

# Synthesis of $\beta$ -alanine from L-aspartate using L-aspartate- $\alpha$ -decarboxylase from *Corynebacterium glutamicum*

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**Abstract**  $\beta$ -Alanine is mainly produced by chemical methods in current industrial processes. Here, *panD* from *Corynebacterium glutamicum* encoding L-aspartate- $\alpha$ -decarboxylase (ADC) was cloned and expressed in *Escherichia coli* BL21(DE3). ADC<sub>C.g</sub> catalyzes the  $\alpha$ -decarboxylation of L-aspartate to  $\beta$ -alanine. The purified ADC<sub>C.g</sub> was optimal at 55 °C and pH 6 with excellent stability at 16–37 °C and pH 4–7. A pH-stat directed, fed-batch feeding strategy was developed for enzymatic synthesis of  $\beta$ -alanine to keep the pH value within 6–7.2 and thus attenuate substrate inhibition. A maximum conversion of 97.2 % was obtained with an initial 5 g L-aspartate/l and another three feedings of 0.5 % (w/v) L-aspartate at 8 h intervals. The final  $\beta$ -alanine concentration was 12.85 g/l after 36 h. This is the first study concerning the enzymatic production of  $\beta$ -alanine by using ADC.

**Keywords**  $\beta$ -Alanine production · Aspartate · L-Aspartate- $\alpha$ -decarboxylase · Enzymatic synthesis · Fed-batch

## Introduction

$\beta$ -Alanine plays an important role in fine chemical and pharmaceutical synthesis. It is a precursor of many types of drugs and intermediates, such as disodium pamidronate and the anti-inflammatory drug, balsalazide (Khan et al. 2010).  $\beta$ -Homoalanine has been used for the design of non-natural ligands for therapeutic application against autoimmune diseases such as rheumatoid arthritis (Reinelt et al. 2001).  $\beta$ -Alanine is also one of the recent evidence-based supplements that has entered the nutraceutical market (Derave et al. 2013). The current methods used for the industrial production of  $\beta$ -alanine are mainly chemical conversions. Recent industrial processes for manufacturing  $\beta$ -alanine is the reaction of acrylic acid with ammonium carbonates and gaseous CO<sub>2</sub> (Ohara et al. 2011).

Enzymatic catalysis is an attractive and environmentally-friendly method of synthesis in contrast to chemical methods that generally need harsh conditions. L-Aspartate- $\alpha$ -decarboxylase (ADC) catalyzes the  $\alpha$ -decarboxylation of L-aspartate and is found in microorganisms such as *Escherichia coli*, *Corynebacterium glutamicum*, *Mycobacterium tuberculosis* (Chopra et al. 2002). An ADC from *C. glutamicum* was overexpressed in *Escherichia coli* for pantothenate production (Dusch et al. 1999). Although easy process and high thermostability of *E. coli* ADC under industrial conditions were estimated, its low operational stability needed to be addressed before large scale applications become feasible (Könst et al. 2009).

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In this study, *C. glutamicum panD* gene encoding ADC was functionally expressed in *E. coli* with activity of 94 U/ml and specific activity of 50 U/mg. After the recombinant enzyme was purified, the specific activity reached 103 U/mg. Biochemical characterization and optimization of ADC-catalyzing  $\beta$ -alanine biosynthesis were studied and a pH-stat directed, fed-batch-feeding strategy was developed. This work is useful to the large-scale enzymatic production of  $\beta$ -alanine from *L*-aspartate, and ADC<sub>*C.g*</sub> shows higher operational stability than ADC<sub>*E.c*</sub>.

## Materials and methods

### Materials

Chemicals and reagents were of the highest grade available. Hitrap DEAE Sepharose fast-flow (FF) column (5 × 1) ml and Superdex 75 10/300GL gel filtration column were purchased from GE Healthcare (Shanghai, China). Amicon Ultra-4 centrifugal filter unit with an Ultracel-3 membrane (3 kDa) was purchased from EMD Millipore (Nanjing, China). Materials for SDS-PAGE were from Bio-Rad (Shanghai). Restriction enzymes, polymerases and DNA ligases were purchased from MBI Fermentas (Shanghai).

### Bacterial strains and growth conditions

*E. coli* BL21(DE3) was transformed with the recombinant pET-24a(+) containing *C. glutamicum panD* gene. Liquid cultures of recombinant *E. coli* were grown in LB broth containing 50  $\mu$ g kanamycin/ml at 37 °C.

