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Improved reutilization of industrial crude lysine to 1,5-diaminopentane by enzymatic decarboxylation using various detergents and organic solvents

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Abstract−World-wide production of L-lysine has rapidly increased in recent years. In the industrial scale production, it is cost effective to minimize waste as many waste materials are generated during downstream processing. Therefore, the conversion of crude lysine to a more valuable product reduces waste emission. In this study, 1,5-diaminopentane (DAP, trivial name: cadaverine) was produced by L-lysine decarboxylation using *Hafnia alvei*. The conditions of enzymatic reaction were determined. In particular, the addition of specific detergent (Brij 56) was significantly affected in the bioconversion system. Addition of hydrophobic organic solvent improved the mixing of the reactants. Finally, an industrial crude form of lysine served as a substrate. The DAP conversion by analytical, feed and industrial crude L-lysine was 93.9%, 90.3%, and 63.8%, respectively.

Keywords: 1,5-Diaminopentane, Cadaverine, Decarboxylation, Detergent, *Hafnia alvei*, Lysine

INTRODUCTION

L-Lysine, an essential amino acid, is a supplement used in animal feed, since the typical feed sources such as corn, wheat or barley contain poor L-lysine. In animal growth, L-lysine is a particularly important amino acid for maximum dressed carcass. An increase in global consumption of meat has led to an enormous market growth of L-lysine during the past years [1]. The global consumption of L-lysine in 2022 is expected to reach nearly 3.0 million metric tons and the market value is estimated at nearly $ 5.6 billion [2,3]. However, the market price of L-lysine is very unstable ($ 2.4 per kg in 2011, $ 1.3 per kg in 2014) due to external factors such as worldwide recession, price of raw materials (corn, cassava, wheat or soybean) and excessive manufacturing capacity [1-5]. Therefore, efforts to reduce manufacturing costs, including technological development, are required. In the downstream processing, product purification and formulation is an important cost factor. Simplified and economical downstream processes have been developed, especially for L-lysine production. Uffmann and Binder [6] produced a liquid lysine sulfate (20%-30% purity) without biomass separation via a single evaporation step. As mentioned, the advantages include significantly reduced waste streams, an increased downstream yield and reduced investment costs. Therefore, planning for direct utilization of the crude form (low purity of lysine) is important since the increasing purity of L-lysine requires additional process costs.

L-Lysine has a potential for conversion to attractive biopolymers via decarboxylation. Several studies report the production of aminovalerate, glutamate or diaminopentane from L-lysine to channel the excess supply of lysine and improve the industrial profits. 1,5-Diaminopentane (DAP, trivial name: cadaverine), an important platform chemical, is most interesting due to its promising industrial applications such as polyurethanes, polyamides and chelating

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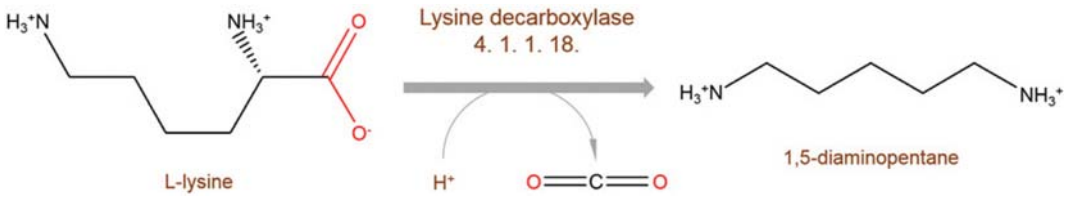

Fig. 1. Simplified pathway for L-lysine decarboxylation.

agents. Nylon is a polyamide with huge market and an annual production of about 6,000,000 tons. Currently, nylon with related polyamides is mostly obtained from oil-based material; however, it is easy to produce from DAP via bioconversion. Therefore, the production of specific polyamide using DAP is an opportunity in the near future [4,7-9].

