Biochemical Engineering Journal 91 (2014) 204–209

Contents lists available at ScienceDirect

Biochemical Engineering Journal

journal homepage: www.elsevier.com/locate/bej

# Efficient synthesis of L-*tert*-leucine through reductive amination using leucine dehydrogenase and formate dehydrogenase coexpressed in recombinant *E. coli*

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ARTICLE INFO

*Article history:*
Received 23 May 2014
Received in revised form 28 July 2014
Accepted 1 August 2014
Available online 11 August 2014

*Keywords:*
L-*tert*-Leucine
Reductive amination
Biocatalysis
Biotransformation
Bioprocess design
Optimization

ABSTRACT

Enantiopure L-*tert*-leucine (L-Tle) was synthesized through reductive amination of trimethylpyruvate catalyzed by cell-free extracts of recombinant *Escherichia coli* coexpressing leucine dehydrogenase (LeuDH) and formate dehydrogenase (FDH). The *leudh* gene from *Lysinibacillus sphaericus* CGMCC 1.1677 encoding LeuDH was cloned and coexpressed with $NAD^+$-dependent FDH from *Candida boidinii* for NADH regeneration. The batch reaction conditions for the synthesis of L-Tle were systematically optimized. Two substrate feeding modes (intermittent and continuous) were addressed to alleviate substrate inhibition and thus improve the space-time yield. The continuous feeding process was conveniently performed in water at an overall substrate concentration up to 1.5 M, with both conversion and ee of >99% and space-time yield of 786 g $L^{-1}$ $d^{-1}$, respectively. Furthermore, the preparation was successfully scaled up to a 1 L scale, demonstrating the developed procedure showed a great industrial potential for the production of enantiopure L-Tle.



## 1. Introduction

Nonproteinogenic L-amino acids are increasingly demanded by the pharmaceutical industry for peptidomimetic and other single-enantiomer drugs [1,2]. The most representative nonproteinogenic L-amino acid probably is L-*tert*-leucine (L-Tle), an important synthetic building block for novel pharmaceutical active ingredients as well as chiral auxiliaries and ligands [3,4]. Owing to its promising applications, a large number of chemical and biocatalytic methods have been developed for the synthesis of L-Tle [4]. Biocatalytic protocols, which can be conducted under mild conditions with high selectivity, usually offer greater benefits than chemical procedures and thus gain more and more attention from organic chemists [5]. For example, several biocatalytic processes based on the kinetic resolution of racemic DL-Tle or its derivatives catalyzed by lipase [6], acylase [7–9], amidase [10] and protease [11] have been developed for the preparation of enantiopure L-Tle. However, most of these resolution procedures are tedious and possess an inherent 50% yield limit.

Enzymatic reductive amination of α-keto acids is an attractive and well established methodology for the preparation of L-amino acids [12]. Several enantiopure L-amino acids have been accomplished through the reductive amination route catalyzed by amino acid dehydrogenase or aminotransferase [13–18]. A branch chain aminotransferase from *Enterobacter sp.* TL3 has been applied for the synthesis of L-Tle through reductive amination of trimethylpyruvate (TMP) using an enzyme coupling system [19]. L-Tle was obtained in an enantiopure form (>99% ee) with 90% conversion at an extremely low concentration of TMP (150 mM). Nevertheless, the relative low substrate concentration does hinder its potential for industrial application. Another reductive amination route for the synthesis of L-Tle was based on the combined use of leucine dehydrogenase (LeuDH) and formate dehydrogenase (FDH) [20]. This efficient but cofactor-dependent route has been successfully operated in an enzyme membrane reactor (EMR) with isolated LeuDH and FDH [21–23]. L-Tle was efficiently produced with excellent conversion, ee value and space-time yield (93%, >99% and

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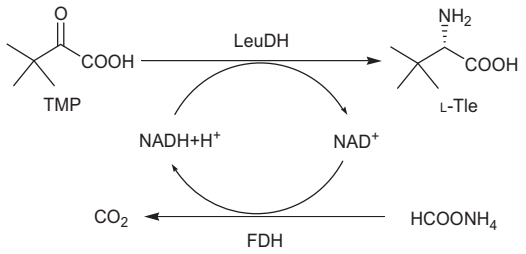
http://dx.doi.org/10.1016/j.bej.2014.08.003
1369-703X/




**Scheme 1.** Reductive amination of TMP to enantipure L-Tle using cell-free extracts of recombinant *E. coli* coexpressing LeuDH and $NAD^+$-dependent FDH.

366 g $L^{-1}$ $d^{-1}$, respectively) in a single continuously operated EMR at a TMP feeding concentration of 0.5 M [22]. As was noticed that the use of isolated and costly enzymes was disadvantageous, a whole-cell catalyst, bearing a LeuDH from *Bacillus cereus* and a FDH from *Candida boidinii*, was developed and used in the synthesis of L-Tle as well as L-neopentylglycine [24,25]. L-Tle was obtained with the whole-cell catalyst in >99% ee and 95% conversion at an impressive substrate concentration of 1.0 M, however, with a low space-time yield of 124 g $L^{-1}$ $d^{-1}$ [24]. In this regard, we became interested in developing a much more efficient procedure for the synthesis of L-Tle based on the combine use of LeuDH and FDH.

