J Ind Microbiol Biotechnol (2015) 42:1427–1437
DOI 10.1007/s10295-015-1672-z

BIOCATALYSIS

Construction of a highly efficient *Bacillus subtilis* 168 whole-cell biocatalyst and its application in the production of L-ornithine

Meizhou Wang[1] · Meijuan Xu[1] · Zhiming Rao[1] · Taowei Yang[1] · Xian Zhang[1]

Received: 14 June 2015 / Accepted: 10 August 2015 / Published online: 29 August 2015

Abstract L-Ornithine, a non-protein amino acid, is usually extracted from hydrolyzed protein as well as produced by microbial fermentation. Here, we focus on a highly efficient whole-cell biocatalyst for the production of L-ornithine. The gene *argI*, encoding arginase, which catalyzes the hydrolysis of L-arginine to L-ornithine and urea, was cloned from *Bacillus amyloliquefaciens* B10-127 and expressed in GRAS strain *Bacillus subtilis* 168. The recombinant strain exhibited an arginase activity of 21.9 U/mg, which is 26.7 times that of wild *B. subtilis* 168. The optimal pH and temperature of the purified recombinant arginase were 10.0 and 40 °C, respectively. In addition, the recombinant arginase exhibited a strong Mn^{2+} preference. When using whole-cell biocatalyst-based bioconversion, a hyper L-ornithine production of 356.9 g/L was achieved with a fed-batch strategy in a 5-L reactor within 12 h. This whole-cell bioconversion study demonstrates an environmentally friendly strategy for L-ornithine production in industry.

Keywords Recombinant arginase · Whole-cell bioconversion · L-Ornithine · *B. subtilis* 168

Introduction

L-Ornithine (L-Orn) is an abundant non-protein amino acid widely used as a food supplement and nutrition product. Pharmacologic dosages of L-ornithine can elicit growth hormone release in the pituitary to strengthen metabolism and prevent obesity or attenuate physical fatigue and is thus an ideal nutritional supplement for bodybuilders and athletes [30, 36]. Oral administration of ornithine was reported to increase the amount of NREM sleep [22]. L-Orn has the ability to promote the wound healing and protect the liver by detoxifying excess ammonia in the human body [6, 26]. In addition, L-Orn is a natural sweetening agent that can be used to reduce bitterness in juice and other beverages [33]. Owing to its multiple useful applications in a worldwide market, a convenient strategy and energy-saving method for L-Orn production is needed.

Arginase (L-arginine amidinase or amidinohydrolase, E.C.3.5.3.1), which catalyzes the hydrolysis of L-arginine (L-Arg) to L-Orn and urea, plays an important role in the urea cycle [4, 20]. Arginase is widely found in organisms as diverse as microorganisms, plants, and animals because of its special activity [18, 20, 41]. In plants, such as tomato, soybean and pea, arginase was demonstrated to have various functions. One of the main functions is nitrogen fixation during fruit development [9, 31, 32]. In mammalian species, arginase expressed in liver depletes arginine levels to help inhibit the growth of various cancer cells [10, 31]. Moreover, in microorganisms, the generation of arginase in *Bacillus brevis*, *Helicobacter pylori*, *Penicillium chrysogenum*, *Bacillus thuringiensis* and *Bacillus subtilis* has been well characterized [7, 15, 40, 42]. Previous studies of the crystallization structure of arginase revealed that arginase from *H. pylori* is a monomer, whereas arginase from *B. brevis* TT02-8 is a tetramer [27, 29]. It has been reported that arginase is a metal-ion-activated enzyme, activated in particular by a divalent cation [2, 3]. As suggested by this review of previous works, most reports have focused more on the characterization and structure of arginase and less on its potential applications.

✉ Zhiming Rao
raozhm@jiangnan.edu.cn

[1] The Key Laboratory of Industrial Biotechnology of Ministry of Education, School of Biotechnology, Jiangnan University, Wuxi 214122, Jiangsu, People's Republic of China

The industrial production of L-Orn is usually obtained as results of microbial fermentation. Various methods have been described for the L-Orn production by the engineered microorganism. Lee et al. obtained 13.2 mg/g dry cell weight (DCW) L-Orn from engineered *E. coli* [17]. Hwang et al. used mutative *C. glutamicum* to produce L-Orn with a production of 16.49 mg/g DCW [13]. Analysis of the L-Orn biosynthetic pathway using metabolic engineering has led to increased production. Hwang et al. improved L-Orn production by increasing NADPH availability [11, 12], and Jiang et al. constructed the resulting strain, *C. glutamicum* ΔAPRE::*rocG*, which increased the level of NADPH, led to the L-Orn production of 14.84 g/L [14]. Kim et al. knocked out the competing branches gene of L-Orn biosynthesis of *C. glutamicum* ATCC 13032, deleted repressor gene *argR* to remove feedback inhibition and overexpressed the L-arginine synthesis gene cluster *argCJBD* from *C. glutamicum* ATCC 21831, which resulted in the production of 7.19 g/L L-Orn. Fed-batch cultivation of the engineered strain YW06 (PSY223) enabled the production of 51.5 g/L L-Orn [16]. Although microbial fermentation offers relatively high absolute production of L-Orn, the long-term fermentation period is inefficient and wasteful of raw materials. Meanwhile, the complex composition of the fermentation broth make product separation difficult. In comparison, the enzymatic method for L-Orn production from L-Arg is much more efficient. Additionally, the price of L-Arg is three times cheaper than L-Orn, which makes it a potential substrate for the production of L-Orn. As pointed out in 2013, Zhang et al. used a purified arginase from *Bacillus thuringiensis* SK20.001 to catalyze L-Arg for the production of L-Orn, obtaining 72.7 g/L within 10 h [40]. However, this approach was still highly uneconomical because of the high cost and time-consuming nature of enzyme extraction and purification. Over-expression of thermophilic arginase (at an optimal temperature of 60 °C) from *Bacillus caldovelox* in *E. coli* increased the production of L-Orn to 112.3 g/L [28]. Nevertheless, poor stability of this arginase at 60 °C restricted for the higher production of L-Orn and be reused as biocatalyst. Furthermore, the potential pathogenicity of *E. coli* limits its application in the food industry. Therefore, a more efficient and safer method for L-Orn production is needed.

In this study, *argI* encoding arginase from *B. amyloliquefaciens* B10-127 was expressed in the GRAS *B. subtilis* 168 strain [34, 43], and the recombinant arginase was characterized. Additionally, a whole-cell bioconversion system was developed in recombinant *B. subtilis* 168 for conversion of L-Arg to L-Orn, and this system was optimized.

Materials and methods

Bacteria strains, plasmids and materials

Bacillus amyloliquefaciens B10-127, used as a gene resource, was previously isolated by our laboratory. (This strain is preserved in the China Center for Type Culture Collection, preservation number CCTCCM2012349.) *B. subtilis* 168, used as a host, and *E. coli* JM109, used for plasmid construction, existed as laboratory stock strains. The expression vector pMA5 was also preserved in our laboratory. The simple vector pMD™18-T, the restriction enzymes, T4 DNA ligase, and *Ex Taq* DNA polymerase were purchased from TaKaRa Co. (Dalian, China). The Mini Chromosome Rapid Isolation kit, Mini Plasmid Rapid Isolation kit and Mini DNA Rapid Purification kit were obtained from Sangon Biotech Co., Ltd. (Shanghai, China). Methanol and tetrahydrofuran (both chromatographic grade) were obtained from J&K Scientific. L-Arg for bioconversion was food grade (purity ≥99 %); all other chemicals were analytical grade.

