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Enzymatic production of L-ornithine from L-arginine with recombinant thermophilic arginase

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ABSTRACT

In this study, to develop a simple and efficient enzymatic production process for the environment-friendly synthesis of L-ornithine from L-arginine, the *Escherichia coli* BL21 (DE3) strain overexpressing arginase (ARG) from *Bacillus caldovelox* was chosen as the potential biocatalyst. The biochemical properties of the recombinant ARG were characterized and compared with those of the native enzyme. The maximal conversion rate of L-arginine to L-ornithine was 87.1% with a final L-ornithine concentration of 112.3 g/L under the following optimal conditions: 170 g/L L-arginine, 12 g/L whole-cell biocatalyst, 10 μM Mn^{2+}, 60 °C, pH 9.0, and 4 h of incubation. When compared with a recent work, the biocatalytic process described in the present study achieved higher average L-ornithine synthesis rate of 26.2 g/L/h, and thus has great potential for large-scale production of L-ornithine.

1. Introduction

L-Ornithine, which plays an important role in the urea cycle, is a non-protein amino acid with various bioactive functions and applications in many fields, including health care, drug manufacturing, and chemical industry [1,2]. Ornithine has the ability to detoxify excess ammonia in the human body and thus has significant effect on liver cells [3]. Furthermore, L-ornithine is effective for treating liver diseases, strengthening the heart, and protecting against endotoxin-induced shock, and can promote protein synthesis and catabolism of sugar and fat [4,5]. Therefore, L-ornithine is an ideal nutritional supplement for bodybuilders and athletes. Owing to its multiple functions in health care, L-ornithine has a sizable market worldwide. Hence, a simple, efficient, and energy-saving method for L-ornithine production is needed.

In recent years, several effective methods have been used for the preparation of L-ornithine, such as chemical synthesis methods [6,7], microbial fermentation [8–10], and enzymatic methods [11,12]. In chemical synthesis methods, acrolein, hydrogen cyanide, ammonia, and CO_2 are used as raw materials, and DL-ornithine is obtained after a multi-step reaction [6]. However, these methods could not be widely used owing to their limitations such as low yield, poor purity, and difficulty in product separation [7]. Microbial fermentation is employed for producing L-ornithine from citrulline or arginine auxotrophic microorganisms such as *Corynebacterium glutamicum* and *Arthro bactericitreus* [8,10]. The auxotrophs resistant to mycophenolic acid or ornithinol are capable of producing higher yields of L-ornithine than conventional auxotrophs, with the highest L-ornithine yield reaching 50 g/L [10]. The use of fed-batch fermentation could further increase the production of L-ornithine to 70 g/L in 60 h [9]. However, the complex composition of the fermentation broth could make subsequent product separation difficult. Hence, some researchers have turned to enzymatic synthesis of L-ornithine involving arginase (ARG; E.C. 3.5.3.1) [11–13]. Purified ARG is rarely used for L-ornithine production [12,13], and 72.7 g/L L-ornithine could be obtained in 10 h. However, enzyme isolation and purification can be relatively expensive and time-consuming. Recently, L-ornithine was produced by using whole-cell (recombinant ARG) biotransformation in the presence of surfactants, with the L-ornithine yield reaching 111.52 g/L after 15 h [11]. However, whole-cell biotransformation always has the problem of cell permeability, and addition of permeability reagent may lead to difficulties in subsequent product separation. Therefore, a simple and effective method for L-ornithine production is required.

In the present manuscript, the ARG-encoding gene from the thermophilic bacterium *Bacillus caldovelox* was expressed in *Escherichia coli* for the conversion of L-arginine to L-ornithine,

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and an attempt was made to shorten the reaction time and solve the problem of cell permeability without adding any permeability reagent. The kinetic parameters of the recombinant thermophilic ARG and the effects of biotransformation conditions were investigated.

2. Materials and methods

2.1. Materials

The expression plasmid pET-28a (+) and the host strain *E. coli* BL21 (DE3) were obtained from Novegen (Masdison, WI). The T vector, restriction enzymes (*Nco*I and *Hind*III), T4 DNA ligase, plasmid mini-preps kit, and agarose gel DNA purification kit were supplied by TaKaRa Biotechnology (Otsu, Japan). L-Ornithine, L-arginine, and phthaldialdehyde were purchased from Sigma-Aldrich (St. Louis, MO).