### Expression and purification of *L*-aspartate- $\alpha$ -decarboxylase (ADC)

ADC was expressed in *E. coli* BL21 (DE3). Cells were grown at 37 °C in shake-flasks (50 ml terrific broth/250 ml). Protein expression was induced by adding 0.5 mM IPTG when biomass reached an OD<sub>600nm</sub> of 0.8–1.2. After 10–14 h at 25 °C, cells were harvested by centrifugation (15 min, 4,000×*g*, 4 °C), washed with 50 mM sodium phosphate buffer (pH 7.5) and disrupted in the same buffer by sonification on ice

(30 min). The crude enzyme was purified with a HiTrap DEAE FF column. The bound protein was washed with 50 mM sodium phosphate buffer (pH 7.5) at 1 ml/min, and eluted with 50 mM sodium phosphate buffer containing 1 M NaCl. The active fractions were pooled and concentrated by ultrafiltration. Samples were fractionated using a Superdex 75 10/300GL column and the proteins were graded eluted with 50 mM sodium phosphate buffer (pH 7.5) containing 0.15 M NaCl at 0.5 ml/min. The purified enzyme was stored at 4 °C or lyophilized at –20 °C. Enzyme purity and expression level were verified by SDS-PAGE (4 % stacking gel and 12 % resolving gel). PageRular Prestained Protein Ladder (Fermentas, MW:10–170 kDa) was used as protein marker.

### Ultrafiltration process

Ultrafiltration was applied to concentrate proteins and wash off the substrate and product. ADC in solution could be retained by ultrafiltration with an Amicon Ultra tube (3 kDa, 30 min, 6,000×*g*, 4 °C). The retained proteins were dissolved by 50 mM sodium phosphate buffer. *L*-Aspartate and  $\beta$ -alanine were completely washed off after 3 cycles of ultrafiltration and dissolution.

### Enzyme assays

ADC activity assays were performed at 37 °C: 1,900  $\mu$ l reaction buffer (1 mM EDTA/50 mM sodium phosphate buffer, pH 6) was mixed with 60  $\mu$ l 50 mM *L*-aspartate. The assay was initiated by the addition of 40  $\mu$ l enzyme. 100  $\mu$ l aliquots were removed after 1 h and heated in boiling water bath to quench the enzyme reaction. Samples were treated with *o*-phthalaldehyde (OPA) for derivatization and analyzed by HPLC with detection at 338 nm) (Bar-tolomeo and Maisano 2006).

### Determination of specific activity

Total protein concentration was determined using the Bio-Rad protein assay, using BSA as standard. One unit of ADC activity is defined as the amount of enzyme that catalyzes 1  $\mu$ mol *L*-aspartate  $\alpha$ -decarboxylated per hour. Specific activity is reported as units per mg protein.

## Batch and fed-batch experiments

Batch and fed-batch enzymatic catalysis were carried out in 50 ml sodium phosphate buffer (pH 6, 50 mM). Batch enzymatic catalysis was initiated by adding 2 % (w/v) solids. Fed-batch enzymatic catalysis was initiated with 1, 0.5 % (w/v) solids loaded with two and four feedings made at 16 and 8 h intervals. After 32 h, the total substrate concentration in reaction system was 20 g/l. Consumption and production rates were both analyzed by sampling 500  $\mu$ l from reaction system every 2 h.