Culture medium and conditions

Luria–Bertani (LB) medium (10 g/L tryptone, 5 g/L yeast extract and 10 g/L NaCl) containing 100 μg/mL ampicillin was used for cultivating the recombinant *E. coli* strains. The recombinant *B. subtilis* 168/pMA5-*argI* strain was cultivated in seed medium containing (in g/L): peptone 10, yeast extract 5, K_2HPO_4 2.3, KH_2PO_4 1.7, $MgSO_4$ 0.75, NaCl 5, at pH 6.8–7.0, and fermentation medium containing (in g/L): soy peptone 10, corn steep liquor 5, ammonium citrate 3, glucose 40, K_2HPO_4 2.3, KH_2PO_4 1.7, $MgSO_4$ 0.75, NaCl 5, at pH 6.8–7.0, with 50 μg/mL of kanamycin supplementation in a 5-L fermenter at 300 r/min and 37 °C for 12 h.

Cloning of *argI* from *B. amyloliquefaciens* B10-127

The *argI* gene encoding arginase was amplified from chromosomal DNA of *B. amyloliquefaciens* B10-127 by PCR amplification using *ExTaq* DNA polymerase, with forward primer 5′-ATCCATATGAACAAGAATATCTCAG-3′ (*Nde*I restriction site underlined) and reverse primer 5′-ACCGGGATCCTTAATGATGATGATGATGATGTAATAGTTTTTTTCCTAAC-3′ (*Bam*HI restriction site underlined). The amplification products were isolated and ligated directly into the simple cloning vector pMD™18-T, which was then transformed into chemically competent *E. coli* JM109 cells. The presence of pMD™18-T-*argI* was verified using *Bam*HI and *Nde*I restriction digestion. The resulting plasmid (pMD™18-T-*argI*) and the expression vector pMA5

were digested with *Bam*HI and *Nde*I. The digested fragments and plasmids were separated using agarose gel electrophoresis and purified from the gel. The purified *argI* fragment was then inserted into pMA5. The nucleotide sequence of the *argI* insert was analyzed by Sangon Biotech Co., Ltd.

The recombinant pMA5-*argI* was transformed into *B. subtilis* 168 competent cells using the procedure described by Spizizen et al. [1]. Transformants were obtained following growth overnight in selective LB medium supplemented with 50 μg/mL kanamycin.

Expression of pMA5-*argI* in *B. subtilis* 168

The recombinant strain *B. subtilis* 168/pMA5-*argI* was cultivated in LB medium at 37 °C for 12 h. Cells were harvested by centrifugation at 10,000×*g* for 10 min at 4 °C and washed twice with 50 mM Tris–HCl (pH 8.0). The washed cells harboring recombinant arginase were then resuspended in 50 mM Tris–HCl. The cell paste was then sonicated for 15 min (2 s worktime with 5 s interval). Cell extracts were centrifuged for 30 min at 10,000×*g* to remove cell debris. The supernatant (crude arginase) was analyzed using an arginase enzyme activity assay and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis (12 % acrylamide).

Purification and SDS-PAGE analysis of arginase

The N-terminal His-tagged arginase was purified in a single step using Ni–NTA affinity chromatography, sequential washing and elution procedures were performed according to the protocol of GE Healthcare Bio-Sciences. The active fractions were collected for SDS-PAGE analysis (12 % acrylamide) and arginase enzyme assay or stored at −40 °C for further analysis.

Arginase activity assay

The arginase assay was carried out spectrophotometrically by measuring the formation of L-Orn at 515 nm with ninhydrin. The standard mixture contained 50 mM sodium carbonate buffer, containing 0.2 M L-Arg at pH 10.0, 50 μL of 20 mM $MnCl_2$, and 50 μL crude arginase solution in a total volume of 1 mL. The reaction mixture was incubated at 40 °C for 5 min and terminated by heating for 5 min. The L-Orn concentration was measured as described by Chinard et al. [5]. One unit of arginase activity was defined as the amount of enzyme that generates 1 μmol L-Orn in 1 min. L-Ornithine monohydrochloride was used as the standard. The concentration of protein was determined using a Bradford Protein Assay Kit (Sangon Biotech Co., Ltd). The specific activity was determined by arginase activity and protein concentration.

Arginase enzyme characterization

Optimal pH and pH stability

The optimal pH for arginase activity was confirmed in the following buffers containing 0.2 M L-Arg: citric acid–Na_2HPO_4 (pH 5.0–6.5), sodium phosphate (pH 6.6–7.5), Tris–HCl (pH 8.0–8.5), glycine–NaOH (pH 8.6–9.3), sodium carbonate (pH 9.4–10.9) and sodium carbonate–NaOH buffer (pH 11.0–12.0). Meanwhile, the pH stability was determined by incubation at different pH levels at 4 °C.

Optimal temperature and thermal stability

The effect of temperature on arginase was determined by measuring enzyme activity between 20 and 65 °C. To test the thermal stability, the enzyme was incubated at various temperatures (4, 20, 30, 40, 45, 50, 60 °C) in the presence of 50 mM Tris–HCl (pH 8.0) for 20 h.

Metal ions preference

The purified enzyme solution was first dialyzed against 10 mM Tris–HCl buffer (pH 8.0) for 12 h at 4 °C. Enzyme activity was then assessed as described above in the presence of the following divalent cations: (Cu^{2+}, Ca^{2+}, Co^{2+}, La^{3+}, K^+, Fe^{2+}, Mg^{2+}, Ba^{2+}, Mn^{2+}, Fe^{3+}, Al^{3+}, Zn^{2+} and Na^+) and EDTA at 1 mM. The assay mixture lacking ions served as the control.

Whole-cell biocatalyst-based bioconversion for L-Orn production

The recombinant strain *B. subtilis* 168/pMA5-*argI* cells were cultivated in LB medium at 37°C for 12 h (OD ≈ 3.2–3.5). Cells were harvested by centrifugation at 10,000×*g* at 4 °C and washed twice with 50 mM sodium phosphate buffer (pH 10.0). The 50 mL cell cultures were then resuspended into a 50-mL bioconversion mixture (0.2 M sodium phosphate buffer, pH 10.0) containing 200 g/L L-Arg and 1 mM $MnCl_2$. The whole-cell bioconversion was performed on a rotary shaker (160 r/min) at 40 °C.

Analysis methods

The concentrations of L-Arg and L-Orn in the bioconversion mixture were measured by HPLC (Agilent 1260, Agilent Technologies, Palo Alto, CA, USA) with UV detector at 338 nm as described by Georgi et al. [8, 25]. ZORBAX

SB-C18 column (4.6 × 150 mm 5-Micron, Agilent) was used. The buffer gradient consisted of Buffer A (0.1 M sodium acetate with 0.023 % triethylamine and 0.5 % tetrahydrofuran, pH 7.2) and Buffer B (40 % methanol, 40 % acetonitrile and 20 % 0.15 M sodium acetate), with gradient elution at 40 °C and a flow rate of 1 mL/min. o-Phthaldialdehyde was used as the pre-column derivation reagent. Yield was defined as the amount of L-Orn produced from a given amount of L-Arg added. Productivity was defined as the mass of L-Orn production per hour and per liter and used to determine the efficiency of bioconversion.

$$\text{Yield} = \frac{m_{\text{L-Orn}}}{132} \div \frac{m_{\text{L-Arg}}}{174} \times 100\,\% \quad (1)$$

$$\text{Productivity} = \frac{m_{\text{L-Orn}}}{t \times v} \quad (2)$$

where $m_{\text{L-Orn}}$ is the mass of L-Orn in the bioconversion mixture, $m_{\text{L-Arg}}$ is the mass of L-Arg that was added; V is the volume of bioconversion mixture; t is the bioconversion time (h), and 132 and 174 are the relative molecular weights of L-Orn and L-Arg, respectively.