2.2. Expression in E. coli

The published sequence of the ARG gene from *B. caldovelox* was modified by using codon optimization. The synthesized ARG gene was inserted into the multicopy plasmid pET-28a (+) by introducing the *Nco*I and *Hind*III restriction sites, and then the obtained plasmid pET28a (+)-ARG was transformed into *E. coli* BL21 (DE3). Luria-Bertani (LB) medium (10 g/L peptone, 5 g/L yeast extract, and 10 g/L NaCl; pH 7.0) was used for seed culture. The seed culture was incubated on a reciprocal shaker (200 rpm) at 37 °C in a 250-mL flask containing 25 mL of LB medium (supplemented with 0.1 g/L kanamycin sulfate) for 6 h. Fermentation was conducted in a 500-mL flask containing 50 mL of Terrific Broth (TB) medium (24 g/L yeast extract, 12 g/L peptone, 5 g/L glucose, 2.31 g/L KH_2PO_4, 16.43 g/L K_2HPO_4, and 0.1 g/L kanamycin sulfate; pH 8.0) at 37 °C and 200 rpm in a rotary shaker. When the optical density at 600 nm (OD_{600}) reached 0.6, IPTG was added to the culture at a final concentration of 0.1 mM for induction. The culture of recombinant strain without IPTG induction was used as the control.

2.3. Enzyme purification and SDS-PAGE analysis

The recombinant ARG was purified by using a facile purification procedure comprising a heat-treatment step [14]. The intact cells were collected and resuspended in lysis buffer (100 mM Tris, 50 mM $MnCl_2$, pH 7.5) and lysed by sonication (power 285 W, ultraphonic 4 s, pause 4 s, total 20 min). After centrifuged at 200,000 × *g* for 1 h at 4 °C, the obtained supernatant was rapidly heated to and incubated at 70 °C for 15 min. The insoluble material was removed by centrifugation at 30,000 × *g* for 15 min at 4 °C. Solid ammonium sulphate was added to the supernatant to 30% saturation and the solution was centrifuged at 30,000 × *g* for 10 min at 4 °C. The supernatant was made 50% saturated by further addition of ammonium sulphate and the solution was centrifuged as before. The pellet was solubilized in 200 mM Tris buffer (pH 7.5) and centrifuged as before. The supernatant was dialysed against 20 mM Tris, pH 7.5, overnight at 4 °C. The purified enzyme was obtained after dialysis and further centrifugation. The SDS-PAGE analysis was performed with 5% and 10% polyacrylamide gel slabs for concentration and separation, respectively (Bio-Rad Laboratories, Hercules, CA). After electrophoresis, the gels were stained with Coomassie brilliant blue R-250, and the protein bands in the gels were visualized after destaining the gels in destaining solution.

2.4. Measurement of ARG activity

To measure the ARG activity, the reaction mixture containing 20 mM L-arginine and enzymes was incubated with 50 mM Tris–HCl buffer (pH 9.0) in a final volume of 2 mL at 60 °C. The reaction was stopped after 15 min by adding 1.5 M hydrochloride. The L-ornithine concentration in the mixture was measured as described by Chinard [15], and L-ornithine monohydrochloride was used as the standard. One ARG unit corresponds to the amount of enzyme that could generate 1 μmol L-ornithine per minute from L-arginine by hydrolysis. The protein concentration was determined by the Bradford method [16], with bovine serum albumin as the standard.

2.5. Determination of the kinetic parameters of the recombinant ARG

2.5.1. Determination of optimal pH and pH stability

The optimal pH and pH stability of the recombinant ARG were determined at a pH range of 6.0–12.0 by using different solutions, including 50 mM sodium phosphate buffer (pH 7.0, 7.5), 50 mM Tris–HCl buffer (pH 8.0, 8.5, 9.0), 50 mM glycine–NaOH buffer (pH 9.5, 10.0, 10.5), 50 mM sodium bicarbonate buffer (pH 10.5, 11.0, 11.5), and 50 mM KCl–NaOH buffer (pH 12.0). For measuring the pH stability of the recombinant ARG, the enzyme was incubated at the indicated pH for 12 h at 4 °C and then the enzyme activity was measured as described previously. The relative activities were expressed as the percentage of maximum enzyme activity.

