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ORIGINAL ARTICLE

Reduction of *m*-chlorophenacyl chloride coupled with regeneration of NADPH by recombinant *Escherichia coli* cells co-expressing both carbonyl reductase and glucose 1-dehydrogenase

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Abstract Both *Sys1* and *Sygdh*, two codon-optimized genes encoding SyS1 and SyGDH, were synthesized based on the carbonyl reductase (S1) and glucose 1-dehydrogenase (GDH) gene sequences, respectively, from *Candida magnoliae* and *Thermoplasma acidophilum*, and co-expressed in *Escherichia coli* BL21(DE3) using two strategies. One strategy involved a recombinant *E. coli* strain (*E. coli*/*Sygdh-Sys1*) constructed by transforming a recombinant plasmid, pETDuet-*Sygdh-Sys1*, into *E. coli* BL21. The other strategy involved another recombinant *E. coli* strain (*E. coli*/*Sys1*/*Sygdh*) obtained by co-transforming the recombinant plasmids pET-22b-*Sys1* and pET-28a-*Sygdh* into *E. coli* BL21. The enzyme activity assays indicated that the activities of recombinant SyS1 and SyGDH (3.7 and 56.3 U/g wet cells) expressed in *E. coli*/*Sygdh-Sys1* were higher than those (2.8 and 44.1 U/g wet cells) in *E. coli*/*Sys1*/*Sygdh*. Accordingly, *E. coli*/*Sygdh-Sys1* was chosen, and its whole cells were used as catalysts for the asymmetric reduction of *m*-chlorophenacyl chloride (*m*-CPC) to the corresponding (*R*)-2-chloro-1-(3-chlorophenyl)ethanol [(*R*)-CCE] coupled with the regeneration of NADPH in situ. Under the optimized reaction conditions of 30 mM *m*-CPC, 50 mg/ml wet cells, 40 mM glucose and 0.2 mM $NADP^+$ at pH 7.0 and 35 °C for 3 h, (*R*)-CCE was obtained with a molar yield of 99.2 % and an enantiomeric excess (e.e.) value of more than 99 %.

Keywords *m*-Chlorophenacyl chloride · (*R*)-2-Chloro-1-(3-chlorophenyl)ethanol · Carbonyl reductase · Glucose 1-dehydrogenase · Co-expression · *Escherichia coli*

Introduction

Enantiopure halohydrins are versatile building blocks for the synthesis of physiologically active compounds such as adrenergic receptor (AR) agonists, bronchodilators and HIV-1 protease inhibitors (Ren et al. 2012). Among them, (*R*)-CCE is an important drug intermediate for β_3-AR agonists with anti-obese, anti-diabetic and anti-depressant activities (Xia et al. 2012). To date, the asymmetric reduction of carbonyl compounds, such as aromatic/alkyl ketones and β-ketoesters, to the corresponding chiral alcohols has been considered as the most promising methodology (Ohkuma et al. 2007; Ni et al. 2012). Nevertheless, the chemical reduction suffered from the problems of using more expensive chiral metal catalysts, as well as lower productivity and enantioselectivity. For example, the asymmetric reduction of *m*-CPC with an oxazaborolidine-based catalyst gave (*R*)-CCE with only 87 % yield and 85 % enantiomeric excess (e.e.) value (Bloom et al. 1992). In addition, the metal residue in products

Tao Yu and Jian-Fang Li, the two first authors, contributed equally to this work.

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from metal catalysts presented another challenge because of ever more stringent restriction on the level of metals allowed in pharmaceuticals and other fine chemicals (Zhu et al. 2006).

The biological way, an alternative to the chemical way, has been applied to produce drug intermediates because of its higher enantioselectivities, mild and environmentally friendly reaction conditions, and because it is void of residual metals in products (Ni and Xu 2012; Solano et al. 2012; Huisman and Collier 2013). There were two technical routes for the preparation of chiral CCE by the biological way. One route was the kinetic resolution of racemic CCE, but the theoretical yield of a desired enantiomer is 50 % (Xia et al. 2012). The other route was the asymmetric reduction of *m*-CPC with a theoretical molar yield of 100 %, requiring the synergistic action of NADH or NADPH (Lin et al. 2009; Berenguer-Murcia and Fernandez-Lafuente 2010; Ni and Xu 2012). The regeneration of NAD(P)H in situ could be accomplished through an enzyme-coupled or substrate-coupled method (Liang et al. 2010; Wu et al. 2011), playing important roles in using an amount of expensive NAD(P)H that is as small as possible and in maintaining the continuous progress of reduction.

