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Optimization of Direct Lysine Decarboxylase Biotransformation for Cadaverine Production with Whole-Cell Biocatalysts at High Lysine Concentration S

Hyun Joong Kim[1], Yong Hyun Kim[1], Ji-Hyun Shin[2], Shashi Kant Bhatia[1], Ganesan Sathiyanarayanan[1], Hyung-Min Seo[1], Kwon Young Choi[3], Yung-Hun Yang[1]*, and Kyungmoon Park[2]*

[1]*Department of Biological Engineering, College of Engineering, Konkuk University, Seoul 143-701, Republic of Korea*
[2]*Department of Biological and Chemical Engineering, Hongik University, Sejong city, Republic of Korea*
[3]*Department of Environmental Engineering, Ajou University, Suwon 443-749, Republic of Korea*

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**Corresponding authors*
Y.H.Y.
Phone: +82-2-450-3936;
Fax: +82-2-3437-8360;
E-mail: seokor@konkuk.ac.kr
K.P.
Phone: +82-44-868-6941;
Fax: +82-44-868-6941;
E-mail: pkm2510@hongik.ac.kr

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Cadaverine (1,5-diaminopentane) is an important industrial chemical with a wide range of applications. Although there have been many efforts to produce cadaverine through fermentation, there are not many reports of the direct cadaverine production from lysine using biotransformation. Whole-cell reactions were examined using a recombinant *Escherichia coli* strain overexpressing the *E. coli* MG1655 *cadA* gene, and various parameters were investigated for the whole-cell bioconversion of lysine to cadaverine. A high concentration of lysine resulted in the synthesis of pyridoxal-5′-phosphate (PLP) and it was found to be a critical control factor for the biotransformation of lysine to cadaverine. When 0.025 mM PLP and 1.75 M lysine in 500 mM sodium acetate buffer (pH6) were used, consumption of 91% lysine and conversion of about 80% lysine to cadaverine were successfully achieved.

Keywords: Biotransformation, whole-cell biocatalyst, lysine decarboxylase, cadaverine, high-concentration reaction, *Escherichia coli*

Introduction

Currently, the worldwide annual consumption of amino acids is estimated to be over 4 million tons, and the annual demand for amino acids, such as monosodium-glutamate-based flavor enhancers or feed additives comprising mainly L-lysine, D,L-methionine, and L-threonine, is estimated to be much higher than 1 million tons of each amino acid for industrial processes [4, 9]. L-Lysine is an essential amino acid and is commonly used as a food additive for humans and animals [4, 17]. Lysine can also be used as a precursor for the synthesis of various industrially valuable products, such as caprolactam, cadaverine, glutaric acid, 5-aminovaleric acid, and so on [1, 11] . Cadaverine is a diamine compound produced by protein hydrolysis during the putrefaction of animal tissue. The biosynthesis of cadaverine is a well-documented process and it is an end-product of decarboxylation of lysine, with immense applications in the drug industry and other industrial sectors [8, 20, 21]. Bioprocessing of cadaverine is an economic process and, specifically, the production of biopolyamides from renewable feedstocks could be an alternative and also replacement for commercial polyamides derived from petrochemicals [5, 6, 10, 25]. In the petroleum industry, 1,5-pentanediamine (cadaverine) can be produced from the starting material of 1,5-dichloropentane (obtainable from

tetrahydropyran), glutarodinitrile, or glutaraldehyde [7]. However, it can also be obtained from the natural lysine decarboxylation process, which exists in several microorganisms such as *Corynebacterium glutamicum* and *Escherichia coli* [16, 22]. So far, many efforts have been focused on the production of cadaverine from the fermentation of glucose and other simple sugars by metabolic engineering. There are only a few reports on the lysine conversion into cadaverine and it is complicated to control the processes to avoid several stages of reaction [6, 15]. However, considering that the conventional production system of lysine is very well established and lysine itself is readily available at a reasonable price, the biotransformation of lysine to cadaverine seems to be quite efficient and economical. In addition, there is also a complication to construct a whole-cell factory that utilizes lysine for the synthesis of cadaverine and to start lysine decarboxylation system optimization for the industrial production of cadaverine. However, recent literature evidenced that the construction of microbial cell factories for lysine decarboxylation could be possible, and they reported that the bioconversion of lysine to cadaverine is merely dependent on the low concentration of lysine as substrate in a metabolically engineered system. [12]. Hence, we hypothesized that the lysine bioconversion could be possible at high-concentration reactions and we have constructed an engineered *E. coli* system for the direct conversion of lysine to cadaverine and to demonstrate the ability to develop an economic bioprocess that can be performed at high substrate concentrations.

