefore, the effects of cell concentration and bead quantity on 1 M L-lysine conversion were investigated. As expected, a higher cell concentration resulted in higher specific activity (Table 1). At more than 16.5 mg/mL, there was no further improvement of conversion rate, suggesting 16.5 mg/mL cells to be the saturation point. The maximum lysine decarboxylase activity and final conversion yield of the saturated beads were 4.5 mmol/min/bead and 80.9%, respectively. When considering total cell mass, however, 16.5 mg/mL cells would not be practical because distributing cells among more beads would increase the reaction surface area (Table S1). Also, as 1.1 mg/mL cells were obtained by the whole-cell biocatalyst preparation, 250 mL culture was required to set up the glass reactor if 16.5 mg/mL of whole-cell immobilized beads were used. Therefore, we performed continuous cadaverine production using beads with 2.2 mg/mL cells.

The effect of feed flow rate in the packed bed column reactor was investigated (Table 2). Approximately 90% conversion yield was maintained up to a flow rate of 0.75 mL/min, but any further increase in flow rate decreased the conversion yield. Only 50% conversion was achieved when the flow rate was 1.5 mL/min. It is of interest to note that the conversion yield at a flow rate of 2 mL/min was similar to that at 1.5 mL/min. This may be explained by the rapidity of the reaction. The maximum productivity (69.5 cadaverine mmol/h) was achieved at a flow rate of 2 mL/min, but cadaverine yield was only 55%, which would result in a large quantity of residual L-lysine. Therefore, 0.75 mL/min was selected for continuous cadaverine production to achieve both high conversion yield and productivity. When compared with a batch reaction, the continuous reaction exhibited 78.3% of activity.

Then, prolonged cadaverine production by the continuous bioconversion system was performed. The reactor was stable for 123 h and 5.5 L of 819 mM cadaverine was produced which is 466.5 g in mass (Figs. 3 and 4). Cadaverine productivity was 3.8 g/h (37.2 mmol/h), as determined by linear regression. Considering productivity at a 0.75 mL/min flow rate (40.6 mmol/h), it is of interest that 91% of productivity was maintained during the operation. The production time course revealed that the system maintained a 91–94% conversion yield for 48 h, which decreased thereafter, and 130.2 g of residual L-lysine was accumulated after 123 h (Fig. 4, Table S2). These results indicated continuous conversion of L-lysine into cadaverine using a packed bed reactor to be feasible. Since many factors await improvement, future studies may further enhance productivity and yield. Especially, engineering the strain to synthesize PLP from pyridoxal (PL) or pyridoxine (PN), less costly precursors, would decrease the operating cost. Also, to produce high-purity cadaverine, studies of recovery and purification methods should be carried out [7,17].

This work describes a continuous cadaverine production system. Using a packed-bed reactor with a 14 mL working volume, 5.5 L of 819 mM cadaverine was produced by L-lysine bioconversion. The results demonstrate possible improvement on reusability of enzyme and removal of enzyme separation step by continuous system with immobilized whole-cell biocatalyst. Considering fermentation of lysine is well established process, this continuous cadaverine production process could give several advantages to

Table 1
Cell concentration and lysine decarboxylase activity of the immobilized whole-cell catalyst.

Cell concentration (mg/mL)	Activity (mmol/min/bead)[a]
5.5	2.67 ± 0.50
11	3.20 ± 0.15
16.5	4.46 ± 0.31
22	4.52 ± 0.83

Activity was measured at 37 °C and pH 6.
The values are representative of three independent experiments.
[a] Bead diameter was 2 mm.

Table 2
Effect of flow rate on cadaverine concentration and productivity.

Flow rate (mL/min)	Cadaverine concentration (mM)	Productivity (mmol/h)
0.5	900.2 ± 24.8	27.4 ± 0.4
0.75	895.6 ± 11.7	40.6 ± 0.3
1	714.7 ± 50.4	44.4 ± 1.5
1.5	503.1 ± 14.0	45.9 ± 0.6
2	554.6 ± 49.8	69.5 ± 3.0

The values are representative of two independent experiments.