The asymmetric reduction of *m*-CPC coupled with the regeneration of NAD(P)H could be catalyzed by some microorganisms (Hamada et al. 2001). However, such a biocatalytic process has not yet been of practical use, because the wild-type strain usually expresses several reductases with different stereoselectivities, which may result in a lower e.e. value of (*R*)- or (*S*)-CCE. In this work, to improve the e.e. value of (*R*)-CCE and eliminate the influence of rare codons on enzyme expression in *E. coli*, the SyS1-encoding gene *Sys1* was synthesized based on the NADPH-dependent carbonyl reductase (S1) gene (GenBank: AB036927) from *Candida magnoliae* (Yasohara et al. 2000). The synonymous codons of *Sys1* were optimized according to the codon database (www.kazusa.or.jp/codon) for biasing towards *Escherichia coli*. Synchronously, another SyGDH-encoding gene, *Sygdh*, with optimized codons biasing towards *E. coli* was synthesized based on the $NADP^+$-dependent glucose 1-dehydrogenase (GDH) gene (AL445065) from *Thermoplasma acidophilum* (Smith et al. 1989). Then, two recombinant *E. coli* strains, *E. coli*/*Sygdh*-*Sys1* and *E. coli*/*Sys1*/*Sygdh*, were constructed by transforming a recombinant plasmid and by co-transforming two recombinant plasmids, respectively, into *E. coli* BL21. The activities of recombinant SyS1 and SyGDH expressed in two recombinant *E. coli* strains were assayed and compared. Finally, the reaction conditions for asymmetric reduction of *m*-CPC to (*R*)-CCE catalyzed by whole *E. coli*/*Sygdh*-*Sys1* cells were optimized using a 'one-parameter-at-a-time' method (Zhang et al. 2014). To our knowledge, this is the first report on the asymmetric reduction of *m*-CPC coupled with regeneration of NADPH in situ by recombinant *E. coli* cells co-expressing both SyS1 and SyGDH.

Materials and methods

Reagents

The restriction enzymes *rTaq* DNA polymerase, T_4 DNA ligase and IPTG were purchased from TaKaRa Biotechnology (Dalian, China). NADPH and $NADP^+$ were from Yuanye Biotechnology (Shanghai, China). *m*-CPC, (*R*)- and (*S*)-CCE were products of Sumitomo Chemical Industries (Osaka, Japan). (*R*,*S*)-CCE was synthesized by $NaBH_4$-dependent reduction of *m*-CPC. All other chemicals used were of analytical grade.

Strains, plasmids and culture media

Escherichia coli JM109 and plasmid pUCm-T (Sangon, Shanghai, China) were used for gene cloning and DNA sequencing. *E. coli* BL21(DE3) and expression plasmids pETDuet-1, pET-22b(+) and pET-28a(+) (Novagen, Madison, WI, USA) were used for the construction of recombinant plasmids and the expression of heterogenous genes. *E. coli* JM109 and BL21(DE3) were cultured with shaking (220 rpm) at 37 °C in the LB medium containing (g/l): tryptone 10, yeast extract 5 and NaCl 10, pH 7.2. If necessary, ampicillin (Amp) and/or kanamycin (Kan) were supplemented to LB medium at a final concentration of 100 μg/ml.

Synthesis of two codon-optimized genes, *Sys1* and *Sygdh*

Both the SyS1- and SyGDH-encoding genes, *Sys1* and *Sygdh* with optimized synonymous codons biasing towards *E. coli*, were designed according to the codon database (www.kazusa.or.jp/codon), and synthesized based on the *C. magnoliae* S1 and *T. acidophilum* GDH genes, respectively, by Sangon (Shanghai, China). The rare codon replacements were listed in Table S1. The synthesized *Sys1* contains *Nde*I and *Xho*I sites at its 5′- and 3′-ends, while *Sygdh* has *Nco*I and *Hind*III sites at its two ends. *Sys1* and *Sygdh* were ligated with pUCm-T, and transformed into *E. coli* JM109, respectively. Both recombinant T-plasmids, pUCm-T-*Sys1* and pUCm-T-*Sygdh,* were confirmed by restriction enzyme analysis and DNA sequencing.

Construction of two recombinant *E. coli* strains

A recombinant *E. coli* strain, *E. coli*/*Sys1*/*Sygdh*, was constructed as described below. *Sys1* was released from pUCm-T-*Sys1* by double digestion with *Nde*I and *Xho*I, and was ligated with pET-22b(+) digested with the same enzymes, forming a recombinant plasmid, pET-22b-*Sys1* (Fig. 1a). Similarly, *Sygdh* from pUCm-T-*Sygdh* digested with *Nco*I and *Hind*III was ligated with pET-28a(+) digested with the same enzymes, forming another recombinant plasmid, pET-

