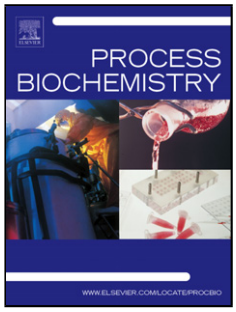


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# Development of a multi-enzymatic desymmetrization and its application for the biosynthesis of L-norvaline from DL-norvaline

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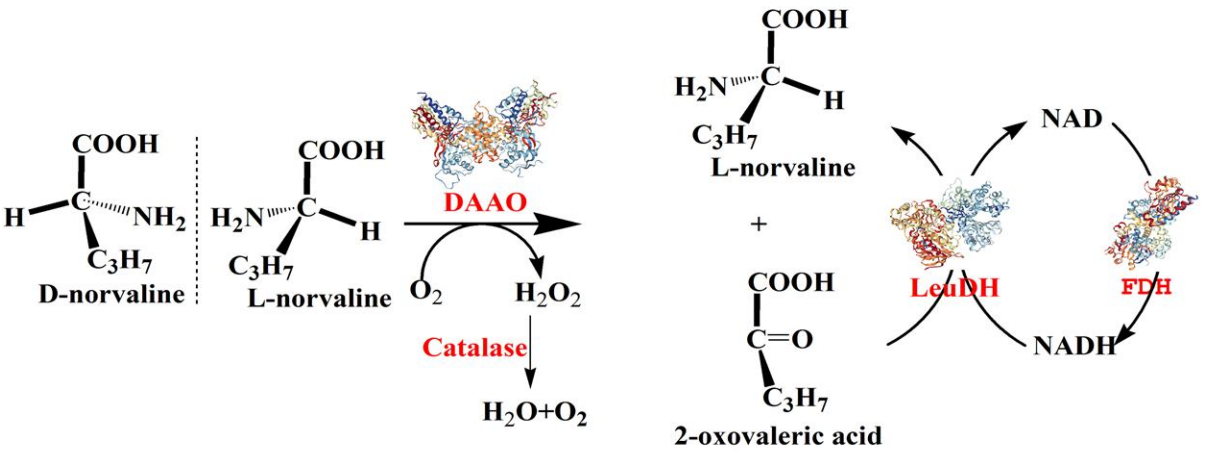
**Graphical Abstract**



Highlights:

- Development of a multi-enzymatic desymmetrization of L-norvaline from DL-norvaline.
- The catalytic system obtained various L-amino acids with varied optical purities.
- The system produced L-norvaline with a high yield, enantiomeric purity and titer.

**Abstract**

Perindopril is an effective antihypertensive drug in strong demand used to treat hypertension. L-norvaline is a vital intermediate of Perindopril production mainly produced by chemical synthesis with low purity. We developed an environmentally friendly method to produce L-norvaline with high purity based on a desymmetrization process. D-Norvaline was oxidized to the corresponding keto acid by D-amino acid oxidase from the substrate DL-norvaline. Asymmetric hydrogenation of the keto acid to L-norvaline was carried out by leucine dehydrogenase with concomitant oxidation of NADH to $NAD^+$. A NADH regeneration system was introduced by overexpressing a formate dehydrogenase. The unwanted $H_2O_2$ by-product generated during D-norvaline oxidation was removed by adding catalase. A total of 54.09 g/L of L-norvaline was achieved, with an enantiomeric excess over 99% under optimal conditions, with a 96.7% conversion rate. Our desymmetrization method provides an environmental friendly strategy for the production of enantiomerically pure L-norvaline in the pharmaceutical industry.

**Keywords:** Desymmetrization; D-amino acid oxidase; Leucine dehydrogenase; NADH regeneration.

**Introduction**

Hypertension, or high blood pressure, is a long-term medical condition that is partially caused by a high-salt, high-fat diet or a stressful lifestyle. Hypertension plagues more than 25% of adults globally and is estimated to be a factor in over 7 million death each year [1]. Several antihypertensive drugs have been developed to treat high blood pressure. Perindopril is an effective antihypertensive drug that is commonly used and is in strong demand [2]. Perindopril treatment decreased the relative risks of strokes and intracerebral hemorrhages in patients by 26% and 49%, respectively, during a 3.9-year period [3]. L-Norvaline is a vital intermediate in the chemical synthesis of Perindopril. Several strategies have been developed for production of L-norvaline. The conventional chemical synthesis of L-norvaline is expensive and has many disadvantages, such as a complicated process, low enantiomeric excess (e.e.<75%) and negative environmental impacts [4] [5]. L-Norvaline can also be produced by bacterial fermentation with an appropriate substrate. Stoynova et al. devised a method to produce L-norvaline using a bacterium from the Enterobacteriaceae family by inactivating its acetohydroxy acid synthases [6]. Soriano et al. reported the production of L-norvaline from racemic N-Formyl-DL-norvaline and N-Carbamoyl-DL-norvaline catalyzed by L-N-carbamoylase and N-succinyl-amino acid racemase [7]. Han et al. employed ω-Transaminase to carry out the asymmetric synthesis of L-norvaline from a keto acid using amino donors [8]. Chiriac et al. developed an enzymatic synthesis of L-norvaline using Leucine dehydrogenase as a catalyst and glucose dehydrogenase plus galactomutarotase as a NADH regeneration system [9].