Optimization of bioconversion conditions

The optimization of whole-cell bioconversion conditions was based on the properties of the arginase. To determine the effect of substrate concentration in the bioconversion mixture, an L-Arg concentration range from 20 to 200 g/L was used. Bioconversion was carried out as previously described. After 1 h, the amount of L-Orn and L-Arg in the bioconversion mixture was quantified by HPLC.

Bioconversion buffer optimization was performed using the following buffers (pH 10.0) containing 200 g/L L-Arg and 1.0 mM $MnCl_2$:sodium phosphate, Na_2HPO_4–citric acid, sodium citrate–citric acid, Tris–HCl, sodium carbonate, and boric acid–borax. Bioconversion was carried out for 2 h as previously described. The concentration of L-Orn in the various bioconversion mixtures was measured. In addition, different concentrations of Mn^{2+} (0.1–1.0 mM) were used in the mixtures to investigate the effect of Mn^{2+} on bioconversion. Relative productivity was used as a measurement of Mn^{2+} influence.

Whole-cell bioconversion under fed-batch strategy

The recombinant cells were cultivated in the fermentation culture for 12 h ($OD_{600} \approx 14.9$–15.6), and 2 L of recombinant *B. subtilis* 168/pMA5-*argI* culture cells was harvested and resuspended into 2 L of bioconversion mixture (0.25 M sodium carbonate buffer at pH 10.0) containing 200 g/L L-Arg and 0.6 mM $MnCl_2$. Whole-cell bioconversion was carried out in a 5 L reactor. L-Arg was added into the reactor to maintain the substrate at a reasonable level. The pH and temperature were maintained automatically by the reactor at 10.0 and 40 °C, respectively.

Results and discussion

Construction of recombinant plasmid pMA5-*argI*

The *argI* gene was successfully amplified from the genomic DNA of *B. amyloliquefaciens* B10-127 by PCR. The amplicon was cloned into the pMD™18-T simple vector and verified by restriction enzyme digestion. Subsequently, the *argI* fragment was inserted into pMA5 and resulted in the recombinant plasmid pMA5-*argI*. This insertion was verified and analyzed by DNA sequencing. The results revealed that the open reading frame (ORF) of *argI* consisted of 891 bases (GenBank accession number KR363261).

Expression of *argI* and SDS-PAGE analysis of arginase

The verified recombinant plasmid pMA5-*argI* was transformed into the expression host strain *B. subtilis* 168 to construct the recombinant strain *B. subtilis* 168/pMA5-*argI*, which expressed *argI* under the control of the *Hpa* II promoter. *B. subtilis* 168 recombinants were selected with kanamycin and verified by DNA sequencing.

The transformant harboring pMA5-*argI* was inoculated in LB medium for 12 h. The recombinant arginase expression was confirmed by 12 % (*w*/*v*) SDS-PAGE analysis. As shown in Fig. 1, *argI* was highly expressed in *B. subtilis* 168. The SDS-PAGE analysis showed that the molecular weight (MW) of the recombinant arginase was approximately 33 kDa (including 6× His-Tag), in agreement with the prediction by gene sequencing.

Purification and activity assay of arginase

The recombinant strain *B. subtilis* 168/pMA5-*argI* was incubated in LB medium for 12 h and cells were harvested. The cell lysate supernatant exhibited an arginase specific activity of 21.9 U/mg, which is 26.7 times that of wild *B. subtilis* 168 and 21.5 times of *B. amyloliquefaciens* B10-127. (Arginase of *B. subtilis* 168 and *B. amyloliquefaciens* B10-127 exhibited an enzyme activity of 0.82 and 1.02 U/mg, respectively.) The recombinant arginase was purified by Ni–NTA and verified by SDS-PAGE analysis. As shown in Fig. 1, purified arginase appeared in a single band with a molecular weight of approximately 33 kDa. The resulting purified arginase exhibited a specific activity of 94.2 U/mg (Table 1) with an enzyme activity yield of 80.7 %.

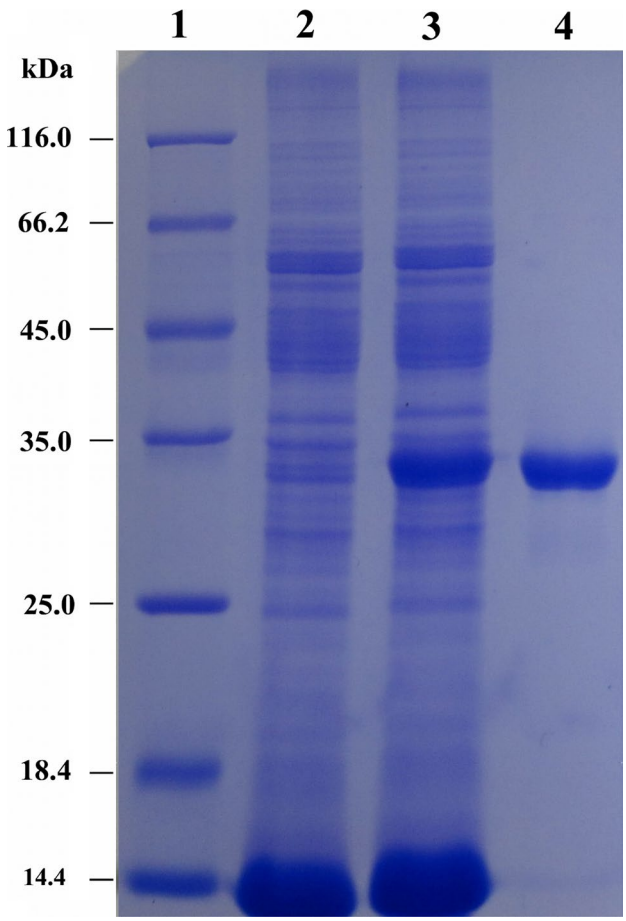

Fig. 1 SDS-PAGE analysis of the recombinant strain *B. subtilis* 168/pMA5-*argI*. *Lane 1* protein marker, *Lane 2* cell extraction of wild *B. subtilis* 168, *Lane 3* cell extraction of recombinant *B. subtilis* 168/pMA5-*argI*, *Lane 4* purified arginase

Characterization of recombinant arginase

Figure 2a shows that the optimal temperature of this recombinant arginase is 40 °C, which is similar to arginase from *B. thuringiensis* SK 20.001 [40]. As previously reported, arginase from different species has an optimal temperature between 30 and 60 °C [19, 23, 37]. Arginase from *B. caldovelox* exhibits the highest optimal temperature measured to date, at 60 °C [7, 23]. The optimal pH of the recombinant arginase is 10.0 (Fig. 2c), which indicates that it is an alkaline enzyme. This finding is quite different from arginase from *H. pylori*, which exhibited an acidic pH preference and an optimal pH of 6.1 as reported by McGee and Zhang et al. [19, 39]. It has a slight difference from arginase from *B. subtilis* 168, which exhibited an optimal pH of 8.4 [35].