Cofactor regeneration is of great importance for cofactor-dependent syntheses due to the high cost and stoichiometric use of cofactor [26]. FDH from *C. boidinii*, which catalyzes the oxidation of formate to $CO_2$ with the concomitant reduction of $NAD^+$ to NADH, has been well characterized and widely utilized for NADH regeration [27,28]. In this work, a recombinant *Escherichia coli* strain was constructed to coexpress LeuDH from *Lysinibacillus sphaericus* CGMCC 1.1677 and FDH from *C. boidinii*. The crude cell extracts of the constructed *E. coli* cells containing both LeuDH and FDH was used to catalyze the reductive amination of TMP yielding enantiopure L-Tle (Scheme 1). After optimization of the batch reaction conditions, the preparation was further implemented with substrate feeding strategy at 100 mL scale to alleviate the substrate inhibition. Moreover, the process was subsequently scaled up to 1 L scale to show the potential applicability of the developed procedure.

## 2. Materials and methods

### 2.1. Materials

*E. coli* BL21 (DE3) was preserved in our laboratory and used as host organism. The plasmids pMD18T-simple, pRSFDuet-1 and pET-21a were purchased from Takara and Novagen, respectively. Restriction enzymes and other DNA-modifying enzymes were obtained from Takara and were used according to the manufacturer's instructions. The recombinant plasmid pUC-fdh containing *fdh* gene of *C. boidinii* was previously constructed in our laboratory [29]. *L. sphaericus* CGMCC 1.1677 were purchased from China General Microbiological Culture Collection Center. Trimethylpyruvate (TMP, >98% HPLC purity) was synthesized in our laboratory. All other regents were commercial available and used without further purification.

### 2.2. Construction of strains

To coexpress LeuDH and FDH, a two plasmid system using the compatible plasmids pRSFDuet-1 and pET-21a was devised. The *leudh* gene of *L. sphaericus* CGMCC 1.1677 was amplified by polymerase chain reaction (PCR) and subcloned into a pMD18T-simple vector to give recombinant vector pMD-leudh. pMD-leudh was then digested with *BamH*I and *Xho*I endonucleases and ligated into pRSFDuet-1 digested with the same enzymes to obtain pRSFDuet-1-leudh. The *fdh* gene was isolated by PCR using pUC-fdh as template and subcloned into a pMD18T-simple vector to give recombinant vector pMD-fdh. pMD-fdh was digested and then subcloned into the *Nde*I-*Xho*I sites of pET-21a which resulted in the construct of pET-21a-fdh. The recombinant pRSFDuet-1-leudh and pET-21a-fdh plasmids were transformed into *E. coli* BL21 (DE3) cells resulted in the kanamycin- as well as ampicillin-resistant strains *E. coli* BL21-(LeuDH-FDH).

### 2.3. Cultivation of strains

Precultures of *E. coli* BL21-(LeuDH-FDH) were grown in 250 mL shaking flasks each filled with 50 mL Luria-Bertani (LB) medium containing 50 mg $L^{-1}$ kanamycin and ampicillin, respectively. After incubation for 20 h at 37 °C and 200 rpm, a total of 100 mL of the cell suspension from two 250-mL flasks were used to inoculate the fermentor. Fermentation of *E. coli* was carried out in a 10 L fermentor containing 7 L of medium operated under the following conditions: aeration 0.5 vvm, agitation 200–500 rpm, DO >20%, pH 7.0 (controlled by adding 12.5% NaOH). The fermentation medium contained (per liter): glycerol 20 g, yeast extract 20 g, $Na_2HPO_4$ 5.08 g, $KH_2PO_4$ 3 g, $K_2SO_4$ 2 g, $MgCl_2{\cdot}6H_2O$ 0.4 g, $CaCl_2$ 0.011 g, sodium citrate 0.5 g, antifoam 3 g, kanamycin 50 mg, ampicillin 50 mg. Cells were grown at 37 °C until the optical density of the cultures at 600 nm ($OD_{600}$) reached 0.6. After addition of 0.2 mM IPTG, the induction was allowed for 20 h at 25 °C. Cells were harvested by centrifugation (12,000 rpm, 10 min) and washed twice with 0.85% NaCl solution. The harvested cells were stored in a freezer at −20 °C.

### 2.4. Preparation of cell-free extracts

The harvested cells were suspended in phosphate buffer (0.1 M, pH 8.5) and disrupted by ultrasonication. The crude extracts were centrifugated at 12,000 rpm and 4 °C for 10 min to remove the cell debris. The supernatants containing both LeuDH and FDH were used as cell-free extracts for enzyme activity assay (see Supplementary material) and reductive amination of TMP.

### 2.5. Batch reductive amination of TMP

The batch reductive amination was typically conducted in a 50 mL flask on a rotary shaker at 200 rpm and 30 °C. TMP was first suspended in deionized water and dissolved with the addition of NaOH. The TMP solution was then added to the cell-free extracts, following with the addition of ammonium formate. The pH of the reaction mixture was adjusted to 8.5 and the final reaction volume was made up to 20 mL. The reaction was initiated with the addition of cofactor $NAD^+$. The detailed reaction conditions were given in the legends of figures and tables. All reactions were carried out at least in triplicate.

### 2.6. Fed-batch reductive amination of TMP

The fed-batch reactions were performed on a rotary shaker at 200 rpm and 30 °C and the final reaction volume was 100 mL. The initial reaction mixture (50 mL, pH 8.5) contained cell-free extracts, ammonium formate and $NAD^+$. The substrate (TMP) solution (50 mL, pH 8.5) was added using different feeding strategies. For intermittent fed mode, substrate solution was fed in 3 batches, 20 mL at beginning and 2 h, 10 mL at 5 h. For continuous fed mode, substrate solution was fed continuously at a feeding rate of 10 mL $h^{-1}$ using a peristaltic pump. All reactions were carried out at least in triplicate.