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FULL PAPER

Production of β-alanine from fumaric acid using a dual-enzyme cascade

Yuanyuan Qian,[a,b] Jia Liu,[a,b] Wei Song,[a,b] Xiulai Chen,[a,b] Qiuling Luo,[a,b] and Liming Liu*[a,b]

Abstract: The aim of this study was to develop an environmentally safe and efficient method for β-alanine production using a dual-enzyme cascade route with L-aspartase (AspA) from *E. coli* and L-aspartate-α-decarboxylase (PanD) from *Corynebacterium glutamicum*. Poor cooperativity in this system due to the divergent catalysis efficiencies of AspA and PanD led to an imbalance between the two reactions. To address this issue, we employed ribosome binding site regulation and gene duplication to coordinate the expression levels of AspA and PanD. Finally, we achieved β-alanine production of 80.4±1.6 g L^{-1} with a conversion rate of 95.3±1.6% in a 5-L bioreactor. The dual-enzyme cascade reported herein represents a promising strategy to meet industrial requirements for large-scale β-alanine production in the future.

Introduction

β-alanine is a naturally occurring β-amino acid. As an important precursor to pantothenic acid (vitamin B5), coenzyme A (CoA), and poly-alanine (nylon-3), β-alanine is widely used in food additives, pharmaceuticals, and nitrogen-containing chemicals.[1] At present, the worldwide market demand for β-alanine relies on industrial production through chemical catalysis, which requires harsh conditions that are ultimately unsustainable from environmental and societal perspectives. Thus, research has focused on the development of biocatalytic processes for β-alanine synthesis.[2] At present, there are two such forms of biocatalysis available. The first type is fermentation with engineered *E. coli* that directly convert glucose into β-alanine through an introduced β-alanine synthetic pathway.[3] Yet, this method requires long periods of time for biocatalyst (whole cell) growth and has limited productivity due to the generation of numerous by-products in the broth.[4] The second type is PanD (EC: 4.1.1.11) dependent, which catalyzes the decarboxylation of L-aspartic acid to generate β-alanine and release CO_2 at the α position.[2b, 5] Unfortunately, high cost of the substrate L-aspartic acid and substrate inhibition have limited the industrialization of this method. As an alternative, L-aspartic acid can be produced from fumaric acid through AspA, which catalyzes a reversible reaction of fumaric acid to L-aspartic acid by amination or deamination.[6] Therefore, fumaric acid is a potential substrate that can be transformed into β-alanine through a linear *in vitro* two-enzyme cascade involving AspA and PanD (Figure 1).

Artificially designed cascades are widely used for the synthesis of high-value chemicals,[7] such as amino acids,[8] amines,[9] and chiral alcohols.[10] This is in part related to several advantages of the enzyme cascades. First, enzyme cascades can shift the reaction equilibrium and circumvent the generation of unstable or toxic intermediates, as these intermediates are generated in situ and directly consumed in the reaction sequence soon thereafter. Second, enzyme cascades avoid the isolation and purification of intermediates, which conserves time, costs, and reagents.[11] Third, enzyme cascade reactions produce less waste as the result of using fewer chemicals. Finally, the cascade approach typically generates higher yields than classical single-step transformations.[12]

Yet, the combination of different enzymes, chemicals, and intermediates into a single volume for multiple reactions requires the precise tuning and compatibility of all reaction conditions. Accordingly, catalyst (chemo)selectivity, compatible reaction conditions, and cooperativity of the reactions represent important bottlenecks for the implementation of cascade pathways, limiting productivity and conversion yield.[12a, 13] Of these, cooperativity within the reaction system can require elaborate fine-tuning, e.g., the precise control of the ratio of activities for individual enzymes in multi-catalytic cascades.[7a] Therefore, additional efforts are necessary to expand the industrial utility of the enzyme cascade approach.

To fine-tune an industrially useful enzyme cascade, four different approaches are considered. The first approach is gene mining for a potential protein sequence with specific properties. For example, genome database mining was used to identify a novel D-mandelate dehydrogenase with high efficiency from *Lactobacillus brevis*, with 4.6-fold of the highest record origin in k_{cat}. Incorporation of this dehydrogenase into a three-enzyme cascade resulted in a space time yield of 50.4 g L^{-1} d^{-1} L-phenyl glycine from rac-mandelic acid.[8a] Second, sequence optimization for enhanced performance. Directed evolution has emerged as a powerful tool for addressing the limitations of natural enzymes. In a previous study of cascade reactions for CO_2 fixation, it was found that the promiscuity of a key enzyme, propionyl-CoA, led to the problematic generation of additional byproducts. To compensate for this limitation, the authors introduced a site mutation that decreased the k_{cat}/K_m of the side-reaction by approximately 500-fold, thereby increasing the overall conversion.[14] Third, the fine-tuning of enzyme expression at the transcriptional level, including promoter optimization and altering the number of gene copies. Li and colleagues reported this approach for the synthesis of chiral α-amino acids, using multi-plasmids with different copies, enzymes were assembled in a single host with specific expression levels and eventually produced 90–98% of the target product with perfect enantiomeric excess (≥ 99%).[8b] Finally, the precise control of enzyme

