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Research article

Biosynthetic L-*tert*-leucine using *Escherichia coli* co-expressing a novel NADH-dependent leucine dehydrogenase and a formate dehydrogenase

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ABSTRACT

Background: L-*tert*-Leucine has been widely used in pharmaceutical, chemical, and other industries as a vital chiral intermediate. Compared with chemical methods, enzymatic methods to produce L-*tert*-leucine have unparalleled advantages. Previously, we found a novel leucine dehydrogenase from the halophilic thermophile *Laceyella sacchari* (*Ls*LeuDH) that showed good thermostability and great potential for the synthesis of L-*tert*-leucine in the preliminary study. Hence, we manage to use the *Ls*LeuDH coupling with a formate dehydrogenase from *Candida boidinii* (*Cb*FDH) in the biosynthesis of L-*tert*-leucine through reductive amination in the present study.

Result: The double-plasmid recombinant strain exhibited higher conversion than the single-plasmid recombinant strain when resting cells cultivated in shake flask for 22 h were used. Under the optimized conditions, the double-plasmid recombinant *E. coli* BL21 (pETDute-FDH-LDH, pACYCDute-FDH) transformed 1 mol·L^{-1} trimethylpyruvate (TMP) completely into L-*tert*-leucine with greater than 99.9% *ee* within 8 h.

Conclusions: The *Ls*LeuDH showed great ability to biosynthesize L-*tert*-leucine. In addition, it provided a new option for the biosynthesis of L-*tert*-leucine.

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1. Introduction

L-*tert*-Leucine is a non-polar amino acid and its *tert*-butyl group has significant hydrophobicity and steric hindrance, which can well control molecular conformation in a chemical reaction to form a chiral compound [1]. Hence, L-*tert*-leucine is commonly used as an inducer or a template in asymmetric synthesis [2,3]. As a vital chiral intermediate, L-*tert*-leucine is widely used in the synthesis of anti-cancer, anti-viral, and biological inhibitors [4,5,6]. In addition, L-*tert*-leucine is an essential additive in the food and cosmetic industry [7].

In earlier studies, the preparation of L-*tert*-leucine using chemical methods was restrained by the low stereoselectivity, high pollution, complicated process, and low yield [8,9,10]. Currently, enzymatic methods have gradually replaced chemical methods. Some previous research on the preparation of L-*tert*-leucine using hydrolase [7,8,9,10,11], acyltransferase [12], and amino acid dehydrogenase [13,14,15,16,17] have been reported. Among them, the preparation of L-*tert*-leucine using NADH-dependent L-leucine dehydrogenase (LeuDH, EC 1.4.1.9) has exhibited the most industrial potential. In the presence of NADH and free ammonium, LeuDH can catalyze trimethylpyruvate (TMP) to L-*tert*-leucine [18,19]. In order to improve catalytic efficiency and reduce costs, the coenzyme regeneration system has been developed. NAD^+-dependent formate dehydrogenase (FDH, EC 1.2.1.2) can convert formate into CO_2, which is accompanied by the production of NADH [20]. Therefore, FDH is usually applied in coenzyme regeneration systems. Among all FDHs from difference sources [20,21], FDH from *Candida boidinii* (*Cb*FDH) has been widely used for the regeneration of NADH and has also been demonstrated on a technical scale [22]. At present, the coupling of LeuDH and *Cb*FDH to form a coenzyme regeneration system for the preparation of L-*tert*-leucine is the focus of research. This method enables NAD^+ to be continuously regenerated and reduces costs [14,15], and Degussa has successfully applied a coupling LeuDH and FDH system to produce L-*tert*-leucine on a large scale (Fig. 1) [23].

In a previous study, we found a novel LeuDH from the halophilic thermophile *Laceyella sacchari* (*Ls*LeuDH) [24]. The *Ls*LeuDH showed

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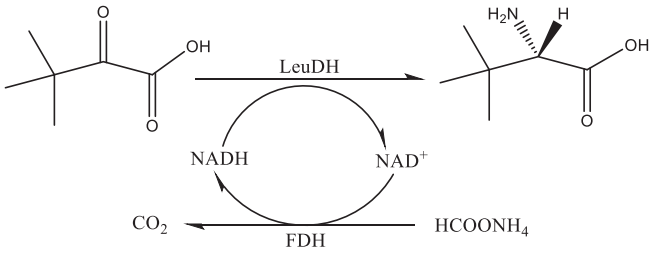

Fig. 1. Enzymatically converting TMP into L-*tert*-leucine using the recombinant *E. coli* coexpressing NADH-dependent LeuDH and NAD^+-dependent FDH.

good thermostability and great potential for the synthesis of L-*tert*-leucine in preliminary study. Hence, in this paper, we constructed a recombinant strain of *E. coli* for co-expression of *Ls*LeuDH and *Cb*FDH to form a bienzymatic system and for use in converting TMP into L-*tert*-leucine.