Enzyme-mediated chiral resolution is a common method for the large-scale production of a single enantiomer of a chiral compound because this method is high efficiency and has high stereo-, regio- and enantioselectivity [10]. Deracemization of chiral compounds via a enantioselective amine oxidases and unspecific reduction process was first introduced by Turner [11], [12]. D-Amino acid oxidase (DAAO, EC 1.4.3.3, GenBank

accession number: AAR98816.1) is a well-known flavoenzyme that catalyzes the oxygen-dependent oxidative deamination of D-amino acids to corresponding α-keto acids with absolute stereo-specificity, which also yields hydrogen peroxide ($H_2O_2$) and $NH_3$ as by-products [13]. DAAO has been attracting considerable attention because of its prospects for the production of 7-aminocephalosporanic acid (7-ACA), the precursor of other semi-synthetic cephalosporins [14]. DAAO is also widely used for chiral resolutions to produce single enantiomers by catalyzing the oxidative deamination of D-amino acids to corresponding α-keto acids [15], [16] and [17]. DAAO from porcine kidneys or *Trigonopsis variabilis* was employed to prepare a keto acid by oxidation of racemic 6-hydroxy norleucine, and subsequent reductive amination of the keto acid catalyzed by glutamate dehydrogenase transformed the mixture completely to give L-6-hydroxy norleucine exclusively [18], [19]. Leucine dehydrogenase (LeuDH, EC 1.4.1.9, GenBank accession number: KPU53554.1) is an NAD(H)-dependent oxidoreductase that is widely used with NADH as a coenzyme for the enantioselective production of L-leucine and other branched-chain L-amino acids [20]. LeuDH can catalyze the conversion of aliphatic 2-oxo-acids to the corresponding L-amino acids via reductive amination [21]. NADH acts as an important coenzyme during catalytic reactions of LeuDH. NADH is costly and its stoichiometric use is economically unfeasible. Various *in situ* NADH regeneration strategies have been developed, such as enzymatical, biological, electrochemical, chemical and photochemical methods [22]. Formate dehydrogenase (FDH, EC 1.2.1.2, GenBank accession number: 013437) is one enzyme capable of regenerating of NADH, which catalyzes the oxidation of formate to carbon dioxide followed by the reduction of $NAD^+$ to NADH. FDH is extensive used as an enzymatic NADH regeneration system in industrial production because formate is an innocuous substrate and the $CO_2$ produced in the regeneration off gasses, which drives the process towards irreversible NADH regeneration [23]. Jiang et al. constructed a regulatory expression system of LeuDH and FDH for the synthesis of L-Tle, which could be used to synthesize other L-amino acid and chiral drug

intermediates [24].

We proposed an alternative strategy for production of L-norvaline through enzyme-mediated kinetic resolution combined with asymmetric hydrogenation of DL-norvaline based on the functions of these three enzymes. In addition, catalase (EC 1.11.1.6, GenBank accession number: KT963080) was added to degrade the unwanted $H_2O_2$ by-product generated during the oxidation of D-norvaline [16], [25] and [26]. This paper provides an effective method for converting DL-norvaline to L-norvaline without any keto acid by-product.

**Materials and methods**

**Chemicals**

DL-Norvaline (content>98.5%, D-norvaline: L-norvaline ratio, 1:1) was purchased from Kebang Chemical Co., Ltd (Henan, China). D-Norvaline (purity 99%) and L-norvaline (purity 99%) were purchased from Sigma-Aldrich Co. LLC (Shanghai, China). All other chemicals were of reagent grade.