The conversion of DAP from L-lysine is conducted by L-lysine decarboxylase (EC: 4.1.1.18) and the simplified pathway is shown in Fig. 1. The action of the decarboxylase generates a diamine based on the extraction of carbon dioxide from L-lysine. The production of L-lysine decarboxylase is well known via cultivation of various microbial strains (*Escherichia coli*, *Selenomonas ruminantium* and *Hafnia alvei*). In previous reports, DAP production was enhanced by overexpression or gene manipulation of the strains [10, 11]. However, these studies have a major problem associated with the low conversion via enzymatic reaction. It is difficult to increase the initial substrate concentration since the biosynthesis or degradation of L-lysine is regulated *in vivo*. Thus, the final product (DAP) concentration depends on the initial substrate concentration. Moreover, in most studies, only the purified L-lysine (for analytical grade) has been used as a substrate in the bioconversion [12,13].

In this study, L-lysine decarboxylation by *H. alvei* was performed in a bioconversion system for DAP production. The effect of reaction conditions such as initial substrate and enzyme loading, temperature and time on DAP conversion was fundamentally investigated. Various detergent additives improved the conversion, and the specific type was selected by the investigation. Furthermore, the organic solvent is a supplemental additive to improve the reactant mixture since the detergent (amphiphilic molecule) contains a non-polar hydrophobic region. Finally, the industrial crude lysine and other purities (for analytical and feed grade L-lysine) were used as substrates in the bioconversion system, and the efficiency was evaluated for further application.

EXPERIMENTAL

1. Materials

Nutrient agar (NA), nutrient broth (NB) and yeast extract peptone dextrose (YPD) broth were purchased from BD Difco (Maryland, MD, USA). Analytical grade (99.5%) L-lysine was purchased from Daejung Chemical and Metals Co. Ltd. (Siheung, Korea). Feed grade (~90%) L-lysine and industrial crude (~50%) lysine were obtained from PKI Co. Ltd. (Gunsan, Korea). 1,5-Diaminopentane (DAP), Brij 56, Triton X-100, Tween 80, Tween 20, and sodium dodecyl sulfate (SDS) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Methyl alcohol, ethyl alcohol, acetone, and tetrahydrofuran (THF) purchased from Junsei (Tokyo, Japan), were used as the solvents. Other reagents used were analytical grade or above.

2. Bacterial Strain and Production of Biocatalyst

Hafnia alvei ATCC 9760 was purchased from American Type Culture Collection (Manassas, VA, USA). The seed culture of *H. alvei* was performed on NA plate at 30 °C for three days. The strain was subcultured on NB at 30 °C in a shaking incubator (150 rpm) for a day. The main cultivation was performed in 250 mL Erlenmeyer flasks containing 50 mL YPD with 5% inoculum at 30 °C and 200 rpm shaking speed for 18 hr. The cultural broth was centrifuged at 12,000 ×g for 10 min, and the cells were washed with phosphate buffer (pH 5.6) after removal of the supernatant.

3. Enzymatic Reaction Using Biocatalyst

The effect of initial concentration of biocatalyst was investigated based on OD (optical density) values of 0.5, 1.0, 2.0, 3.0, 4.0 and 5.0 at a wavelength of 600 nm. The enzymatic reaction was carried out in a 50 mL Erlenmeyer flask with 5 mL total working volume at 25 °C with shaking speed of 200 rpm for 8 hr. The initial concentration of substrate was maintained from 50 to 200 mM L-lysine. The effect of initial concentration of substrate was determined at 50 mM, 100 mM, 150 mM and 200 mM L-lysine. The initial concentration of biocatalyst was maintained at 4.0 (OD_{600}). The effect of reaction temperature on DAP conversion was investigated at various temperatures (25, 35, 45 and 55 °C). The initial concentration of biocatalyst and substrate was maintained at 4.0 (OD_{600}) and 150 mM L-lysine, respectively. The effects of additives (detergents and organic solvents) on DAP conversion were investigated. Five detergents (Brij 56, Triton X-100, Tween 80, Tween 20, and SDS) and four organic solvents (acetone, ethyl alcohol, methyl alcohol and THF) were used in the bioconversion system. In the experiments, each of 10% (v/v) detergent or organic solvent was added. The initial concentration of biocatalyst and substrate were maintained at 4.0 (OD_{600}) and 150 mM L-lysine, respectively. The reaction was performed at 35 °C in a shaking incubator at 200 rpm for 8 hr.