## Results and discussion

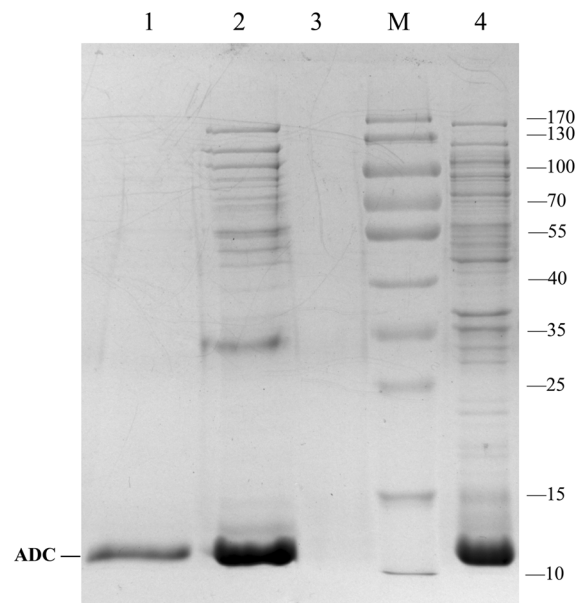
### Expression and purification of ADC

The gene *panD* amplified from genomic DNA of *C. glutamicum* ATCC13032 was cloned into pET-24a(+) and identical with that published on GenBank (Gene ID: 3343673) by DNA sequencing. Recombinant ADC was expressed in *E. coli* BL21 (DE3) with the enzyme activity up to 94 kU/l culture broth and the specific activity reached 50.2 U/mg (50.2  $\mu$ mol/h/mg). Two steps of gel filtration chromatography yielded the purified protein, as seen on SDS-PAGE (Fig. 1, lane 1). Specific activity of the purified ADC was 103 U/mg (Table 1). *C. glutamicum panD* was firstly cloned and overexpressed in *C. glutamicum* with a specific activity of ADC at 2.08  $\mu$ mol/h/mg (Dusch et al. 1999). Our results clearly showed that the *E. coli* BL21 (DE3)-pET system provided higher expression efficiency compared with that of homologous expression, which resulted in a 24-fold increase in specific activity of crude enzyme.

### Temperature- and pH-optima, stability

ADC had the highest activity at 55 °C but a significant loss of activity (62 % residual) was observed after 12 h at this temperature (Fig. 2a). Raising temperature at the range of 16–55 °C results in greater enzymatic activity but thermo-denaturation was observed above 37 °C and the activity would not be restored by cooling after thermo-denaturation.

ADC had highest activity at pH 6 in sodium phosphate buffer and more than 80 % activity was retained between pH 4 and 7 (Fig. 2b). The



**Fig. 1** SDS-PAGE analysis of purified ADC. 1 fraction from the Superdex 75 10/300GL column, 2 fraction from the HiTrap DEAE FF column, 3 blank control, M protein marker, 4 crude enzyme

decarboxylase tolerates wide pH range but prefers acidic conditions. The substrate, carboxylate in pyruvoyl-dependent decarboxylases, is in an environment predominated with hydrophobic and negatively charged residues. This acidic environment destabilizes the ground state of the reactant and promotes the release of the carboxyl group (Li et al. 2012).

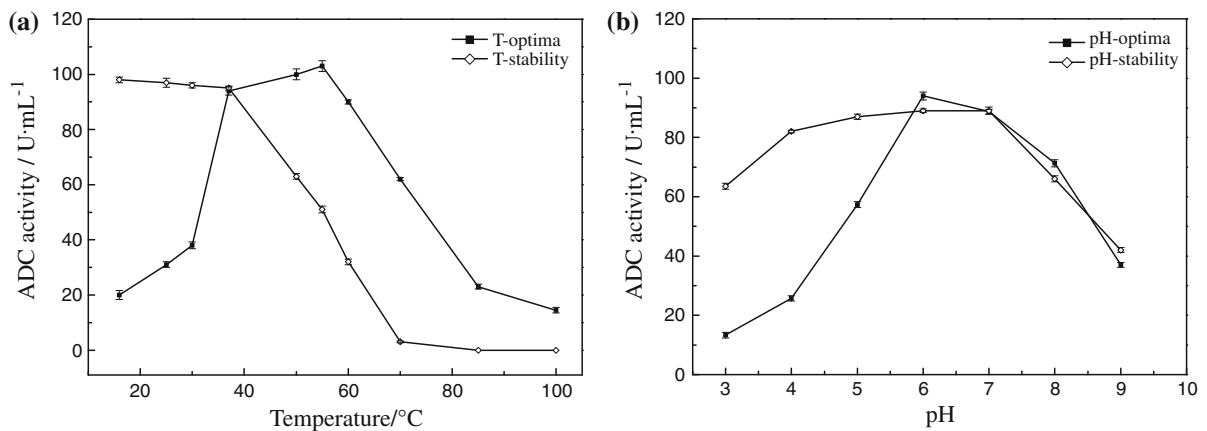
### Effect of temperature and pH on enzymatic synthesis of $\beta$ -alanine

Enzyme activity and stability both affected the enzymatic reaction conversion rate. ADC exhibited the highest activity at 55 °C but a significant loss of activity could be found due to thermal inactivation (Fig. 2a). We carried out a temperature versus conversion rate experiment that revealed 37 °C was an appropriate option (Fig. 3). 95 % of 5 g L-aspartate/l were  $\alpha$ -decarboxylated at 37 °C after 6 h while the conversion rate at 55 °C was 80 % after 12 h with an equal ADC load. Conversion rate of catalytic reaction and deactivation rate of the ADC itself were temperature-sensitive especially when substrate concentration was high.

