Process Biochemistry xxx (2014) xxx–xxx

Contents lists available at ScienceDirect

Process Biochemistry

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

Enzymatic preparation of *t*-butyl-6-cyano-(3*R*, 5*R*)-dihydroxyhexanoate by a whole-cell biocatalyst co-expressing carbonyl reductase and glucose dehydrogenase

Xuri Wu [1], Xudong Gou [1], Yijun Chen *

State Key Laboratory of Natural Medicines and Laboratory of Chemical Biology, China Pharmaceutical University, 24 Tongjia Street, Nanjing, Jiangsu Province 210009, People's Republic of China

ARTICLE INFO

Article history:
Received 5 June 2014
Received in revised form
24 September 2014
Accepted 27 October 2014
Available online xxx

Keywords:
Carbonyl reductase
Glucose dehydrogenase
Nicotinamide cofactor
Whole-cell biocatalysis
Statin side chains

ABSTRACT

Statins are the most effective drugs for hyperlipidemia-related diseases by competitively inhibiting 3-hydroxy-3-methylglutaryl coenzyme A reductase. Because of the difficulty and environmental concerns associated with chemical preparation of the chiral diols of statin side chains, different biocatalytic approaches have been explored and the two-step bio-reduction process for the introduction of two chiral hydroxyl groups has been industrialized. However, the high costs and poor stability of nicotinamide cofactors in the process was a major limiting factor. In the present study, a whole-cell biocatalyst simultaneously expressing carbonyl reductase and glucose dehydrogenase was constructed. This biocatalyst was then used to synthesize *t*-butyl-6-cyano-(3*R*, 5*R*)-dihydroxyhexanoate *via* enzymatic reduction of *t*-butyl-6-cyano-(5*R*)-hydroxy-3-carboxylhexanoate, which involves in the self-recycling of endogenous cofactors. After systematic optimization, the bioconversion was complete with a productivity of 120 g l^{-1} day^{-1} without exogenous addition of cofactors after 7 h at 35 g/L substrate concentration. Thus, the present system has simplified the process and improved the overall efficiency for the preparation of statin side chains.

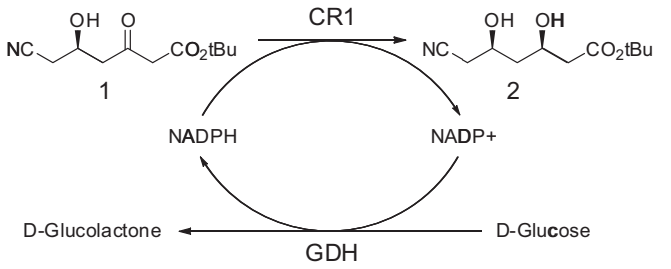
1. Introduction

3-Hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, also known as statins, were discovered from fungal secondary metabolites. The stereochemical similarity between the 3-hydroxy-3-methylglutaryl group of HMG-CoA and (3*R*, 5*S*/*R*)-dihydroxy ester in the side chain allows that statins competitively inhibit HMG-CoA reductase, making them the most effective drugs for the treatment of hyperlipidemia-related diseases as well as coronary heart disease [1–3]. Given the existence of two asymmetric hydroxyl groups in statin side chain, chemical synthesis of this chiral diol has resulted in poor atom-economy due to complicated synthetic route, large energy consumption and environmental pollution from harsh processing conditions [4–6]. In addition, it is difficult to meet the high standards on optical purity of chiral drugs by chemical synthesis [7]. Consequently, biocatalysis has emerged as a powerful approach for the synthesis of statin drugs owing to obvious benefits such as mild reaction condition, high catalytic efficiency, excellent stereoselectivity and environmental friendliness. This far, a number of biocatalytic approaches has been developed for the synthesis of statin side chains to replace or complement the existing chemical processes [8–11]. The employment of different biocatalysts including alcohol dehydrogenase, lipase, nitrilase, dehalogenase, aldolase and ketoreductase has resulted in various biosynthetic routes [12–17]. Nonetheless, very few of them have been applied in commercial practice, due mainly to the shortcomings of these biocatalytic approaches, including low substrate loading, high cost of cofactors and the complicated process for biocatalyst preparation.

Previously, a novel NADPH-dependent α-keto reductase from *Saccharomyces cerevisiae* (designated as CR1 in this study) was reported to exhibit strong activity on reducing aliphatic α-keto esters including *t*-butyl-6-cyano-(5*R*)-hydroxy-3-carboxylhexanoate (**1**) with enantiomeric excess (*ee*) and diastereomeric excess (*de*) values both greater than 99.5% [18,19]. The resulting product *t*-butyl-6-cyano-(3*R*, 5*R*)-dihydroxyhexanoate (**2**) was then used as a key intermediate for the synthesis of atorvastatin. With a series of improvements, this biocatalytic approach has been the only industrialized process for the production of chiral side

* Corresponding author. Tel.: +86 25 83271045; fax: +86 25 83271031.
E-mail address: yjchen@cpu.edu.cn (Y. Chen).
[1] These authors contributed equally to this work.