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expression at a translational level using ribosome binding site (RBS) regulation. This approach was recently employed to maximize fructosylated chondroitin production by combining different RBS strengths with multi-genes and balancing gene expression to yield a 5.2-fold increase in the ratio of precursors.[15] Combinations of these four approaches are often used to fine-tune the overall activities of pathway enzymes. One report described the successful synthesis of (−)-menthol by controlling three enzymes: ketosteroid isomerase (KSI), pulegone reductase, and (−)-menthone:(−)-menthol reductase (MMR). To address the low catalytic activity of KSI, the authors introduced four active site mutations that yielded a 4.3-fold increase in activity compared to the wild-type enzyme. After optimization, a RBS approach was employed to regulate the ratio of KSI to MMR. As a result, the authors achieved a near 2.5-fold increase in (−)-menthol production.[16] The use of these approaches on rate-limiting enzymes can benefit the coordination of complex biocatalytic cascade reactions and improve overall efficiency.[17]

In the present study, we combined strategies regulating enzyme expression at the transcriptional and translational levels in an attempt to solve the issue of intermediate inhibition in β-alanine production. After identifying divergent catalytic efficiency in our two target enzymes, we optimized co-expression of these enzymes in *E. coli* strains using RBS regulation with AspA and gene duplication with PanD. Finally, the enzyme cascade pathway was well balanced and produced β-alanine with high efficiency.

Results

Cascade design and *in vitro* reconstruction of β-alanine biosynthesis

β-alanine synthesis from fumaric acid requires two enzymes: AspA and PanD. First, fumaric acid is transformed into L-aspartic acid by AspA through an ammonation reaction with the aid of NH_3. Then, β-alanine and CO_2 are generated by PanD through a decarboxylic reaction (Figure 1).

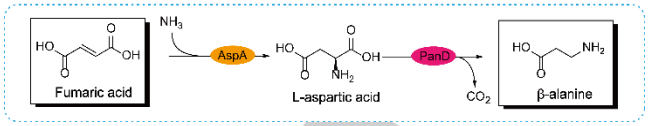

Figure 1. *In vitro* design and reconstruction of β-alanine biosynthesis pathway from fumaric acid, by the dual-enzyme system containing AspA and PanD.

To reconstruct this system *in vitro*, we selected eight different AspA and PanD enzymes from the BRENDA database (Table S2, Table S3). Next, the *aspA* and *panD* genes were amplified, overexpressed, and purified. To reconstruct the enzyme cascade *in vitro*, we randomly selected EcAspA (AspA from *E. coli*) and BsPanD (PanD from *Bacillus subtilis*) and combined these enzymes in a molar ratio of 1:1 with 100 mM fumaric acid. After reaction for 2 h, we confirmed the synthesis of our target product, β-alanine, by mass spectrometry (Figure 2a). The concentration of β-alanine was 70.3±2.0 mM as determined by HPLC (Figure S1).

In order to optimize the *in vitro* system, we next measured the specific activities of all AspAs and PanDs and selected the top three for each enzyme: TbAspA, EcAspA, PfAspA, BsPanD, CgPanD, and StPanD (Table S2, Table S3). Next, a series of AspA and PanD expression cassettes were designed and combined in a molar ratio of 1:1 to produce nine combinations for conversion (Table S4). Number five, consisting of EcAspA and CgPanD, yielded the highest β-alanine concentration of 87.6±1.8 mM. For further optimization, we combined different activity ratios of AspA to PanD (from 0.5:1 to 10.0:1) with 40 g L^{-1} fumaric acid (PanD activity was fixed at 4 U mL^{-1} of substrate). The highest β-alanine production was obtained with an AspA/PanD activity ratio of 1.0:1–1.5:1, yielding a β-alanine concentration and conversion rate of approximately 28.5±0.8 g L^{-1} and 92.7±0.8%, respectively. Beyond this range, the β-alanine concentration and conversion rate decreased, suggesting that a ratio of 1.0:1–1.5:1 was the optimal activity ratio for AspA/PanD (Figure 2b).

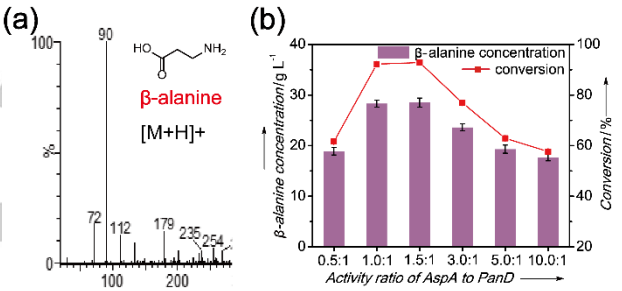

Figure 2. Optimization of activity ratio of AspA/PanD *in vitro*. (a) Analysis of the *in vitro* reconstructed system with LC-MS; (b) Effect of different activity ratio of AspA/PanD on β-alanine production. The dual-enzyme system was supplemented with fumaric acid (40 g L^{-1}), with PanD activity fixed at 4 U mL^{-1}. The ratio of AspA/PanD was changed from 0.5:1 to 10.0:1.