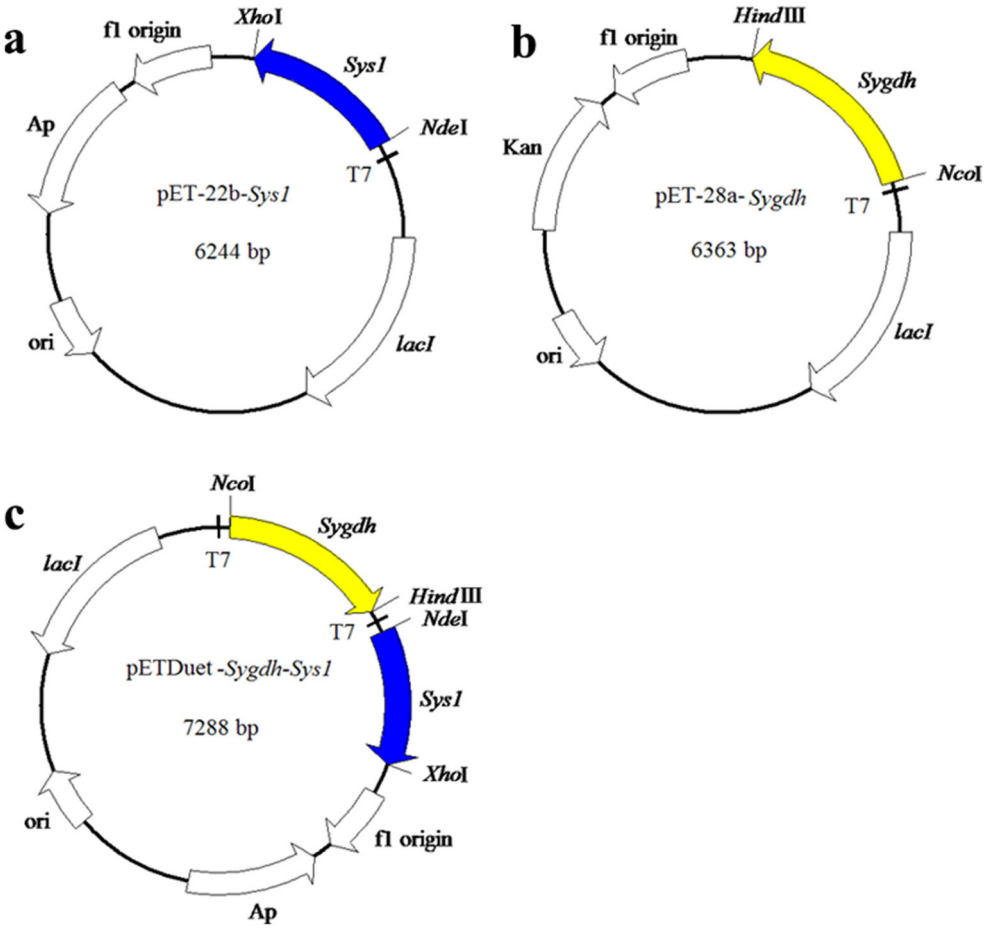

Fig. 1 Construction of the three recombinant expression plasmids. **a** The recombinant expression plasmid pET-22b-*Sys1* was constructed by ligating *Sys1* with pET-22b(+). **b** Similarly, the recombinant expression plasmid pET-28a-*Sygdh* was constructed by ligating *Sygdh* with pET-28a(+). **c** The recombinant expression plasmid pETDuet-*Sygdh-Sys1* was constructed by co-inserting both *Sys1* and *Sygdh* into pETDuet-1, an expression plasmid with double T7 promoters

28a-*Sygdh* (Fig. 1b). Both recombinant expression plasmids were confirmed by restriction enzyme analysis, and were then co-transformed into *E. coli* BL21.

Another recombinant *E. coli* strain, *E. coli*/*Sygdh-Sys1*, was constructed by the following steps. Firstly, *Sys1* from pUCm-T-*Sys1* was ligated with a double-promoter plasmid, pETDuet-1, digested with *Nde*I and *Xho*I, transformed into *E. coli* JM109, and this was followed by restriction enzyme analysis. Secondly, the recombinant plasmid pETDuet-*Sys1* was digested with *Nco*I and *Hind*III, and ligated with *Sygdh* from pUCm-T-*Sygdh*, forming a recombinant plasmid, pETDuet-*Sygdh-Sys1* (Fig. 1c). And finally, pETDuet-*Sygdh-Sys1* was confirmed by restriction enzyme analysis, and then transformed into *E. coli* BL21.

Co-expression of both *Sys1* and *Sygdh* in *E. coli* BL21

The recombinant *E. coli* strain was inoculated into LB medium supplemented with 100 μg/ml Amp for *E. coli*/*Sygdh-Sys1* or with 100 μg/ml Amp and Kan for *E. coli*/*Sys1*/*Sygdh*, and grown at 37 °C for 12 h. Then, a fresh LB medium was inoculated with 1 % (*v*/*v*) seed culture and cultured at 37 °C until the OD_{600} reached 0.6. Co-expression of *Sys1* and *Sygdh* was induced by the addition of 0.6 mM IPTG at 25 °C for 10 h. *E. coli* cells were harvested from 25 ml of induced broth by centrifugation, washed with 20 mM K_2HPO_4–KH_2PO_4 buffer (pH 7.0), and suspended in 5 ml of the same buffer. After the cells were disrupted by ultrasonication, the debris was removed, while the supernatant was collected and used as cell-free extract.

Enzyme activity and protein assays

Both SyS1 and SyGDH activities were measured as reported previously (Kizaki et al. 2001), with slight modification. SyS1 activity was assayed at 30 °C in a 96-well plate, where each well consisted of 1 mM *m*-CPC and 0.1 mM NADPH in 100 mM K_2HPO_4–KH_2PO_4 buffer (pH 6.5). The reaction, in a volume of 200 μl, was initiated by adding an appropriate amount of cell-free extract, and monitored for the decrease in absorbance at 340 nm. Similarly, for the SyGDH activity assay at 30 °C, each well consisted of 50 mM glucose and 0.4 mM $NADP^+$ in 100 mM K_2HPO_4–KH_2PO_4 buffer (pH 7.5) and a certain amount of cell-free extract. The reaction, in a volume of 200 μl, was monitored for an increase in absorbance at 340 nm. One activity unit (U) was defined as the amount of enzyme catalyzing the oxidation of 1 μmol NADP

H per minute (for SyS1) or the reduction of 1 μmol $NADP^+$ per minute (for SyGDH) under their assay conditions.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method of Laemmli (1970). The separated proteins were visualized by staining with Coomassie Brilliant Blue R-250 (Sigma, St. Louis, MO, USA). Apparent molecular weights and amounts of the expressed recombinant SyS1 and SyGDH were estimated using Quantity One software and BandScan 5.0 software, respectively, based on the standard marker proteins on a 12.5 % gel of SDS-PAGE.