2.5.2. Determination of optimal temperature and temperature stability

The optimal temperature of the recombinant ARG was determined by measuring the enzyme activity at various temperatures (30–90 °C) as described earlier. To measure the temperature stability of the recombinant ARG, the enzyme activity was determined after incubating it at the indicated temperature for 0.5 h. The activity of ARG at each time point was normalized as the percent of enzyme activity at time zero at each corresponding temperature.

2.5.3. Effect of metal ions on enzyme activity

To determine the effect of metal ions on the recombinant ARG, the enzyme activity was measured as described earlier in the presence of various metal ions (Cu^{2+}, Ca^{2+}, Co^{2+}, Mn^{2+}, Ni^{2+}, Fe^{2+}, Mg^{2+}, and Zn^{2+}) at a concentration of 0.1 mM. The relative activity determined in the absence of metal ions at 60 °C for 15 min was taken as 100%.

2.5.4. Determination of K_m and V_{max}

The kinetic parameters of the recombinant ARG were determined by using reaction mixtures containing variable amounts of L-arginine (2–40 mM) in Tris–HCl buffer (50 mM, pH 9.0) at 60 °C. The K_m and V_{max} values were calculated by the Lineweaver–Burk plotting method.

2.6. Production of recombinant ARG in E. coli

The frequently used TB medium was employed to determine the ARG activity, and the conditions for the expression of the recombinant ARG were examined.

2.7. Production of L-ornithine by transformation of L-arginine with ARG

The transformation reaction was optimized under the following conditions: 25 mL of 0.1 M carbonate buffer solution (pH 9.0) in a 250-mL flask on a rotary shaker (150 rpm) at 60 °C. To optimize L-ornithine production, different concentrations of L-arginine (120–200 g/L), intact cells (0–21 g/L, wet cell weight), and Mn^{2+} (10^{-2}–10^{-6} M) were employed and the reactions were analyzed.

Table 1
Characters of the ARG from different strains.

Strains	K_m (mM)	V_{max} (μmol/min/mg)	Optimum pH	Optimum temperature (°C)	References
Bacillus caldovelox	3.4	4.3×10^3	9.0	60	[17]
Bacillus brevis	12.8	–	10.0	37	[24]
Bacillus subtilis KY 3281	13.5	–	10	37	[25]
Bacillus thuringiensis	15.6	538.9	10.0	40	[12]
Saccharomyces cerevisiae	15.7	887.5	9.5	30	[26]
Helicobacter pylori	22.0	0.2	6.1	37	[27]
Neurospora crassa	131.0	–	9.5	37	[28]
E. coli BL21 (pET28a (+)-ARG)	3.5	4.5×10^3	9.0	60	This work

The optimized transformation reaction was subsequently amplified in a 30-L fermentor (New Brunswick Scientific, Enfield, CT, USA).

2.8. Analysis of L-arginine and L-ornithine

The total amount of L-arginine and L-ornithine in the reaction mixture was determined by HPLC [12]. The conversion rate was calculated as follows:

$$\text{Conversion rate} = \frac{m_{Orn}}{m_{Arg}} \times \frac{174.20}{132.19} \times 100\% \quad (1)$$

where m_{Orn} is the mass of L-ornithine in the conversion system, m_{Arg} is the initial mass of L-arginine, 132.19 is the relative molecular mass of L-ornithine, and 174.20 is the relative molecular mass of L-arginine.

3. Results

3.1. Thermophilic ARG: a potential enzyme for L-ornithine production from L-arginine

L-Arginine and L-ornithine, which are alkaline amino acids, have the similar molecular structure, except for the guanidine group at the end of the side chain. According to the KEGG database, L-arginine can be directly converted to L-ornithine in the urea cycle by enzyme ARG (http://www.genome.jp/dbget-bin/www_bget?R00551). The BRENDA database could indicates that ARG widely exists in procaryotic and eucaryotic microorganisms. The characteristics of ARG from different microorganisms are illustrated in Table 1. It can be noted that the ARG from the thermophilic bacterium *B. caldovelox* has a low K_m (3.4 mM) and high V_{max} (4.3×10^3 μmol/min/mg), when compared with the corresponding values from other strains. Therefore, this thermophilic ARG can be chosen as a potential catalyst for L-ornithine production. However, as *B. caldovelox* is a gram-positive bacterium, its cytoderm could block the contact of the substrate and enzyme, resulting in low L-ornithine yield in wild strains. Hence, in the present study, this enzyme was overexpressed in *E. coli*.