As shown in Fig. 2b, the recombinant arginase exhibited good stability below 40 °C, it retained about 88.42 % of its residual activity after incubation at 20 °C for 20 h, and showed about 80.33 % of retention of activity after 20 h incubation in 30 °C. However, enzyme stability decreased rapidly when the temperature reached 60 °C (approximately 50 % decrease in activity after incubation in 60 °C for 3 h). Additionally, the arginase exhibited good stability at pH values ranging from 8.0 to 10.0. As shown in Fig. 2d, 89.96 % of the maximum activity was retained after incubation for 25 h at pH 9.0. However, the stability decreased rapidly when the pH is above 11.0.

The activity of recombinant arginase in the presence of different metal ions is shown in Fig. 2e. The results indicate that Mn^{2+} improved enzyme activity considerably. The activity of arginase with 1 mM $MnCl_2$ added was almost twofold higher than that without the addition of ions. Other divalent ions promoted activity: the addition of Cu^{2+} and Ba^{2+} resulted in 122.9 and 116.7 % of the relative activity compared to the control, respectively. Trivalent ions (Fe^{3+} and Al^{3+}) also exhibited a positive effect on recombinant arginase activity. In contrast, K^+ slightly inhibited the activity of arginase. Finally, EDTA, acting as a metal chelator, strongly inhibited the activity of the recombinant enzyme, indicating that arginase is a metal-ion-activated enzyme.

Recombinant whole-cell bioconversion for the production of L-Orn

In this study, whole cells of the recombinant strain *B. subtilis* 168/pMA5-*argI* were used as a biocatalyst, and L-Arg was employed as a substrate. *B. subtilis* 168/pMA5-*argI* was inoculated and cultivated in 50-mL LB medium for 12 h ($OD_{600} \approx 3.5$), and the cells were harvested and resuspended into 50 mL of the standard bioconversion mixture (0.2 M phosphate buffer, pH 10.0) containing 200 g/L L-Arg and 1 mM $MnCl_2$. The whole-cell bioconversion was carried out at 40 °C and 160 r/min in a rotary shaker. As a result, 200 g/L L-Arg was almost depleted after 4 h. As shown in Fig. 3, 148.56 g/L L-Orn and 2.3 g/L L-Arg were detected

Table 1 Arginase enzyme activity assay of wild and recombinant strains

Strains	Total activity (U)	Total protein (mg)	Specific activity (U/mg)
B. amyloliquefaciens B10-127	36.3 ± 1.58	35.6 ± 0.98	1.02 ± 0.17
B. subtilis 168	26.8 ± 1.34	32.7 ± 0.93	0.82 ± 0.13
B. subtilis 168/pMA5-*argI*	746.8 ± 8.27	34.1 ± 0.87	21.9 ± 0.92
Purified arginase	602.8 ± 7.47	6.4 ± 0.43	94.2 ± 2.36

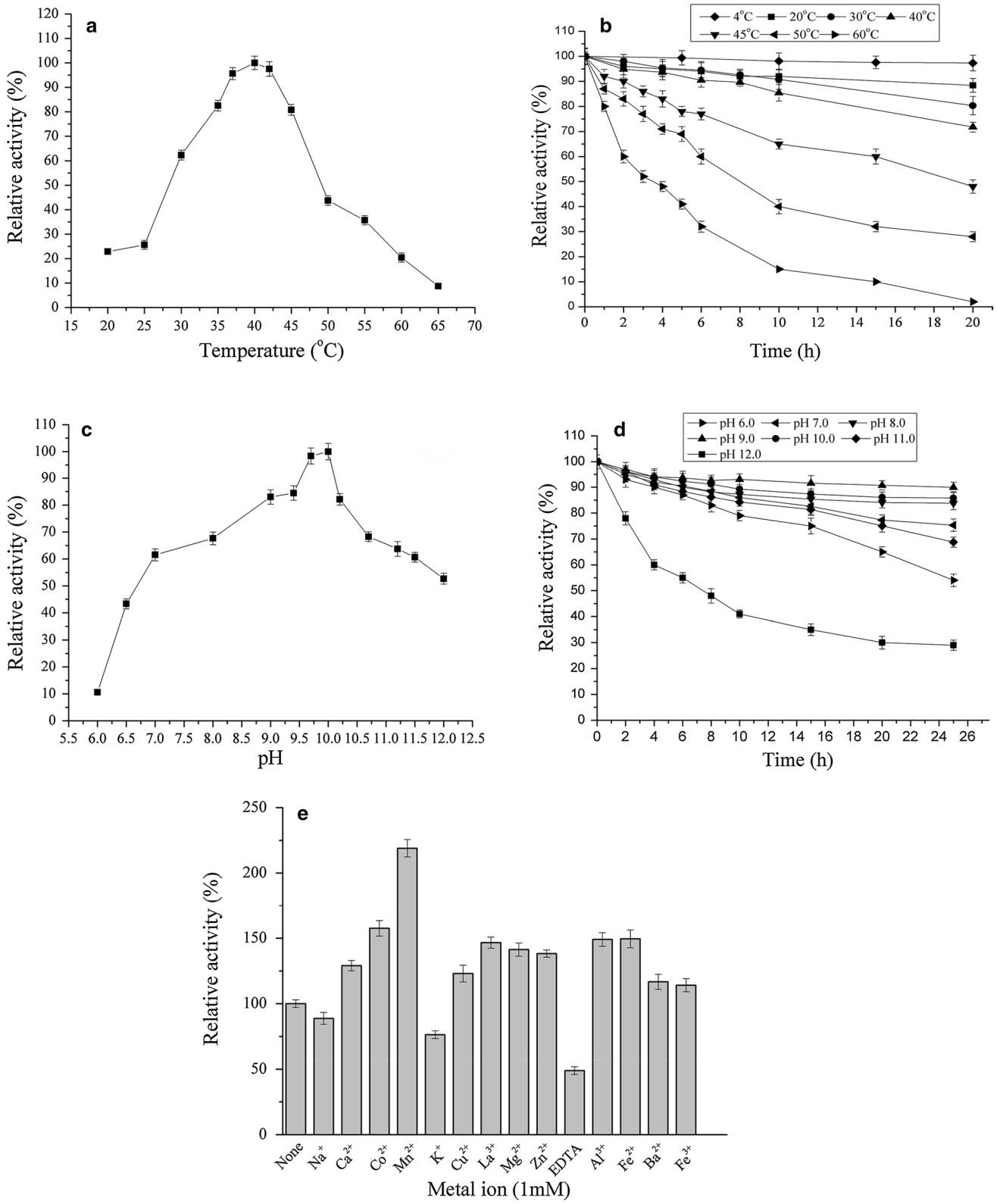

Fig. 2 Characterization the recombinant arginase. **a** Optimal temperature of the recombinant arginase; **b** thermal stability; **c** optimal pH of the recombinant arginase; **d** pH stability; **e** metal ion preference of the recombinant arginase. Data are mean ± SD for three replicates. *Bars* indicate standard deviations. The following pH buffers were employed for the optimal pH experiment: 0.2 M citric acid–Na_2HPO_4 (pH 5.0–6.5); sodium phosphate (NaH_2PO_4–Na_2HPO_4) (pH 6.6–7.5); Tris–HCl (pH 8.0–8.5); glycine–NaOH (pH 8.6–9.3); sodium carbonate ($NaCO_3$–$NaHCO_3$) (pH 9.4–10.9) and sodium carbonate–NaOH (pH 11.0–12.0)