2. Materials and methods

2.1. Materials

Restriction endonuclease, T4 DNA ligase, DNA polymerase, and *E. coli* BL21 (DE3) were purchased from Vazyme (Nanjing, China). The plasmids were purchased from MiaoLingBio (Wuhan, China), and primers used in this study were synthesized by GenScript (Nanjing, China). All chemicals were of analytical grade and purchased from Aladdin (Shanghai, China), unless otherwise noted.

2.2. Plasmid construction and co-expression of LsLeuDH and CbFDH

The plasmid pET-22b-LeuDH containing the *leudh* gene encoding *Ls*LeuDH (GenBank accession number: WP_040387131) from *L. sacchari*, was constructed in our previous work [24]. The gene *fdh* encoding FDH (GenBank accession number: ABE69165) from *C. boidinii* was codon-optimized and synthesized by GenScript (Nanjing, China) and it was inserted into the *Nco*I/*Eco*RI site of the plasmid pACYCDute-1 to construct the plasmid pACYCDute-FDH. Then the gene of FDH and LeuDH were subcloned into the *Nco*I/*Eco*RI and *Nde*I/*Xho*I sites of the plasmid pETDuet-1 to generate the plasmid pETDuet-FDH-LeuDH. All the constructed plasmids were verified by double digestion and DNA sequencing. The pETDuet-FDH-LeuDH were transformed into *E. coli* BL21 (DE3) to create recombinant bacteria *E. coli* BL21 (pETDute-FDH-LDH). Subsequently, the plasmid pACYCDute-FDH was transformed into *E. coli* BL21 (pETDute-FDH-LDH) to form the double-plasmid recombinant bacteria *E. coli* BL21 (pETDute-FDH-LDH, pACYCDute-FDH). The positive transformants were verified by colony PCR.

To co-express *Ls*LeuDH and *Cb*FDH, the recombinant *E. coli* BL21 (pETDute-FDH-LDH) was grown on an auto-inducing medium containing: 1.5% (w/v) peptone, 2.5% (w/v) yeast extract, 1% (w/v) NaCl, 0.2% glucose, 0.3% lactose, and 100 mg·L^{-1} ampicillin. The cultures were incubated at 37°C and 180 rpm for 1 h, and then were continually oscillated at 180 rpm at 30°C for 20 h. Then, the cultures were collected and centrifuged at 4 °C and 4000 × *g* for 5 min. The pellets were then washed with 20 mL phosphate buffer (100 mmol·L^{-1}, pH 7.0) and stored at -20°C. The recombinant *E. coli* BL21 (pETDute-FDH-LDH, pACYCDute-FDH) has the same cultivation conditions and steps, but it should be noted that it is a double-plasmid strain, and the cultures should be supplemented with chloramphenicol (34 mg·L^{-1}).

2.3. Bioconversion of TMP into L-tert-leucine

Bioconversion of TMP into L-*tert*-leucine was carried out using the resting cells of the two recombinant strains co-expressing *Ls*LeuDH and *Cb*FDH. The reaction mixture containing 60 g·L^{-1} wet cells, 1 mol·L^{-1} TMP, 3 mol·L^{-1} ammonium formate, 1.5 mol·L^{-1} NAD^+, 0.1% (v/v) triton, and 100 mmol·L^{-1} phosphate buffer (pH 8.5) was incubated at 35°C and 200 rpm. The pH was adjusted to 8.5 with 2 mol·L^{-1} NaOH.

2.4. Analysis

TMP was analyzed by Agilent1260 (Agilent Technologies, Santa Clara, CA, USA) HPLC system equipped with an InertSuatain® C18 column (3.5 μm, 4.6 mm × 100 mm) at 244 nm and 25°C. The gradient elution profile is as follows: 96% A and 4% B at 0–5 min; rapidly switching to 4% A and 96% B at 5–8 min and remaining 2 min; and then rapidly switching to 96% A and 4% B at 10–13 min and remaining 3 min (A: 30 mmol·L^{-1} ammonium acetate; B: acetonitrile). The flow rate of mobile phase was 0.8 mL·min^{-1}.