4. HPLC Analysis

After reaction, the cells were removed by centrifugation at 9,000 ×g for 10 min and the supernatant was filtered. The DAP concentration was analyzed by HPLC system (Agilent 1200 series, USA) and the assay was performed with o-phthaladehyde. The Zorbax eclipse XDB C18 column with mobile phase consisted of 60% 40 mM acetate buffer (pH 5.6) and 40% acetonitrile and diode array detector (DAD) were used. The detection wavelength was 338 nm with a 10 nm bandwidth and the reference wavelength was 390 nm with a 20 nm bandwidth. The column temperature was maintained at 40 °C, and the flow rate was 1.0 mL/min. The DAP conversion was calculated using Eq. (1).

$$\text{DAP conversion (\%)} = \frac{\text{produced 1.5-diaminopentane (mM)}}{\text{initial L-lysine (mM)}} \times 100(\%) \quad (1)$$

RESULTS AND DISCUSSION

1. Determination of Bioconversion System

DAP conversion from L-lysine is known as "decarboxylation." Wang et al. [11] reported theoretically that 1 mole L-lysine is converted to 1 mole DAP via decarboxylation. In this study, *H. alvei* strain, which is a well-known L-lysine decarboxylase producer [14], was utilized as the whole cell biocatalyst with a higher catalytic efficiency than other strains. K_{cat} (turnover number) and K_m (Michaelis constant) of lysine decarboxylase (LDC) from *H. alvei* were 175.0 (1/s) and 0.37 (mM), respectively, when L-lysine was used as a substrate. By contrast, LDC from *E. coli* (K_{cat}: 30.0 1/s, K_m: 0.42 mM) and *S. ruminantium* (K_{cat}: 2.6 1/s, K_m: 3.60 mM) showed lower catalytic efficiency than *H. alvei* [15,16].

In general, the initial concentration of enzyme and substrate is highly affected by the enzymatic reaction system. Thus, the effect of initial concentration of whole cell biocatalyst and L-lysine was investigated in the bioconversion system. Fig. 2(a) shows the effect of initial concentration of biocatalyst on DAP conversion. The bioconversion was performed using a biocatalyst at OD_{600} 0.5 to 5.0 with a substrate concentration of 50 mM L-lysine. The DAP conversion was enhanced by increasing the biocatalyst from OD_{600} 0.5 to 4.0. The maximized conversion (81.41%) at OD_{600} 4.0, however, decreased to 72.67% at OD_{600} 5.0 due to contact inhibition (i.e., cell inhibition), which is a type of avoidance behavior where a reaction pauses due to contact with other cells [17]. Thus, the contact inhibition increases with increased biocatalyst concentration. In our bioconversion system, the contact inhibition was confirmed at more than 5.0 (OD_{600}) of biocatalyst loading. Therefore, the optimum biocatalyst concentration was determined as 4.0 (OD_{600}) for further experiments.