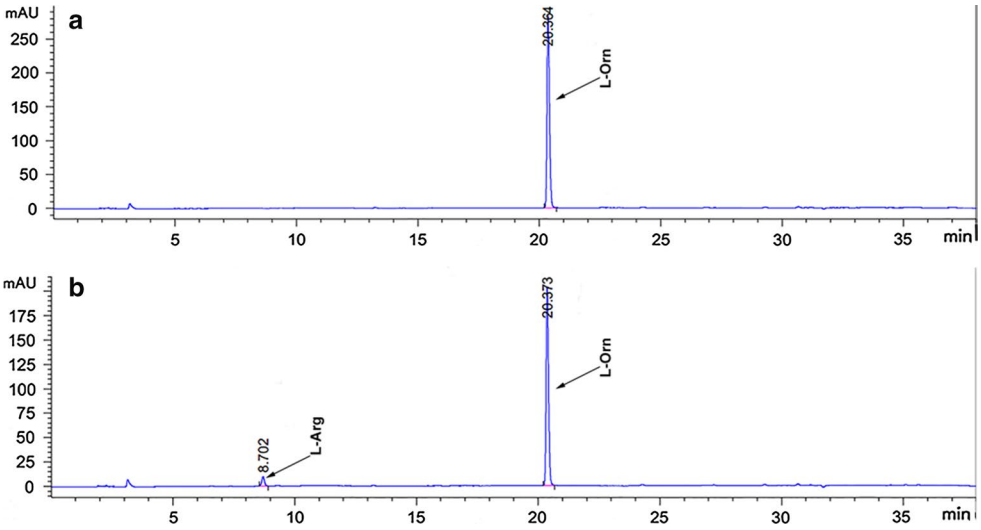

Fig. 3 HPLC analysis of L-ornithine and L-arginine. **a** HPLC analysis of standard L-ornithine monohydrochloride (1 g/L); **b** HPLC analysis of bioconversion mixture (dilution factor = 300)

Table 2 Concentration of L-Orn and L-Arg in the bioconversion mixture

Strains	L-Orn concentration (g/L)	L-Arg concentration (g/L)
B. amyloliquefaciens B10-127	15.8 ± 1.02	179.7 ± 2.03
Bacillus subtilis 168	12.5 ± 0.92	183.6 ± 1.23
Bacillus subtilis 168/pMA5-*argI*	148.7 ± 1.45	2.3 ± 0.43

in the bioconversion mixture. The yield was 97.9 %. The titre is highly efficient compared with those obtained from the more primitive strains. Under the same conditions, *B. subtilis* 168 and *B. amyloliquefaciens* B10-127 could produce only 12.5 and 15.8 g/L L-Orn, respectively (Table 2). According to the previous study, the activity of arginase is inhibited by a high L-Orn concentration [15, 27]. However, we observed that L-Orn had a lower inhibitive effect on this recombinant arginase compared with the previous study [28]. The effect of L-Orn concentration on bioconversion was thus investigated further. As shown in Fig. 4, the $K_{i(\text{L-Orn})}$ (the L-Orn concentration producing 50 % inhibition on bioconversion) of bioconversion is 152.6 g/L.

Optimization of whole-cell bioconversion conditions

Substrate concentration

In this study, concentrations of L-Arg ranging from 20 to 200 g/L in the bioconversion mixture were investigated. The results showed that L-Arg does not inhibit the bioconversion; in contrast, productivity improved with increasing L-Arg concentration. After 1 h, 53.1 g/L L-Orn was obtained when the initial L-Arg concentration was 200 g/L, whereas 13.6 g/L L-Orn was obtained when the initial L-Arg concentration was 20 g/L. These results, shown in Fig. 5a, indicate that a concentration of L-Arg maintained at 120–200 g/L is likely more suitable for high productivity.

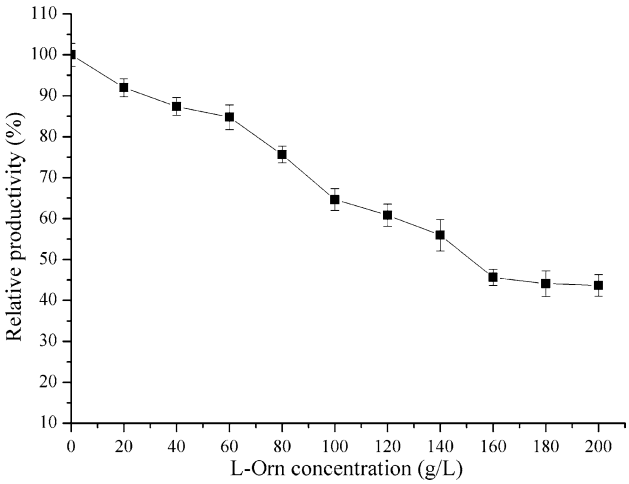

Fig. 4 The effect of L-Orn concentration on whole-cell bioconversion. Bioconversion was carried out under a standard bioconversion mixture (0.2 M sodium phosphate buffer, 200 g/L L-Arg, 1 mM $MnCl_2$, pH 10.0) at 40 °C for 1 h. The concentration of L-Orn in the bioconversion mixture was assayed by HPLC. The group with zero initial concentration of L-Orn is defined as the control. *Bars* indicate standard deviations of three replicates

Mn^{2+} concentration

Our previous study revealed that arginase was sensitive to Mn^{2+}. To further improve production, the concentration of Mn^{2+} in the bioconversion mixture was optimized. As