Materials and Methods

Chemical Reagents

The chemical reagents used in this study, such as cadaverine, pyridoxal-5-phosphate, sodium acetate anhydrate, and L-lysine monohydrochloride, were purchased from Sigma Aldrich Co. (USA). The derivatization reagent, diethyl ethoxymethylenemalonate (DEEMM), was purchased from Fluka Co. (Japan), and sodium borate decahydrate was purchased from Sigma Aldrich Co. (USA). To examine direct application to a lysine-based fermentation system, liquid lysine produced from fermentation was used and kindly donated by Paekwang Industry Co. (Korea).

Bacterial Strain and Media

For plasmid construction, *E. coli* strains were grown at 37°C in Luria–Bertani (LB) medium (10 g/l tryptone, 5 g/l yeast extract, and 5 g/l sodium chloride) supplemented with 50 μg/ml kanamycin (Km) for selection and screening purposes. The genomic DNA of *E. coli* K12 MG1655 was used for cloning *cadA*. The coding region of *cadA* was amplified by PCR using the primers cadAF (5′-CGTCGTGGATCCATGAACGTTATTGC AATATTAATCACA-3′) and cadAR (5′-ATATAAGCTTTCCCGCGATTTTTAGGAC TCG-3′). The PCR product was then digested with restriction enzymes (*Bam*HI and *Hin*dIII), and inserted into the vector pET24ma using the same restriction enzymes. The pET24ma vector (constructed by Dr. David Sourdive, Pasteur Institute, France) contains a p15A replication origin [18]. The constructed plasmid was transformed into *E. coli* BL21 (DE3) competent cells. After transformation, the cells were cultivated at 30°C in a shaking incubator (Han-Beak Science Co., Korea) at 200 rpm. The pre-culture was prepared by inoculating a single transformant colony from an agar plate into 5 ml of LB medium in a 14 ml round-bottom tube, prior to incubating for 16 h with shaking. One milliliter of the pre-culture was used as the seed for production in 50 ml of LB medium (15 μg/ml kanamycin and 0.1 mM iso-propyl-β-D-thiogalactopyranoside) in a 250 ml Erlenmeyer flask and it was incubated at 30°C with shaking. After 16 h, the culture was harvested by centrifugation at 13,000 rpm for 5 min at 4°C, and the cell pellet was washed with 50 mM sodium acetate buffer (pH 6.0) and then suspended in the same buffer. The cell suspension was stored at 4°C until further use, and referred to as whole cells.

Whole-Cell Reaction

The activity of lysine decarboxylase was determined using the whole cells as a catalyst. The assay was performed at 37°C in a total volume of 500 μl, containing 500 mM sodium acetate buffer (pH 6.0), 1 M L-lysine, 0.1 mM pyridoxal-5-phosphate, and 20 μl of the whole-cell suspension, at 37°C in a water bath. The reaction was stopped by heating the reaction mixture at 100°C for 5 min. One unit of activity (mmol/cell dry weight (mg)/min) was defined as the amount of enzyme producing 1 mmol of cadaverine per minute at 37°C. The whole cells used in the following experiments had 30.27 units of activity.

Derivatization and HPLC Analysis

Diamine derivatives were obtained in a mixture of 300 μl of borate buffer (50 mM, pH 9), 100 μl of methanol, 47 μl of distilled water, 50 μl of target sample, and 3 μl of DEEMM without pretreatment [2]. The sample was then heated at 70°C for 2 h to allow complete degradation of excess DEEMM and reagent by-products. Analysis using a high-performance liquid chromatography (HPLC, YL-9100; Korea) was performed after derivatization of reaction products with DEEMM. The equipment consisted of a binary pump, an in-line degasser, an autosampler, and a column thermostat. Chromatographic separation was done using a reverse-phase C18 column (Agilent ZORBAX SB-C18 column, 4.6 × 250 mm, 5 μm particle size) and the column temperature was maintained at 35°C. 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 and the composition of the mobile phase was programmed (as shown in Fig. S1).