In vivo construction of β-alanine biosynthesis in *E. coli*

To further simplify the cascade reaction, we co-expressed AspA and PanD in a single *E. coli*. Based on the above results, we inserted the *C. glutamicum panD* and *E. coli aspA* genes into a pET-28a(+) plasmid and transformed the plasmid into *E. coli* BL21(DE3), resulting in the strain pCgPA (Figure 3a). Analysis of pCgPA cells by protein gel electrophoresis of cell-free lysates confirmed the expression of both enzymes (Figure 3b). To observe the conversion performance of the enzyme cascade in a whole-cell system, we tested pCgPA cells with substrate (fumaric acid) concentrations from 10 to 60 g L^{-1} added at a fixed substrate/whole-cell biocatalyst (wet) ratio of 1.5 g/1.0 g at 37°C. As shown in Figure 3c, the β-alanine titer increased from 7.31±0.35 to 18.2±0.9 g L^{-1} as the fumaric acid concentration was increased from 10 to 30 g L^{-1}; however, higher substrate concentrations (> 30 g L^{-1}) did not result in higher production. Overall, the β-alanine conversion rate decreased from 95.2±1.0% to 18.9±0.8% as the substrate concentration increased from 10 to 60 g L^{-1}. In contrast, L-aspartic acid accumulation increased from 0.10±0.01 to 53.3±1.0 g L^{-1} as the substrate concentration

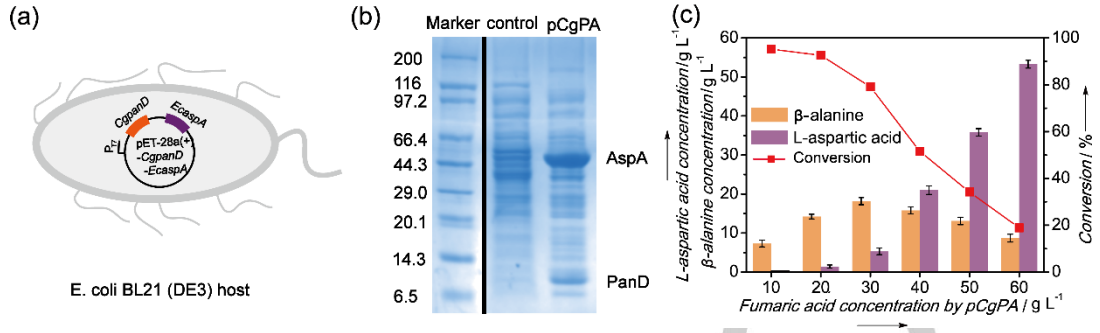

Figure 3. Construction of pCgPA. (a) Construction of the plasmid used for pCgPA; (b) SDS-PAGE analysis of strain pCgPA from cell-free extracts; (c) Effect of substrate loading on β-alanine production by strain pCgPA. The reactions were supplemented with varying concentrations of fumaric acid from 10 to 60 g L^{-1} added at a fixed substrate/whole-cell biocatalyst ratio of 1.5 g/1.0 g at 37°C.

increased from 10 to 60 g L^{-1}, potentially explaining the observed decrease in β-alanine generation when fumaric acid was provided at a concentration > 30 g L^{-1}. We attributed this result to the divergent catalytic efficiency of the two enzymes: that is, an excess of intermediate inhibited the activity of PanD. To test this hypothesis, we measured the activity of AspA and PanD in pCgPA cells and determined that AspA showed a high activity of 705±15 U g^{-1} whereas PanD exhibited an activity of 27.4±0.8 U g^{-1}, resulting in a ratio of 26:1. This was in agreement with the divergence in kinetic parameters of AspA and PanD (Table 1), which had specific enzyme activities of 103±4 and 2.70±0.10 U mg^{-1} protein, respectively (approximately 40-fold). Therefore, it was necessary to control the activity ratio of AspA to PanD between 1.0:1 and 1.5:1 in order to avoid excess L-aspartic acid accumulation.

Table 1. Kinetic constants of EcAspA and CgPanD

Parameters	EcAspA	CgPanD
K_m (mM)	3.1±0.2	3.9±0.1
k_{cat} (s^{-1})	39±1	1.2±0.1
k_{cat}/K_m (mM^{-1} s^{-1})	13	0.30
Specific enzyme activity (U mg^{-1} protein)	103±4	2.70±0.10