The effect of initial substrate concentration on DAP production and the conversion was investigated. The results are shown in Fig. 2(b). The enzymatic reaction was performed using 50, 100, 150, and 200 mM L-lysine at 4.0 (OD_{600}) biocatalyst loading. As a result, the DAP concentration of 40.3 mM, 70.4 mM, 99.7 mM and 97.6 mM was measured at L-lysine concentrations of 50, 100, 150, and 200 mM, respectively. Thus, DAP production was improved by increasing the L-lysine concentration, and the production was maximized at 150 mM L-lysine. However, the DAP conversion was 81.5%, 70.4%, 66.3% and 48.8% at L-lysine concentrations of 50, 100, 150, and 200 mM, respectively. This result shows inhibition of the enzymatic reaction with increased substrate concentration. Han and Levenspiel [18] reported substrate inhibition at higher concentrations of substrate loaded in the reaction system. The lowest inhibition was confirmed (the highest conversion) experimentally at 50 mM L-lysine loading; however, the overall production was low (40.3 mM) due to the lowest substrate loading. Low concentration or production is a drawback of industrial application. In L-lysine loading of 150 mM, the conversion was lower than at 50 mM L-lysine. However, the overall DAP production was increased about 2.5-fold. Thus, the optimum loading of substrate was determined as 150 mM L-lysine for further experiments.

The reaction temperature directly affects the bioconversion. Thus, determination of the reaction temperature is a way to increase the conversion and reaction rate. Li et al. [10] reported that the LDC derived from *H. alvei* or *E. coli* has a similar activation tempera-

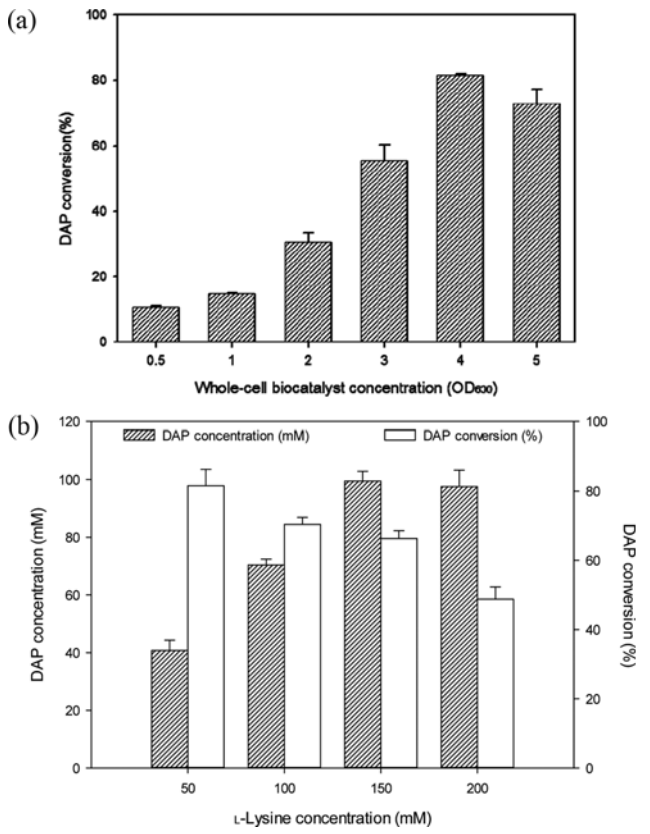

Fig. 2. Effect of initial concentration of whole cell biocatalyst on 1,5-diaminopentane conversion (a), and effect of initial L-lysine concentration on 1,5-diaminopentane production and conversion (b). The reaction was performed in a shaking incubator (200 rpm) at 25 °C for 8 hr.

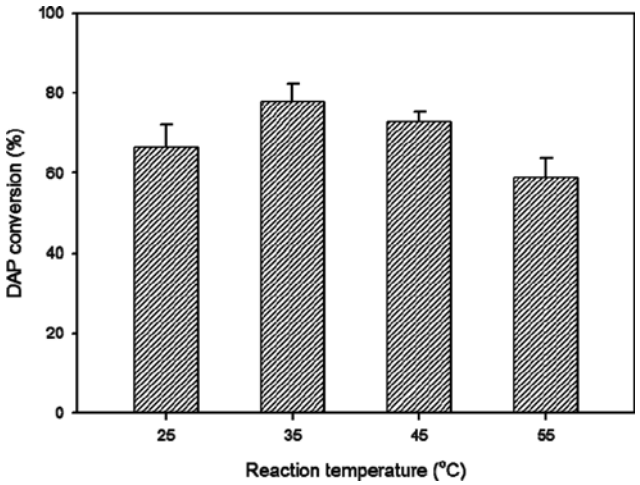

Fig. 3. Effect of reaction temperature on 1,5-diaminopentane conversion.