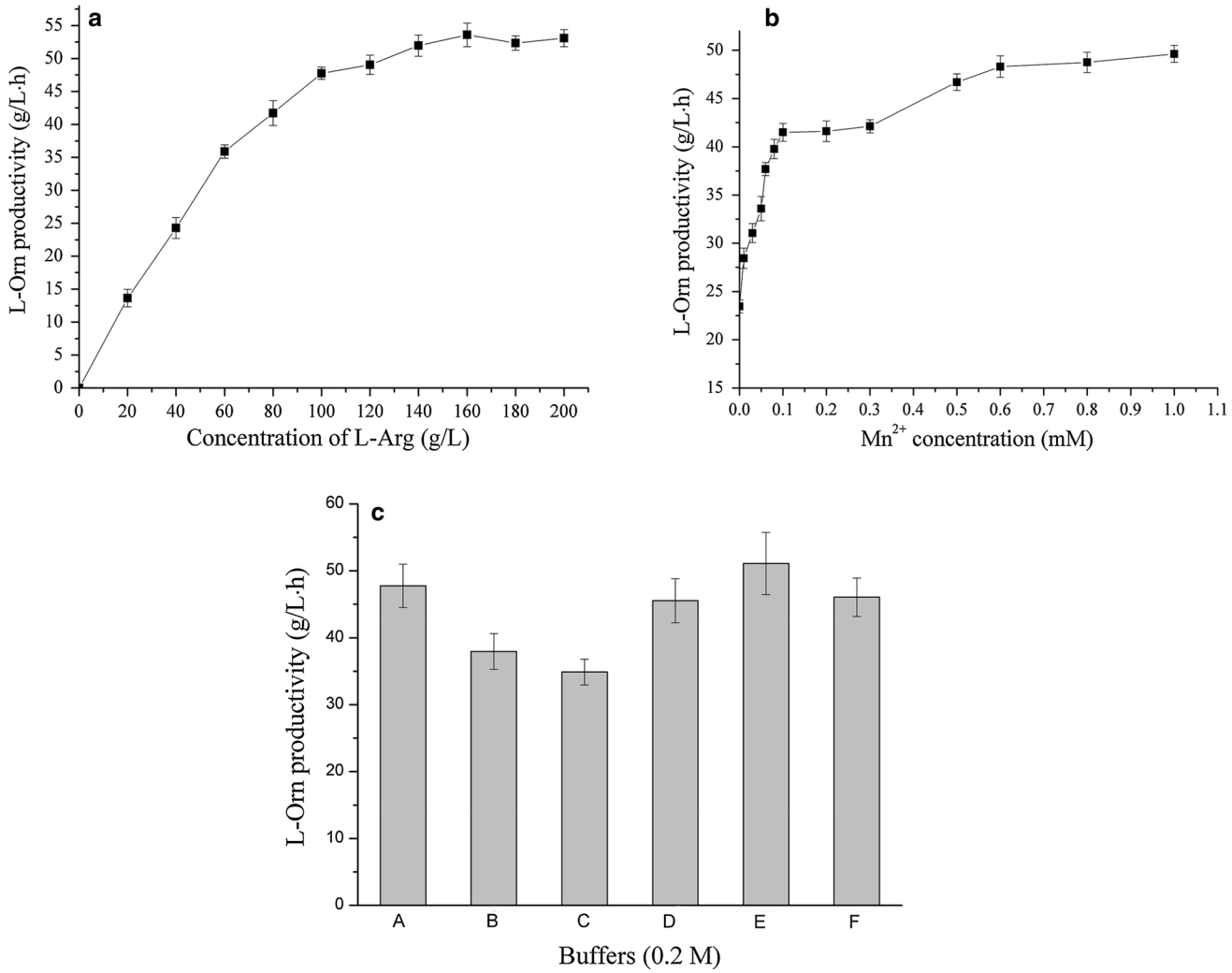

Fig. 5 Optimization of whole-cell bioconversion the bioconversion conditions. Bioconversion was carried out under a standard bioconversion mixture at 40 °C. **a** Optimization of the concentration of substrate; **b** optimization of the concentration of Mn^{2+}; **c** optimization of whole-cell bioconversion buffer. Data are mean ± SD for three replicates. *Bars* indicate standard deviations. Bioconversion was carried out under a standard bioconversion mixture at 40 °C for 2 h. The buffers were *A* sodium phosphate; *B* Na_2HPO4–citric acid; *C* sodium citrate–citric acid; *D* Tris–HCl; *E* sodium carbonate; *F* boric acid–borax

shown in Fig. 5b, the productivity improved as the Mn^{2+} concentration increased; in fact, with the presence of 1 mM Mn^{2+} during the bioconversion, the productivity [49.6 g/(L h)] was more than twice as high as that of the control [23.4 g/(L h)]. However, further increases in the Mn^{2+} concentration of the bioconversion mixture are unproductive because of saturation under alkaline conditions (pH 10.0). The increasing concentrations of Mn^{2+} eventually generate the precipitate $Mn(OH)_2$, which adversely affects L-Orn production. The optimal concentration of Mn^{2+} should ideally promote productivity while ameliorating this potential negative effect. When the concentration of Mn^{2+} was 0.6 mM, the corresponding L-Orn productivity was 48.3 g/(L h), which is 97.3 % of the maximum value. Because 0.6 mM Mn^{2+} is less prone to form $Mn(OH)_2$ compared with 1.0 mM Mn^{2+}, the concentration of 0.6 mM Mn^{2+} was selected for bioconversion.

Bioconversion buffer

L-Arg (pI 10.76) is a basic amino acid that has a significant influence on the pH of the bioconversion mixture. In our study, a decrease in the pH of the bioconversion mixture was observed as L-Arg was converted to L-Orn and urea. The reason for the pH alteration can be explained by the presence of the positively charged guanidinium group of L-Arg, which makes it more basic compared with L-Orn (pI 9.7). When 200 g/L L-Arg was converted to L-Orn and urea, the pH of the bioconversion mixture decreased from 10.0 to 9.24. As a result, the recombinant arginase exhibited

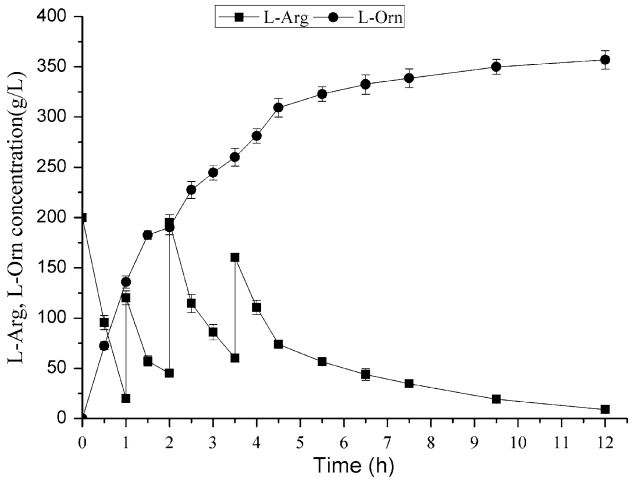

Fig. 6 Fed-batch strategy of whole-cell bioconversion for the production of L-Orn. *Bars* indicate standard deviation

approximately 84 % of the maximum activity found in our previous study (Fig. 3c). A more efficient conversion buffer was required to maintain the pH of the bioconversion mixture at a reasonable level so that the conversion could continue at high efficiency. Different buffers (0.2 M) were tested under identical conditions. It was shown that bioconversion using alkaline buffer (e.g., sodium carbonate, sodium phosphate) was more efficient compared with acidic or neutral buffers (e.g., Na_2HPO_4–citric acid buffer, sodium citrate–citric acid buffer). With the using of sodium carbonate buffer for bioconversion for 2 h, 102.2 g/L L-Orn was achieved with a productivity of 51.1 g/(L h). This was the highest production of all the buffers used in bioconversion (Fig. 5c). In this regard, the sodium carbonate buffer is potentially the optimal buffer for bioconversion. Further optimization was carried out using different concentrations of sodium carbonate. The results indicate that the optimal concentration of sodium carbonate is 0.25 M.