**Table 1** Purification of recombinant ADC

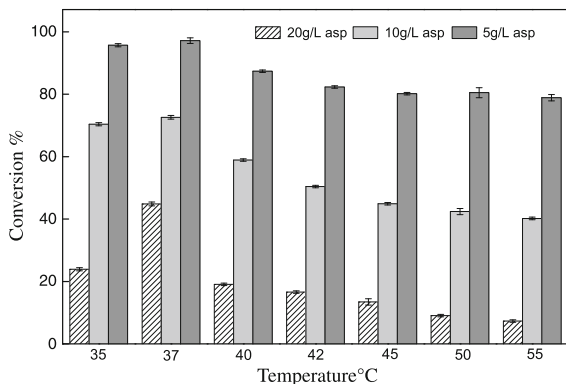
Purification steps	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Fold purification	Yield (%)
Crude cell extract*	4,710	93.8	50.2	1	100
DEAE anion exchange chromatography	1,960	23.2	84.5	1.7	41.7
Superdex G75 column chromatography	866	8.4	103.2	2.1	18.4

\* Obtained from 50 mL TB broth of recombinant *E. coli* BL21 (DE3)



**Fig. 2** Temperature and pH dependency of ADC activity. ADC had the highest activity at 55 °C with a significant loss of activity (62 % residual activity after 12 h) due to thermostability. At 37 °C ADC implied a higher stability and

relatively high enzyme activity. ADC showed best activity in sodium phosphate buffer at pH 6 and more than 80 % activity remained between pH 4–7



**Fig. 3** Conversion rate of reaction solutions containing L-aspartate at 5, 10, 20 g/l and at different temperatures with 1,000 U/g enzyme load. ADC had the highest activity at 55 °C but 37 °C was the optimum temperature for  $\beta$ -alanine synthesis

ADC showed highest activity in sodium phosphate buffer at pH 6 and more than 80 % activity remained between pH 4–7 (Fig. 2b). During ADC assays, the pH of reaction buffer system changed slightly over 1 h

**Table 2** pH changes and conversion rate after 12 h with 5 g L-aspartate/l and 1,000 U/g enzyme load at different initial pH values

Initial pH	Final pH	$\beta$ -alanine concentration (g/l)	Conversion rate (%)
5.25	6.73	0.36 $\pm$ 0.04	10.83
5.65	7.05	2.67 $\pm$ 0.03	79.13
6.00	7.18	3.15 $\pm$ 0.04	93.3
6.35	7.52	2.11 $\pm$ 0.03	62.5

though, in the long-time decarboxylation conversion, L-aspartate was mostly converted to  $\beta$ -alanine with the pH value raised. Different initial pH values could make a difference in the conversion rates after 12 h catalysis. Table 2 shows that the reaction system with an initial pH 6.0 reached a high yield and the pH value was relatively stable. Decarboxylation is a chemical reaction that removes a carboxyl group and releases CO<sub>2</sub>. With this loss of CO<sub>2</sub>, the pH value of the

**Table 3** Specific activity of ADC retained by ultrafiltration after 12 h reaction initial with different concentration of L-aspartate

L-aspartate concentration (g/l)	Specific activity of ADC after ultrafiltration (U/mg)	Activity loss (%)	Conversion rate (%)
0	101.4	3.78	–
5	77.0	24.07	98.0
10	47.0	53.62	63.4
20	28.1	72.29	39.1
50	17.5	82.76	17.0