**Bacterial strains and plasmids construction**

The bacterial strains, plasmids and primers used in this study are listed in Table s1. The gene *daao*, encoding D-amino acid oxidase (DAAO), was cloned from *T. variabilis* using primers P1/P2 or P3/P4 (primers P3/P4 were for pXMJ19-*daao* construction). The gene *leudh*, encoding LeuDH, was amplified from *Bacillus cereus* using primers P5/P6. The gene *fdh*, encoding FDH, was cloned from *Candida boidinii* with primers P7/P8. The plasmids pET-28a (+)-*leudh* and pET-28a (+)-*fdh* were separately transformed into *Escherichia coli* BL21 (DE3) cells by the calcium chloride method. The plasmid pXMJ19-*daao* was transformed into *Corynebacterium glutamicum* ATCC 13032 using the method described by Xu et al. [27].

**Preparation of DAAO, LeuDH and FDH**

The recombinant *E. coli* and *C. glutamicum* strains were cultivated in 100 mL of Terrific Broth (TB) medium (24 g/L yeast extract, 12 g/L peptone, 4 g/L glycerol, 2.31 g/L $KH_2PO_4$, 12.54 g/L $K_2HPO_4$) at 37 °C

and 30 °C, respectively, until the $OD_{600}$ reached 0.6–0.8. Isopropyl β-D-Thiogalactoside (IPTG, 0.8 mM) was added and incubation continue for 12 h. Cells were harvested by centrifugation at 10000 rpm and 4 °C for 10 min and washed twice with 0.1 M sodium phosphate buffer (pH 7.5). The cells were resuspended with 10 mL of 0.1 M sodium phosphate buffer (pH 7.5). The dry cell weights of ATCC13032/pXMJ19-*daao*, BL21 (DE3)/pET-28a (+)-*leudh* and BL21 (DE3)/pET-28a (+)-*fdh* were 6.32, 5.14 and 3.06 g/L, respectively. Lysozyme (100 mg/mL) was used to aid *C. glutamicum* cell lysis before ultrasonic decomposition. Cells were sonicated for 3 s 100 times with 7 s cooling intervals. Cell disruption by high-pressure homogenizer was conducted at a pressure of 1000 bar for 20 min at 4 °C. The lysed cells were centrifuged at 12,000 rpm and 4 °C for 25 min, and the supernatant was used for enzyme assays.

**Assays of DAAO, LeuDH and FDH activities**

DAAO activity was assessed spectrophotometrically by measuring the formation of 2-oxovaleric acid at 550 nm [28]. LeuDH activity was measured by spectrophotometrically monitoring the consumption of NADH in the reductive amination at 340 nm ($\varepsilon$=6220 $M^{-1}cm^{-1}$) [21]. FDH activity was assayed by the method described by Berrios-Rivera et al. [29]. A Bradford Protein Assay Kit (Sangon Biotech Co, Ltd) was used to determine protein concentration.

**Production of L-norvaline by enzyme-mediated desymmetrization**

DAAO, LeuDH and FDH were added according to the optimal dosage ratio of the enzymes into a 50-mL enzymatic conversion mixture (0.1 M sodium phosphate buffer, pH 7.0) containing 1 M ammonium formate, 5% glycerol, 5 g/L DL-norvaline and 30 U/mL catalase (prepared by our laboratory [30]). The enzymatic resolution was performed on a magnetic stirrer at 25 °C and 160 rpm. Formate (20%) or ammonium hydroxide (50%) were added to maintain the pH at 7.0 during the process.

**Production of L-norvaline by fed-batch**

The three crude enzymes, DAAO, LeuDH and FDH, were released using a high-pressure homogenizer. The crude enzymes were added according to their optimal dosages to a 5-L reactor containing 2 L of 0.1 M sodium phosphate buffer (pH 7.5). An initial DL-norvaline charge of 5.0 g/L was added to the reactor, with a further 5.0 g/L added into the transformation system to continue the enzymatic reaction when almost all the D-norvaline was converted to L-norvaline. The temperature, pH and rotor speed were kept at 30 °C, 7.5 and 160 rpm, respectively.

**Analysis of products**

The concentrations of D- and L-norvaline were assayed by HPLC (Agilent 1260, Agilent Technologies, Palo Alto, CA, USA) with a UV detector at 338 nm as described by Denton et al. [31] using a Chiralcel OD-3R column (150×4.6 mm, 3-Micron, Daicel). The eluent was composed of buffer A (0.1% $H_3PO_4$ in water) and buffer B (100% MeCN), with gradient elution at 40 °C and a flow rate of 1 mL/min [31]. Conversion rate was calculated by the following equation:

$$\text{Conversion rate} = \frac{\text{produced } m_{\text{L-norvaline}}}{m_{\text{D-norvaline}}} \times 100\%$$

where $m_{\text{L-norvaline}}$ is the titer of L-norvaline produced from D-norvaline and $m_{\text{D-norvaline}}$ is the titer of D-norvaline contained in the substrate DL-norvaline.

