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Research Article

Metabolic engineering of *Escherichia coli* for agmatine production

Agmatine is a kind of important biogenic amine. The chemical synthesis route is not a desirable choice for industrial production of agmatine. To date, there are no reports on the fermentative production of agmatine by microorganism. In this study, the base *Escherichia coli* strain AUX4 (JM109 $\Delta speC \Delta speF \Delta speB \Delta argR$) capable of excreting agmatine into the culture medium was first constructed by sequential deletions of the *speC* and *speF* genes encoding the ornithine decarboxylase isoenzymes, the *speB* gene encoding agmatine ureohydrolase and the regulation gene *argR* responsible for the negative control of the *arg* regulon. The *speA* gene encoding arginine decarboxylase harboured by the pKK223-3 plasmid was overexpressed in AUX4, resulting in the engineered strain AUX5. The batch and fed-batch fermentations of the AUX5 strain were conducted in a 3-L bioreactor, and the results showed that the AUX5 strain was able to produce 1.13 g agmatine L⁻¹ with the yield of 0.11 g agmatine g⁻¹ glucose in the batch fermentation and the fed-batch fermentation of AUX5 allowed the production of 15.32 g agmatine L⁻¹ with the productivity of 0.48 g agmatine L⁻¹ h⁻¹, demonstrating the potential of *E. coli* as an industrial producer of agmatine.

Keywords: Agmatine production / Batch and fed-batch fermentations / *Escherichia coli* / Gene deletion / Gene overexpression

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1 Introduction

Agmatine, also known as 1-amino-4-guanidinobutane, is a kind of important biogenic amine that is widely present in living organisms [1]. Agmatine has been used as a dietary ingredient for health care products and has great potential for applications in treating many complex diseases including central nervous system disorders, cardiovascular diseases, and cancers [2, 3]. Currently, the industrial production of agmatine relies on a five-step chemical process with agmatine sulfate or hydrochloride as final products [4, 5]. Due to the use of nonrenewable petrochemical raw materials, harsh reaction conditions, high potential production safety issues, and serious environment pollution, the chemical synthesis route is not a desirable choice for industrial production of agmatine. A biotechnological approach is safe, efficient,

and environment-friendly and has been widely used for industrial production of various compounds. Therefore, it is quite necessary to develop a biotechnological approach to produce agmatine.

Escherichia coli is an attractive microbial cell factory and has been used to produce various substances of interest by metabolic engineering [6]. In *E. coli*, agmatine is synthesized via a series of reactions with L-glutamate as initial substrate [7–9] (Fig. 1). L-Arginine is the direct precursor of agmatine, and agmatine is formed via the decarboxylation of L-arginine catalyzed by arginine decarboxylase (ADC) encoded by the *speA* or *adiA* genes. The biosynthesis of L-arginine from L-glutamate is regulated by the *arg* regulon composed of the arginine genes *argA*, *argD*, *argF*, *argI*, *argG*, *argR*, *carAB*, and gene cluster *argECBH* [10]. The arginine repressor ArgR can be activated by the product L-arginine and repress the transcription of each gene of the *arg* regulon by its binding to the operator sites. Agmatine can be converted to putrescine via agmatine ureohydrolase (AUH) encoded by the *speB* gene, and putrescine can also be directly formed via the decarboxylation of ornithine catalyzed by ornithine decarboxylase (ODC) encoded by the *speC* or *speF* genes.

In this study, based on metabolic and regulatory information of agmatine, metabolically engineered *E. coli* strains were

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Abbreviations: ADC, arginine decarboxylase; CDW, cell dry weight; OPA, O-phthalaldehyde

Figure 1. The metabolic pathways involved in agmatine biosynthesis in *E. coli*. (D), deletion of the genes in the chromosome; (O), overexpression of the genes on the recombinant plasmid; AST pathway, the arginine succinyltransferase pathway.

developed to produce agmatine by sequential deletions of the genes *speC*, *speF*, *speB*, and *argR* and overexpression of the *speA* gene on the recombinant plasmid. The production of agmatine using the final engineered strain was performed by batch and fed-batch fermentations. The fermentative production by metabolically engineered *E. coli* strains is an attractive approach to produce agmatine.