http://dx.doi.org/10.1016/j.procbio.2014.10.023
1359-5113/



Scheme 1. Combination of CR1 and GDH for the biosynthesis of compound **2**. **1**: *t*-butyl-6-cyano-(5*R*)-hydroxy-3-carboxylhexanoate; **2**: *t*-butyl-6-cyano-(3*R*, 5*R*)-dihydroxyhexanoate; CR1: carbonyl reductase from *Saccharomyces cerevisiae*; GDH: glucose dehydrogenase from *Bacillus megaterium*.

chain of atorvastatin using lyophilized CR1 and glucose dehydrogenase (Scheme 1). However, the requirements of exogenous addition of cofactors in the asymmetric reduction and complicated operation, such as cell disruption and enzyme lyophilization, have made this process economically inefficient [20]. Therefore, construction of a more economical and simpler biosynthetic process is a valuable and necessary improvement on the preparation of compound **2**.

To date, whole-cell biocatalysts with co-expression of an oxidoreductase and an NAD(P)H regeneration enzyme including formate dehydrogenase (FDH) or glucose dehydrogenase (GDH) or alcohol dehydrogenase have been utilized in various asymmetric bio-reductions to eliminate or to reduce the exogenous addition of cofactors [21]. Unfortunately, in most cases, the process still requires the addition of sufficient amount of expensive cofactors to initiate the enzymatic transformation and to achieve complete conversion of the substrate on large scales [17,22,23], which could be a result of lower catalytic efficiency of the biocatalyst or the incompatibility between a reductase and a cofactor regeneration system in the host cells. Previously, a close correlation between intracellular cofactor concentration and biocatalytic efficiency was observed when we coupled diketoreductase with GDH as a whole-cell biocatalyst for the preparation of a chiral diol (ethyl 3*R*, 5*S*-dihydroxy-6-benzyloxy hexanoate). Further analysis revealed that the order of genes cloned in the same vector under different promoters could still affect enzyme expression and enzymatic activity [24]. Therefore, due to sequence diversity of various genes and different properties of genes and promoter, the compatibility of co-expressed enzymes could be an issue to affect their functional expression, especially the order of genes in a co-expression vector, in order to identify more valuable whole-cell biocatalyst. In the present study, to completely eliminate the addition of exogenous NAD(P)(H) and simplify the operation for the preparation of **2**, we established a biocatalytic process with whole-cell biocatalyst coupling CR1 and GDH-cofactor regeneration system.

A recombinant *Escherichia coli* strain simultaneously overexpressing CR1 (GenBank no. NP_010159.1) from *S. cerevisiae* and glucose dehydrogenase (GDH) (GenBank no. YP_003563827.1) from *Bacillus megaterium* was constructed by co-expression vector pETDuet-1 with two independent T7 promoters. Subsequently, after comparing two *E. coli* strains expressed both enzymes in different orders and optimizing the biocatalysis conditions, an efficient *in situ* cofactor-regenerating system was established to improve the biocatalytic efficiency with a productivity of 120 g l^{-1} day^{-1}, and the complete elimination of cofactor addition significantly reduced the preparation costs for the chiral side chain of atorvastatin.

2. Materials and methods

2.1. Materials

Co-expression vector pETDuet-1 was obtained from Novagen, USA. All restriction endonuclease were obtained from TaKaRa Bio Inc., Japan. *t*-Butyl-6-cyano-(5*R*)-hydroxy-3-carboxylhexanoate and *t*-butyl-6-cyano-(3*R*, 5*R*)- dihydroxyhexanoate were purchased from J&K Scientific Ltd. China. Chromatographic grade acetonitrile used for HPLC was purchased from Tedia Company Inc., USA. Other biological and chemical reagents used in this study were of analytical grade.

2.2. HPLC analysis

Achiral HPLC method was performed with mobile phase A (0.25% acetic acid in water) and mobile phase B (acetonitrile) at 30 °C with UV detection at 220 nm. The analyses were achieved on a Thermo ODS-2 HYPERSIL column (5 μm, 250 mm × 4.6 mm) with an injection volume of 20 μL and a flow rate of 1 mL/min. The conversion catalyzed by CR1 was determined by an isocratic elution 25% B in 20 min. The retention times for **2** and **1** were 6.9 min and 10.2 min, respectively. Meanwhile, the *de* value of compound **2** was also analyzed according to reported method [20].

2.3. Constructions of *E. coli* (pETDuet-cr1-gdh) and *E. coli* (pETDuet-gdh-cr1)

DNAs with the nucleotide sequences of *cr1* and *gdh* were synthesized by Generay Biotech Ltd., China. The pETDuet-1 vector with two multiple cloning sites (MCS), each of which is preceded by a T7 promoter was selected to construct co-expression system for CR1 and GDH. The DNA fragment of *cr1* was then cloned into the MCS1 of pETDuet-1 vector between *Nco* I and *Bam*H I restriction sites while *gdh* fragment was cloned into the MCS2 of pETDuet-1 vector between *Nde* I and *Xho* I restriction sites. The constructed plasmid was then designated as pETDuet-*cr1-gdh*. With the same strategy, pETDuet-*gdh-cr1* was built accordingly. Constructed plasmids were transformed into *E. coli* BL21 (DE3) respectively to obtain recombinant *E. coli* containing pETDuet-*cr1-gdh* and *E. coli* containing pETDuet-*gdh-cr1*. After screening by PCR and DNA sequencing, the strains with correct plasmids were subsequently used and named as pCG and pGC, respectively.

