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Selective recovery of cadaverine from lysine decarboxylase bioconversion solution using methyl ethyl ketone

Yun-Gi Hong[a,1], Hyun-Joong Kim[a,1], Jong-Min Jeon[a], Yu-Mi Moon[a], Ju-Won Hong[a], Jeong-Chan Joo[c], Bong-Keun Song[c], Kyung-Mooon Park[d], Sang-Hyun Lee[a], Yung-Hun Yang[a,b,*]

[a] Department of Microbial Engineering, College of Engineering, Konkuk University, 120 Neungdong-ro, Gwangjin-gu, Seoul 05029, Republic of Korea
[b] Institute for Ubiquitous Information Technology and Applications, Konkuk University, Seoul 143-701, Republic of Korea
[c] Center for Bio-based Chemistry, Division of Convergence Chemistry, Korea Research Institute of Chemical Technology, 141 Gajeong-ro, Yuseong-gu, P.O. Box 107, Taejon 305-600, Republic of Korea
[d] Department of Biological and Chemical Engineering, Hongik University, Sejong Ro 2639, Jochiwon, Sejong City, Republic of Korea

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ABSTRACT

Efficient extraction and purification process is still a major bottleneck for economical production of cadaverine. We examined 10 different organic solvents to determine which one might be a suitable solvent for cadaverine extraction. Methyl ethyl ketone (MEK) was a very selective solvent for cadaverine. High pH was critical factor for cadaverine extraction with high purity. Cadaverine extraction efficiency of more than 70.1% with 99% of purity was successfully achieved by applying optimized extraction condition (pH 13.5, 58 °C, 200 rpm incubation for 6 h). Cadaverine extraction using MEK seems quite feasible and promising for the preparation of polyamide monomers for environmental process.

Introduction

Cadaverine is a diamine compound produced by protein hydrolysis during putrefaction of animal tissue. Biosynthesis of cadaverine is a well-documented process. It is a metabolite of lysine degradation by lysine decarboxylase which can be produced by several microorganisms such as *Hafnia alvei* and *Escherichia coli* [1,2]. To produce cadaverine, various fermentation approaches have been tried using renewable resources like glucose [3] and other simple sugars by metabolic engineering [4,5]. By using *E. coli* that over-expresses lysine decarboxylase as a biocatalyst, high concentration cadaverine production has been recently achieved due to high lysine biotransformation [6,7]. Considering that lysine as a cadaverine precursor is readily available at a reasonable price [3], biotransformation of lysine to cadaverine seems to be quite efficient [8,9]. As a result, producing cadaverine by both fermentation and bioconversion seems feasible. It might be suitable for large-scale production. However, to obtain cadaverine of high purity in large scale, downstream process such as purification needs improvement.

Cadaverine has a relatively low boiling point. It is relatively easy to evaporate and distillate. However, its raw production mixture may contain nonvolatile inorganic and organic impurities that are soluble and dispersible in water [10]. Such impurities may significantly interfere with cadaverine evaporation and distillation. Since liquid–liquid extraction can achieve energy efficient separation of product from broths, a suitable solvent might be useful for cadaverine extraction. Replacing direct distillation by solvent extraction or adding a solvent extraction step could significantly reduce the cost of energy for product separation [11]. Considering the nature of liquid–liquid extraction which preferentially dissolves a chemical to be separated to one of the solvent used, this method requires relatively low temperature. In addition, mixtures involving substances with close boiling temperature can

* Corresponding author at: Department of Microbial Engineering, College of Engineering, Konkuk University, 120 Neungdong-ro, Gwangjin-gu, Seoul 05029, Republic of Korea.
E-mail address: seokor@konkuk.ac.kr (Y.-H. Yang).
[1] The authors equally contributed to this study.

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be separated by solvent extraction at low cost [12,13]. Although the equilibrium relationship in liquid extraction is generally more complicated than other separation methods because various factors could affect the extraction process [14], once a suitable solvent is selected, it might lead to improvement of the recovery process by reducing time and cost.

Therefore, the objective of this study was to examine whether solvent extraction system could be used to achieve higher recovery of cadaverine. We examined 10 different organic solvents to determine which one might be a suitable solvent for cadaverine extraction. Our solvent screening experiments showed that methyl ethyl ketone (MEK) had good selectivity and solubility for cadaverine. We also evaluated various factors affecting extraction yield and purity such as pH, temperature, and cadaverine to lysine ratio to determine the optimal condition for MEK extraction.

Materials and methods

Chemical reagents

Chemical reagents used in this study such as cadaverine, pyridoxal-5-phosphate, sodium acetate anhydrate, 1-butanol, 1-pentanol, 1-hexanol, chloroform, dichloromethane, toluene, isoamyl alcohol, hexane, methyl ethyl ketone (MEK), methyl isobutyl ketone (MIBK), and L-lysine monohydrochloride were purchased from Sigma–Aldrich (USA). Diethyl ethoxymethylene malonate (DEEMM), the derivatization reagent, was purchased from Fluka (Japan). Sodium borate decahydrate was purchased from Sigma–Aldrich (USA).