2 Materials and methods

2.1 *E. coli* strains, plasmids, and growth condition

E. coli strains and plasmids used in this study are listed in Table 1. JM109 was used as a starting *E. coli* strain to develop agmatine overproducing strains. During the constructions of engineered

Table 1. *Escherichia coli* strains and plasmids used in this study

Strain or plasmid	Description	Source
Strains		
JM109	<i>endA1, recA1, gyr96, thi, hsdR17(rk⁻, mk⁺), relA1, supE44, λ⁻, Δ(lac-proAB), [F', traD36, proAB, lacI^qZΔM15]</i>	Promega
AUX1	JM109 Δ <i>speC</i>	This work
AUX2	JM109 Δ <i>speC</i> Δ <i>speF</i>	This work
AUX3	JM109 Δ <i>speC</i> Δ <i>speF</i> Δ <i>speB</i>	This work
AUX4	JM109 Δ <i>speC</i> Δ <i>speF</i> Δ <i>speB</i> Δ <i>argR</i>	This work
AUX5	AUX4 (pKK223-3- <i>speA</i>), AUX5 harbouring the recombinant plasmid pKK223-3- <i>speA</i>	This work
Plasmids		
pKD3	λ-Red recombination plasmid, Ap ^R , Cm ^R , FRT-Cm ^R -FRT cassette	[11]
pKD46	λ-Red recombination plasmid, Ap ^R , λ Red recombinase under arabinose-inducible <i>araBAD</i> promoter, temperature sensitive origin	[11]
pCP20	λ-Red recombination plasmid, Ap ^R , Cm ^R , thermal induction of FLP recombinase, temperature sensitive origin	[11]
pKK223-3	<i>E. coli</i> expression vector, Ap ^R , IPTG-inducible <i>tac</i> promoter	Pharmacia
pKK223-3- <i>speA</i>	pKK223-3 (<i>EcoRI/HindIII</i>) Ω <i>speA</i> , a recombinant pKK223-3 plasmid harbouring the <i>speA</i> gene	This work

strains, *E. coli* was grown in LB media comprising 10 g tryptone L⁻¹, 5 g yeast extract L⁻¹, and 10 g NaCl L⁻¹. The cultivations of all *E. coli* strains in shake flasks were carried out at 37°C and 220 rpm. When needed, final concentration 50 µg kanamycin mL⁻¹ or 30 µg chloramphenicol mL⁻¹ were added.

2.2 Experimental manipulation in molecular biology

Plasmid DNA was prepared and purified using the corresponding kits. The sequences of primers used in this study are listed in Table 2, and the PCR amplification of DNA was conducted using PrimeSTARTM HS DNA Polymerase according to the manufacturer's protocol (TaKaRa, Dalian, China). DNA cleavage by restriction enzymes, ligation, transformation, and screening of transformants were performed according to the routine methods.

2.3 Agmatine tolerance test

Agmatine tolerance test with the *E. coli* JM109 strain was performed in a fermentation medium used in the production of agmatine at 37°C and 220 rpm. The fermentation medium (pH 6.80) contains (L⁻¹): 10 g glucose, 3 g (NH₄)₂SO₄, 300 mg MgSO₄·7H₂O, 15 mg CaCl₂·2H₂O, 3 g KH₂PO₄, 2 g (NH₄)₂HPO₄, 100 mg NaCl, 1 g Na citrate·3H₂O,

Table 2. Sequences of primers used for PCR experiments in this study

Names	Sequences
<i>speC</i> -F	<u>tccacctgtccggtattcttactccccgaaacgggttgcgcttgtaggctggagctgcttc^{a)}</u>
<i>speC</i> -R	<u>gcaaagaaaaacgggtcgccagaaggtgacccgtttttttattcatggtccatatgatatcctcctta^{b)}</u>
<i>speF</i> -F	<u>tgcgaaattgaggacgtctattactctaaataaagagatgaaaagttaggctggagctgcttc^{a)}</u>
<i>speF</i> -R	<u>tggtaactgaacgacgcccattttgttcgatttagcctgactcatggtccatatgatatcctcctta^{b)}</u>
<i>speB</i> -F	<u>cgcggaagggttttttatatcgactttgtaataaggagtcctccgtgtaggctggagctgcttc^{a)}</u>
<i>speB</i> -R	<u>tccgacattaatggcagcttttaccgtgcgcacgcacatggtgcatggtccatatgatatcctcctta^{b)}</u>
<i>argR</i> -F	<u>tctgtatgcacaat aatgtgtatcaaccacatcggtgactgtgtaggctggagctgcttc^{a)}</u>
<i>argR</i> -R	<u>ggataagcaacatttccccgcgcagaaacgacggggcagagacatggtccatatgaatatcctcctta^{b)}</u>
<i>speA</i> -F	attagaattcatgtctgacgacatgtctatgggt
<i>speA</i> -R	acataagcctttactcatcttcaagataagataaccgtac