ture, and shows the highest enzyme activity in the temperature range of 35 °C-40 °C. The effect of reaction temperature on DAP conversion was investigated and the results are shown in Fig. 3. The bioconversion was performed at a temperature range of 25 °C-55 °C under 4.0 (OD_{600}) biocatalyst with 150 mM L-lysine. The DAP conversion at 25, 35, 45 and 55 °C was 66.2%, 77.7%, 72.7%, and 58.8%, respectively. The highest conversion was achieved at a temperature of 35 °C. Therefore, the optimum temperature was 35 °C in our bioconversion system.

2. Effect of Specific Additive on Bioconversion

Detergents can easily extract protein via lysis of cell membrane by forming micelles and phosphatides with the cell membrane [19]. Detergents include nonionic, anionic, cationic and zwitterionic surfactants according to their hydrophilic solubility as described by Manaargdoo-Catin et al. [20]. The hydrophilic-lipophilic balance (HLB, a balance between hydrophilicity and hydrophobicity exists in surfactant molecules) is an important factor of detergent, which depends on the molecular structure and type. The micelle, an aggregate of surfactant molecules, is easily formed by increasing the hydrophobicity of detergent [21] and the hydrophobicity is increased with decreasing HLB value [22].

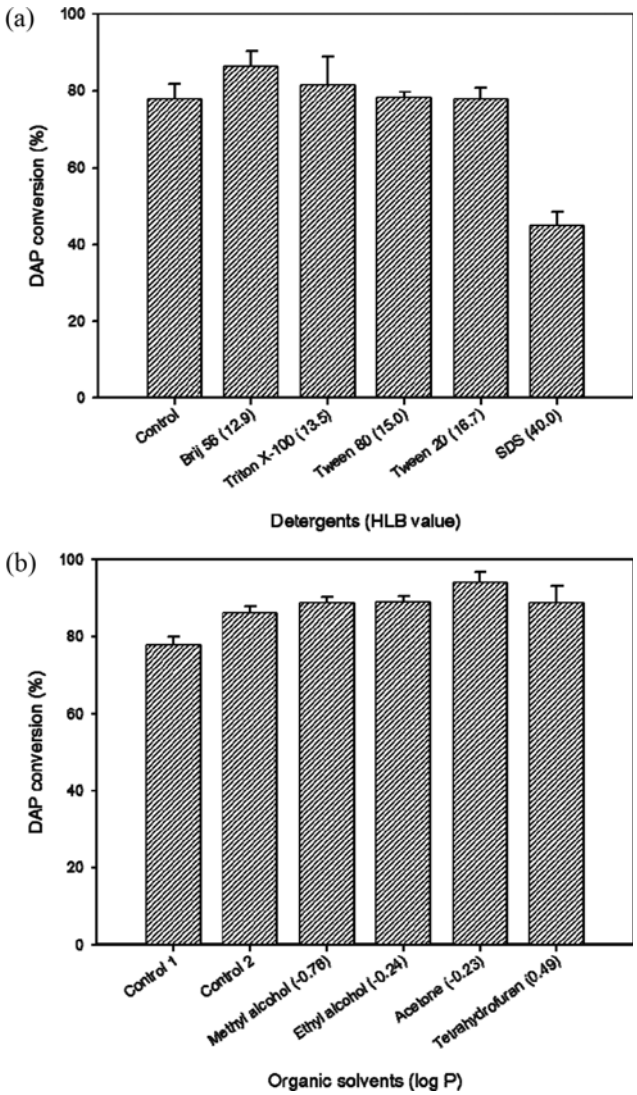

Fig. 4. Effect of different types of detergent (a) and organic solvent (b) on 1,5-diaminopentane conversion.