Verification of coenzyme regeneration in situ

The regeneration of NADPH by whole *E. coli*/*Sygdh-Sys1* cells was investigated. Aliquots of 1 ml mixture, having 2 mM *m*-CPC, 1 mM NADPH or $NADP^+$, 0 or 50 mM glucose and 10 mg/ml wet cells in 100 mM K_2HPO_4–KH_2PO_4 buffer (pH 7.0) (Table 1) were incubated at 30 °C for 6 h, followed by centrifugation. The resulting supernatant of 0.6 ml was extracted with 1 ml of ethyl acetate (containing 1 mM n-hexanol used as internal standard). The organic phase was dried with $MgSO_4$, and analyzed by gas chromatography (GC) using a GC-2014 apparatus (Shimadzu, Tokyo, Japan) equipped with a chiral CP-Chirasil-DEX CB column (0.25 mm×25 m, 0.25-μm film, Agilent, Santa Clara, CA, USA) and a flame ionization detector. Nitrogen gas was used as carrier and the split ratio was 1:50. The temperatures of injector and detector were set at 220 and 250 °C, respectively. The column temperature was set at 145 °C for 2 min, raised up to 200 °C at a rate of 5 °C/min, and kept at 200 °C for 5 min. The e.e. value of (*R*)-CCE = [(R-S)/(R+S)]×100 %, and the molar yield = $(R/CPC_0)\times 100$ %. R and S express the final molar concentrations of (*R*)- and (*S*)-CCE, respectively, while CPC_0 the initial molar concentration of *m*-CPC.

Optimization of the asymmetric reduction conditions

It has been reported that various parameters for the asymmetric reduction of carbonyl compounds catalyzed by isolated enzymes or whole microbial cells have significant effects on catalytic efficiency and productivity (Itoh et al. 2002; Ni et al. 2012). In this work, whole *E. coli*/*Sygdh-Sys1* cells were used as catalysts and the molar yield of (*R*)-CCE was used as an index. The parameters, such as *m*-CPC concentration, pH value and glucose concentration (for NADPH regeneration), for the asymmetric reduction of *m*-CPC to (*R*)-CCE, were optimized individually using a 'one-parameter-at-a-time' method, which maintains all parameters, except the one being investigated, at constant levels. The primary reaction conditions for the reduction of *m*-CPC were set as follows (in a volume of 1 ml): 10 mM *m*-CPC, 40 mg/ml wet cells, 50 mM glucose and 0.1 mM $NADP^+$ in 100 mM K_2HPO_4–KH_2PO_4 buffer (pH 7.0) at 30 °C for 4 h.

Data analysis

Calculation of the theoretical molecular weight and homology alignment between two amino acid sequences or nucleotide sequences were carried out, respectively, using DNAMAN 6.0 software (Lynnon Biosoft, USA). All data obtained were from three independent experiments or parallel measurements, and expressed as the mean±standard deviation (SD). Statistical comparison was made by Student's *t*-test using a computerized statistical package. The level of statistical significance was defined as $p<0.05$ or $p<0.01$.

Results and discussion

Analysis of two synthesized genes, *Sys1* and *Sygdh*

Two synthesized *Sys1* and *Sygdh* DNA sequences reported in this work have been deposited in the GenBank database under the accession numbers of KJ522844 and KF739810, respectively. The homology alignment indicated that the nucleotide sequences of *Sys1* and *Sygdh* shared 84.5 and 82.8 % identities with those of the *C. magnoliae* S1 and *T. acidophilum* GDH genes, respectively. As desired, the amino acid sequences of SyS1 and SyGDH separately deduced from *Sys1* and *Sygdh* were entirely identical to those of S1 and GDH. One gene *Sys1* is 882 bp in length, containing *Nde*I and *Xho*I sites at its 5′- and 3′-ends and a 6-aa His tag-encoding DNA fragment at its 5′-end. The other gene *Sygdh* is 1118 bp in

Table 1 Verification of NADPH regeneration in situ catalyzed by whole-cell *E. coli*/*Sygdh-Sys1* in the different reaction systems

Reaction system	Yield (%)[a]	e.e. (%)[a]
1: *E. coli*/*Sygdh-Sys1* + *m*-CPC	ND[b]	–
2: *E. coli*/*Sygdh-Sys1* + *m*-CPC + NADPH	43.2±0.8	>99
3: *E. coli*/*Sygdh-Sys1* + *m*-CPC + NADPH+ glucose	98.7±2.1	>99
4: *E. coli*/*Sygdh-Sys1* + *m*-CPC + $NADP^+$	ND[b]	–
5: *E. coli*/*Sygdh-Sys1* + *m*-CPC + $NADP^+$+ glucose	98.0±2.3	>99

[a] Determined by GC analysis

[b] Not detected

length, containing an *Nco*I site, a 6-aa His tag-encoding DNA fragment at its 5′-end and a *Hind*III site at its 3′-end. The sites at the 5′-ends of *Sys1* and *Sygdh*, *Nde*I (CAT**ATG**) and *Nco*I (CC**ATG**G), contain the strat codons (ATGs) of three plasmids pET-22b(+), pET-28a(+) and pETDuet-1. As a result, both recombinant SyS1 and SyGDH, co-expressed in *E. coli* BL21 mediated by those plasmids, consist of 290 and 369 amino acid residues with theoretical molecular weights of 31,374.2 and 41,260.9 Da, respectively.