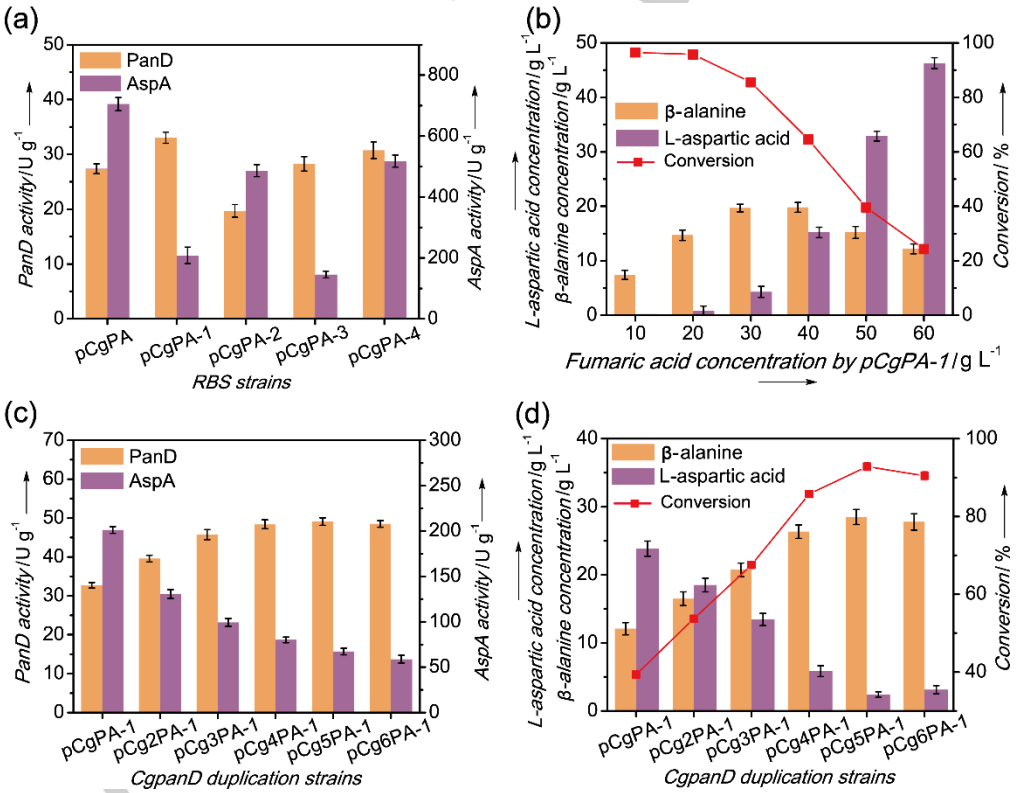

Figure 4. Optimization of co-expressed strain in the activity ratio of AspA/PanD *in vivo*. (a) The assay of enzymes activity in recombinant strains with different RBS sequences of *E. coli aspA* gene; (b) Effect of substrate loading on β-alanine production by strain pCgPA-1; (c) The assay of enzymes activity in recombinant strains with *C. glutamicum panD* gene duplicated for different copies; (d) Effect of different *panD* copies on β-alanine production with fumaric acid (40 g L^{-1}), and the wet whole-cell biocatalysts were added with 27 g L^{-1} (substrate/whole-cell biocatalyst ratio=1.5 g/1.0 g).

Optimization of the AspA/PanD ratio for β-alanine production

The goal of optimization was to decrease the supply of L-aspartic acid by controlling AspA expression or increase L-aspartic acid consumption by promoting PanD expression. For this purpose, we applied RBS regulation (Figure S3). We first selected RBS sequences with lower protein expression levels. Among the RBS strains, pCgPA-1 showed the best performance: AspA activity was significantly decreased by 70.5% (208±4 U g^{-1}) and PanD activity was increased by 20.4% (33.0±0.7 U g^{-1}) compared to the control strain (Figure 4a). As a result, the activity ratio of AspA / PanD decreased from 26:1 to 6.3:1. We repeated the enzymatic synthesis of β-alanine catalyzed by pCgPA-1 and the highest obtained titer was 40 g L^{-1}, producing 19.8±0.9 g L^{-1} β-alanine (4.0 g L^{-1} higher than that of pCgPA) with a low conversion rate of 64.5±0.9% (Figure 4b).

We next implemented gene duplication to further decrease the activity ratio of AspA/PanD. We increased the number of *C. glutamicum panD* gene copies from 2 to 6, yielding the strains pCg2PA-1, pCg3PA-1, pCg4PA-1, pCg5PA-1, and pCg6PA-1 (Figure S3). As a result, PanD activity was increased to 39.6±0.8, 45.7±1.2, 48.4±1.1, 49.1±1.0, and 48.2±0.8 U g^{-1} and AspA activity was decreased to 131±5, 99.3±4.2, 80.1±3.0, 67.3±2.1, and 58.7±2.1 U g^{-1}, respectively (Figure 4c). The resultant activity ratios of AspA/PanD were 3.3:1, 2.2:1, 1.7:1, 1.4:1, and 1.2:1, respectively. Subsequently, we performed transformation experiments using the 6 strains supplemented with fumaric acid (40 g L^{-1}). As shown in Figure 4d, β-alanine titers increased as the number of *panD* gene duplicates increased from 2 to 5 copies, such that pCg5PA-1 offered maximal β-alanine production of 28.3±1.1 g L^{-1}, minimal L-aspartic acid accumulation of 1.83±0.08 g L^{-1}, and the highest conversion rate of 92.2±1.1%. Furthermore, we examined accumulation of L-aspartic acid during transformation by pCg5PA-1 and detected less than 2 g L^{-1}, indicating that the balance between AspA and PanD was unobstructed. Yet, pCg6PA-1 showed less β-alanine compared to pCg5PA-1, possibly due to limited PanD expression. Therefore, pCg5PA-1 was used in subsequent experiments.