The derivatization of L-*tert*-leucine with *o*-nitrobenzenesulfonyl chloride was required before HPLC analysis [25]. The concentration of L-*tert*-leucine was also analyzed on C18 column with the same mobile phase as described above at 275 nm and 25°C (Fig. S1). The gradient elution profile is as follows: 96% A and 4% B at 0–5 min; gradually switching to 42% A and 58% B at 5–29 min; continually switching to 10% A and 90% B at 29–32 min and remaining 2 min; and then rapidly switching to 96% A and 4% B at 34–38 min and remaining 2 min. L-*tert*-Leucine was derivatized and then analyzed for chirality on a CHIRALPAK® WH column (50 mm × 4.6 mm,10 μm) at 254 nm, eluted with 2.5 mmol·L^{-1} copper sulfate solution [14].

3. Results

3.1. Bioconversion of TMP into L-tert-leucine by the two recombinant strains

We first investigated the catalytic ability of the *E. coli* BL21 (pETDute-FDH-LDH) and the *E. coli* BL21 (pETDute-FDH-LDH, pACYCDute-FDH) to biosynthesize L-*tert*-leucine. The resting cell bioconversion system was carried out for 12 h and sampled periodically. Both recombinant strains have the ability to convert TMP in>to L-*tert*-leucine; however, the two recombinant strains presented

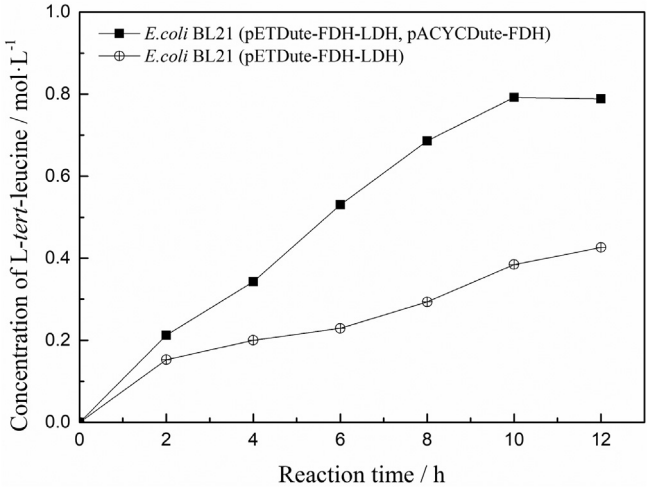

Fig. 2. Biosynthetic L-*tert*-leucine ability of the two recombinant strains.

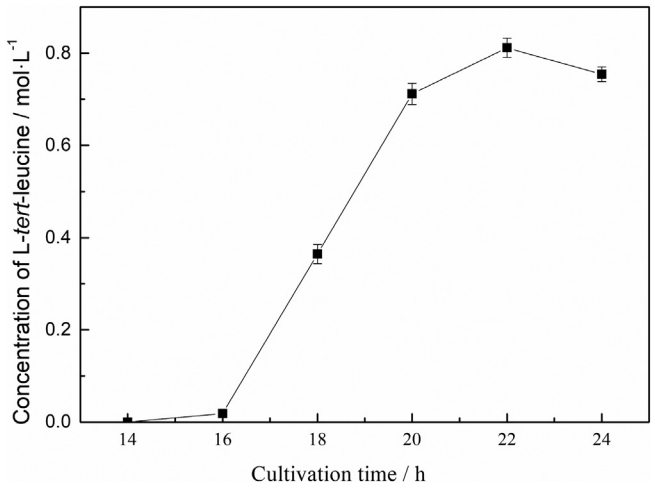

Fig. 3. Effect of cultivation time of the recombinant *E. coli* BL21 (pETDute-FDH-LDH, pACYCDute-FDH) on the synthesis of L-*tert*-leucine.

significant differences in the production of L-*tert*-leucine. The titer of L-*tert*-leucine converted by the *E. coli* BL21 (pETDute-FDH-LDH, pACYCDute-FDH) rose rapidly, reaching around 0.8 mol·L^{-1} within 12 h, which was more than twice that of the *E. coli* BL21 (pETDute-FDH-LDH) (Fig. 2). According to HPLC analysis, the enantiomeric excess (*ee*) of L-*tert*-leucine produced by the two recombinant strains was greater than 99.9% (Fig. S2). Therefore, *E. coli* BL21 (pETDute-FDH-LDH, pACYCDute-FDH) was applied to biosynthesize L-*tert*-leucine in the subsequent studies.