Co-expression of both genes, *Sys1* and *Sygdh*, in *E. coli* BL21

A total of 30 *E. coli* transformants in a single-plasmid system were randomly picked out for flask expression tests (Zhou et al. 2008). The strain *E. coli*/*Sygdh-Sys1*, co-expressing the highest activities of recombinant SyS1 and SyGDH (3.7 and 56.3 U/g wet cells), was selected from among the 30 transformants tested. Similarly, another strain in a double-plasmid system, *E. coli*/*Sys1*/*Sygdh*, with maximum activities of SyS1 and SyGDH (2.8 and 44.1 U/g wet cells) was selected from among 30 *E. coli* transformants tested. Accordingly, the strain *E. coli*/*Sygdh-Sys1* was chosen, and its whole cells, induced by IPTG at 25 °C for 10 h, were used as catalysts for the asymmetric reduction of *m*-CPC to (*R*)-CCE coupled with NADPH regeneration. Although the two strategies of using single-plasmid and double-plasmid systems to co-express two enzymes were reported previously (Wu et al. 2011; Zhang et al. 2011; Su et al. 2012), no work was conducted on the co-expression of both *Sys1* and *Sygdh* with optimized synonymous codons in *E. coli* BL21.

SDS-PAGE analysis displayed that, compared with that of uninduced *E. coli*/*Sygdh-Sys1* (Fig. 2, line 1), the cell lysate of the induced strain exhibited two extra and clear protein bands with apparent molecular weights of about 31.8 kDa (SyS1) and 41.4 kDa (SyGDH) (Fig. 2, line 2), extremely close to their theoretical ones, respectively. The amounts of expressed SyS1 and SyGDH were estimated to account for 36.5 and 31.8 % of the total protein amount of *E. coli*/*Sygdh-Sys1*. Similarly, the cell lysate of induced *E. coli*/*Sys1*/*Sygdh* also exhibited two extra and clear protein bands (Fig. 2, line 4) with the same apparent molecular weights as those of induced *E. coli*/*Sygdh-Sys1*.

Regeneration of NADPH in situ by whole *E. coli/Sygdh-Sys1* cells

NAD(P)H regeneration catalyzed by dehydrogenase is necessary for the sustainable progress of reduction owing to the limited amount of coenzyme added to a reaction system. Here, glucose was used as co-substrate for NADPH regeneration. The asymmetric reduction of *m*-CPC to (*R*)-CCE coupled with regeneration of NADPH in situ was

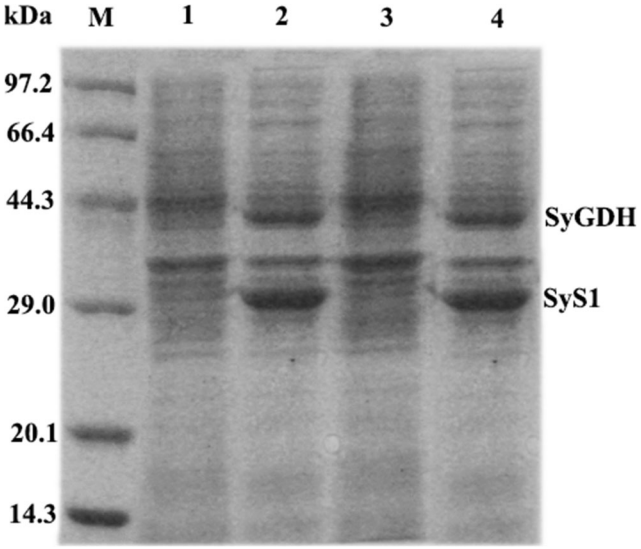

Fig. 2 SDS-PAGE analysis of both recombinant SyS1 and SyGDH co-expressed in *E. coli*. *Lane M* protein marker. *Lane 1* the cell lysate of uninduced *E. coli*/*Sygdh-Sys1*. *Lane 2* the cell lysate of *E. coli*/*Sygdh-Sys1* induced by 0.6 mM IPTG at 25 °C for 10 h. *Lane 3* the cell lysate of uninduced *E. coli*/*Sys1*/*Sygdh*. *Lane 4* the cell lysate of *E. coli*/*Sys1*/*Sygdh* induced by IPTG

experimentally tested using the whole *E. coli*/*Sygdh-Sys1* cells co-expressing both SyS1 and SyGDH as catalysts. As shown in Table 1, no (*R*)-CCE was detected in the reaction systems 1 and 4, owing to their being void of NADPH, a donor of hydrogen. The molar yield of (*R*)-CCE (98.7 %) in the reaction system 3 was approximately 2.3-fold higher than that (43.2 %) in the system 2, indicating that the regeneration of NADPH resulted from the oxidation of glucose catalyzed by SyGDH co-expressed in *E. coli*/*Sygdh-Sys1*. In addition, there was no distinct difference in molar yields of (*R*)-CCE between the reaction systems 3 and 5. Therefore, $NADP^+$ was used instead of NADPH, due to the higher price of NADPH.