The underlined sequences illustrate homology patch chosen to match sequences in

^{a)}the upstream region and

^{b)}the downstream region of the chromosomal integration site. The italic sequences are the priming sequences

75 mg FeSO₄·7H₂O, 14.25 mg spermidine, 5 mg thiamine-HCl (vitamin B1) as well as the trace elements 3 mg Al₂(SO₄)₃·18H₂O, 1.05 mg CoCl₂·3H₂O, 3.75 mg CuSO₄·5H₂O, 0.75 mg H₃BO₃, 30 mg MnCl₂·4H₂O, 4.5 mg Na₂MoO₄·2H₂O, 3 mg NiSO₄·6H₂O, and 22.5 mg ZnSO₄·7H₂O. The seed culture was prepared by inoculating 10 mL LB medium in a 50-mL shake flask with a small aliquot of cell glycerol stock of JM109 and incubation overnight. One hundred microliter of seed culture was inoculated into 5 mL fermentation medium in test tubes and cultured. After cultivation for 4 h (OD₆₀₀ ≈ 0.4), different final concentrations of agmatine sulfate (0.1, 0.2, 0.3, 0.4, 0.5, and 0.6 M, respectively) were added into the JM109 cultures. Culture samples were periodically taken for the determinations of cell dry weights (CDWs). The experiment was carried out in triplicates with two parallel samples.

2.4 Deletion of genes

Deletion of the *speC* gene in the JM109 chromosome was performed using the method described by Datsenko and Wanner [11]. The first step was that the linear DNA fragment composed of two homology arms and FRT-Cm^R-FRT cassette was generated by PCR using pKD3 as the template and primers *speC*-F and *speC*-R. The second step was that the linear DNA fragment was electroporated into the *E. coli* JM109 cells harboring pKD46, and colonies with double crossover homologous recombination were selected on LB agar plates containing chloramphenicol and confirmed by direct colony PCR. The third step was that the Cm^R marker was eliminated by the helper plasmid pCP20. Deletion of the gene in the chromosome was confirmed

by DNA sequence analysis. The resulting strain JM109 $\Delta speC$ was designated as AUX1. Deletion of the *speF* gene in the AUX1 chromosome was performed in the same manner as described for the *speC* gene, except that primers *speF*-F and *speF*-R were used in PCR reaction in the first step and AUX1 harboring pKD46 was used as the recipient strain in the second step, and the resulting strain JM109 $\Delta speC \Delta speF$ was designated as AUX2. Similarly, the *speB* gene in the AUX2 chromosome was deleted using the primers *speB*-F and *speB*-R in the first step and AUX2 harboring pKD46 in the second step, and the resulting strain JM109 $\Delta speC \Delta speF \Delta speB$ was designated as AUX3. The *argR* gene in the AUX3 chromosome was deleted using the primers *argR*-F and *argR*-R in the first step and AUX3 harboring pKD46 in the second step, and the resulting strain JM109 $\Delta speC \Delta speF \Delta speB \Delta argR$ was designated as AUX4.

2.5 Construction of a gene expression system

The ORF of *speA* was generated by PCR using JM109 genomic DNA as a template and the forward primer *speA*-F and reverse primer *speA*-R. The forward primer *speA*-F harbors an *EcoRI* site at its 5'-terminal end, and the reverse primer *speA*-R harbors a *HindIII* site at its 5'-terminal end. The *speA* fragment was digested with *EcoRI* and *HindIII*, and ligated into pKK223-3 which was similarly digested, generating the recombinant expression vector pKK223-3-*speA*. pKK223-3-*speA* was transformed into the strain AUX4, and the resulting strain was designated as AUX5. The expression of the *speA* gene encoding ADC was determined by SDS-PAGE and activity analysis.