The cultivation time is very important for the activity of the resting cells for recombinant strains. We collected cells that were cultivated at different times for bioconversion experiments. The production of L-*tert*-leucine was very low for resting cells of the recombinant *E. coli* BL21 (pETDute-FDH-LDH, pACYCDute-FDH) cultivated for 16 h, but the production of L-*tert*-leucine was at maximum when the recombinant strain was cultivated for 22 h (Fig. 3). The results showed that the optimal cultivation time of the recombinant strain was 22 h.

3.2. Effect of substrate and cosubstrate on the reaction

Substrate concentration is one of the important factors affecting the conversion of enzymatic reaction. The effect of TMP concentration on the reaction was presented in Fig. 4a. The concentration of L-*tert*-leucine first increased with the increase of the TMP concentration to 1

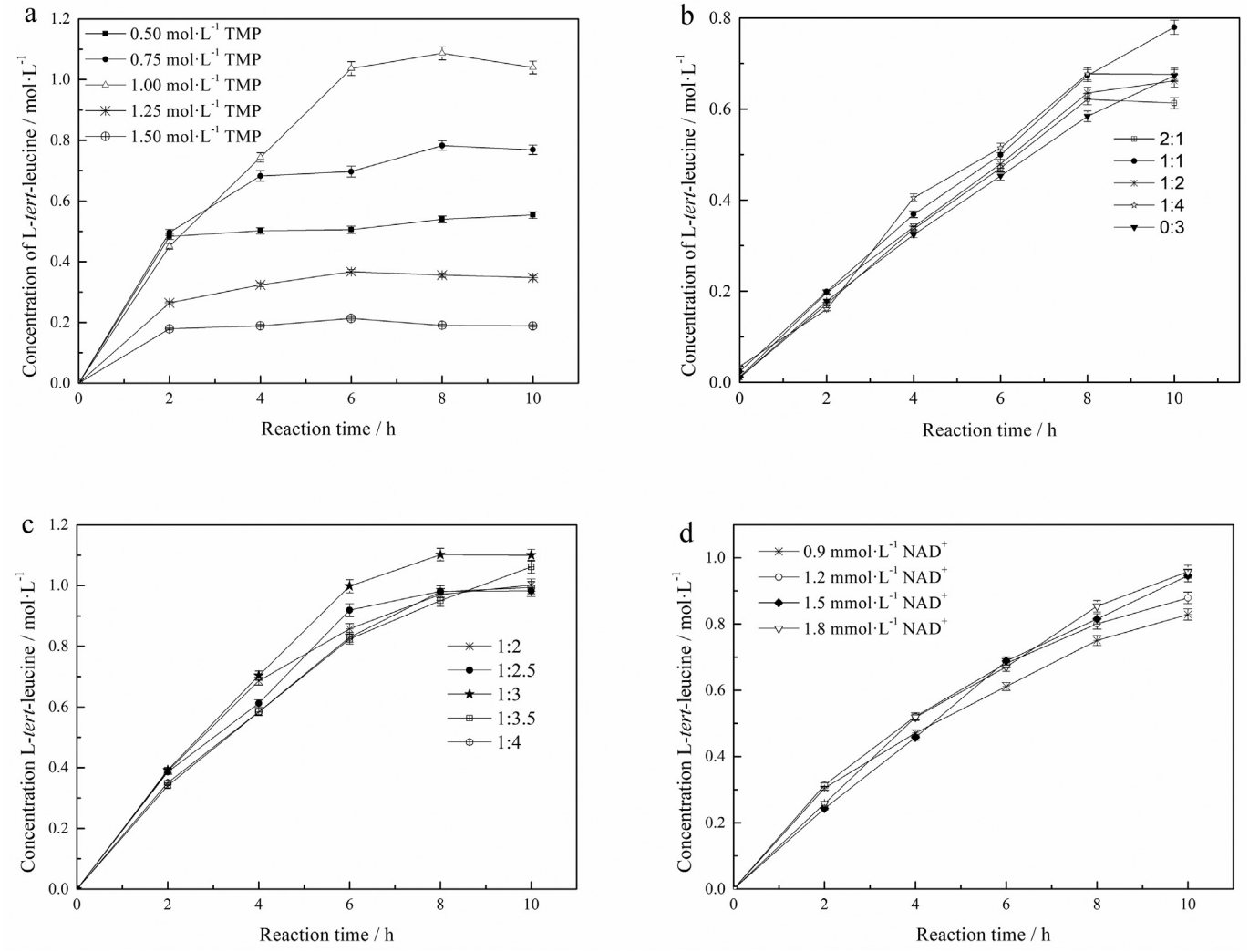

Fig. 4. Effect of substrate and cosubstrate on the synthesis of L-*tert*-leucine. a) The concentrations of TMP; b) the ratios of sodium formate to ammonium formate; c) the ratios of TMP to formate; d) the concentrations of NAD^+.

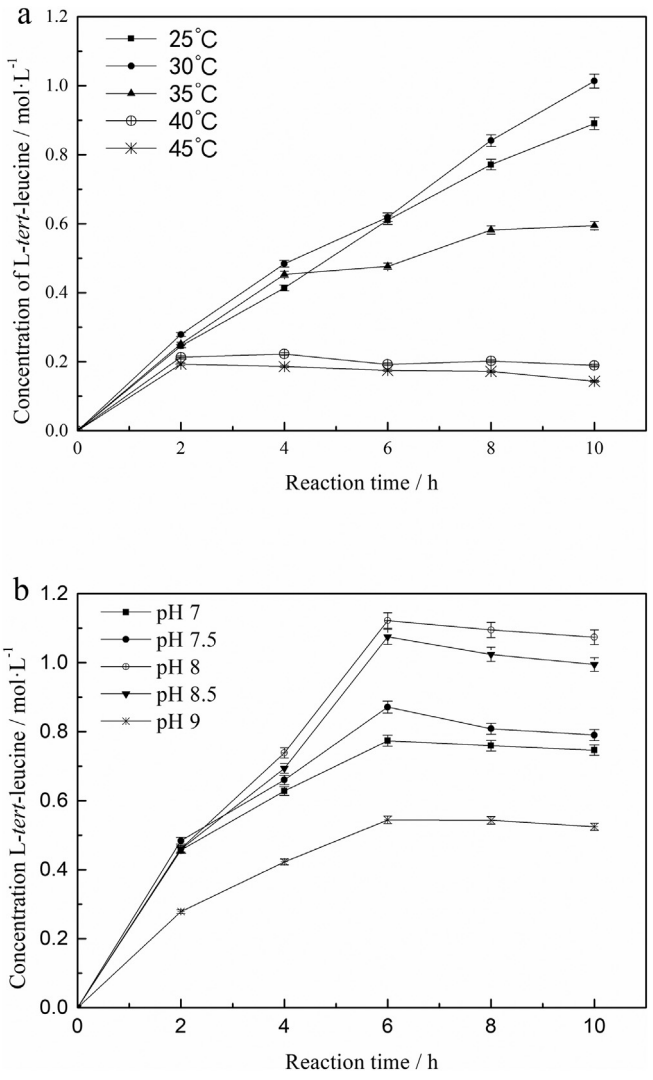

Fig. 5. Effect of temperature and pH on the synthesis of L-*tert*-leucine. a) Temperature; b) pH.