Results and Discussion

Effects of Initial pH on Whole-Cell Reaction

Initially, the enzyme reaction was carried out with purified CadA, which had high enzymatic activity. The high concentration of CadA protein was easily precipitated at 4°C after His-tag purification. Consequently, several steps were performed for the purification to make the whole cell reaction preferable. The optimal pH of lysine decarboxylase was reported to be around pH 6 [19]. However, the new optimal conditions for the lysine biotransformation system needed to be determined. The whole-cell reactions were conducted with the initial pH range of 5–10 in the reaction buffer, and it was found that pH 6 to 8 showing the highest enzymatic activity, at which most lysine added was used by the whole cells and about 95% of lysine was consumed and converted into cadaverine (Fig. 1). The final pH was around 8.5 after 2 h of reaction, regardless of the initial pH. The conversion of lysine into cadaverine was finished within 15 min when 500 mM of lysine was used. The overall pH increase was not a sudden process because the reaction mixture uses a high-concentration buffer (around 500 mM sodium acetate buffer) and there is a diffusion limitation in the small-scale reaction. The difference between lysine consumption and cadaverine production may be ascribed to leftover lysine and cadaverine in the cells, and to the production of by-products such as *N*-acetyldiaminopentane. Our current results agree with those of Kind *et al*. [14].

Examination of Substrate Inhibition

The reaction was carried out at 37°C at pH 6 using 500 mM sodium acetate buffer and 0.1 mM pyridoxal-5-phosphate with different concentrations of substrate. The substrate conversion profiles are shown in Fig. 2. After 2 h of reaction, lysine was fully converted to cadaverine up to 1 M concentration (data not shown). However, substrate inhibition was observed from 1.25 M, resulting in some residual lysine (Fig. 2). To achieve higher lysine concentration of the reaction, the amount of whole cell was increased by adding 50% and 100% of enzyme to the initial whole-cell concentration (30.27 units). Although, some lysine remained after 2 h of reaction at high concentrations of lysine, it was observed that higher amounts of lysine decarboxylase could increase the conversion ratio of lysine into cadaverine and the overall consumption ratio of lysine was estimated around 80% at 1.25 M substrate concentration. The maximum substrate conversion by whole cells was 95%, 94%, and 92% from 1.25, 1.5, and 1.75 M concentration of substrate lysine, respectively.

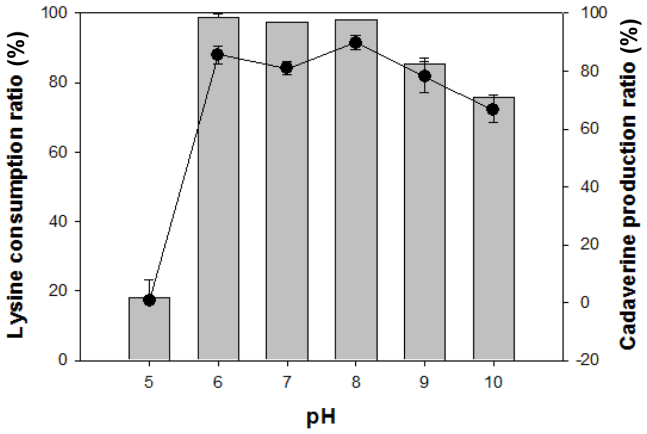

Fig. 1. Effects of initial pH on lysine consumption (▮) and cadaverine conversion (●) after 2 h reaction of 500 mM lysine at different initial pHs.

Effects of Adding Exogenous PLP to the Reaction Buffer

The requirement of PLP for the activity of lysine decarbolxyase is well known, especially for enzyme reactions [13, 23]. However, there are no reports on the direct effect of adding exogenous PLP to a mixture of whole cells expressing lysine decarboxylase. Therefore, the effect of PLP concentration was examined (Fig. 3). When no PLP was added, lysine consumption was only 20% for the 1 M lysine to cadaverine reaction. When more than 0.025 mM of PLP was added, the lysine consumption was recovered, suggesting that the whole-cell reaction also