One-pot production of β-alanine through a dual-enzyme cascade

To obtain higher enzyme activity and cell yield, we investigated a number of variables for protein expression and cell growth in a 5-L bioreactor. Firstly, the Isopropyl-β-D-thiogalactopyranoside (IPTG) addition point varied between 1 and 4 h; we identified the optimal activity of PanD with addition at 2 h (47.0±1.2 U g^{-1}), at which point the activity of AspA was 65.3±2.7 U g^{-1} (Figure 5a). Next, we compared the effects of different inducers (IPTG and lactose, 5 g L^{-1}) on enzymes activity and cell growth. As shown in Figure 5b, lactose induction for 14 h yielded an optimal profile, similar PanD activity (48.8±1.0 U g^{-1}) to IPTG (48.6±1.4 U g^{-1}) and AspA activity of 69.7±1.9 U g^{-1} (Figure S4), although there were obvious early disadvantages compared to IPTG. Cell growth with lactose (OD_{600} = 48.9±1.4) was 59.8% higher than that in an IPTG-induced control experiment (OD_{600} = 30.6±0.7); therefore, lactose was selected as the inducer. We next tested the effect of lactose concentration on transformation. PanD activity increased as the lactose concentration increased from 1 to 5 g L^{-1}, but declined thereafter. Thus, 5 g L^{-1} was selected for catalyzing the desired biotransformation; this concentration yielded a maximum PanD activity of 48.9±1.2 U g^{-1} with AspA activity of 64.2±1.4 U g^{-1} (Figure S5). Then, we investigated the optimal induction temperature for enzyme activity and cell growth. PanD activity increased significantly as the temperature increased from 16 to 25°C and exhibited optimal activity (57.9±1.6 U g^{-1}) at 25°C with AspA activity of 72.1±1.8 U g^{-1} (Figure 5c). At this temperature, the OD_{600} was 43.4±1.2, representing a decrease of just 7.5%

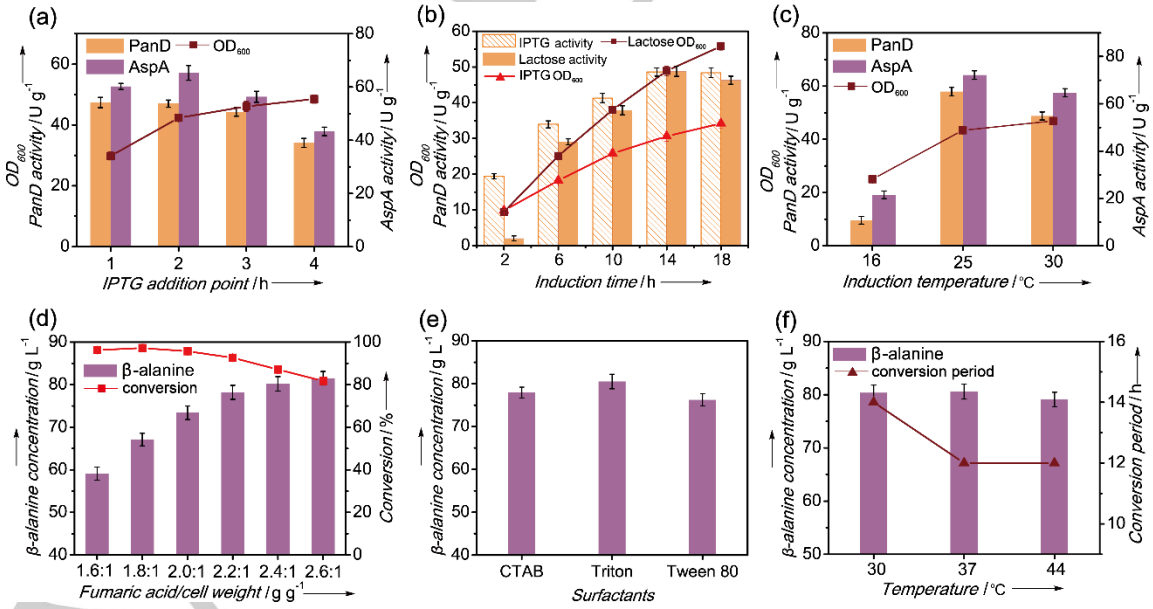

Figure 5. Optimizations during fermentation and conversion process by strain pCg5PA-1. (a) Effect of time of IPTG addition on enzymes activity and cell growth; (b) Effect of inducers (IPTG and lactose) and induction time on PanD activity and cell growth; (c) Effect of induction temperature on enzymes activity; (d) Effect of mass ratio of fumaric acid and wet cell weight on β-alanine concentration; (e) Effect of surfactants on β-alanine concentration; (f) Effect of conversion temperature on β-alanine concentration.

compared to the initial temperature of 30°C (OD_{600} = 46.9±1.4). Based on these results, the optimized conditions were included 14 h expression of protein in BL21(DE3) at 25°C with 5 g L^{-1} lactose added for induction after culturing at 37°C for 2 h in a 5-L fermentation. The cells were subsequently harvested for conversion experiments.