mol·L^{-1} and then decreased at higher TMP concentration. This indicates that the optimum TMP concentration is 1 mol·L^{-1}.

Cosubstrates ammonium formate and NAD$^+$ in this reaction plays an indispensable role in the efficiency of NADH regeneration [26,27]. In particular, ammonium formate not only provides formate ions as a substrate for FDH, but also an amino donor for the synthesis of L-*tert*-leucine by LeuDH. In general, an excess amount of ammonium formate had no inhibitory effect on FDH [14]; instead, it was beneficial to FDH-catalyzed reaction, which improved NADH regeneration efficiency [28]. However, this directly caused the production of more ammonium ions, which affects the pH of the reaction mixture. A feasible solution is to replace a certain amount of ammonium formate with sodium formate. The effect of the molar ratio of sodium formate to ammonium formate on the reaction was investigated by keeping formate concentration at 3 mol·L^{-1} (Fig. 4b). When the molar ratio of sodium formate to ammonium formate reached up to 1:1, the biosynthesis of L-*tert*-leucine was more efficient. In addition, we also investigated the effect of the molar ratio of TMP to formate on the reaction (Fig. 4c). Approximately 1 mol·L^{-1} of TMP was completely converted into L-*tert*-leucine at 8 h when the molar ratio of TMP to formate reached up to 1:3 which was significantly more advantageous than other ratios.