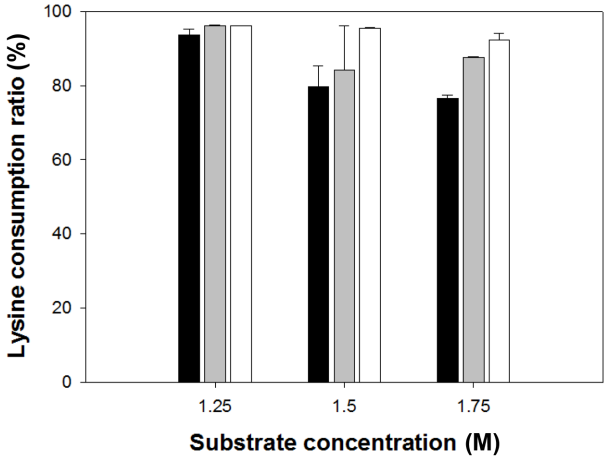

Fig. 2. Effects of lysine concentration and biocatalyst concentration on lysine consumption ratio.
Concentrations of 30.27 units (black bar), 45.41 units (gray bar), and 60.54 units (white bar) were tested.

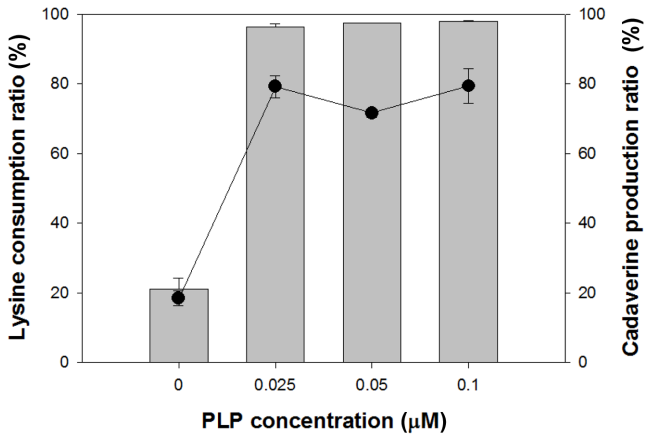

Fig. 3. Effects of PLP concentration on lysine consumption ratio (▮) and cadaverine conversion ratio (●).

requires a certain amount of PLP as a cofactor to enable the 1 M lysine to cadaverine reaction. PLP is one of the critical control factors for the production of cadaverine at high concentrations of lysine, unlike the growth-based production of cadaverine through fermentation [21, 22].

Effect of Buffer on pH and Activity

The reaction buffer is normally used to reduce the fluctuations in pH caused by the final product and to improve the enzymatic reaction [3, 24]. In this study, we examined the effect of the buffer in the whole-cell reactions. To monitor the time-dependent conversion of lysine to cadaverine and to evaluate the effects of the buffer on pH change, the reaction and time-dependent data were compared in 500 μl reaction volumes containing 1 M lysine with and without 500 mM of sodium acetate buffer (pH 6.0). Unlike our initial expectation, the pH was increased by cadaverine production and the experimental results showed similar patterns of cadaverine production with and without buffer (Figs. 4A and 4B). Almost 50% of lysine was consumed within 20 min and monitoring of the reaction revealed a dramatic increase of the pH to over pH 8 without buffer. Although pH differences were shown, the overall conversion ratio was quite similar. This seems to be due to the fast enzymatic reaction in the small-scale reaction, and the robustness of the whole-cell catalysis to resist the pH changes. When pH optimization was performed, the cadaverine conversion rate was negatively affected at above pH 9. However, the results of non-buffered experiments revealed that there was no significant increase of the pH above 9. As we obtained the results from this study, it is possible to produce cadaverine from the