To provide a better environment for conversion, we fine-tuned other parameters that benefited β-alanine generation. First, we examined the effect of the substrate/whole-cell catalyst ratio on the β-alanine titer in a range from 1.6:1 to 2.6:1 with a fixed cell weight of 50 g L^{-1}. It appeared that as this ratio increased from 1.6:1 to 2.2:1, β-alanine concentration also increased, but with a sustaining decrease in conversion rate. A ratio of 2.2:1 provided both a relatively high β-alanine concentration (78.2±1.6 g L^{-1}) and conversion rate (92.7±1.6%). Subsequently increases in substrate did not significantly improve β-alanine production, which was attributed to an insufficient supply of whole-cell catalyst (Figure 5d). Next, we investigated the effects of different surfactants including CTAB, Tween 80, and Triton at a concentration of 1 g L^{-1} in the transformation. The highest β-alanine concentration (80.4±1.6 g L^{-1}) was detected when the transformation was performed in the presence of Triton, 2.6 g L^{-1} higher than the CTAB control (Figure 5e). Finally, we evaluated the transformation temperature and selected 37°C as 30°C required a longer transformation period (additional 2 h) and 42°C offered no advantage over 37°C (Figure 5f). The final optimized conditions (the ratio of substrate/whole-cell catalyst was 2.2:1 in existence of Triton (1 g L^{-1}) at a conversion temperature of 37 °C) yielded 80.4±1.6 g L^{-1} of β-alanine within 12 h with a conversion rate of 95.3±1.6% and productivity of 6.7 g L^{-1} h^{-1} in a 5-L reactor under mild conditions. After subsequent isolation and purification process, a purity >97% of β-alanine was obtained, with the isolated yield reaching 82%.

Discussion

In the present study, we designed and reconstructed a dual-enzyme cascade consisting of AspA and PanD for the efficient transformation of fumaric acid to β-alanine in a 5 L-bioreactor. Our optimized method achieved β-alanine production of 80.4±1.6 g L^{-1} with conversion rate of 95.3±1.6%, which was higher than the concentration of previous studies by biosynthesis (from 12.8 to 32.3 g L^{-1}).[3, 5, 18] In this cascade pathway, we eliminated excess L-aspartic acid accumulation and subsequent PanD inhibition by implementing RBS regulation and gene duplication to successfully decrease AspA activity from 705±15 to 67.3±2.1 U g^{-1} and increase PanD activity from 27.4±0.8 to 49.1±1.0 U g^{-1}. After optimizing this cascade in a 5-L reactor, the activity ratio of AspA/PanD decreased to 1.2:1 and was controlled between 1.0:1 and 1.5:1, which contributed to a higher efficiency of β-alanine production.

Various methods have been reported for the biosynthesis of β-alanine, mainly including microbial fermentation and enzymatic conversion. Previously, aldehyde dehydrogenases *ALD2* and *ALD3* were identified for β-alanine biosynthesis in *Saccharomyces cerevisiae*, but there were no subsequent studies.[19] Recently, a strain of *E. coli* was engineered to produce β-alanine directly from glucose with a titer of 32.3 g L^{-1} in 39 h;[3] however, this approach is limited by a long fermentation time and by-product accumulation (acetic acid, reaching about 6 g L^{-1}). Additionally, β-alanine accumulation > 48 g L^{-1} severely inhibited cell growth during fermentation. Therefore, research attention has favored enzymatic conversion. In a recent attempt, L-aspartic acid was transformed into β-alanine with a titer of 24.8 g L^{-1} and a conversion rate of 92.6% in 20 h by overexpressing PanD.[5] Yet, L-aspartic acid is an expensive substrate and substrate inhibition is an important bottleneck for improving efficiency.

Here, we designed a dual-enzyme (AspA and PanD) cascade pathway for the conversion of fumaric acid to β-alanine by expressing optimal enzymes in a strain of *E. coli* under optimized conditions for β-alanine generation *in vivo*. However, Divergent catalyzing efficiencies of different enzymes often result in imbalance of the cascade, leading to too much accumulation or insufficient of intermediates, which slows down the reaction or even eventually stops the entire cascade system.