**Results and Discussion**

**Preparation of DAAO, LeuDH and FDH**

LeuDH from *Bacillus cereus* and FDH from *Candida boidinii* were successfully overexpressed in *E. coli* BL21 (DE3). The specific activity of crude LeuDH in the recombinant strain was 2.96 U/mg, which was 59.2-fold higher than that of the parent strain (Table s2). This activity was a little lower than that of LeuDH from *Bacillus sphaericus* (GenBank accession number: BAD11020) [32] likely due to the differences of amino acid sequence (sequence identity was 79.12%). The specific activity of crude FDH from the engineered *E. coli* was

0.39 U/mg, and no FDH activity was detected in the wild type *E. coli* BL21 (DE3). DAAO expression failed in *E. coli* BL21 (DE3). Expression of DAAO in *E. coli* BL21 (DE3) has previously been reported to resulted in a marked decrease of cell viability owing to the toxicity of DAAO activity toward D-alanine, an essential amino acid for cell wall construction [33], [34]. We screened other species for a candidate to host DAAO production and the gram-positive bacteria *C. glutamicum* ATCC 13032 was chosen as a suitable host for DAAO expression. The activity of crude DAAO in the engineered *C. glutamicum* was 0.3 U/mL, which was slightly lower than that of other DAAOs likely due to the different substrates and amino acid sequence differences [35], [36]. No DAAO activity was detected in the parent *C. glutamicum* ATCC 13032 strain.

**Substrate spectrum of the multi-enzymatic desymmetrization system**

Both DAAO and LeuDH have broad substrate spectrums, including various D-amino acids and α-keto acids [37], [38]. Desymmetrizations of a variety of DL-amino acids were attempted to produce enantiomeric pure L-amino acids with our system. L-Methionine, L-valine, L-arginine, L-leucine, L-histidine L-norvaline and L-phenylalanine were all produced form their respective racemates (Table 1). The system was highly effective for the production of L-valine, obtaining an e.e of >99%. However, DAAO was more active towards D-valine than D-histidine, and the conversion rate of D-histidine to L-histidine was much slower than that of D-valine to L-valine [37].

**Production of L-norvaline by the enzyme-mediated desymmetrization**

The lysate supernatants of recombinant strains *E. coli* BL21 (DE3)/pET-28a (+)-*fdh*, *E. coli* BL21 (DE3)/pET-28a (+)-*leudh* and *C. glutamicum* ATCC 13032/pXMJ19-*daao* were used as catalysts. The dosages of DAAO, LeuDH, and FDH in the conversion mixture were 0.6, 1 and 0.8 U/mL, respectively. Due to little activity of LeuDH was detected in the parent strain *E. coli* BL21 (DE3), so a reaction blank without the recombinant LeuDH extract was also investigated, D-norvaline was oxidased to corresponding keto acid

completely but only 0.04 g/L L-norvaline was produced from D-norvaline during 2 h of biocatalysis. When recombinant LeuDH extract was employed as catalyst, 0.48 g/L D-norvaline was transformed to L-norvaline at 2 h. It is probably that there were some LeuDH homologs produced in *E. coli* BL21 resulting in LeuDH activity detected in the parent strain, but these LeuDH homologs showed much lower catalytic capacity for the keto acid oxidased from D-norvaline than that of the recombinant LeuDH. However, by-product $H_2O_2$ was generated during the oxidation of D-norvaline by DAAO, which has deleterious effects on enzyme catalysts and decarboxylates the intermediate keto acid [39], [40]. This degradation of the intermediate keto acid would have decrease the product yield drastically. Catalase (30 U/mL) was added to degrade the unwanted by-product $H_2O_2$, eliminating its toxic effects on the catalysts and intermediate. With this addition, 1.66 g/L D-norvaline was converted into L-norvaline after 2 h of biocatalysis.