2.4. Expression of CR1 and GDH and preparation of cell-free extract

Recombinant *E. coli* cells were grown in LB medium containing 100 μg/mL ampicillin at 37 °C on a rotary shaker (220 rpm). When OD_{600} value reached 0.8 ± 0.1, co-expression of CR1 and GDH was induced by addition of IPTG for 16 h. Temperatures from 15 to 35 °C were applied to examine the effects on CR1 and GDH expression at 0.2 mM IPTG. IPTG concentrations from 0.2 to 1.2 mM were employed for optimal induction. Cells were harvested *via* centrifugation at 5000 × *g* for 10 min and washed twice with 0.1 M potassium phosphate buffer (pH 7.0). The cells were resuspended in same buffer for high pressure cell disruption (35 kpsi). After centrifugation at 15,000 × *g* for 30 min, cell-free extract was obtained for SDS-PAGE and enzyme activity assay.

2.5. Enzyme activity assays of CR1 and GDH

Enzyme activity of CR1 and GDH in the cell-free extract was assayed *via* spectrophotometric method. Assay mixture for CR1 composed of 0.1 mM NADPH, 5 mM **1**, 0.1 M potassium phosphate buffer (pH 7.0) and 10 μL cell-free extract in a final volume of

Please cite this article in press as: Wu X, et al. Enzymatic preparation of *t*-butyl-6-cyano-(3*R*, 5*R*)-dihydroxyhexanoate by a whole-cell biocatalyst co-expressing carbonyl reductase and glucose dehydrogenase. Process Biochem (2014), http://dx.doi.org/10.1016/j.procbio.2014.10.023

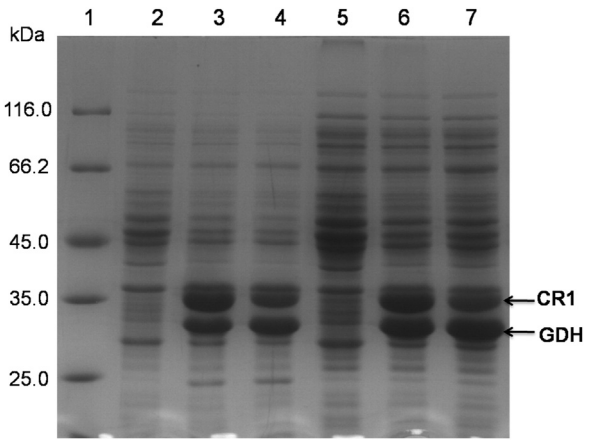


Fig. 1. SDS-PAGE for co-expression of CR1 and GDH in *E. coli*. SDS-PAGE was performed on a 15% gel under reduced condition. The subunit weights of CR1 and GDH are approximately 35 and 28 kDa, respectively. Lane 1, molecular weight markers; Lane 2, soluble proteins from *E. coli* cells without plasmid; Lane 3, soluble proteins from pGC strain co-expressed CR1 and GDH; Lane 4, soluble proteins from pCG strain co-expressed CR1 and GDH; Lane 5, total proteins from *E. coli* cells without plasmid; Lane 6, total proteins from pGC strain; Lane 7, total proteins from pCG strain.

1.0 mL. Assay mixture for GDH contained 0.6 mM $NADP^+$, 5 mM glucose, 0.1 M potassium phosphate buffer (pH 7.0) and 10 μL cell-free extract in a final volume of 1.0 mL. The CR1 and GDH activity were assayed by measuring absorbance change at 340 nm for the decrease or the increase of NADPH at 37 °C. For all assays, enzyme activity was defined as one unit representing the oxidation of one μmole of NADPH or the reduction of one μmole of $NADP^+$ per minute per min per gram cell dry weight (CDW). In addition, the turnover number of NADP(H) catalyzed by endogenous enzymes was measured and evaluated using *E. coli* cells with the same procedures in the absence of recombinant CR1 and GDH.

2.6. Biocatalytic efficiency of pGC and pCG

The whole-cell biocatalytic activity was assessed by monitoring the increase of **2** in the reaction mixture *via* HPLC analysis. The reaction mixtures comprising 30 mL fresh culture of pGC or pCG, 5 g/L or 50 g/L of **1**, 10% DMSO (v/v) and glucose (2 equiv. of substrate) were incubated at 25 °C on a 200 rpm rotary shaker for 12 h. pH value of the mixture was monitored and adjusted to 7.0 using 5 N NaOH and 3 N HCl. 30 mL of acetonitrile was added into resulting mixture to terminate the enzymatic reaction. At the same time, the formation of **2** catalyzed by native enzymes in *E. coli* cells was also investigated using the same procedures. After centrifugation at 15,000 × *g* for 10 min, supernatant was subjected to HPLC analysis.