3.2. Overexpression and biochemical characterization of the recombinant ARG

To achieve high-level expression of ARG in *E. coli*, the sequence of the ARG-encoding gene from *B. caldovelox* was modified by replacing relatively low-usage codons with high-usage codons in *E. coli*. The synthesized ARG gene was ligated with the expression plasmid pET28a (+), and the obtained recombinant plasmid pET28a (+)-ARG was transformed into *E. coli* BL21 (DE3). Thus, the recombinant strain *E. coli* BL21 (pET28a (+)-ARG) was obtained. The recombinant ARG is an intracellular protein, which makes the enzyme more conducive for collection. The crude enzyme was obtained after the collected cells were disrupted and centrifuged, and its specific activity was 439.2 U/mg protein (9.2-fold higher than that of the wild strain). The recombinant ARG was purified (Fig. 1) by using a facile purification procedure comprising a heat-treatment step [14] and its specific activity was 9.8-fold higher than that of the crude enzyme (Table 2).

The optimal pH of the purified recombinant ARG was determined to be 9.0 (Fig. 2A). The recombinant ARG was stable at the pH range of 7.0–12.0 and retained more than 80% of its maximal activity between pH 7.5 and 10.0 (Fig. 2A). The optimal temperature of the recombinant ARG was 60 °C (Fig. 2B). The thermostability experiments showed that the recombinant enzyme was stable below 40 °C (Fig. 2B); that is, the enzyme was stable at the recombinant *E. coli* culture temperature (37 °C). The effects of different metal ions on the enzyme activity are illustrated in Fig. 2C. It was found that Mn^{2+}, Co^{2+}, and Ni^{2+} showed stimulatory effect, and that 0.1 mM Mn^{2+} and Co^{2+} increased the ARG activity toward L-ornithine by 28% and 20%, respectively. Furthermore, Ga^{2+}, Mg^{2+}, Fe^{2+}, and Zn^{2+} exhibited inhibitory effect on the recombinant ARG activity. The V_{max} and K_m values of the purified recombinant protein were 4.5 mM/min/mg and 3.5 mM, respectively, which were essentially identical to those of the native enzyme [17], the other biochemical property comparison data were illustrated in Table 1. The above results indicated that the expression of ARG did not significantly affect the kinetic parameters of ARG. Considering the fact that both L-arginine and L-ornithine are alkaline amino acids stable at 60 °C, the recombinant thermophilic ARG exhibited a great potential for L-ornithine production.

3.3. Production of the recombinant ARG in E. coli

Different expression conditions were investigated in the TB medium to determine the ARG activity. Fig. 3A and B shows that the optimal induction stage was the exponential growth phase (the maximum ARG activity was similar and approximately 130 U/mL at 2–6 h of ARG fermentation). Maximum ARG activity (133.4 U/mL)

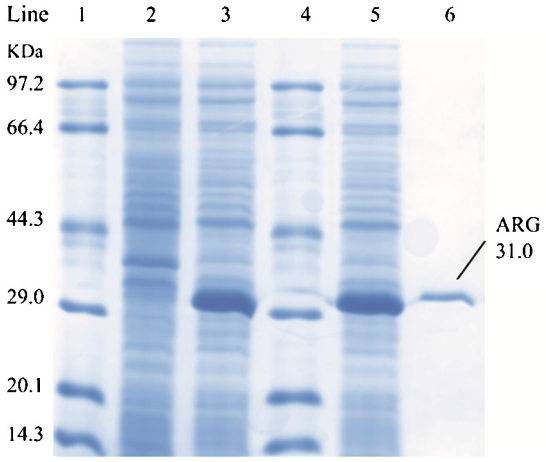

Fig. 1. SDS-PAGE analysis of ARG. Lane1: marker protein molecular weight standard, Lane 2: control cell, Lane 3: expressed cell, Lane 4: marker protein molecular weight standard, Lane 5: crude enzyme, Lane 6: purified ARG.

Table 2
Purification of recombinant enzyme from *E. coli* BL21 (DE3) carrying pET28a-ARG.