**Optimization of enzyme-mediated L-norvaline biocatalyzed desymmetrization conditions**

Each enzyme catalyst involved in the conversion has its own optimal pH and temperature conditions. The optimal temperatures of DAAO and LeuDH are 25 °C and 55 °C, respectively, and the optimal pH of DAAO and LeuDH are 8.0 and 8.5, respectively [41], [42], [43]. The optimal pH of FDH is around 7.5–8.5, with an optimal temperature of 37 °C [44]. The pH and temperature of the biocatalysis system should be optimized to achieve the highest product yield and productivity because the optimal pH and temperature conditions are not the same for all the enzyme catalysts. The effects of pH on the enzymatic resolution were investigated between 5.5–10.5. The conversion rate improved as pH increased until the maximum conversion rate was reached at pH 7.5, then the conversion rate declined gradually with further increases in pH (Fig. 1a). Temperature can also affect the efficiency of enzymatic resolutions processes. The effect of temperature on our enzymatic resolution process was investigated by carrying out six enzymatic resolutions at temperatures varying from 20 to 45 °C at pH 7.5. The conversion rate gradually increased with temperature up to 30 °C and then decreased at higher

temperatures, probably because of thermal inactivation of the enzymes at high temperature (Fig. 1b). The optimal conversion conditions were at pH 7.5 and a temperature of 30 °C, which were employed in the following experiments.

DAAO and LeuDH act as the main catalysts that participate in the kinetic resolution process directly. The catalytic rates of DAAO and LeuDH were different, and the dosage ratio of DAAO and LeuDH in the conversion mixture should be optimized in order to balance the individual catalytic steps. The activities of DAAO, LeuDH and FDH were considered stable according to previous reports [41], [45], [46]. The initial rates of DAAO and LeuDH are shown in Fig. s1a and b, respectively, and the slopes of these plots were used to calculate the conversion rates and compare the catalytic rates of DAAO and LeuDH. The catalytic rates of DAAO to convert D-norvaline to the keto acid and LeuDH to convert the keto acid to L-norvaline were 0.0015 g/(L·min·U) and 0.003 g/(L·min·U), respectively. Thus, the optimal ratio of DAAO to LeuDH activity was theoretically 2:1 because the ratio of catalytic rates was 1:2, and the highest conversion rate was achieved at this ratio (Fig. 2a). The rate dropped gradually when the ratio exceeded 2:1 because DAAO suffered from product inhibition at a high concentration of keto acid (Fig. s2) [47], and a high concentration of the keto acid also inhibited the LeuDH activity (Fig. s3) [48]. The optimal dosage ratio of DAAO to LeuDH activity of 2:1 was employed in the following experiments.

The optimal dosage ratio of LeuDH to FDH activity was also investigated, with the ratio of DAAO to LeuDH activity kept at 2:1. Only 0.7 g/L of L-norvaline was produced in 2 h when no FDH was added (Fig. 2b-f). The amount of L-norvaline produced in 2 h was 2.55 g/L when the dosage ratio of LeuDH to FDH activity was 1:0.5, which was 3.5-fold higher than that of no FDH added. This indicates that FDH activity is the rate-limiting step. However, further increases in the proportion of FDH activity failed to improve the production of L-norvaline. There is a sequentially ordered ternary/binary kinetic mechanism for the reductive amination

process catalyzed by LeuDH, in which NADH, keto acid and ammonia bind to LeuDH in that order [49]. NADH showed noncompetitive inhibition with the keto acid and a high concentration of NADH prevented the keto acid binding to LeuDH, leading to a low production of L-norvaline. We used the optimal dosage ratio of LeuDH to FDH activity of 1:0.5 in subsequent experiments.

**Fed-batch conversion under optimal conditions for biocatalysis of L-norvaline**

A fed-batch strategy could effectively improve the concentration of the desired product. High DL-norvaline concentration inhibits the conversion, and high L-norvaline production could be attained using a fed-batch strategy to mitigate the inhibition. We used an initial DL-norvaline concentration of 5.0 g/L and added a further 5 g/L charge into the conversion system when the D-norvaline was all almost converted to L-norvaline. As shown in Fig. 3, the L-norvaline yield increased rapidly during the nine fed-batches and 23.74 g/L of D-norvaline was converted into L-norvaline, with an average productivity of 1.187 g/(L·h). However, total L-norvaline production increased slowly after the tenth fed-batch and the productivity gradually decreased because of inactivation of the enzyme catalysts (Fig. s4) and limitation caused by high product concentration (Fig. s5). Almost no intermediate keto acid accumulated during the conversion process, which is favorable for separation and purification of the product. A total of 26.49 g/L of D-norvaline was consumed in 25 h, and the L-norvaline titer reached 54.09 g/L with a conversion rate of 96.3% and an e.e. over 99%.