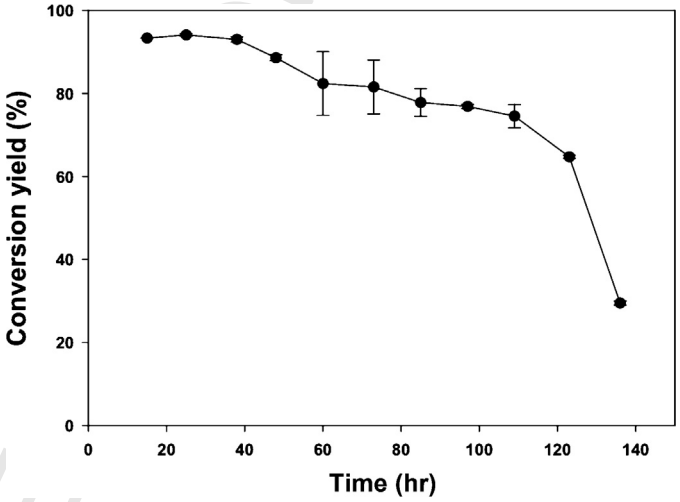

Fig. 3. Time-dependent conversion yield of the continuous cadaverine production system. The *error bars* represent standard deviations of two independent experiments.

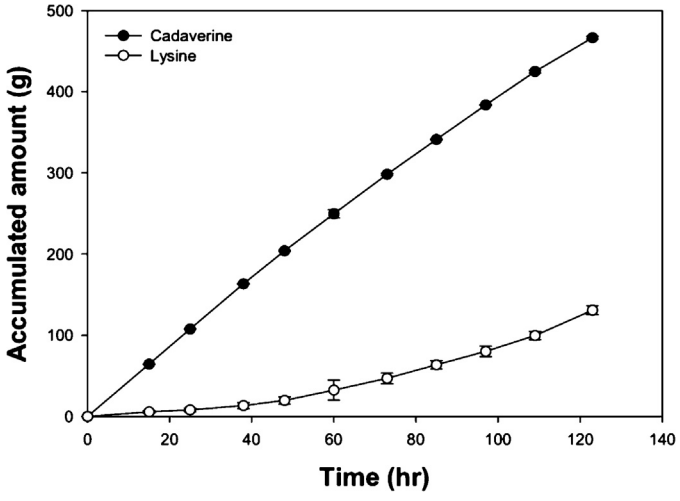

Fig. 4. Accumulation of cadaverine and residual L-lysine by the continuous cadaverine production. The *error bars* represent standard deviations of two independent experiments.

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direct bioconversion by increasing reusability of immobilized enzyme and producing cadaverine with higher purity.

Experimental

Chemical reagents

Barium chloride was purchased from Ducksan Science (Seoul, Korea) and sodium alginate was purchased from Kanto Chemical (Tokyo, Japan). The other reagents used in this study, such as cadaverine, sodium acetate anhydrate, and pyridoxal-5-phosphate (PLP), were purchased from Sigma Aldrich (MO, USA). L-Lysine monohydrochloride was a gift from Paik Kwang Industrial Corp. (Seoul, Korea). Some alginate and bacterial agar were supplied from the Microbial carbohydrate resource bank at Konkuk University, Korea.