Whole-cell bioconversion using fed-batch strategy under optimum conditions

Whole-cell bioconversion was conducted under optimal conditions in a 5-L reactor. A 2-L culture volume of recombinant *B. subtilis* 168/pMA5-*argI* cells was harvested and resuspended into a 2-L bioconversion mixture (0.25 M sodium carbonate buffer, 0.6 mM $MnCl_2$, 200 g/L L-Arg, pH 10.0). L-Arg was added into the bioconversion mixture to maintain the substrate at a reasonable concentration as described above. As shown in Fig. 6, after bioconversion for 1 h, 135.8 g/L L-Orn and 20.3 g/L L-Arg were detected in the bioconversion mixture. At this point, 100 g/L L-Arg was added into the bioconversion mixture to maintain conversion with high efficiency. Over the first 2.5 h, 227.5 g/L L-Orn was produced with an average productivity of 91.0 g/(L·h). However, the productivity subsequently decreased because of arginase inhibition in response to the high concentration of L-Orn. The average productivity from 2.5 to 5.5 h was 31.7 g/(L h). During the final 6.5 h (5.5–12 h), the average L-Orn productivity was a relatively low 5.2 g/(L h) because of the low substrate concentration and feedback inhibition of arginase. The concentration of L-Arg in the bioconversion mixture decreased from 56.5 to 9.4 g/L in the final 6.5 h. Additionally, the yield decreased with increasing concentration (>190 g/L) of L-Orn in the bioconversion mixture. The reason is that a high concentration of L-Orn promotes lactamization to form 3-aminopiperidine-2-one under extreme alkaline conditions [21, 24]. Ultimately, 356.9 g/L L-Orn was obtained within 12 h with a yield of 85.5 %.

In previous studies, bioconversion for L-Orn production was reported using recombinant *E. coli* or the purified arginase [38, 40]. Although L-Orn could be applied as a food supplement and food additive, food safety must be taken into account in the food industry. Compared with *E. coli*, *B. subtilis* is a GRAS strain widely used in the industry. In this study, a recombinant *B. subtilis* 168 was used for high L-Orn production and constitutes a safer production method compared with the previous study. Song et al. reported that 112.3 g/L L-Orn was obtained within 4 h, which is nearly the highest production rate of L-Orn ever reported [28]. However, the high concentration of residual substrate (>30 g/L) made product separation difficult. In this study, within 12 h, 356.9 g/L L-Orn was achieved with only 9.4 g/L L-Arg residue, which demonstrates it to be a more efficient L-Orn production method—one that is suitable for industry implementation.

Conclusion

Bacillus subtilis is a GRAS strain that is widely used in the food industry, as noted above. To our knowledge, we are the first to successfully construct recombinant *B. subtilis* 168 that expresses *argI* from *B. amyloliquefaciens* B10-127. An enzyme activity assay demonstrated that the recombinant strain exhibited a relatively high arginase activity. For a better understanding of the arginase properties, the recombinant arginase was characterized. The recombinant strain cells (i.e., the whole cells) were harvested and used as a biocatalyst for the production of L-Orn. The results indicated that this was a highly efficient method, producing 356.9 g/L L-Orn within 12 h using the fed-batch strategy. This is the highest L-Orn production reported to date, to our knowledge. Furthermore, only 9.4 g/L L-Arg remained as residual material. This low substrate concentration makes it easier to separate the product (L-Orn) from the substrate

(L-Arg). The use of whole-cell bioconversion for the production of L-Orn in this study has promising implications for L-Orn production in industry. However, further improvement of production is limited because L-Orn inhibits the activity of arginase. In our present study, the whole-cell biocatalyst was recycled for additional future conversion. As a result, the enzyme activity of arginase recovered from its high inhibition by L-Orn. It is proposed that further enzyme stability improvement combined with cell immobilization could increase L-Orn production even further.

Acknowledgments This work was supported by the High-tech Research and Development Programs of China (2015AA021004), the National Natural Science Foundation of China (31300028), the Research Project of Chinese Ministry of Education (113033A), the Jiangsu Provincial National Basic Research Program (BK20130137), the Fundamental Research Funds for the Central Universities (JUSRP51306A), the Project Funded by the Priority Academic Program Development of Jiangsu Higher Education Institutions, the 111 Project (No. 111-2-06) and the Jiangsu province "Collaborative Innovation Center for Advanced Industrial Fermentation" industry development program.

References

1. Anagnostopoulos C, Spizizen J (1961) Requirements for transformation in *Bacillus Subtilis*. J Bacteriol 81:741–746
2. DE Ash (2004) Structure and function of arginases. J Nutr 134:2760S–2764S **(discussion 2765S–2767S)**
3. Bewley MC, Lott JS, Baker EN, Patchett ML (1996) The cloning, expression and crystallisation of a thermostable arginase. FEBS Lett 386:215–218. doi:10.1016/0014-5793(96)00459-0
4. Callery EM, Elinson RP (1996) Developmental regulation of the urea-cycle enzyme arginase in the direct developing frog *Eleutherodactylus coqui*. J Exp Zool 275:61–66. doi:10.1002/(SICI)1097-010X(19960501)275:1<61:AID-JEZ9>3.0.CO;2-8
5. Chinard FP (1952) Photometric estimation of proline and ornithine. J Biol Chem 199:91–95
6. Dakshayani KB, Velvizhi S, Subramanian P (2002) Effects of ornithine alpha-ketoglutarate on circulatory antioxidants and lipid peroxidation products in ammonium acetate treated rats. Ann Nutr Metab 46:93–96. doi:10.1159/000063076
7. El-Sayed AS, Shindia AA, Diab AA, Rady AM (2014) Purification and immobilization of L-arginase from thermotolerant *Penicillium chrysogenum* KJ185377.1; with unique kinetic properties as thermostable anticancer enzyme. Arch Pharm. doi:10.1007/s12272-014-0498-y
8. Georgi T, Rittmann D, Wendisch VF (2005) Lysine and glutamate production by *Corynebacterium glutamicum* on glucose, fructose and sucrose: roles of malic enzyme and fructose-1,6-bisphosphatase. Metab Eng 7:291–301. doi:10.1016/j.ymben.2005.05.001
9. Goldraij A, Polacco JC (1999) Arginase is inoperative in developing soybean embryos. Plant Physiol 119:297–303
10. Gotoh T, Sonoki T, Nagasaki A, Terada K, Takiguchi M, Mori M (1996) Molecular cloning of cDNA for nonhepatic mitochondrial arginase (arginase II) and comparison of its induction with nitric oxide synthase in a murine macrophage-like cell line. FEBS Lett 395:119–122
11. Hwang GH, Cho JY (2012) Implication of gluconate kinase activity in L-ornithine biosynthesis in *Corynebacterium glutamicum*. J Ind Microbiol Biotechnol 39:1869–1874. doi:10.1007/s10295-012-1197-7
12. Hwang GH, Cho JY (2014) Enhancement of L-ornithine production by disruption of three genes encoding putative oxidoreductases in *Corynebacterium glutamicum*. J Ind Microbiol Biotechnol 41:573–578. doi:10.1007/s10295-013-1398-8
13. Hwang JH, Hwang GH, Cho JY (2008) Effect of increased glutamate availability on L-ornithine production in *Corynebacterium glutamicum*. J Ind Microbiol Biotechnol 18:704–710
14. Jiang LY, Zhang YY, Li Z, Liu JZ (2013) Metabolic engineering of *Corynebacterium glutamicum* for increasing the production of L-ornithine by increasing NADPH availability. J Ind Microbiol Biotechnol 40:1143–1151. doi:10.1007/s10295-013-1306-2
15. Kanda M, Ohgishi K, Hanawa T, Saito Y (1997) Arginase of *Bacillus brevis* Nagano: purification, properties, and implication in gramicidin S biosynthesis. Arch Biochem Biophys 344:37–42. doi:10.1006/abbi.1997.0174
16. Kim SY, Lee J, Lee SY (2015) Metabolic engineering of *Corynebacterium glutamicum* for the production of L-ornithine. Biotechnol Bioeng 112:416–421. doi:10.1002/bit.25440
17. Lee YJ, Cho JY (2006) Genetic manipulation of a primary metabolic pathway for L-ornithine production in *Escherichia coli*. Biotechnol Lett 28:1849–1856. doi:10.1007/s10529-006-9163-y
18. Lisowska-Myjak B, Tomaszewski L, Hryckiewicz L (1978) Intestinal arginase in vertebrates and invertebrates. Comp Biochem Physiol Part B 61:545–552
19. McGee DJ, Zabaleta J, Viator RJ, Testerman TL, Ochoa AC, Mendz GL (2004) Purification and characterization of *Helicobacter pylori* arginase, RocF: unique features among the arginase superfamily. Eur J Biochem 271:1952–1962. doi:10.1111/j.1432-1033.2004.04105.x
20. Morris SM Jr, Bhamidipati D, Kepka-Lenhart D (1997) Human type II arginase: sequence analysis and tissue-specific expression. Gene 193:157–161
21. Murray K, Rasmussen PS, Neustaedter J, Luck JM (1965) The hydrolysis of arginine. J Biol Chem 240:705–709
22. Omori K, Kagami Y, Yokoyama C, Moriyama T, Matsumoto N, Masaki M, Nakamura H, Kamasaka H, Shiraishi K, Kometani T, Kuriki T, Huang ZL, Urade Y (2012) Promotion of non-rapid eye movement sleep in mice after oral administration of ornithine. Sleep Biol Rhythms 10:38–45. doi:10.1111/j.1479-8425.2011.00515.x
23. Patchett ML, Daniel RM, Morgan HW (1991) Characterisation of arginase from the extreme thermophile *Bacillus caldovelox*. Biochim Biophys Acta 1077:291–298
24. Rivard DE (1955) Chemical preparation of L-ornithine from L-arginine. J Am Chem Soc 5:1260–1261
25. Schneider J, Niermann K, Wendisch VF (2011) Production of the amino acids L-glutamate, L-lysine, L-ornithine and L-arginine from arabinose by recombinant *Corynebacterium glutamicum*. J Biotechnol 154:191–198. doi:10.1016/j.jbiotec.2010.07.009
26. Shi HP, Fishel RS, Efron DT, Williams JZ, Fishel MH, Barbul A (2002) Effect of supplemental ornithine on wound healing. J Surg Res 106:299–302. doi:10.1006/jsre.2002.6471
27. Shimotohno KW, Iida J, Takizawa N, Endo T (1994) Purification and characterization of arginine amidinohydrolase from *Bacillus brevis* TT02-8. Biosci Biotechnol Biochem 58:1045–1049. doi:10.1271/bbb.58.1045
28. Song W, Niu PQ, Chen XL, Liu LM (2014) Enzymatic production of L-ornithine from L-arginine with recombinant thermophilic arginase. J Mol Catal B-Enzym 110:1–7. doi:10.1016/j.molcatb.2014.09.005
29. Srivastava A, Sau AK (2010) Biochemical studies on *Helicobacter pylori* arginase: insight into the difference in activity compared to other arginases. IUBMB Life 62:906–915. doi:10.1002/iub.401