NADH determined the total production of L-*tert*-leucine. Although it can be produced intracellularly, the amount was significantly lower than the requirements of LeuDH [14,17]. The use of a proper amount of NAD$^+$ directly affects cofactor regeneration, and it is a necessity for this reaction. Fig. 4d displays the effect of NAD$^+$ concentration on the reaction. As the increase of NAD$^+$ concentration, the concentration of the product also increased within 10 h; however, the increase in the concentration of L-*tert*-leucine can be completely negligible when the concentration of NAD$^+$ is higher than 1.5 mmol·L^{-1}. In consideration of the cost, the initial concentration of NAD$^+$ in the reaction is more reasonable at 1.5 mmol·L^{-1}.

3.3. Effect of temperature and pH on the reaction

*Ls*LeuDH exhibited high thermostability at 55°C with a half-life of 12 h and its optimal temperature was about 60°C [24]. Although the optimal temperature of *Cb*FDH being about 60°C [29], the half-life was only 75 min at 50°C [30]. The reaction accelerates as temperature increases, but higher temperature will lead to the gradual deactivation of *Ls*LeuDH. In particular, the thermostability of *Cb*FDH was poor and it would deactivate earlier than *Ls*LeuDH at high temperature which will enable the regeneration of NADH to be blocked. As illustrated in Fig. 5a, the TMP was completely converted within 10 h at 30°C, but the reaction stopped at 2 h when the temperature exceeds 40°C. The possible reason for this was the inactivation of *Cb*FDH which can not provide the required reducing power for *Ls*LeuDH.

The optimal pH of the *Ls*LeuDH was 10.5 [24], and the *Cb*FDH displayed a broad pH optimum from 6.5 to 8.5 [20]. The key to the biosynthesis of L-*tert*-leucine by the double-enzyme system is that the NADH regeneration cycle is well-functioning. Results showed that the optimal pH of the system is 8.0, which is close to that of *Cb*FDH (Fig. 5b).

3.4. Effect of triton concentration on the reaction

Triton, a surfactant, can effectively increase cell permeability. The addition of triton might enhance the mass transfer between cells and solution in the reaction which leads to the improved conversion. The effect of triton concentration on the biosynthesis of L-*tert*-leucine is depicted in Fig. 6. Different concentrations of triton had no significant effect on the reaction within 6 h, but the difference began to appear later. TMP was almost completely converted in the different concentrations of triton except 0.025% for 10 h. A concentration of 0.05% triton is more suitable for the reaction.

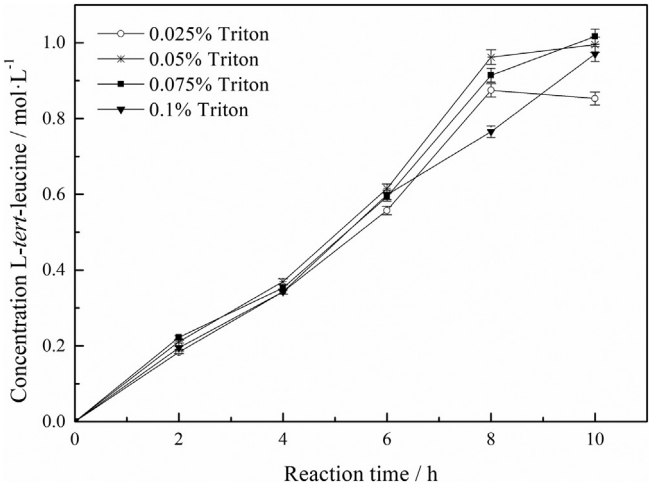

Fig. 6. Effect of triton concentration on the synthesis of L-*tert*-leucine.