2.7. Effects of pH and temperature on the bioconversion

Reactions with different initial pH values ranging from 5.0 to 9.0 were formulated with 30 mL fresh culture (0.25 g-CDW of cells), 50 g/L of **1**, 10% DMSO (v/v) and glucose (2 equiv. of substrate). After incubating at 25 °C on a 200 rpm rotary shaker for 12 h, reaction mixtures were then subjected to HPLC analysis. As for temperature assay, serials of reactions with the optimized pH value were incubated under different temperatures ranging from 15 to 35 °C. Resulting mixtures were then subjected to HPLC analysis.

2.8. The effects of substrate concentrations on biocatalytic efficiency

Serials of reactions were conducted under the optimized pH value and temperature, and different concentrations of **1** ranging from 10 to 55 g/L were used to determine the maximal loading of **1** within 12-h of reaction period. For glucose concentration, reactions with different glucose concentrations ranging from 2 equivalents to 1 equivalent of **1** were performed with the optimized concentration of **1**. All reactions were then assessed by HPLC.

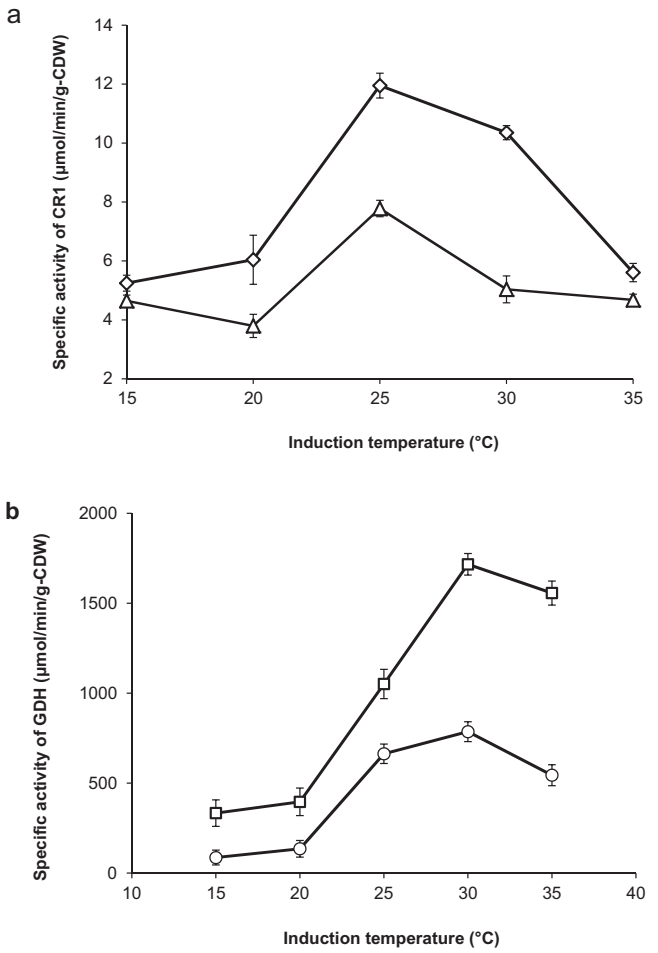


Fig. 2. Effects of induction temperature on enzyme activity. (a) Effects of induction temperature on the expression of CR1. Open diamond: specific activity of CR1 in pGC strain; Open triangle: specific activity of CR1 in pCG strain; (b) Effects of induction temperature on the expression of GDH. Open square: specific activity of GDH in pCG strain; Open circle: specific activity of GDH in pGC strain. All data are averages of 3 independent experiments with standard deviations.

2.9. Biosynthesis of **2**

To scale up the reaction for the preparation of **2**, 0.5 L scale reaction containing 4.1 g cells and 17.5 g of substrate **1** was performed with the optimized parameters. Samples were taken every hour and subjected to HPLC analysis. 1 L ethyl acetate was added into resulting mixture for product extraction. Vacuum rotary evaporation was performed to remove the organic solvent after the separation of aqueous and organic phases by centrifugation at 12,000 × *g* for 10 min. After purification, 14.4 g of **2** was obtained as oil.