To improve the bioconversion, five different types of detergent were prepared and their HLB values were investigated. Hait and Moulik [21] showed that the HLB value of Brij 56, Triton X-100, Tween 80, Tween 20, and SDS was 12.9, 13.5, 15.0, 16.7, and 40.0, respectively. Fig. 4(a) shows the effect of detergent on DAP conversion, and the experiments were carried out via detergent loading of 10% (v/v). The control group means that no detergent is added. The conversion increased by the addition of Tween 80 and Tween 20 was 78.2% and 77.7%, respectively, which was similar to control (77.7%). The DAP conversion was improved by the addition of Brij 56 (86.2%) and Triton X-100 (81.3%). Luche et al. [23] reported that the selection of detergent mostly depends on the empirical aspect. Brij 56 and Triton X-100, which have appropriate HLB values, are efficient in dissociating cell membranes [23]. However, addition of SDS (anionic detergent) was achieved a low conversion of 44.8%, which is about two-fold decrease compared with that of the control group. The most effective Brij 56 is known as a colorless non-ionic detergent that is mainly used for isolation of membrane proteins. It can be assumed that detergent containing a large amount of lipophilic regions is advantageous for dissociating cell membranes of *H. alvei*.

Detergent, an amphiphilic substance, contains partially hydrophilic (polar) and hydrophobic (non-polar) region. In particular, Brij 56, the selected detergent, contains relatively more hydrophobic region due to its lower HLB value compared to other detergents. It was confirmed that the addition of Brij 56 to our bioconversion system was not adequately mixed in the liquid phase (buffer: mainly composed of water). To improve the reactant mixture, four different types of organic solvents (methyl alcohol, ethyl alcohol, acetone, and THF) were added to the reaction system. The effect of organic solvents on DAP conversion was investigated and the results are shown in Fig. 4(b). Control 1 means that no detergent (Brij 56) is added, and control 2 contains only Brij 56 (without organic solvent) in the reaction system. As a result, the conversion via addition of methyl alcohol, ethyl alcohol, acetone, and THF was 88.9%, 88.7%, 93.9% and 88.7%, respectively. Addition of organic solvents improved the conversion than the control group (86.2%). The value of log P is used to determine the hydrophobicity, which is the main factor that affects the solvent dissolution. The value of log P refers to the octanol-water partition coefficient and represents an index of the hydrophobic and hydrophilic properties of an organic solvent [24]. Laane et al. [25] showed that log P values of methyl alcohol, ethyl alcohol, acetone, and THF were −0.76, −0.24, −0.23, and 0.49, respectively. Among the organic solvents, acetone showed the highest DAP conversion, with a log P value of −0.23 and is closest to the solvent containing both hydrophobic and hydrophilic region. Since our bioconversion system with Brij 56 contains a relatively high lipophilic region, the addition of an appropriate solvent such as acetone can improve the reaction by mixing well in two immiscible phases.

3. Application of Industrial Crude Lysine to Bioconversion System

In the industrial process for L-lysine production, downstream

processing including purification and formulation is a major cost factor. Thus, various technologies have been developed. In general, the separation of biomass from the product stream is the precursor to purification. In typical purification stages, chromatography, concentration and crystallization technologies are used. Chromatographic methods provide usually higher product qualities, although the lower concentrations and increased waste streams lead to higher costs in waste liquor. Crystallization, a chemical solid-liquid separation technique, is used to obtain high purity crystals after impurity removal processes including the chromatography. In particular, product properties such as crystal size and size distribution are determined by various crystallization conditions (crystallizer type, feeding mode, temperature, pressure and time, etc.) and downstream processing efficiency and the cost are highly dependent. On the other hand, the waste streams are significantly reduced by simplification suggesting that a liquid lysine sulphate is produced by a single evaporation step without biomass separation. However, the disadvantage of this technique is that low lysine purity is obtained such as liquid lysine, liquid lysine sulfate or granulated lysine sulfate [4,6,26-28].