Step	Total activity (U)	Total protein (mg)	Specific activity (U/mg protein)	Purification (Fold)	Yield (%)
Crude enzyme	33,864.9	77.1	439.2	1	100
Heat-treatment	11,476.3	3.62	2892.6	6.59	33.9
Salting-out	5853.7	0.43	3978.3	9.05	17.3
Purified	3185.3	0.34	4304.5	9.8	9.4

was observed when the ARG fermentation time at induction was 6 h and the induction period was 6 h (Fig. 3B). In addition, it was also found that the maximum recombinant ARG activity (177.3 U/mL, with the specific productivity of 5.5×10^4 U/g DCW), which was 47.9-fold higher than that of the native enzyme (3.7 U/mL), was achieved with 0.4 mM IPTG (Fig. 3C). Following optimization, the ARG activity and specific productivity improved by 52.5% and 66.7%, respectively, when compared with those before optimization.

3.4. Optimization of whole-cell biocatalysis of L-arginine to L-ornithine

As the recombinant ARG protein is an intracellular protein, the intact cells were collected by centrifugation and then used for biotransformation. Different concentrations (0–21 g/L, wet cell

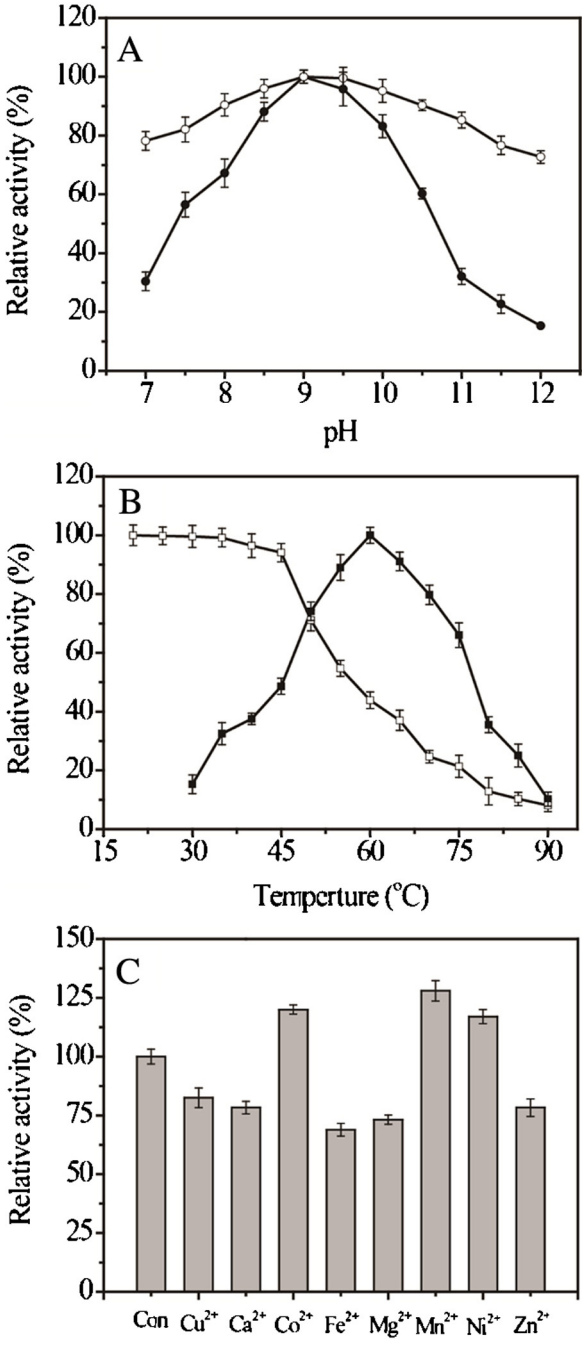

Fig. 2. Effect of pH, temperature and metal ions on the activity of recombinant ARG. (A) ○ pH, ● pH stability. (B) □ temperature, ■ temperature stability. (C) Metal ions.