Several chemical synthesis and biosynthesis methods for L-norvaline production are listed in Table 2. ω-Transaminase was adopted for the asymmetric synthesis of L-norvaline from a keto acid using the amino donor isopropylamine and gave almost 50 mM (5.8 g/L) of L-norvaline in 2 h with a conversion rate of 99.3% [8]; however, an unwanted byproduct isopropanol was generated during the deamination of amino donor isopropylamine, which would increase the cost of product purification. Taking advantage of the catalytic promiscuity of L-N-carbamoylase and N-succinyl-amino acid racemase, Soriano et al evaluated the production

of L-norvaline starting from racemic N-formyl-DL-norvaline and N-Carbamoyl-DL-norvaline [7], but only 7.5 mM (0.87 g/L) of substrate was converted in 3 h with a conversion rate of 95%. Furthermore, a stereospecific enzymatic synthesis of L-norvaline from the corresponding keto acid catalyzed by Leucine dehydrogenase combined with glucose dehydrogenase/galactose mutarotase has also been reported and 0.11M (12.3 g/L) keto acid was transformed to L-norvaline with the yield of 92% in 2-3h [9]. Galkin et al described a method for the conversion of keto acid to L-norvaline with recombinant *E.coli* cells which contain plasmids with *leudh* and *fdh* genes necessary for biotransformation, 0.38 M (44.4 g/L) L-norvaline was synthesized in 12 h [50]. In this study, we employed DL-norvaline as substrate for biosynthesis of L-norvaline, and the titer of L-norvaline reached to 54.09 g/L in 25 h with a productivity of 1.06 g/ (L·h) and a conversion rate of 96.7%. Furthermore, no byproduct was generated. The L-norvaline titer, and yield obtained in this work could be comparable to the most efficient strategy (Table 2).

In conclusion, an enzyme-mediated desymmetrization system for L-norvaline production was successfully developed by applying DAAO, LeuDH, FDH and catalase. Optimization of conversion conditions and a fed-batch strategy were investigated to improve the conversion yield. This study provides a promising bioconversion method for enantiomerically pure L-norvaline production. However, the preparation of the conversion system is time-consuming because bacteria do not naturally secrete these enzymes extracellularly and cell wall disruption is necessary. Extracellular expression of the enzyme catalysts is more desirable and our group is undertaking work to achieve this goal.

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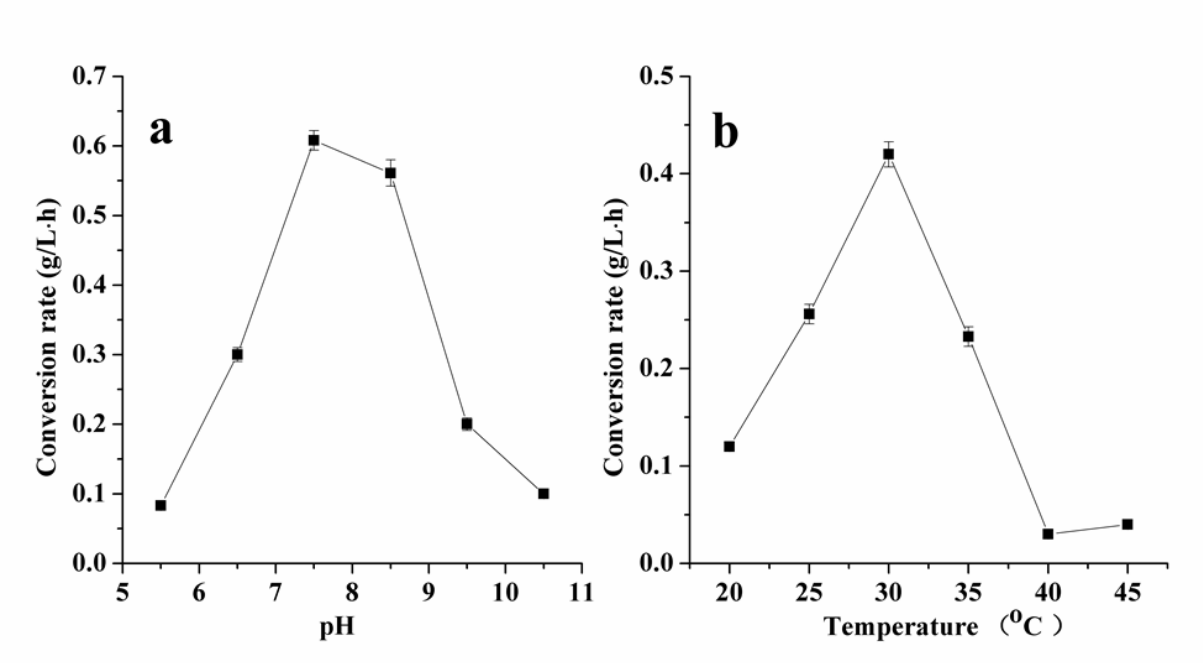