3. Results and discussion

3.1. Construction of the in situ cofactor recycling biocatalyst

The poor long-term stability of crude CR1 and the requirement of cofactor recycling are the limiting factors for the previous biocatalytic process to prepare atorvastatin side chain. Compared to

Please cite this article in press as: Wu X, et al. Enzymatic preparation of *t*-butyl-6-cyano-(3*R*, 5*R*)-dihydroxyhexanoate by a whole-cell biocatalyst co-expressing carbonyl reductase and glucose dehydrogenase. Process Biochem (2014), http://dx.doi.org/10.1016/j.procbio.2014.10.023

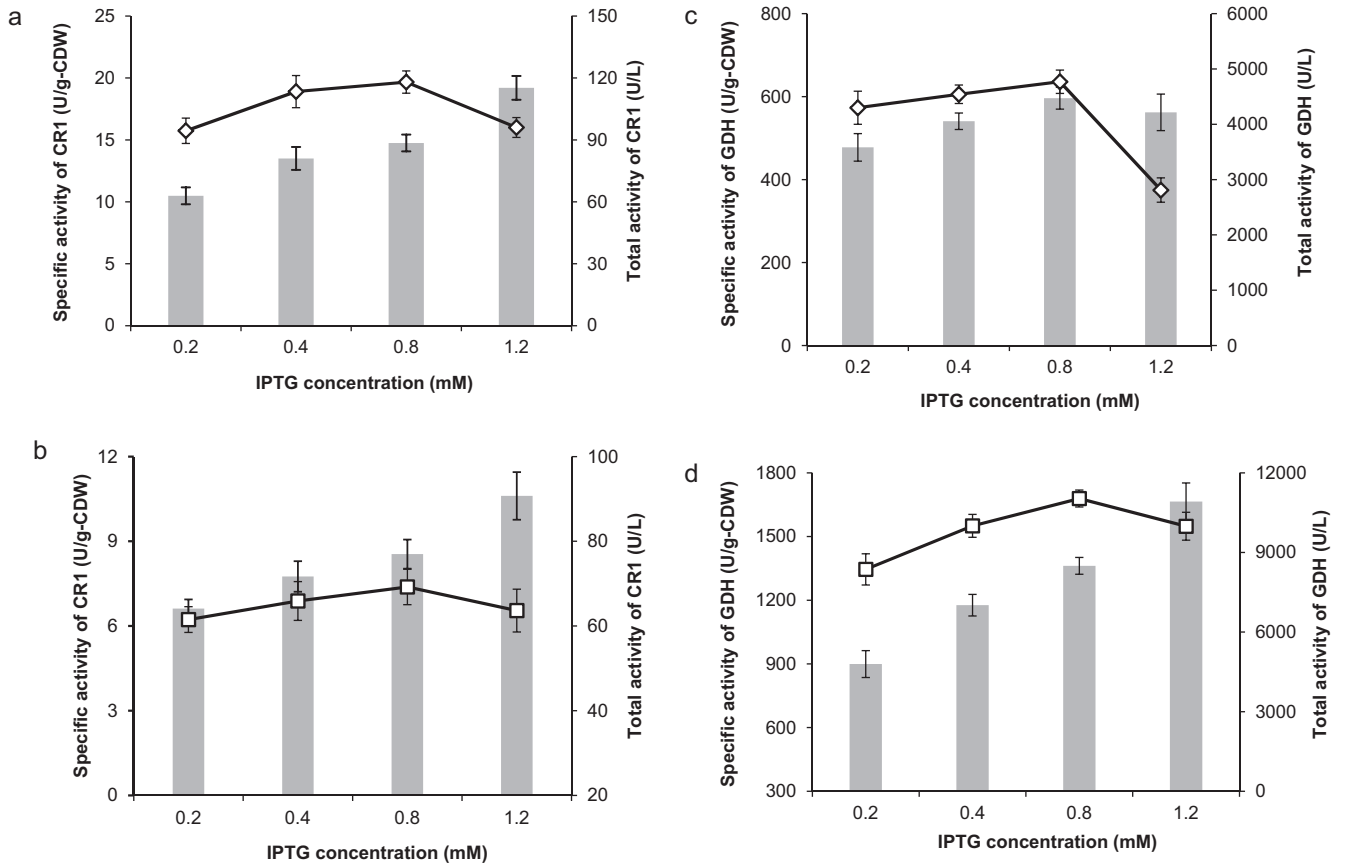


Fig. 3. Effects of IPTG concentration on enzyme activity. Effects of IPTG on CR1 activity in pGC strain. Gray columns: specific activity of CR1. Open diamond: total activity of CR1 in 1 L culture. (b) Effects of IPTG on CR1 activity in pCG strain. Gray columns: specific activity of CR1; Open square: total activity of CR1 in 1 L culture. (c) Effects of IPTG on GDH activity in pGC strain. Gray columns: specific activity of GDH; Closed diamond: total activity of GDH in 1 L culture. (d) Effects of IPTG on GDH activity in pCG strain. Gray columns: specific activity of GDH; Closed square: total activity of GDH in 1 L culture. Total activity was obtained by specific activity × cell dry weight in 1 L culture. All data are averages of three independent experiments with standard deviations.

cell-free extracts and lyophilized enzymes, whole cells showed obvious advantages to improve the stability of unstable biocatalysts by protecting cell wall to reserve endogenous cofactors that can be recycled by coupling with another exogenous enzyme and native enzymes on cell surface or within the host cells [17,25]. Therefore, whole-cell biocatalysts have been employed in many asymmetric reduction systems [26–28]. Also, previous study revealed that CR1 possessed a cofactor preference for NADPH [18], which could be efficiently regenerated by GDH with readily available glucose. Accordingly, the cheaper and simpler whole cells overexpressing CR1 along with *in situ* cofactor regeneration system mediated by GDH would be a preferable biocatalyst for the preparation of **2**.