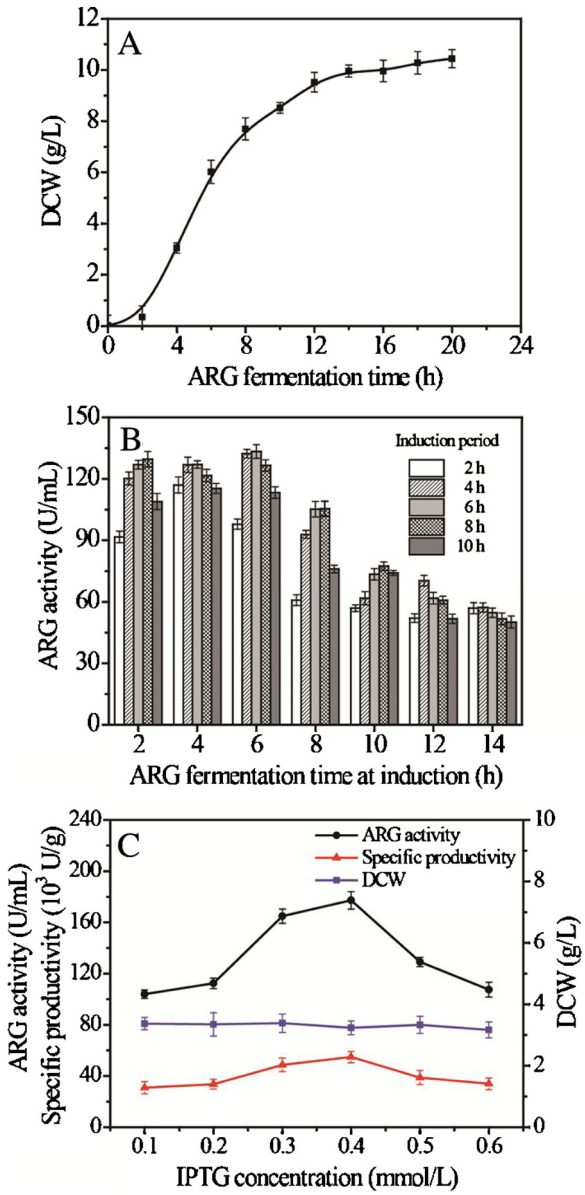

Fig. 3. Expression condition optimized on recombinant ARG production. (A) Growth curve of uninduced recombinant *E. coli* BL21 (DE3). (B) Growth phase at induction and induction period. (C) IPTG concentration.

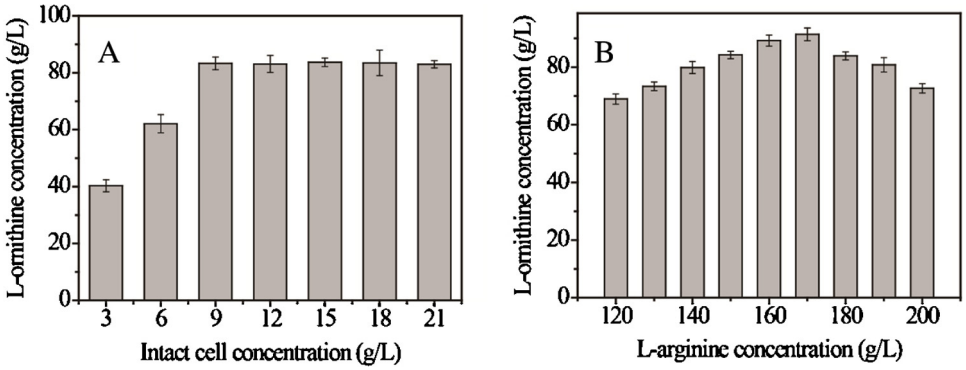

Fig. 4. Different concentration of intact cell (A) and L-arginine (B) on L-ornithine production.

Table 3
Orthogonal array design for L-ornithine production.

Run	Factors		A	B	L-Ornithine titer (g/L)
	A	B	L-Arginine (g/L)	Intact cell (g/L)	
1	1	1	150	90	82.8
2	1	2	150	12	83.7
3	1	3	150	15	83.9
4	2	1	170	9	92.6
5	2	2	170	12	105.3
6	2	3	170	15	105.0
7	3	1	190	9	87.1
8	3	2	190	12	99.7
9	3	3	190	15	100.1
Delt			17.5	8.8	
Ran			1	2	

weight) of intact cells were employed, and the maximum L-ornithine titer obtained from 150 g/L L-arginine and 9 g/L intact cells was 83.3 g/L (Fig. 4A). The effects of different L-arginine concentrations (120–200 g/L) on the L-ornithine titer are shown in Fig. 4B, 91.4 g/L L-ornithine was obtained from 170 g/L L-arginine. Subsequently, the optimum concentrations of L-arginine and intact cells were examined by using an orthogonal array design (Table 3). The highest L-ornithine titer was observed to be 105.3 g/L when 12 g/L intact cells and 170 g/L L-arginine were employed (experimental combination 5), and the conversion rate was 81.3% at this end.