**Fig. 1** Optimization of enzymatic conversion conditions for enantiomerically pure L-norvaline production **a** Effects of pH on the enzyme-mediated desymmetrization. The following pH buffers were employed for the optimal pH experiments: 0.1 M acetate-sodium acetate (pH 4.5–5.5); sodium phosphate ($Na_2HPO_4$-$NaH_2PO_4$) (pH 5.5–8.5); $Na_2B_4O_7$-NaOH (pH 8.5–10.5). **b** Effects of temperature on the enzymatic desymmetrization. All assays were performed in triplicate with three independent measurements. Results are presented as the mean with standard deviation error bars for each condition.

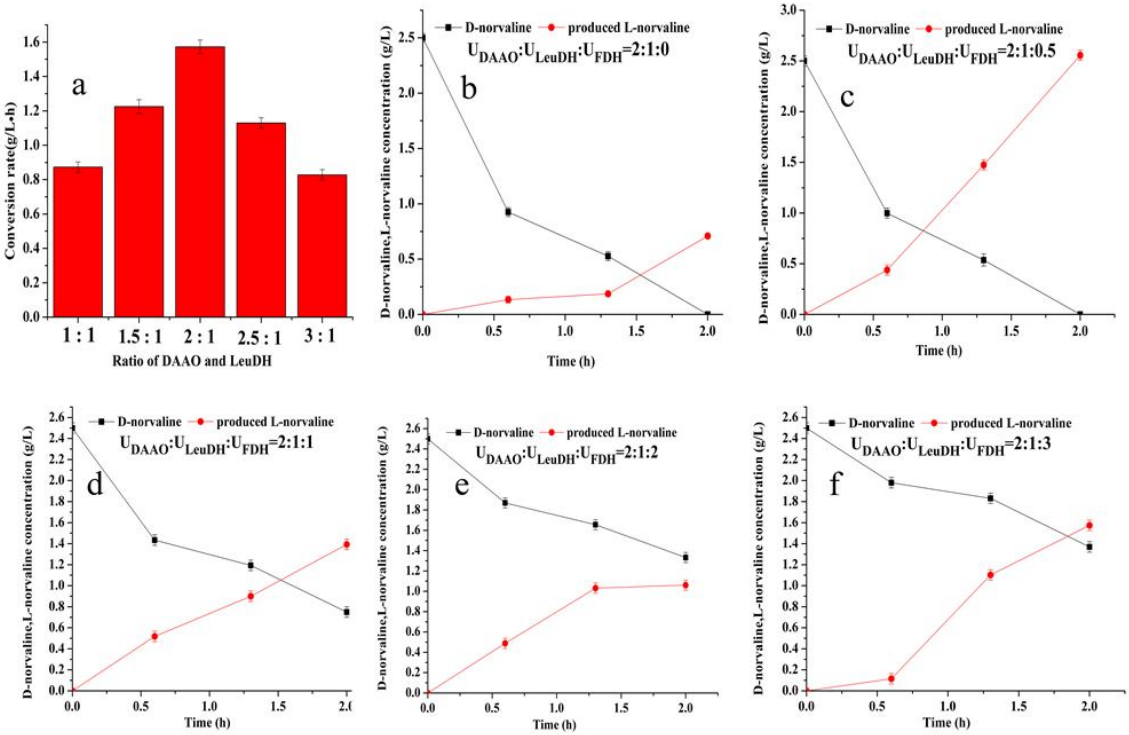


**Fig. 2** Optimization of dosage of DAAO, LeuDH and FDH **a** Effects of different DAAO to LeuDH ratios on the conversion rate of L-norvaline. The conversion was carried out with various dosages of DAAO (1.2 U·mL$^{-1}$, 1.8 U·mL$^{-1}$, 2.4 U·mL$^{-1}$, 3 U·mL$^{-1}$, 3.6 U·mL$^{-1}$), 1.2 U·mL$^{-1}$ LeuDH, 1.2 U·mL$^{-1}$ FDH, 30 U·mL$^{-1}$ catalase and 5 g/L DL-norvaline. **b-f** Effects of different ratios of LeuDH to FDH activity on L-norvaline production. The conversion was carried out with 2.4 U·mL$^{-1}$ DAAO, 1.2 U·mL$^{-1}$ LeuDH, 30 U·mL$^{-1}$ catalase and various dosages of FDH (0 U·mL$^{-1}$, 0.6 U·mL$^{-1}$, 1.2 U·mL$^{-1}$, 2.4 U·mL$^{-1}$, 3.6 U·mL$^{-1}$). Ratios of DAAO, LeuDH and FDH: **b**, 2:1:0; **c**, 2:1:0.5; **d**, 2:1:1; **e**, 2:1:2; **f**, 2:1:3.