2.6 Shake flask cultivations

The seed culture was prepared by inoculating 10 mL LB medium in a 50-mL shake flask with a small aliquot of cell glycerol stock of JM109, AUX1, AUX2, AUX3, AUX4, or AUX5 and incubation overnight. The shake flask cultivations of JM109, AUX1, AUX2, AUX3, AUX4, and AUX5 were carried out in a 500-mL shake flask containing 100 mL fermentation medium or spermidine-free fermentation medium inoculated with 2 mL corresponding seed culture for testing the growth tendencies and the fermentative production of agmatine. For the incubation of the AUX5 strain for agmatine production, the cultures were supplemented with IPTG (final concentrations, 0.01, 0.02, 0.04, 0.06, 0.08, and 0.1 mM, respectively) after 4-h cultivation. For all strains, after 16-h cultivation periods, culture samples were taken from the final cultures and centrifuged at $13200 \times g$ and 4°C for 5 min, the resulting culture supernatants were used for determination of agmatine concentration. If needed, the cell pellets were used for enzymatic activity analysis. The shake flask experiment for each strain was carried out in triplicates with two parallel samples.

2.7 Batch and fed-batch fermentations

Batch and fed-batch fermentations of the AUX5 strain were conducted at 37°C in a 3-L bioreactor (BLBIO-3GJ, Shanghai Bailun Biological Technology Co., Ltd., Shanghai, China) containing 1 L

fermentation medium. The seed culture was prepared by inoculating 10 mL LB medium in a 50-mL shake flask with a small aliquot of cell glycerol stock of AUX5 and incubation overnight. One milliliter seed culture was inoculated into 100 mL fermentation medium in a 500-mL shake flask and cultured until the maximum OD_{600} value, resulting in the fermentation seed liquid. In accordance to 1:10 volume ratio of fermentation seed liquid to fermentation medium, the fermentation seed liquid was added into the bioreactor. After cultivation for 4 h, the culture was supplemented with IPTG (final concentration, 0.02 mM) and then further cultured. pH was maintained at 6.80 by automatic addition of 6 M KOH except for the short periods of pH increase due to glucose depletion. The dissolved oxygen concentration was maintained at 20% of air saturation by automatically increasing the agitation speed. Using the pH-stat feeding strategy for fed-batch fermentation [12], the feeding solution comprising 500 g glucose L^{-1} , 150 g $(\text{NH}_4)_2\text{SO}_4$ L^{-1} , and 7 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ L^{-1} was automatically added into the bioreactor to increase the glucose concentration to 2 g L^{-1} when the pH rose to 6.82 due to glucose depletion. Culture samples were periodically taken for analyzing CDWs and concentrations of glucose and agmatine. The batch fermentation and fed-batch fermentation experiments were both carried out in duplicates.

2.8 Analytical methods

In accordance to 1:20 volume ratio of buffer to culture, the cell pellet of the AUX5 shake flask culture was washed and resuspended in 50 mM different pH Tris-HCl buffer (pH 7.5). The resuspended cells were disrupted by ultrasound and centrifuged, generating the cell homogenate supernatant for enzymatic activity analysis. ADC activity was measured by monitoring the formation of agmatine from L-arginine. The enzymatic reaction of ADC was assayed according to a modified method of Song et al. [13]. The reaction was initiated by adding the cell homogenate supernatant into 1.5 mL activity assay mixture containing 67 mM Tris-HCl (pH 7.5), 3.3 mM EDTA, 1 mM pyridoxal-5'-phosphate, 3.3 mM dithiothreitol, 10 mM L-arginine, and incubated for 15 min at 37°C . Reaction was stopped by adding one-fifth volume of 40% trichloroacetic acid. The concentration of the agmatine product was determined by HPLC. One unit of ADC activity is defined as the amount of enzyme required to produce 1 μmol of agmatine per min from L-arginine at 37°C and pH 7.5. Protein concentration was determined using the Protein dotMETRIC™ Kit (Sangon, Shanghai, China).

CDW (g L^{-1}) was determined from the OD_{600} value according to the formula: $Y = 0.47X + 0.18$ (Y represents the CDW values, X represents the OD_{600} values).

Glucose concentration was measured using a glucose analyser (M-100, Shenzhen Sieman Technology Co., Ltd., Shenzhen, China).