As exogenous Mn^{2+} ions exhibited a positive effect on the recombinant ARG activity, their impact (0–10^{-2} M) on the production of L-ornithine was ascertained. As shown in Fig. 5A, the optimal concentration of Mn^{2+} was 10 μM. By using 170 g/L L-arginine and 12 g/L intact cells under the optimal conditions of 60 °C and pH 9.0, the highest titer of L-ornithine increased to 112.3 g/L, which was 6.6% higher than that obtained without Mn^{2+} addition. From the conversion time profiles (Fig. 5B), it can be noted that the maximum titer of L-ornithine was reached at 4 h.

However, the L-ornithine synthesis rate decreased rapidly with the increasing reaction time (Fig. 5B), which might be due to product inhibition. To verify this possibility, the effect of products (L-ornithine and urea) on the biotransformation reaction was investigated (Fig. 6). As shown in Fig. 6A, L-ornithine exhibited an apparent inhibition on the biotransformation reaction, while urea had no obvious effect on L-ornithine production (Fig. 6B). Furthermore, the conversion rate was only 35.1% when the L-ornithine concentration was 100 g/L.

3.5. *Production of L-ornithine by using recombinant ARG in a 30-L fermentor*

The L-ornithine production system was amplified in a 30-L fermentor under the optimal conditions of 60 °C, pH 9.0, 170 g/L L-arginine, 12 g/L intact cells, and 10 μM $MnCl_2$, and the

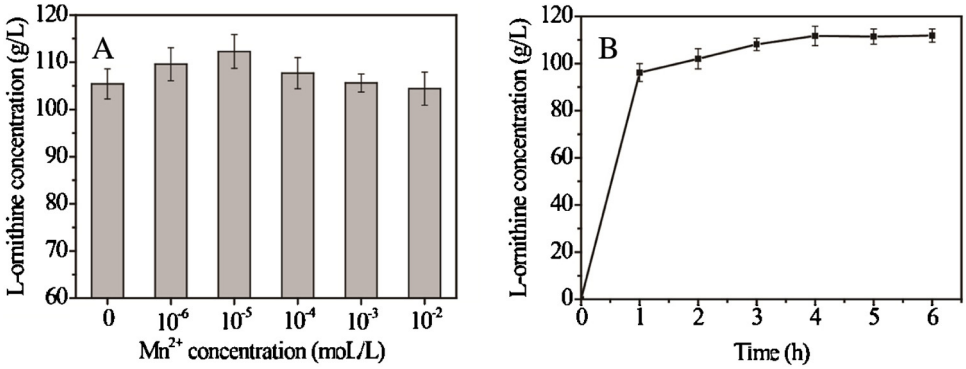

Fig. 5. The effect of Mn^{2+} on L-ornithine production (A) and time course of L-ornithine production (B).

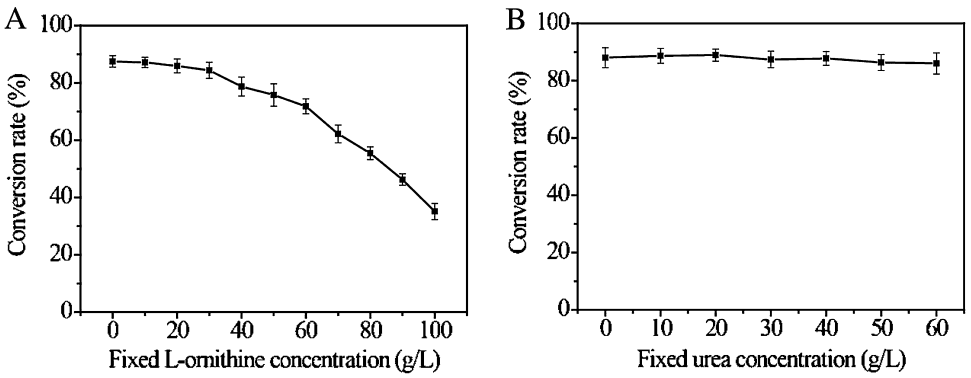

Fig. 6. The production inhibition of arginase by L-omithine (A) and urea (B).

conversion-time plot is presented in Fig. 7. It was found that the initial L-ornithine production rate was about 344.3 g/L/h, and the average L-ornithine synthesis rate was 26.2 g/L/h. After incubation for 4 h, the titer of L-ornithine reached 104.7 g/L and the conversion rate was 81.2%.