After the construction of both recombinant co-expression vectors of pETDuet-*cr1-gdh* and pETDuet-*gdh-cr1* and transforming them to *E. coli* cells, the co-expression of the enzymes was achieved by addition of 0.5 mM IPTG at 25 °C, which was analyzed by SDS-PAGE. As indicated in Fig. 1, both strains showed soluble expression of CR1 and GDH, indicating that pETDuet-1 is suitable to express both enzymes. By comparing the amount of both enzymes, pGC and pCG strains showed similar expression of GDH, but the expression of CR1 in pGC was obviously more than that in pCG, suggesting that the order of the two genes can significantly affect the expression level of the enzymes. Importantly, the resulting whole-cell biocatalyst pGC strain could be used for the bioconversion substrate **1**, which would be further investigated.

3.2. Optimization of cofactor-regenerating system for the whole-cell biocatalyst

Temperature plays an important role during protein expression by affecting the folding of the nascent polypeptide chain. When temperature is high, protein is likely to be expressed in the form of inclusion body, which is typically inactive [29]. On the other hand, catalytic activity of the biocatalyst may dramatically decrease due to retardant protein expression caused by hypothermia. To reach the optimal induction, different temperatures ranging from 15 to 35 °C were investigated in the same batch of cultures in the presence of 0.2 mM IPTG for induction. Then, cell-free extracts obtained from resulting cells were subjected to activity assays, and the supernatant of *E. coli* cells without the vector was employed as a control. As shown in Fig. 2a, CR1 in both pGC and pCG was more active at higher temperatures and reached a maximum at 25 °C with a specific activity at 11.9 and 7.8 μmol/min/g-CDW, respectively. On contrary, the maximal activity of GDH in both strains was observed at 30 °C (Fig. 2b), which was different from that for CR1. Because CR1 activity was the rate-limiting factor for the biosynthesis of **2** and GDH activity in both combinations was high enough to drive the reaction (Fig. 2), 25 °C was selected as the optimal temperature for co-expression of both enzymes. Furthermore, the CR1 activity in pGC strain under all temperatures was markedly higher than that in pCG strain.

IPTG concentration used for induction also impacts the expression of the enzymes. At relatively low concentrations, the increase

Please cite this article in press as: Wu X, et al. Enzymatic preparation of *t*-butyl-6-cyano-(3*R*, 5*R*)-dihydroxyhexanoate by a whole-cell biocatalyst co-expressing carbonyl reductase and glucose dehydrogenase. Process Biochem (2014), http://dx.doi.org/10.1016/j.procbio.2014.10.023

of IPTG could boost enzyme expression regulated by *lac* operon. However, the toxicity of IPTG would impair the growth of bacteria to affect the biocatalytic efficiency of the resulting cultures. Although Fig. 3 displayed different specific activity of CR1 in pGC and pCG strains at various IPTG concentrations, 0.8 mM IPTG gave the highest total enzyme activity in both cultures. Meanwhile, similar results were also observed for GDH in both strains (Fig. 3c and d). Since total enzyme activity is the decisive factor to reflect biocatalytic efficiency, IPTG with a final concentration of 0.8 mM was subsequently used for the induction. In addition, same as temperature, CR1 activity in pGC strain was also much higher than that in pCG strain at all tested IPTG concentrations, indicating that pGC strain should be more favorable as a biocatalyst for the biosynthesis of **2**.

3.3. Comparison of co-expression systems on the biocatalytic efficiency

As stated above, the translation level could be altered when a gene was inserted into different cloning sites of pETDuet-1 vector. Therefore, it was necessary to compare the stains of pGC and pCG in order to identify which one is more useful and efficient for the biocatalytic preparation of **2**. Different concentrations of **1** were employed to compare the catalytic efficiency between pGC and pCG. HPLC analysis revealed that > 99% of substrate was converted by both strains at a substrate concentration of 5 g/L with *de* value > 99.5%. However, when the substrate **1** was increased to 50 g/L, the 12-h *in situ* bioconversion of **1** by pGC and pCG cultures with the same biomass (8.2 g/L) was dropped to 82.4% and 10.5% respectively. This distinct catalytic efficiency was in agreement to above comparisons of enzyme activity between pGC and pCG strains (Fig. 3). Better catalytic efficiency by pGC also confirmed previous speculation that CR1 activity is the rate-limiting factor for the bioconversion. Moreover, the results strongly indicated that the order between CR1 and GDH is crucial to the biocatalytic efficiency and the placement of GDH before CR1 could achieve better overall activity for both enzymes. Therefore, pGC strain was induced by 0.8 mM IPTG for 16 h to result in a more active and preferable whole-cell biocatalyst for the preparation of **2**.