Agmatine concentration was determined by HPLC (1260 Infinity, Agilent Technologies, Santa Clara, CA) using the method described by Yildirim et al. [14] with minor modifications. Agmatine was first derivatized by O-phthalaldehyde (OPA) derivatization reagent before HPLC. Potassium borate buffer

(0.2 M) was prepared by dissolving 3.09 g boric acid in water and adjusting the pH to 9.40 with a saturated solution of potassium hydroxide in a final volume of 250 mL. The OPA derivatization reagent was prepared by dissolving 27 mg OPA in 1 mL methanol, then adding 9.0 mL 0.20 M potassium borate buffer (pH 9.4) and 26.5 μ L 2-mercaptoethanol. For derivatization, 50 μ L sample of the culture supernatant or the ADC enzyme reaction mixture and 450 μ L water were added to 400 μ L methanol. Following the addition of 100 μ L OPA reagent, the mixture was filtered through a 0.2- μ m PVDF syringe filter, and 20 μ L the filter was immediately injected into HPLC. An AElchrom-C18C-5 column operating at 25°C was used for separation. Stock A solution (ten-fold concentrated, 0.1 M) was prepared by dissolving 13.61 g anhydrous potassium dihydrogen-phosphate in 950 mL of water and adjust the pH to 4.05 at 20°C in a final volume of 1 L. After filtering, the solution was stored at 4°C in the dark. Eluent A was 1:1:8 v/v/v mixture of stock A, methanol, and water. Eluent B was 5:3 v/v mixture of acetonitrile and methanol. Both eluents A and B were vacuum-degassed for 30 min before use. The gradient was applied as follows: 0 min 80% A, 4 min 73% A, 8 min 50% A, 12 min 30% A, 16 min 25% A, 20 min 20% A, 24 min 40% A, 28 min 60% A, and 32 min 80% A. A flow rate of 1 mL min⁻¹ was maintained throughout. Fluorescent derivatives were detected by excitation at 330 nm and emission at 465 nm.

3 Results and discussion

3.1 Agmatine tolerance test

The biogenic amines, such as spermidine and putrescine, are toxic to the producer cells if they accumulate at a high concentration [15, 16]. It is necessary for the fermentative production of agmatine to carry out agmatine tolerance test in *E. coli*. The results were shown in Fig. 2. The positive effect of low concentration agmatine (0.1 M) on cell growth was unexpected. Despite of low growth rate, the cells were still able to uninterruptedly grow in the presence of 0.4 M agmatine. The lysis of partial cells immediately occurred when 0.5 M agmatine was added, and the unlysed cells continued to grow after recovery of cell vitality. The results illustrated that the JM109 strain can tolerate up to 0.4 M agmatine (55.2 g agmatine L⁻¹). The high tolerance to agmatine provides a prerequisite for developing an agmatine high-producing *E. coli* strain by metabolic engineering.

3.2 Construction of an agmatine-producing base strain

L-ornithine is an important node compound for the overproduction of agmatine. *E. coli* possesses two isoenzymes (the biosynthetic ornithine decarboxylase encoded by *speC* and the degradative ornithine decarboxylase encoded by *speF*) responsible for the direct conversion of ornithine to putrescine [17]. In *E. coli*, agmatine is further converted to putrescine via AUH encoded by the *speB* gene [8]. To accumulate agmatine, the decarboxylation of ornithine to putrescine and the ureohydrolysis of agmatine to putrescine should be blocked. Based on the JM109 strain, the *speC*, *speF*, and *speB* genes were sequentially deleted, generating the strains AUX1 (JM109 Δ *speC*), AUX2 (JM109

Figure 2. *Escherichia coli* tolerance to various concentrations of agmatine. After cultivation for 4 h ($OD_{600} \approx 0.4$) in the fermentation media, different final concentrations of agmatine sulfate were added into the JM109 cultures. Data shown are the averages of three independent experiments with the standard deviations.

Δ *speC* Δ *speF*), and AUX3 (JM109 Δ *speC* Δ *speF* Δ *speB*), respectively. The growth test in the spermidine-free fermentation medium showed that the growth rates of AUX1, AUX2, and JM109 were consistent, while the growth rate of AUX3 was significantly lower than that of JM109. Chattopadhyay et al. reported that putrescine was not essential for the aerobic growth of *E. coli*, but the lack of putrescine led to a big decline of its growth rate due to no further spermidine synthesis [18]. Here, the deficiency of the ability to grow of the AUX3 strain in the spermidine-free fermentation medium confirmed the collective deletion of the *speC*, *speF*, and *speB* genes. The arginine repressor ArgR encoded by the *argR* gene, in conjunction with L-arginine, negative controls transcription of the arginine regulon [10]. In order to increase the direct precursor L-arginine of agmatine, it is required to remove the transcriptional repression of the arginine genes in *E. coli*. Therefore, the *argR* gene was deleted from the AUX3 strain, generating the strain AUX4 (JM109 Δ *speC* Δ *speF* Δ *speB* Δ *argR*).