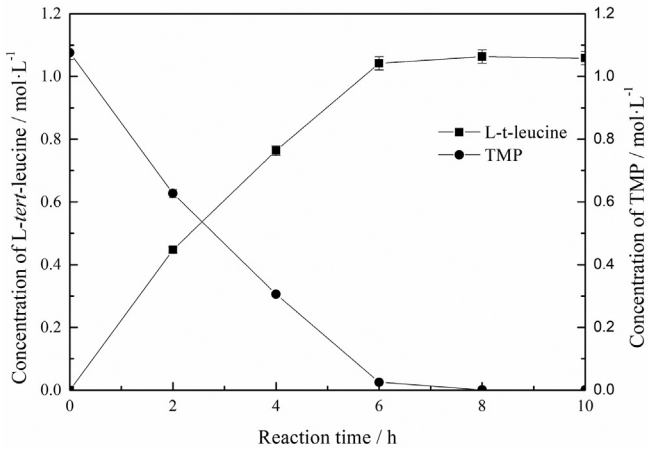

Fig. 7. Biosynthetic L-*tert*-leucine from the coupling system of the *Ls*FDH and *Cb*FDH.

3.5. Biosynthetic L-tert-leucine under optimized conditions

Under the optimized conditions, we performed the biosynthesis of L-*tert*-leucine using the recombinant *E. coli* BL21 (pETDute-FDH-LDH, pACYCDute-FDH) which was cultivated for 22 h in a shaker (Fig. 7). The conversion of TMP was 97%, and the yield of L-*tert*-leucine was 96.84% (136.08 $g \cdot L^{-1}$) within around 6 h. At 8 h, the conversion of TMP reached 99.9%, and the yield of L-*tert*-leucine was 98.4% (139.43 $g \cdot L^{-1}$) with >99.9% *ee*. Compared to the work by Xu and his coworkers [6], the yield of L-*tert*-leucine catalyzed by *Ls*LeuDH was nearly twice that of the *Es*LeuDH from *Exiguobacterium sibiricum*.

4. Discussion and conclusions

Compared to FDH, the specific activity of LeuDH for cofactors is much higher [17], which directly causes the consumption of NADH by LeuDH much faster than the production of NADH by FDH in cells. Therefore, the insufficient supply of reducing power is a vital reason for poor L-*tert*-leucine production. Compared to the single-plasmid recombinant strain, the copy number of the *fdh* gene in the double-plasmid recombinant strain increases. The increased copy number of the *fdh* gene directly affects the expression level of FDH which enhanced the production speed of NADH in the reaction. In addition, the stability of FDH is worse than that of LeuDH, and increasing the copy number of FDH can ensure a sufficient amount of coenzyme in the reaction [17]. Therefore, the conversion of TMP into L-*tert*-leucine catalyzed by the double-plasmid recombinant strain was faster than that catalyzed by single plasmids recombinant strain, and the concentration of L-*tert*-leucine was 0.8 $mol \cdot L^{-1}$ at 12 h catalyzed by the double-plasmid strain which was more than twice as high as that catalyzed by the single-plasmid strain. These studies showed the improvement of the regeneration capacity of NADH is very important for the biosynthesis of L-*tert*-leucine by *Ls*LeuDH.

As TMP has a substrate inhibitory effect on LeuDH [14], the optimal concentration of TMP is 1 $mol \cdot L^{-1}$ in this work, which limits the final L-*tert*-leucine production to not exceed 1 $mol \cdot L^{-1}$ in theory. *Ls*LeuDH exhibited better stability [24] than LeuDH from *Lysinibacillus sphaericus* CGMCC 1.1677 [14]. *E. coli* co-expressing LeuDH from *L. sphaericus* CGMCC 1.1677 and *Cb*FDH was applied to converting TMP into L-*tert*-leucine via a substrate continuous feeding strategy with a concentration of 195 $g \cdot L^{-1}$, and the stability of the LeuDH is worse than *Ls*LeuDH [14]. If the feeding strategy is adopted, the substrate inhibition effect of TMP can be avoided, and the yield of L-*tert*-leucine could be improved.

In summary, we have demonstrated the possibility of the biosynthesis of L-*tert*-leucine through reductive amination of TMP catalyzed by the recombinant *E. coli* co-expressing *Ls*LeuDH and *Cb*FDH. The results showed that TMP (1 $mol \cdot L^{-1}$) was completely transformed into L-*tert*-leucine with 99.9% *ee* by resting cells of the double-plasmid recombinant strain under the optimized reaction conditions. The NADH-dependent *Ls*LDH showed good industrial potential for the production of L-*tert*-leucine.

Conflict of interest

The authors declare no competing interests.

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Supplementary material

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