3.4. Effects of pH and temperature on the biosynthesis of **2**

In large-scale bioprocesses, slight shift of environmental pH could lead to drastic variation of productivity, and the enzymatic reaction would proceed faster and more complete at optimal pH value. In the present study, the drift of pH value during the process caused by H^+ production during glucose dehydrogenation may exceed the homeostasis capacity of the cells, which requires to adjust pH value in the media to an adequate level. Fig. 4a shows pH variations on the bioconversion rate on 50 g/L substrate **1** in a reaction period of 12 h. The results indicated that neutral pH gives the best catalytic efficiency, which is consistent with the optimal pH for CR1 [18].

Temperature also plays important role during the biosynthesis, not only because of its impact on enzyme activity and stability, but also because of the effects on the solubility of substrates and energy consumption during industrial applications. Thus, an appropriate reaction temperature is required to achieve the best catalytic efficiency. When different temperatures were used at pH 7.0, the highest bioconversion rate on 50 g/L substrate **1** was observed after 12 h (Fig. 4b). The conversion rose when temperature was elevated from 15 to 20 °C and slightly dropped when temperature further went up to 25 °C. There was a dramatic decrease of conversion between 25 and 30 °C. Therefore, the optimal temperature for pGC was 20 °C, which is close to room temperature to save energy during the industrial process. Hence, the combination of pH 7.0 and reaction temperature of 20 °C provided the best conditions for the biosynthesis of **2**.

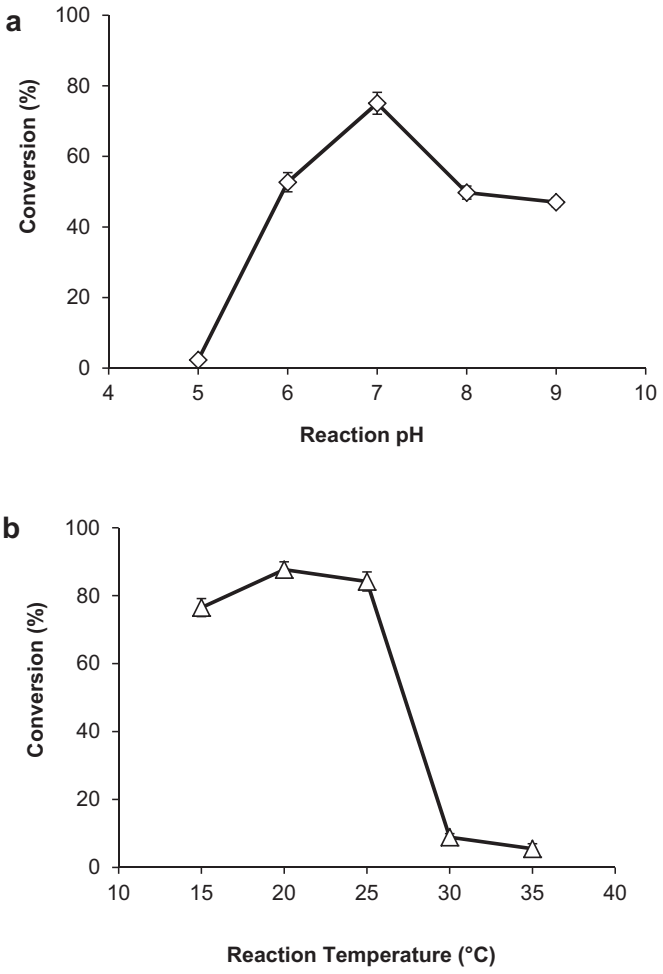


Fig. 4. Effects of pH and temperature on the biocatalytic efficiency. (a) Effects of pH on the biocatalytic efficiency. (b) Effects of temperature on the biocatalytic efficiency. Data are averages of three independent experiments with standard deviations ($n = 3$).

3.5. Optimization of substrate and co-substrate concentration

The amount of substrate loading is a critical factor for a biocatalytic process, and generally high substrate loading can result in high productivity. However, substrate inhibition on biocatalysts should be taken into consideration at the same time [30]. Hence, the concentration of substrate **1** was investigated at optimal conditions to establish an efficient and economic *in situ* bioconversion system. As shown in Fig. 5a, the bioconversion was not able to complete when substrate concentration exceeded 35 g/L. Therefore, the maximal substrate loading in this biocatalytic process was set up at 35 g/L, at which pGC was able to produce **2** with yield > 99% and de > 99.5% after 12 h without addition of exogenous cofactor.