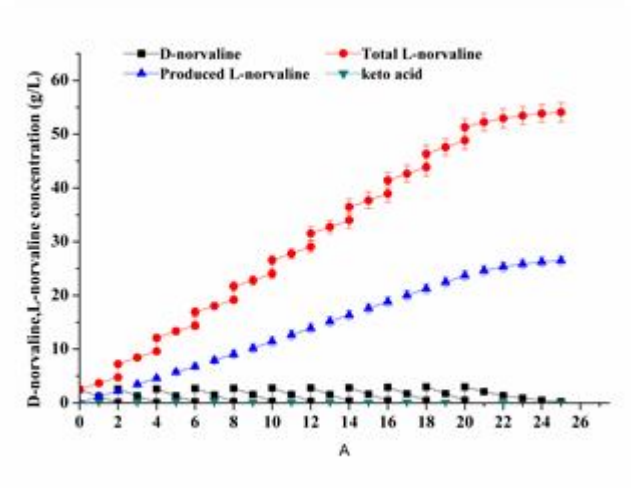


**Fig. 3** Time profiles of a fed-batch enzyme-mediated desymmetrization of DL-norvaline to L-norvaline

**Table 1.** Substrate spectrum of the multi-enzymatic desymmetrization system

| Substrate | Product | Conversion rate (%) |
|---|---|---|
| D-met | L- met | 56.8 |
| D-val | L-val | 99.7 |
| D-arg | L-arg | 92.8 |
| D-leu | L-leu | 30.4 |
| D-his | L-his | 16.4 |
| D-phe | L-phe | 32.0 |
| D-nor | L-nor | 77.4 |

Conversions were carried out with 25 mM of the different substrates in 0.1 M buffer (pH 7.5) at 30 °C for 2 h, 2.4 $U \cdot mL^{-1}$ DAAO, 1.2 $U \cdot mL^{-1}$ LeuDH, 0.6 $U \cdot mL^{-1}$ FDH and 30 $U \cdot mL^{-1}$ catalase were contained in the conversion mixture.

**Table 2**. Different strategies for production of L-norvaline

| Methods | Substrate | Conditions | Conversion rate or yield (%) | Titer (g/L) | Productivity (g/L/h) | e.e. (%) |
|---|---|---|---|---|---|---|
| Chemical synthesis | Racemic α-valeric acid | (S)-2-[(Nalkylprolyl)amino]-benzophenones as chiral auxiliary | 70 | nm | nm | 72 |
| | (1S,2S)-2-(1-phenylethylamino) butyronitrile | S-α-Aminophenethylamine as chiral auxiliary and Cyanide, Methanol were necessary | 75 | nm | nm | n.m. |
| | Valeric acid | α-brominevaleryl chloride and $Br_2$ were required. | 26.7 | 23.3 | nm | <75 |
| Biosynthesis | N-acyl-α-amino-valeric acid | Aminoacylase from pig kidney was employed as catalyst | 77 | nm | nm | >90 |
| | α-keto acid | Transaminases from different sources were used as catalysts and amino donor was necessary | 99 | 5.8 | 2.9 | 99 |
| | D-Formyl-D, L -norvaline and N-Carbamoyl-D, L-norvaline | Immobilized L-N-carbamoylase and N-succinyl -amino acid racemase was acted as the catalyst | >95 | 0.87 | 0.29 | >99 |
| | α-keto acid and ammonium formate | LeuDH and FDH were simultaneous expressed in *E. coli* and used as catalysts | 95 | 44.4 | 3.70 | 100 |
| | α-keto acid | Couple the reactions catalyzed by LeuDH and GluDH/galactose mutarotase | 92 | 12.3 | 4.10 | n.m. |
| | Glucose | Fermentation of bacterium from the Enterobacteriaces family whose acetohydroxy acid synthases were inactivated | n.m. | 0.9 | 0.012 | n.m. |
| | DL-norvaline | DAAO, LeuDH combined with FDH were actd as catalysts, catalase was used to remove unwanted $H_2O_2$ | 96.3 | 54.09 | 1.06 | >99 |

n.m. not mentioned in the reference, e.e. enantiomeric excess, DAAO D-amino acid oxidase; LeuDH leucine dehydrogenase, FDH formate dehydrogenase, GluDH glucose dehydrogenase