30. Sugino T, Shirai T, Kajimoto Y, Kajimoto O (2008) L-Ornithine supplementation attenuates physical fatigue in healthy volunteers by modulating lipid and amino acid metabolism. Nutr Res 28:738–743. doi:10.1016/j.nutres.2008.08.008
31. Taylor AA, Stewart GR (1981) Tissue and subcellular localization of enzymes of arginine metabolism in *Pisum sativum*. Biochem Biophys Res Commun 101:1281–1289
32. Todd CD, Cooke JEK, Gifford DJ (2001) Purification and properties of *Pinus taeda* arginase from germinated seedlings. Plant Physiol Bioch 39:1037–1045
33. Tokuyama E, Shibasaki T, Kawabe H, Mukai J, Okada S, Uchida T (2006) Bitterness suppression of BCAA solutions by L-ornithine. Chem Pharm Bull Tokyo 54:1288–1292
34. Westers L, Westers H, Quax WJ (2004) *Bacillus subtilis* as cell factory for pharmaceutical proteins: a biotechnological approach to optimize the host organism. BBA Mol Cell Res 1694:299–310. doi:10.1016/j.bbammcr.2004.02.011
35. Yu JJ, Park KB, Kim SG, Oh SH (2013) Expression, purification, and biochemical properties of arginase from *Bacillus subtilis* 168. J Microbiol 51:222–228. doi:10.1007/s12275-013-2669-9
36. Zajac A, Poprzecki S, Zebrowska A, Chalimoniuk M, Langfort J (2010) Arginine and ornithine supplementation increases growth hormone and insulin-like growth factor-1 serum levels after heavy-resistance exercise in strength-trained athletes. J Strength Cond Res 24:1082–1090. doi:10.1519/JSC.0b013e3181d321ff
37. Zakalskiy AE, Zakalska OM, Rzhepetskyy YA, Potocka N, Stasyk OV, Horak D, Gonchar MV (2012) Overexpression of (His)6-tagged human arginase I in *Saccharomyces cerevisiae* and enzyme purification using metal affinity chromatography. Protein Expr Purif 81:63–68. doi:10.1016/j.pep.2011.09.001
38. Zhan Y, Liu J, Mao P, Zhang H, Liu Q, Jiao Q (2013) Biotransformation of L-ornithine from L-arginine using whole-cell recombinant arginase. World J Microbiol Biotechnol 29:2167–2172. doi:10.1007/s11274-013-1382-5
39. Zhang J, Zhang X, Wu C, Lu D, Guo G, Mao X, Zhang Y, Wang DC, Li D, Zou Q (2011) Expression, purification and characterization of arginase from *Helicobacter pylori* in its apo form. PLoS One 6:e26205. doi:10.1371/journal.pone.0026205
40. Zhang T, Guo YJ, Zhang H, Mu WM, Miao M, Jiang B (2013) Arginase from *Bacillus thuringiensis* SK 20.001: purification, characteristics, and implications for L-ornithine biosynthesis. Process Biochem 48:663–668
41. Zhang X, Shen L, Li F, Meng D, Sheng J (2011) Methyl salicylate-induced arginine catabolism is associated with up-regulation of polyamine and nitric oxide levels and improves chilling tolerance in cherry tomato fruit. J Agric Food Chem 59:9351–9357. doi:10.1021/jf201812r
42. Zhang X, Zhang J, Zhang R, Guo Y, Wu C, Mao X, Guo G, Zhang Y, Li D, Zou Q (2013) Structural, enzymatic and biochemical studies on *Helicobacter pylori* arginase. Int J Biochem Cell Biol 45:995–1002. doi:10.1016/j.biocel.2013.02.008
43. Zhang X, Zhang R, Bao T, Rao Z, Yang T, Xu M, Xu Z, Li H, Yang S (2014) The rebalanced pathway significantly enhances acetoin production by disruption of acetoin reductase gene and moderate-expression of a new water-forming NADH oxidase in *Bacillus subtilis*. Metab Eng 23:34–41. doi:10.1016/j.ymben.2014.02.002