Bacterial strains, media, and culture conditions

E. coli YH91 and *E. coli* BL21 (DE3) strains overexpressing lysine decarboxylase (CadA), were used as whole-cell biocatalysts [15,17]. The strains were cultivated in lysogeny agar and/or liquid lysogeny broth (LB), which consists of 10 g of tryptone, 5 g of yeast extract, 10 g of NaCl, and 20 g of bacterial agar in 1 L distilled water, supplemented with 50 mg/L kanamycin for plasmid stability. A single colony from an agar plate was inoculated into 5 mL of LB and then cultivated overnight in a shaking incubator at 37 °C and 200 rpm. The cells were inoculated at 1:100 (v/v) dilutions into 50 mL of fresh LB medium in a 250 mL baffled flask and continually cultivated. After 4–5 h, 0.1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to the culture to induce protein expression and the culture was transferred to a 30 °C and 200 rpm shacking incubator. After 20 h, cells were harvested and washed twice with 0.1 M phosphate-buffered saline (PBS). The prepared cells were used for immobilization.

Whole-cell immobilization

Alginate beads were obtained by dropping sodium alginate solution into 0.2 M of divalent cation solution using a 5 mL plastic syringe with a 22G1 1/4 needle. For metal divalent cation tests, $MgCl_2$, $MnCl_2$, $BaCl_2$, $CuCl_2$, $ZnSO_4$, $SnCl_2$, and $CoCl_2$ were used. For alginate concentration optimization, 4% sodium alginate was prepared by vigorous stirring at 100 °C. After cooling, an appropriate amount of concentrated whole cell was added to adjust the alginate and cell concentrations. The resultant hydrogel beads contained identical numbers of cells with different alginate concentrations. For large-scale bead preparation, a syringe pump (LSP01-2A, Longer Pump, China) was used at a rate of 76 L/min. The resultant beads were added drop-wise into 0.2 M $BaCl_2$ and were of 2 mm average diameter. Then, the hydrogel beads were cured in 0.2 M $BaCl_2$ solution at 4 °C for 30 min before use.

HPLC analysis

Concentration of cadaverine and L-lysine was analyzed by high-performance liquid chromatography (HPLC, YL-9100, Korea). Samples were prepared by derivatization with diethyl ethoxymethylenemalonate (DEEMM) [29]. A reverse-phase C18 column (Agilent ZORBAX SB-C18, 4.6 × 250 mm, 5 μm particle size) was used and the temperature was maintained at 35 °C during analysis. The mobile phase was composed of 100% acetonitrile (A) and 25 mM acetate buffer (pH 4.8, B). The flow rate was maintained at 1 mL/min with the following composition gradient: 0–2 min, 20–25% A; 2–32 min, 25–60% A; 32–40 min, 60–20% A. Detection was carried out at 284 nm using UV detector.

Lysine decarboxylase activity

The lysine decarboxylase activity of alginate beads was measured by calculating the amount of cadaverine produced. The reaction consisted of 1 M L-lysine-HCl, 500 mM acetate buffer (pH 6), and whole-cell immobilized alginate beads. The total volume was 0.5 mL in a 1.5 mL microtube and the reaction was performed in a 37 °C water bath. PLP (0.1 mM) was added to start the reaction. After 20 min of incubation, the reaction was stopped by heating the mixture at 95 °C for 5 min. Then, the mixture was diluted 1/100 for analysis.

Reactor setup and operation

The glass column was packed with about 1500 whole-cell immobilized alginate beads. Continuous cadaverine production using the reactor was conducted in a 37 °C incubator. The flow rate was controlled using a peristaltic pump (Ismatec, Germany). For flow rate optimization, 1 M L-lysine-HCl containing 0.1 mM PLP was injected into the reactor at an appropriate flow rate. After 1 h of operation, the output mixture was analyzed. Before each reaction, the reactor was washed with a sufficient volume of 1 M L-lysine-HCl without PLP for 20 min. For continuous cadaverine production, 1 M L-lysine-HCl without buffer was used as the substrate. 0.1 mM PLP was pre-mixed into the L-lysine solution and the mixture was fed into the packed bed reactor at a constant flow rate. Product solution passing through the reactor was sampled at 11–13 h intervals. Finally, we used about 1004.6 g of L-lysine-HCl, 0.16 g of PLP in total 5.5 L reaction solution for 136 h continuous cadaverine production system.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jiec.2016.09.038.

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