Here, we applied the crude lysine as a substrate in our bioconversion system. The DAP conversion is unstable when different types of L-lysine are loaded into the system, since the substrates have different purities. In this stage, three different purities of L-lysine were prepared as follows: analytical grade (AG) of 99.5%, feed grade (FG) of ~90% and industrial crude (IC) of ~50%. They were applied in the system to evaluate DAP conversion. Fig. 5 shows the effect of different grades (purity) of L-lysine on DAP conversion. The enzymatic reaction was conducted at the determined conditions (OD_{600} 4.0 biocatalyst, 150 mM L-lysine, 10% Brij 56 and 10% acetone at 35 °C for 8 hr). As a result, the DAP conversion using AG, FG and IC L-lysine was about 93.9%, 90.3% and 63.8%, respectively. Therefore, crude form (low purity) lysine can be also utilized in this bioconversion system. However, the conversion is relatively lower than AG or FG due to the substrate specificity. To improve the use of crude lysine in the bio-industrial fields, additional studies such as reaction optimization by statistical method, removal of inhibitory compounds, reactor design, scale up, and so forth are needed. The current study serves as a valuable foundation for further investigations in the future.

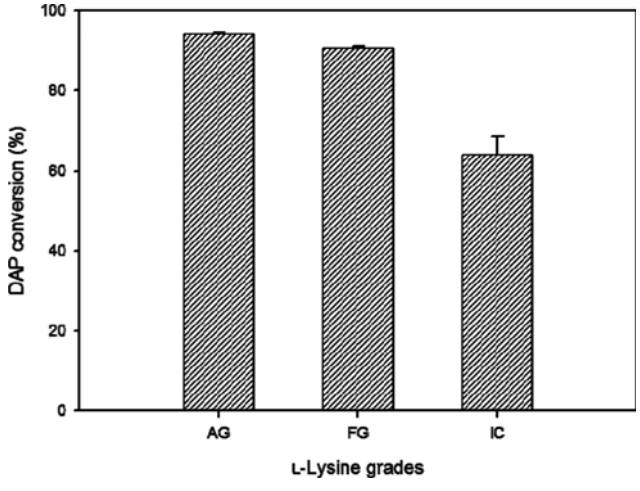

Fig. 5. Effect of different grades of L-lysine on 1,5-diaminopentane conversion (AG: analytical grade (99.5%), FG: feed grade (~90%) and IC: industrial crude grade (~50%)).

CONCLUSIONS

We designed a bioconversion system using L-lysine decarboxylation for DAP production. *H. alvei* represents an attractive biocatalyst producer, and whole cells were used in the conversion system. To improve the DAP conversion, the conditions for enzymatic reaction such as initial concentration of substrate, loading concentration of biocatalyst, temperature and time were fundamentally investigated, and the optimum conditions were determined. Addition of a specific detergent has a significant impact on the bioconversion system. Brij 56 containing hydrophobic portion was selected as a proper detergent and acetone was adopted to improve the mixing of the reactants as an effective organic solvent. The final conditions were as follows: initial L-lysine of 150 mM, biocatalyst loading of OD_{600} 4.0, 10% Brij 56 and 10% acetone at 35 °C for 8 hr. DAP conversion to about 94% was achieved under the optimum condition. To reduce the process costs at industrial lysine production, a simplified process is used by most manufacturers entailing a single evaporation step without biomass separation, resulting in lysine of low purity such as liquid lysine or liquid lysine sulfate. Here, the animal feed grade (~90% purity) and industrial crude (~50% purity) lysine were applied as a substrate and the conversion was found to be 90.3% and 63.8%, respectively. Therefore, the crude form lysine can also be reused as a feedstock in the bioconversion process. This work provides guidance to resolve the challenges associated with excessive manufacturing capacity of lysine and crude lysine emission from downstream processing.

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