Optimization of the asymmetric reduction conditions

The optimum *m*-CPC concentration for its asymmetric reduction by whole *E. coli*/*Sygdh-Sys1* cells was determined under the primary reaction conditions, except with *m*-CPC concentrations ranging from 10 to 60 mM. The result show that the molar yield of (*R*)-CCE remained at more than 90 % until *m*-CPC concentration was up to 30 mM, over which the yield of (*R*)-CCE decreased sharply (Fig. 3). A too-high concentration of *m*-CPC may be toxic to *E. coli* cells, or it may denature enzymes (Itoh et al. 2002). Subsequently, the effect of *E. coli* cell dosage on yield of (*R*)-CCE was estimated, at the optimum *m*-CPC concentration of 30 mM, at varied dosages from 30 to 80 mg/ml wet cells (data not shown). The data analysis indicates that the difference in (*R*)-CCE yields over a range from 50 to 80 mg/ml wet cells was not significant ($p>0.05$), but the difference between 40 and 50 mg/ml was significant

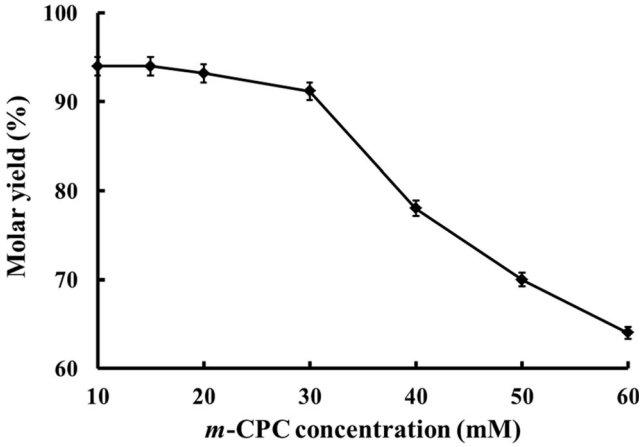

Fig. 3 The effect of *m*-CPC concentration on the molar yield of (*R*)-CCE. The asymmetric reduction of *m*-CPC was performed under the primary reaction conditions, except with *m*-CPC concentrations ranging from 10 to 60 mM. The statistical comparison over a range from 10 to 30 mM *m*-CPC was not significant at $p>0.05$, but between 30 and 40 mM, *m*-CPC was significant at $p<0.01$

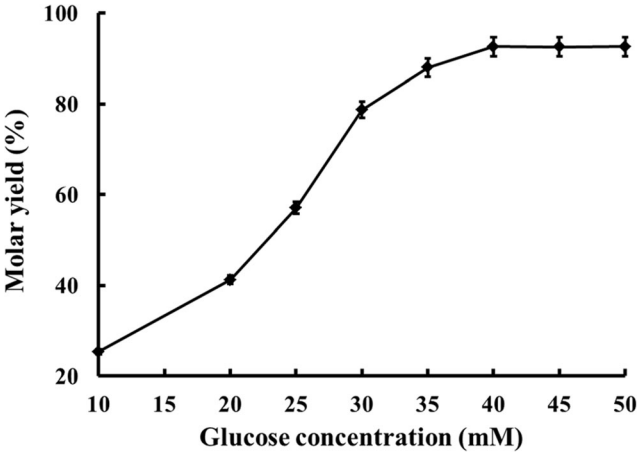

Fig. 5 The effect of glucose concentration on the molar yield of (*R*)-CCE. The asymmetric reduction of *m*-CPC was performed at glucose concentrations ranging from 10 to 50 mM. The statistical comparison over a range of 40 to 50 mM glucose was not significant at $p>0.05$, but between 35 and 40 mM glucose, it was significant at $p<0.05$

($p<0.05$). Accordingly, the optimum dosage for the reduction of 30 mM *m*-CPC was set at 50 mg/ml wet cells. It was reported that the optimum dosage of *C. ontarioensis* was 200 mg/ml wet cells for the preparation of (*R*)-CCE (Ni et al. 2012), much higher than that of *E. coli*/*Sygdh-Sys1*.

The optimum pH for the reduction of *m*-CPC catalyzed by whole *E. coli*/*Sygdh-Sys1* cells was assayed, at 30 mM *m*-CPC and 50 mg/ml wet cells, at different pH values (100 mM potassium acetate buffer, pH 5.0–6.5; potassium phosphate buffer, 6.5–8.0). The yields of (*R*)-CCE were over 90 % over a pH range of 6.5–7.5, with the highest yield of 92.1 % at pH 7.0 (Fig. 4). Thereafter, the effect of temperature on (*R*)-CCE yield was evaluated, at 30 mM *m*-CPC, 50 mg/ml wet cells and pH 7.0, at temperatures from 20 to 55 °C. The data analysis indicated that the difference in (*R*)-CCE yields over a range of 25 to 50 °C was not significant ($p>0.05$), but the difference between 20 and 25 °C or 50 and 55 °C was significant ($p<0.05$). In view of energy consumption and catalytic efficiency, 35 °C was chosen as the optimum temperature.