This difficulty can be addressed in two groups: by altering enzymes properties and by regulating enzyme expression. (i) The first case, improving enzymes properties, is mainly accomplished by screening target genes with a desired functionality and optimizing the protein sequence by protein engineering. Gene mining can be used to identify new enzymes with higher activity and stability from unexplored origins either by screening and analyzing numerous samples from different environments in nature, which is time consuming, or by screening databases that predict the functionality of genes.[20] Protein engineering is an effective tool for improving enzyme properties and generally includes conventional directed evolution based on random mutation, rational design based on computational simulation, and semi-rational design in combinations. These approaches have various benefits and drawbacks; random mutation may not cover all sequences and requires a high-throughput screening method for large mutation libraries. In contrast, rational design is more targeted but requires a basis of knowledge about enzyme structures and catalytic mechanisms. These two methods complement one another and have been used together to overcome the limitations of natural enzymes in enzyme cascades.[20b, 21] For example, Gong and colleagues dramatically enhanced catalytic efficiency of esterase by combining multi-target evolution (including site-directed mutagenesis and random mutagenesis) as a way to enhance L-menthol synthesis from DL-menthyl benzoate;[22] (ii) The second group, regulating enzyme expression, can be accomplished by optimization at the transcriptional or translational levels. Regulation can be accomplished through a number approaches such as promoter replacement, RBS regulation, multi-plasmid systems, and gene copies modification. Promoter replacement and RBS regulation can achieve precise control of gene expression, while multi-plasmid systems are more flexible for balancing different reactions. An important issue with multi-plasmid systems is that cell growth and protein expression rates may be partly inhibited by higher metabolic burden, like chromosomal integration.[9c, 23] Nevertheless, these methods are important tools for balancing cascade pathways. In one report, a two-plasmid system and

genome-integrated methods were simultaneously applied to achieve α-amino acid conversion up to 96%.[24]

In our study, intermediate accumulation was the main problem. We resolved this issue with a combination of RBS regulation and gene duplication. RBS regulation alters the initiation rate of translation, and has previously been implemented for pathway optimization; in one study, balancing the RBS strength of pathway genes resulted in a 46% increase in total fatty acid production compared to the control.[25] In our study, lower AspA expression was achieved by employing a weak RBS. In contrast, gene duplication is not often used for optimization but can enhance the speed of slow steps in a cascade reaction. Lorna et al. duplicated a gene encoding a rate-limiting enzyme to increase amine production by 14%.[9b] Here, we first determined the optimal activity ratio of AspA/PanD *in vitro* to be 1.0:1–1.5:1 and subsequently used RBS regulation and gene duplication *in vivo* to decrease AspA expression and increase PanD expression, ultimately decreasing the activity ratio from 26:1 to 1.4:1.

In summary, we successfully engineered a dual-enzyme cascade pathway for β-alanine biosynthesis to overcome high substrate costs and low catalysis efficiency. RBS regulation of the *aspA* gene and duplication of the *panD* gene solved imbalance of the cascade reactions *in vivo*. A combination of fermentation and transformation conditions was further used to optimize β-alanine synthesis. Finally, we obtained a recombinant *E. coli* strain pCg5PA-1 co-expressing PanD and AspA to produce β-alanine from fumaric acid in a whole-cell system with high conversion efficiency (β-alanine concentration of 80.4±1.6 g L^{-1} and conversion rate of 95.3±1.6% in a 5-L reactor). These results lay an important foundation for the industrialization of β-alanine biosynthesis.

Experimental Section

Strains and media

The expression plasmid pET-28a(+) and the host strain *E. coli* BL21(DE3) were purchased from Novagen (Madison, WI, USA). Cultivation for gene manipulation and plasmid construction was performed in Luria-Bertani (LB) broth or agar plates (2% agar, w/v). Cultivation for *E. coli* cell and enzymes expression for recombinant were performed in the Terrific Broth (TB) medium with 5 g L^{-1} glycerol, 1 g L^{-1}L $MgSO_4$, and 2 g L^{-1} K_2HPO_4•$3H_2O$, and among them, Angel yeast (Angel Yeast Co., Ltd., Hubei, China) and soy peptone were used as alternative to yeast extract and peptone. The final antibiotics concentrations in medium were 50 mg L^{-1} kanamycin.

Construction of the co-expressed strains

Main primers used for constructing co-expressed strains are summarized in Table S1. *C. glutamicum panD* was first inserted into the pET28a(+) using the restriction sites *Nde*I and *BamH*I, followed with an insertion of *E. coli aspA* gene (added with RBS sequence) using the *Hind*III and *Xho*I site. The plasmids of RBS strains were constructed similarly by replacing different RBSs with *E. coli aspA* gene, and the plasmids of gene duplication strains were constructed using ClonExpress Entry One Step Cloning Kit (Vazyme Biotech Co., Ltd., Nanjing, China).

Analytical methods

The optical density at 600nm (OD_{600}) was measured using a spectrophotometer. Fumaric acid levels were determined by high-performance liquid chromatography (HPLC) using a VWD detector with an Aminex HPX-87H column (7.8 × 300 mm).[26] L-aspartic acid and β-alanine levels were determined by HPLC using a FLD detector with an Agilent Zorbax SB-Aq column (4.6× 150 mm).[27] Samples were centrifuged at 12,000 × g for 20 min and then the supernatants were filtered using 0.22-μm filter membrane.