The analysis of agmatine in the shake flask cultures showed that no agmatine was determined in the culture supernatants of the strains JM109, AUX1, AUX2, AUX3, while AUX4 excreted 0.02 g agmatine L⁻¹ into the culture medium, suggesting that deletion of *argR* plays a critical role on agmatine production and the base strain AUX4 that is able to produce agmatine was successfully constructed.

3.3 Effect of different expression levels of the *speA* gene on agmatine production

E. coli possesses two ADCs: the biosynthetic arginine decarboxylase SpeA encoded by the *speA* gene and the acid-inducible biodegradative arginine decarboxylase AdiA encoded by the *adiA* genes. SpeA is important for the agmatine biosynthesis, whereas

Table 3. Effects of different final concentrations of IPTG on expression levels of ADC and agmatine production in the shake flask cultivation of the AU5 strain. Data shown are the averages of three independent experiments with which the standard deviations are not displayed

IPTG concentration (mM)	AU4		AU5					
	0	0	0.01	0.02	0.04	0.06	0.08	0.1
Specific activity (U mg ⁻¹ protein)	0.01	0.02	0.06	0.1	0.16	0.21	0.24	0.26
Agmatine concentration (g L ⁻¹)	0.02	0.05	0.19	0.19	0.19	0.18	0.18	0.17

AdiA is critical for the function of the acid resistance system 3 [19]. To increase the conversion of L-arginine to agmatine, the *speA* gene was overexpressed under the control of IPTG-inducible *tac* promoter on the plasmid pKK223-3 in AUX4, generating the final engineered strain AUX5 (AUX4 (pKK223-3-*speA*)) in this study.

For the metabolic engineering of bacteria, overexpression of the genes encoding necessary enzymes may lead to increases in the target metabolites, while excessive overexpression can bring significant metabolic burden to the host and lead to decreases in the precursor metabolites available to produce the target metabolites [20]. Therefore, it is necessary for achieving a desired yield of the target metabolite to optimize expression levels of the genes encoding necessary enzymes using promoters of different strengths. In this study, ADC is the key enzyme for agmatine production and its expression levels in the strain AUX5 were optimized by adding different final concentrations of IPTG.

Enzymatic activity analysis showed that the ADC specific activities of the cell homogenate supernatants of the shake flask cultures of AUX5 continuously increased with the increase of IPTG concentrations (Table 3). Agmatine analysis showed that the productions of agmatine in the shake flask cultures of AUX5 maintained at the maximal value (0.19 g L⁻¹) at the presence of 0.01, 0.02, and 0.04 mM of IPTG and began to decrease at the presence of 0.06 mM of IPTG (Table 3). These results demonstrated that the overexpression of *SpeA* markedly increased agmatine production and suitable expression levels of *SpeA* led to the highest production of agmatine.

3.4 Agmatine production by batch and fed-batch fermentations

To test the potential of agmatine production, the batch and fed-batch fermentations of the engineered *E. coli* strain AUX5 were performed at the presence of 0.02 mM of IPTG. As shown in Fig. 3A–C, agmatine was continuously accumulated in the medium during cell propagation, and the cells still produced a small quantity of agmatine after the fermentable carbon source was depleted in the batch fermentation; the AUX5 strain was able to finally produce 1.13 g agmatine L⁻¹ with the yield of 0.11 g agmatine g⁻¹ glucose in the batch fermentation. As shown in Fig. 3D, the maximal concentration of agmatine obtained in AUX5 was 15.32 g agmatine L⁻¹ in 32 h and the productivity was 0.48 g agmatine L⁻¹ h⁻¹ in the fed-batch fermentation.

In 2015, Sun et al. [21] reported the enzymatic synthesis of agmatine using L-arginine as reaction substrate and the recombinant ADC. Compared to the enzymatic method, the microbial fermentation is a more attractive biotechnological approach to

produce agmatine. In this study, *E. coli* was metabolically engineered for agmatine production and the final engineered strain AUX5 was able to produce a good yield of agmatine by batch fermentation. To the best of our knowledge, this is the first report on the fermentative production of agmatine by microorganism.