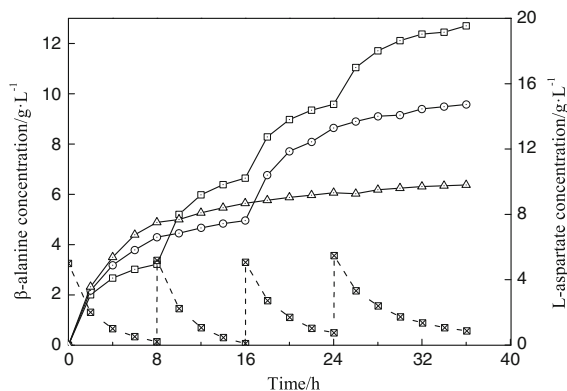
The ADC loaded into the reaction systems has the same specific activity of 105 U/mg. A 3.78 % thermo-loss of activity for 12 h incubation at 37 °C was observed

reaction solution rises with the reaction time. Keeping the reaction system within pH 6–7 was therefore propitious to achieving a relatively high enzyme activity and conversion rate.

#### Effect of substrate concentration on enzymatic synthesis of $\beta$ -alanine

Specific activity of ADC before and after 12 h reaction were assayed to estimate activity loss in the catalysis process. The results showed that the enzyme was stable at 37 °C, with only ~4 % initial activity loss after 12 h. The influence of product on activity could be excluded as incubation with 5 g  $\beta$ -alanine/l at 37 °C for 12 h did not cause enzyme inactivation and the specific activity loss was equal to the thermal loss. However, 5 g L-aspartate/l would significantly impair ADC activity as only 76 % of initial activity was retained after 12 h. The loss in activity increased considerably with the rise in substrate concentration. As shown in Table 3, 50 g L-aspartate/l caused an inhibition of over 80 % on ADC but even when the L-aspartate was removed by ultrafiltration, ADC activity would not be restored. This suggested irreversible substrate inhibition.

Although raising the substrate concentration was considered a direct way to achieve a high concentration of product and also reduce to production costs, it also brought about inactivation of the enzyme. Substrate inhibition has also been observed with decarboxylases, such as L-lysine decarboxylase (Phan et al. 1982) and L-DOPA decarboxylase (Lee et al. 1999). Glutamate decarboxylase also showed substrate inhibition and its



**Fig. 4** Time course of batch and fed-batch enzymatic catalysis. (white square  $\beta$ -alanine with four feedings, white triangle  $\beta$ -alanine with two feedings, white circle  $\beta$ -alanine in batch, dash line L-aspartate with four feedings) Time versus L-aspartate concentration curves of batch and two feedings fed-batch are not shown to avoid confusion. The substrate added into reaction system all reached 20 g/l and 1,000 U/g ADC were added into reaction systems simultaneously

production rate fitted with the substrate inhibition kinetic equation (Ueno et al. 2013). These problems can be avoided if the catalysis carried out in fed-batch mode, adding the substrate gradually to maintain the velocity at relatively high level.

#### Fed-batch-feeding of substrate based on pH-stat

As L-aspartate is slightly soluble at pH 6, we directly added solid L-aspartate into the reaction system. Batch enzymatic catalysis was initiated with 2 % (w/v) solids. Fed-batch enzymatic catalysis was initiated with 1 % or 0.5 % (w/v) solids, with two or four cycles of feeding made at 16 and 8 h intervals, respectively. After 32 h, the final substrate concentration in both reaction systems was 20 g/l. The initial pH of three reaction system were all adjusted to 6.0 and raised up with the release of CO<sub>2</sub>. Fed-batch of L-aspartate solid could bring down the reaction pH keeping reaction system within the optimum pH range. Fed-batch with four feedings made smaller fluctuation of pH value than two feedings and conversion rate of L-aspartate to  $\beta$ -alanine reached 97.2 %. Fed-batch of L-aspartate solid improved the  $\beta$ -alanine concentration compared to that of the enzymatic catalysis carried out in batch (Fig. 4). Employing the proper feeding strategy kept the reaction at a constant pH and attenuated substrate inhibition, which was propitious to relatively high enzyme activity and conversion rate.

The present pathway for  $\beta$ -alanine biosynthesis is  $\beta$ -aminopropionitrile hydrolyzation. The conversion is carried out in a bubble column reactor at 30 °C and pH 7.5 with a final  $\beta$ -alanine concentration of 9 g/l after 2 h (Liang et al. 2008). This route has a high productivity and its custom-designed reactor equipment improves the yield effectively but the high-cost and hazardous feedstock,  $\beta$ -aminopropionitrile, limits the scale of  $\beta$ -alanine production.

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