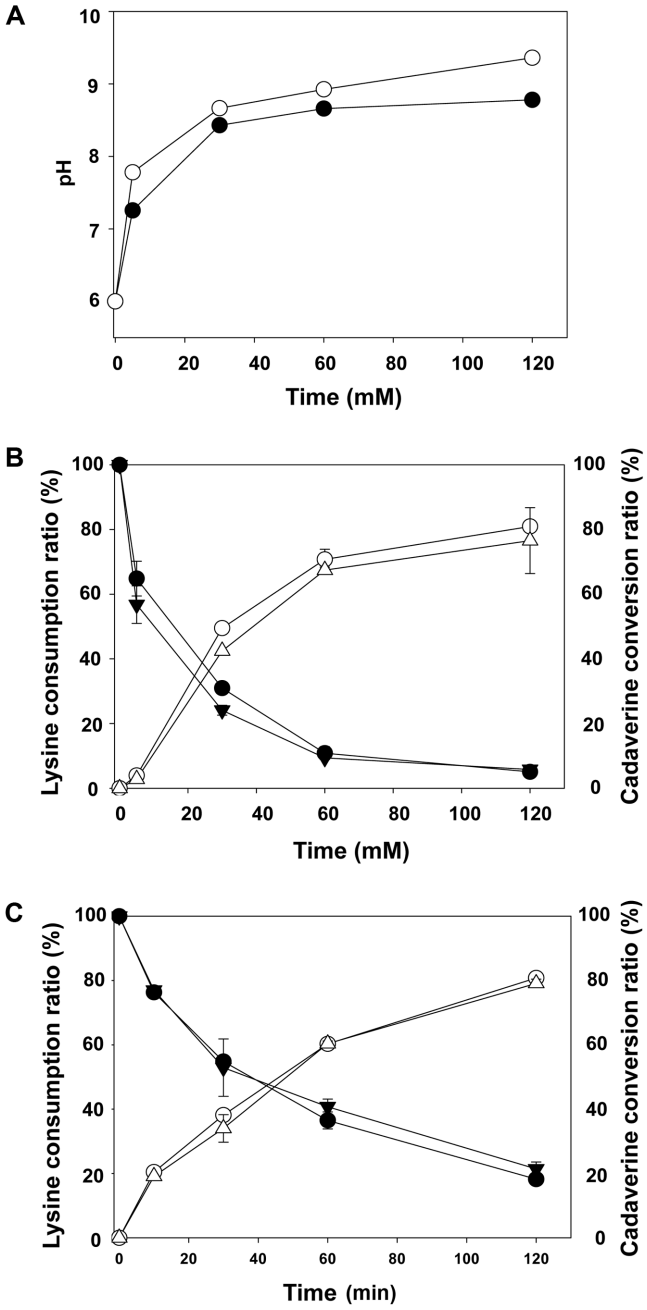

Fig. 4. Effect of buffer on reaction pH and cadaverine production.

Change of pH with (black circle) or without buffer (white circle) (**A**), and the production of cadaverine (white) and the consumption of lysine (black) from purified lysine at different time points with (circle) or without buffer (Triangle) (**B**). Production of cadaverine (white) and the consumption of lysine (black) from lysine-containing fermentation liquid with (circle) or without buffer (Triangle) (**C**).

lysine decarboxylation reaction without adding buffer in a small-scale level, although further optimization may be

needed to scale up the reaction without buffer.

Bioconversion of Fermented Lysine Liquid into Cadaverine Using Whole-Cell System

Bioconversion of liquid lysine obtained through fermentation was evaluated using a whole-cell engineered system. The color of the liquid lysine is dark brown and it has pH 10.2 with 4.35 M of lysine content. The fermented liquid also possesses 165 mM of cadaverine produced from an industrial *Corynebacterium* strain. During the whole-cell reaction, the pH was decreased to 6 by the addition of 1 N HCl and the solution was diluted to 1 M of lysine to avoid possible substrate inhibition and 30.27 units of whole cells was used. The whole-cell reaction demonstrated 80% of lysine consumption after 2 h of reaction (Fig. 4C), which was relatively slower than the previous reaction with purified lysine (Fig. 4B). This may be due to the presence of inhibitors produced during the lysine fermentation process. However, quite a similar pattern was observed as with the previous data (Fig. 4C). Although, the cadaverine productivity was relatively low compared with the case using purified lysine substrate, it demonstrated the possible application of the whole cells to the direct utilization of crude lysine solution for cadaverine production.

In conclusion, we showed here the direct conversion of lysine to cadaverine at high concentrations of substrate, and proved it is a quite feasible and practical approach based on the well-established engineered *E. coli* system for cadaverine production from lysine. Following optimization of several factors such as initial pH, substrate concentration, and PLP concentration, the exogenous addition of PLP was found to be one of the key factors to promote a high molar cadaverine production from the whole-cell reactions. Considering that the conventional production system of lysine is very well established and lysine itself is readily available at a reasonable price, then the biotransformation of lysine to cadaverine is a completely greener, efficient, and economical bioprocess and also an alternative for chemical synthesis.

Acknowledgments

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