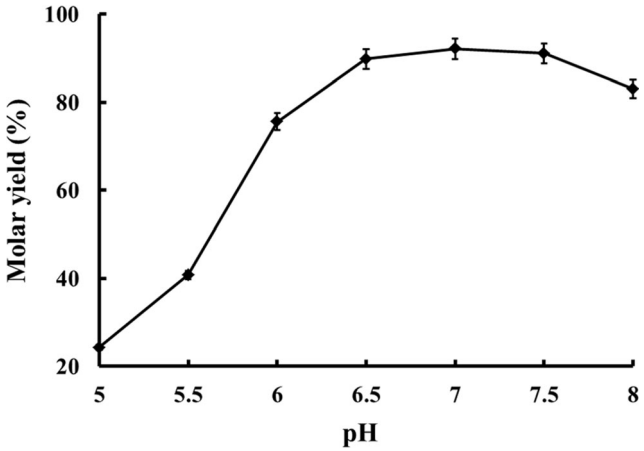

Fig. 4 The effect of pH value on the molar yield of (*R*)-CCE. The asymmetric reduction of *m*-CPC was carried out, at 30 mM *m*-CPC and 50 mg/ml wet cells, at various pH values (100 mM potassium acetate buffer, pH 5.0–6.5; 100 mM potassium phosphate buffer, 6.5–8.0). The statistical comparison over a range of pH 6.5–7.5 was not significant at $p>0.05$, but between pH 6.0 and 6.5 or pH 7.5 and 8.0, it was significant at $p<0.05$

The yield of (*R*)-CCE increased as glucose concentration rose, and remained at about 92 % after exceeding 40 mM glucose (Fig. 5). Subsequently, the effect of $NADP^+$ concentration on the yield was evaluated over a range of 0.1–0.5 mM. An (*R*)-CCE yield of near 100 % was obtained at 0.2 mM $NADP^+$; this was much lower than that (1 mM NAD^+) reported by Itoh et al. (2002). The data analysis displayed that the difference in (*R*)-CCE yields over a range from 0.2 to 0.5 mM $NADP^+$ was not significant ($p>0.05$), but the difference between 0.1 and 0.2 mM was significant ($p<0.01$). Based on the

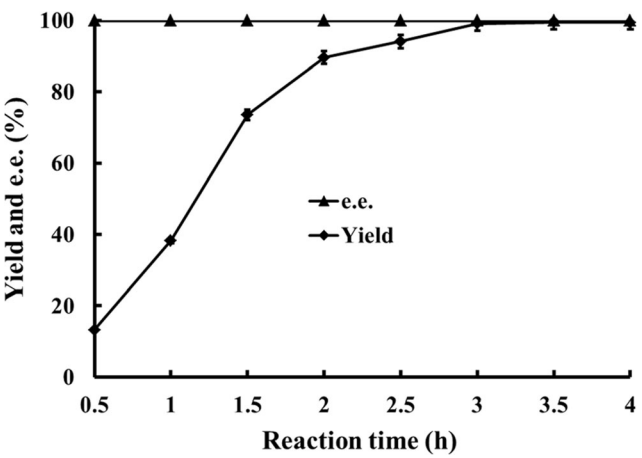

Fig. 6 Efficient preparation of the product (*R*)-CCE from the asymmetric reduction of *m*-CPC catalyzed by whole *E. coli*/*Sygdh-Sys1* cells. Under the optimized reaction conditions, the yield of (*R*)-CCE reached 99.2 % with an e.e. value more than 99 % at 35 °C for 3 h