Bacterial strain and media

During plasmid construction, *E. coli* strains were grown at 37 °C in Luria–Bertani (LB) medium (10 g l^{-1} tryptone, 5 g l^{-1} yeast extract, 5 g l^{-1} sodium chloride). The medium was supplemented with 50 μg ml^{-1} kanamycin (Km) for selection when required. *E. coli* K12 MG1655 was used for cloning *cadA*. The coding region of cadA was amplified by PCR using primers cadAF (5′-CGTCGTGGATCCATGAACGTTATTGCAATATT AATCACA-3′) and cadAR (5′-ATATAAGCT TTCCCGCGATTTTTAGGACTCG-3′). PCR product was then digested with restriction enzymes BamHI/Hind III, after which it was inserted into vector pET24ma that had been cut with the same restriction enzymes. pET24ma vector (constructed by Dr. David Sourdive, Pasteur Institute, France) contained a p15A replication origin. The plasmid was transformed into BL21 (DE3) *E. coli* competent cells. Cultivation was carried out at 30 °C in a shaking incubator (Han-Beak Science Co., Korea) at 200 rpm. Preculture 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 followed by incubation at 30 °C for 16 h with shaking. One ml of the preculture was used to seed 50 ml of LB medium (50 μg/ml Kanamycin, 0.1 mM iso-propyl-β-D-thiogalactopyranoside) using 250 ml Erlenmeyer conical flask followed by incubation at 30 °C with shaking for 16 h. The culture was harvested by centrifugation at 4000 rpm for 10 min at 4 °C. Cell pellet was washed with 50 mM sodium acetate buffer (pH 6.0) and resuspended in the same buffer. These cells were stored at −70 °C until use. They were referred to as whole cells.

Whole cell reaction

Lysine decarboxylase activity was determined using whole cell as catalyst. Assays were performed in a total volume of 50 ml containing 1 M L-lysine, 0.1 mM pyridoxal-5-phosphate, and 2 ml of cell extract. The mixture was incubated at 37 °C for 2 h in a shaking (200 rpm) incubator. The reaction was stopped by heating at 100 °C for 5 min. One unit of activity (mmol/wet weight (mg)/min) was defined as the amount of enzyme producing 1 mM of cadaverine in one min at 37 °C. The whole cell used in the following experiment had 18.52 Units of lysine decarboxylase activity. For 50 ml scale extraction, 100 ml of MEK and same volume of lysine–cadaverine solution were mixed in 250 ml screw capped erlenmeyer flasks at 63 °C 200 rpm incubation for 6 h.

Extraction and sample preparation

Whole cell reaction solution was centrifuged at 4000 rpm for 10 min. The supernatant was collected in 50 ml falcon tube and its pH was adjusted to 13.5 with sodium hydroxide. Then 1 ml of the pH adjusted reaction solution and 1 ml of organic solvent were mixed in a 15 ml falcon tube. Cadaverine liquid–liquid extraction was then carried out at a temperature controlled shaking incubator (Han-Beak Science Co., Korea) at speed of 200 rpm for 120 min. The reaction was then kept still for separation of solvent phase and aqueous phase. After phase separation, 50 μl of solvent phase was then transferred to 1.7-ml eppendorf tube. The solvent was evaporated using a N_2 evaporator (MG-2200, Eyela, Japan), after which 1 ml of distilled water was added to the tube for 20-fold dilution. All experiments were performed in duplicates.

Derivatization and HPLC analysis

Cadaverine and lysine derivatives were obtained by mixing 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 diethyl ethoxymethylenemalonate (DEEMM) without pretreatment. The sample was then heated at 70 °C for 2 h to allow complete degradation of excess DEEMM and reagent by-products. After derivatization with DEEMM, high performance liquid chromatography (HPLC, YL-9100, Korea) analyses were then performed. The equipment consisted of a binary pump, an in-line degasser, an autosampler, and a column thermostat. Chromatographic separation was done using reverse-phase chromatography with a C18 column using water as eluent. The column temperature was maintained at 35 °C. Mobile phase composed of acetonitrile (solvent A) and 25 mM sodium acetate buffer pH 4.8 (solvent B) was supplied at 1 ml/min and the composition of solvent A to B (A:B, v/v) was changed with following gradient program: 0 min (20:80), 2 min (25:75), 32 min (60:40), 37 min (20:80), 40 min (20:80).

Large scale extraction and methyl ethyl ketone (MEK) recovery

To examine optimized extraction condition for large scale extraction of cadaverine, 50 ml of pH adjusted reaction solution was mixed with 100 ml of methyl ethyl ketone (MEK) in a screw capped 250 ml Erlenmeyer flasks (Scott Duran, USA). Cadaverine liquid–liquid extraction was then carried out at 58 °C in a shaking incubator (Han-Beak Science Co., Korea) at 200 rpm for 2 h. The mixture was then kept still until solvent phase and aqueous phase were separated. Solvent phase was transferred to two 50 ml falcon tube. Separated aqueous phase was mixed with 100 ml of MEK in a 250-ml screw-capped Erlenmeyer flask. Extraction and solvent separation procedure mentioned above were repeated four times. To calculate MEK recovery rate for cadaverine, rotary evaporate system. Each 100 ml of cadaverine extracted MEK was transfer to 500 ml Round-bottom flasks with conical ground joint. Consequently, using vacuumed rotary evaporator (N-1100, EYELA, Japan), MEK was evaporated in 60 °C water bath. Condensed MIBK was recovered and its volume was measured using pipette aid (Drummond, USA).

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