Enzyme expression and purification

AspA and PanD were individually overexpressed and purified from *E. coli* BL21(DE3) with pET28a(+) plasmid. Cells were harvested by centrifugation at 6,000 × g for 7 min and resuspended in Tris-HCl buffer. The cell suspensions were sonicated by Ultrasonic Cell Disruptor at 4°C and centrifuged at 12,000 × g for 20 min. The recombinant strains were purified by an AKTA pure system (GE Healthcare Life Science, USA) with a nickel-affinity column.

Enzyme assay

AspA activity on fumaric acid was assayed by coupling L-aspartic acid formation. The reaction system contained fumaric acid (15 g L^{-1}), pH 7.0 with ammonium hydroxide. One unit of AspA activity (U) was calculated as the amount of enzyme producing 1 μmol of L-aspartic acid in 1 min. PanD activity on L-aspartic acid was assayed by coupling β-alanine formation. One unit of PanD activity (U) was calculated as the amount of enzyme producing 1 μmol of β-alanine in 1 min. The activity of the recombinant strains expressed single enzyme was measured using purified enzyme, while the activity of the co-expressed strains was measured using wet whole-cell catalysts. The two reactions both sustained for 10 min and stopped by boiling water for 5 min.

Strategy in fed-batch fermentation

Seed cultures were grown in 500 mL of LB medium and then inoculated in the 5-L bioreactor fermentation with a 3-L working volume for whole-cell biocatalyst, with an inoculation with 5% volume of the seed culture. The pH, agitation rate, and aeration rate were adjusted to 7.0, 400 rpm, and 1.0 vvm, respectively. The temperature for cell growth was 37°C, but after induced with optimized lactose, the temperature was adjusted to optimized temperature for better enzymes expression. When the dissolved oxygen (DO) level increased rapidly (demonstrating glycerol in the medium was completely exhausted), the system was supplied with yeast (60 g L^{-1}), soy peptone (20 g L^{-1}), and glycerol (400 g L^{-1}) at a rate of 8 mL h^{-1}.

Production of β-alanine from fumaric acid

The strains were harvested by centrifugation at 6,000 × g for 7 min after overexpression. The conversion experiments were carried out in a 500 mL shake flask with 100mL working volume and a 5-L bioreactor with 1-L working volume. Because the cascade reactions by PanD was pH-increasing, in shake flask, the fumaric acid was added into the system by one-time, but needed to control pH of the system in 7.0 with 30 % hydrochloric acid every 2 h. In a 5-L reactor, fumaric acid was fed-batched to control pH of the system in about 7.0 in the early, but after all substrate added into the system, 30% hydrochloric acid was used to control pH automatically. The concentration of fumaric acid, L-aspartic acid, and β-alanine was determined using the HPLC method as described above.

Isolation protocols

The mixture of β-alanine and L-aspartic acid were purified using a Dowex 50WX8 cation exchange column. First, the resin was conditioned by washing with aqueous NH_3 (2 M, 2×30 ml), HCl (2 M, 2×30 ml) and H_2O (4×30 ml). Second, the crude reaction mixture was acidified with HCl (1 M) and loaded onto the column. Finally, the column was washed with HCl (1 M, 2×30 ml), H_2O (4×30 ml) and eluted with NH_3 (2 M, 4 ×30 ml). Fractions containing product (β-alanine) were combined and lyophilized to remove water, and then purified by preparation thin liquid chromatography (PTLC) (^{n}BuOH/AcOH/H_2O, 4:1:1). Silica gel containing target product was collected and eluted with ^{n}BuOH/H_2O (2:1). After filtration, the organic solvent was removed by evaporation, and the product was dried overnight under vacuum. The so obtained solid was washed with (EtOH/H_2O, 9:1) affording the corresponding β-alanine in high chemical purity. The purified β-alanine were further identified by HPLC analysis.

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Keywords: β-alanine • enzyme cascade • L-aspartase • L-aspartate-α-decarboxylase • cooperativity

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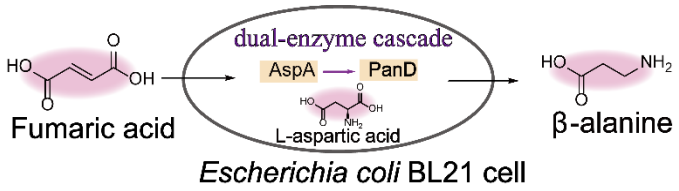

In this study, we developed a dual-enzyme cascade reactions for the generation of β-alanine from fumaric acid, employing the enzymes AspA and PanD. Limited by poor cooperativity between the two enzymes, imbalance of the pathways became a bottleneck when producing β-alanine in whole-cell system. It was eliminated by RBS regulation of the *aspA* gene and duplication of the *panD* gene, well fine-tuned the activity ratio of AspA to PanD. .

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Production of β-alanine from fumaric acid using a dual-enzyme cascade