L-glutamate is the initial substrate for agmatine biosynthesis, and it is of importance for agmatine production to improve the L-glutamate pool concentration in *E. coli*. *Escherichia coli* possesses two pathways for glutamate synthesis: the glutamine dehydrogenase (GDH) pathway encoded by the *gdhA* gene and the glutamine synthetase-glutamate synthase (GOGAT) pathway encoded by the *glnA* and *gltBD* genes [22] (Fig. 1). Enhancement of the conversion of α -ketoglutarate to L-glutamate by overexpressing GDH or GOGAT would contribute to agmatine production. L-Arginine is the direct precursor of agmatine. N-Acetylglutamate synthetase (NAGS) encoded by the *argA* gene is the rate-limiting enzyme of L-arginine biosynthesis and subjected to feedback inhibition by L-arginine. The relief of feedback inhibition of NAGS should contribute to L-arginine biosynthesis. However, it has been reported that introduction of the feedback resistant *argA* led to *E. coli* strains instability even death, due to L-glutamate starvation resulting from continuous increase in the metabolic flux through this enzymatic step [12, 23]. In addition, the instability also occurred in the *E. coli* strain in which the wild-type *argA* was overexpressed under the control of IPTG-inducible strong promoter on the plasmid [12]. Therefore, chromosomal introduction of a promoter with suitable strength, that both ensures as much metabolic flux as possible toward L-arginine synthesis and does not cause glutamate starvation for the wild-type *argA* need to be further explored.

Unlike producing recombinant protein that needs high-level gene expression, metabolic engineering usually involves many genes whose expression levels have to be balanced, and these genes just require moderate level overexpression. The highest production of agmatine in the shake flask cultures of AUX5 was achieved by regulating the expression levels of *SpeA* using different concentrations of IPTG. Due to its cost, IPTG is not suitable for industrial production. The overexpression of the *speA* gene with a constitutive promoter with suitable strength may be considered as a further choice to engineer *E. coli* for agmatine production.

The metabolic flux through L-arginine may also enter the arginine succinyltransferase (AST) pathway that functions on arginine degradation in *E. coli* [9]. The AST pathway contributes to arginine degradation only during nitrogen limited growth. Due to ample nitrogen supply during the fermentation process, block of the AST pathway is unnecessary. In addition to L-glutamate, L-glutamine, and L-aspartate participate in agmatine synthesis, and improvement of the contents of L-glutamine

Figure 3. Batch cultivation (A–C) and fed-batch cultivation (D) of the AUX5 strain. (A) cell growth; (B) residual sugar content; (C) agmatine production; and (D) cell growth and agmatine production. Data shown are the averages of two independent experiments.

and L-aspartate could positively affect agmatine production in engineered *E. coli* strains. The supply of cofactor NADPH generated from the pentose phosphate pathway can become a critical factor for the efficient biosynthesis of fermentative products when the enzyme levels are no longer limiting [24, 25]. For the transport of agmatine, only the arginine: agmatine antiporter encoded by the *adiC* gene is identified [26], other transport reactions mediating the uptake or secretion of agmatine is still unknown in *E. coli*. The arginine: agmatine antiporter AdiC may also be considered as a target for improving agmatine production. In sum, in order to obtain a desired agmatine high-producing strain, it is worthwhile to further investigate known engineering targets involved in agmatine synthesis in *E. coli*.

4 Concluding remarks

In this study, metabolically engineered *E. coli* strains were constructed for agmatine production and the final strain AUX5 (JM109 $\Delta speC$, $\Delta speF$, $\Delta speB$, $\Delta argR$ (pKK223-3-*speA*)) had a good yield of agmatine (0.11 g agmatine g⁻¹ glucose). A relatively high production of agmatine (15.32 g agmatine L⁻¹) of AUX5 was obtained by the fed-batch fermentation, demonstrating the potential of *E. coli* as an industrial producer of agmatine.

Practical application

Agmatine has been used as a dietary ingredient for health care products and has great potential for applications in treating many complex diseases. Currently, the industrial production of agmatine relies on a five-step chemical process. Due to the shortcomings of the chemical method, it is quite necessary to develop a biotechnological approach to produce agmatine. In this study, the successful construction of the metabolically engineered *E. coli* strain AUX5 with a good fermentable yield of agmatine demonstrates that agmatine can be efficiently produced by microorganism fermentation. The *E. coli* strains constructed may be further engineered to develop new strains with industry-level yield of agmatine.

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