4. Discussion

In the present study, the ARG from *B. caldovelox* was overexpressed in *E. coli* BL21 (DE3), and 177.3 U/mL recombinant ARG activity was achieved in the TB medium. By incubating 12 g/L recombinant *E. coli* with 170 g/L L-arginine and 10 μM $MnCl_2$ at 60 °C and pH 9.0 for 4 h, the maximal yield of L-ornithine reached 112.3 g/L and the conversion rate of L-arginine was 87.1%. Thus, this reaction can be considered as an efficient bioprocess for the enzymatic production of L-ornithine from L-arginine at a high ratio.

When compared with the multi-step chemical synthesis methods [6], which are harmful to the environment, the enzymatic production of L-ornithine described in the present study is more simple and environment-friendly. With regard to microbial fermentation, the difficulties in subsequent product separation limit the extraction and purification of L-ornithine [18]. In contrast, in the enzymatic L-ornithine production process, the amount of byproducts produced is low and the separation of L-ornithine is easy. In the enzymatic production methods including whole-cell transformation, the intracellular enzymes are often more stable in a protected environment, but mass transfer has always been the restriction factor. Although surfactants are used in L-ornithine production to improve cell permeability, they may lead to difficulties in subsequent product separation [11]. In the present study, the problem of cell permeability was easily overcome by employing high reaction temperature (60 °C).

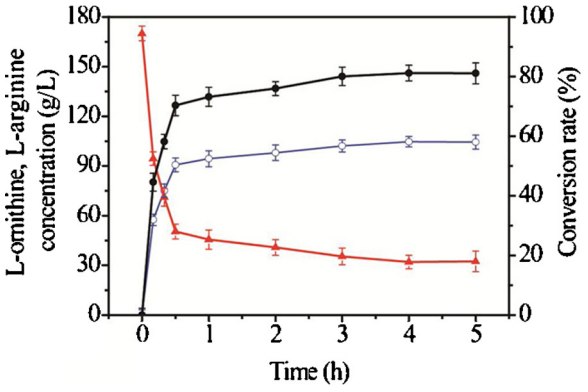

Fig. 7. Time course of L-ornithine production in 30 L fermentor (○ L-ornithine concentration, ▲ L-arginine concentration, ● conversion rate).

In earlier studies on L-ornithine production, the L-ornithine synthesis rate ranged from 0.8 to 7.4 g/L/h [9,11,12]. In the present study, a high L-ornithine synthesis rate of 28.4 g/L/h was achieved. Furthermore, when compared with microbial fermentation and other enzymatic methods exhibiting a transformation time of 70 h or longer and a minimum of 10 h, respectively, the whole-cell catalysis presented a transformation time of only 4 h. The use of a higher operation temperature has several advantages, including favorable equilibrium displacement in endothermic reactions, higher reaction rates owing to decrease in viscosity and increase in the diffusion coefficient of the substrates, and higher process yield owing to increased solubility of the substrates and products [19–23]. Therefore, in the present study, a higher process yield (112.3 g/L) and higher production rate (28.4 g/L/h) were obtained. Finally, L-ornithine production was amplified successfully in a 30-L fermentor, thus demonstrating an improvement in the enzymatic synthesis of L-ornithine, which has significant potential for industrial applications.

Nevertheless, there are still several limiting factors that restrict the L-ornithine conversion rate. It can be noted that the poor stability of the enzyme at 60 °C is one of the reasons for the decrease in the reaction rate in the later stage of the reaction, which could be improved by directed evolution. In addition, the results also showed that the product L-ornithine had an inhibitory effect on ARG activity (Fig. 7A). This inhibitory effect of L-ornithine on the ARG activity could be eliminated by protein engineering to further improve the transformation efficiency. Currently, related works are being carried out by our group, and the enzyme variants with new and evolved properties will be developed by using modern methods of protein engineering.

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