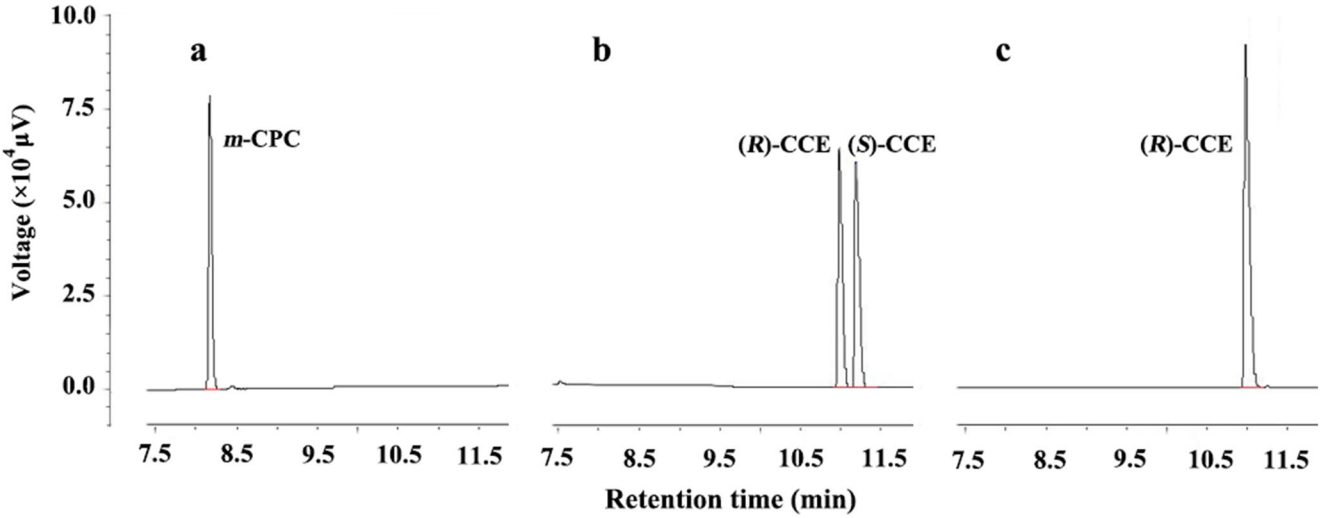

Fig. 7 GC analysis of various molecules under the conditions as described in the 'Materials and methods' section. **a** The retention time of the standard molecule *m*-CPC was 8.172 min. **b** The retention times of the standard molecules (*R*)- and (*S*)-CCE were 10.976 and 11.184 min, respectively. **c** The retention time of the reaction sample was 10.975 min

above experimental results, the optimized reaction conditions for the asymmetric reduction of *m*-CPC to (*R*)-CCE were as follows: 30 mM *m*-CPC, 50 mg/ml wet cells, 40 mM glucose and 0.2 mM $NADP^+$ in 100 mM K_2HPO_4–KH_2PO_4 buffer (pH 7.0) at 35 °C.

Efficient preparation of (*R*)-CCE from *m*-CPC

Under the optimized reaction conditions, the molar yield of (*R*)-CCE reached 99.2 % with an e.e. value more than 99 % at 35 °C for 3 h (Fig. 6). Analyzed by GC, the retention times of the standard molecules *m*-CPC, (*R*)- and (*S*)-CCE were 8.172, 10.976 and 11.184 min, respectively, while that of the reaction sample was 10.975 min (Fig. 7), indicating that the product from asymmetric reduction of *m*-CPC by whole cells of *E. coli*/*Sygdh-Sys1* was (*R*)-CCE, an important drug intermediate for the synthesis of β_3-AR agonists.

The biocatalytic process for the asymmetric reduction of *m*-CPC to (*R*)-CCE coupled with coenzyme regeneration in this study was compared with those in the previous studies (Itoh et al. 2002; Lin et al. 2009; Ni et al. 2012). As shown in Table 2, *E. coli*/*Sygdh-Sys1*, a recombinant *E. coli* strain co-expressing both carbonyl reductase (SyS1) and glucose 1-dehydrogenase (SyGDH), displayed the lowest cell dosage and the shortest reaction time (that is, the highest catalytic efficiency). Therefore, the strain *E. coli*/*Sygdh-Sys1* may be a good candidate for industrial use, owing to its high stereoselectivity and catalytic efficiency.

Conclusion

Both the SyS1-encoding and SyGDH-encoding genes, *Sys1* and *Sygdh* with optimized synonymous codons biasing towards *E. coli*, were synthesized, respectively, as designed theoretically. Subsequently, a recombinant strain *E. coli*/*Sygdh-Sys1* was obtained by transforming a recombinant expression plasmid with double promoters, pETDuet-*Sygdh-Sys1*, into *E. coli* BL21(DE3), while another recombinant strain *E. coli*/*Sys1*/*Sygdh* was constructed by co-transforming two

Table 2 Comparison of several biocatalytic processes for the preparation of (*R*)-CCE

Strain	*Saccharomyces cerevisiae*	*Candida ontarioensis*	*E. coli*/*par*	*E. coli*/*Sygdh-Sys1*
Reaction volume (ml)	10	1000	1	1
m-CPC concentration (mM)	148	159	159	30
Cell dosage (mg/ml)	100	200	180	50
Reaction time (h)	48	24	24	3
Coenzyme concentration (mM)	–	–	1 (NAD^+)	0.2 ($NADP^+$)
Molar yield of (*R*)-CCE (%)	97	97.5	93.2	99.2
e.e. value of (*R*)-CCE (%)	>99	99.9	99.5	>99
References	Lin et al. 2009	Ni et al. 2012	Itoh et al. 2002	This study

recombinant plasmids, pET-22b-*Sys1* and pET-28a-*Sygdh*, into *E. coli* BL21. The strain *E. coli*/*Sygdh-Sys1* was chosen because the activities of recombinant SyS1 and SyGDH expressed in *E. coli*/*Sygdh-Sys1* were higher than those in *E. coli*/*Sys1*/*Sygdh*. Before the whole cells of *E. coli*/*Sygdh-Sys1* were applied to catalyze the asymmetric reduction of *m*-CPC to (*R*)-CCE, the feasibility of NADPH regeneration in situ was investigated by using an enzyme-coupled method. Under the optimized reaction conditions, the molar yield of (*R*)-CCE reached 99.2 % with an e.e. value more than 99 % at a concentration of 30 mM *m*-CPC. This work provided a practical approach for the efficient preparation of (*R*)-CCE.

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