As for the use of co-substrate of glucose, an equal molar ratio of glucose to **1** would theoretically sufficient to drive the bioconversion by the combination of CR1 and GDH. However, due mainly to the existence of multiple pathways to catabolize glucose in *E. coli* cells, it usually requires excess amount of glucose added into the reaction media in order to efficiently complete the bioconversion by the reductase. Therefore, the minimum requirement of glucose was examined for the biocatalytic preparation of **1** by the whole cell biocatalyst pGC. As shown in Fig. 5b, under optimal conditions

Please cite this article in press as: Wu X, et al. Enzymatic preparation of *t*-butyl-6-cyano-(3*R*, 5*R*)-dihydroxyhexanoate by a whole-cell biocatalyst co-expressing carbonyl reductase and glucose dehydrogenase. Process Biochem (2014), http://dx.doi.org/10.1016/j.procbio.2014.10.023

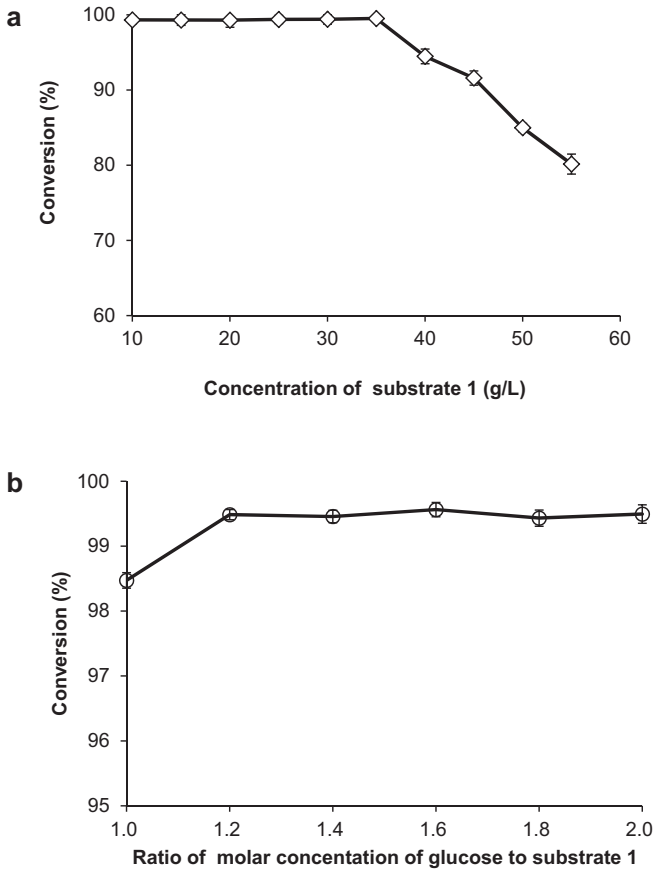


Fig. 5. Effects of substrate **1** concentration and glucose concentration on the biocatalytic efficiency. (a) Effects of substrate **1** concentration on the biocatalytic efficiency. (b) Effects of glucose concentration on the biocatalytic efficiency. Open circle represents the conversion rate of substrate **1** at different ratios of molar concentration of glucose to **1**. All data are averages of three independent experiments with standard deviations.

of pH 7.0 and 20 °C, addition of 1.2 equivalents of glucose could result in >99% conversion of **1** at the concentration of 35 g/L after 12 h. Based on the substrate loading, the minimum requirement for glucose addition was 33.3 g/L, which was in agreement with the process using lyophilized enzyme [20].

3.6. Preparation of compound **2**

To examine the feasibility of the present whole-cell biocatalytic process, 0.5 L scale reaction was conducted using 35 g/L of **1** as substrate under the optimized conditions without addition of expensive cofactors. Samples were taken every hour and subjected to HPLC analysis to monitor the reaction progress. As shown in Fig. 6, the conversion rate of **1** increased by the reaction time and reached >99% at 7 h with a productivity of 120 g l^{-1} day^{-1}. After extraction and purification, the oily diol product **2** was obtained to give a yield of 82.4%. Compared to current industrial bioprocess with lyophilized enzymes [20], the present biocatalytic system showed relatively lower substrate loading and daily volumetric productivity. However, simple operation without cell disruption and lyophilization to prepare biocatalyst and reduced costs by omitting the use of exogenous NAD(P)H make the present process more industrially feasible and attractive for the preparation of **2**, indicating that whole cell biocatalysis could be an alternative approach in the industrial settings.

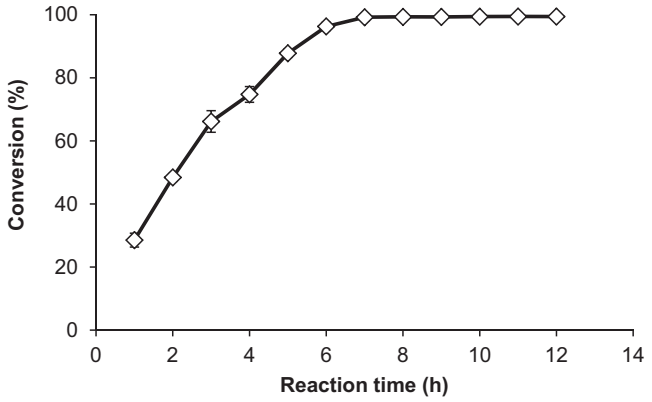


Fig. 6. Time course on the bioconversion of substrate **1** at 0.5 L-scale.

4. Conclusion

A whole-cell biocatalyst co-expressing carbonyl reductase and glucose dehydrogenase was constructed, and the strain containing a vector to harbor *gdh* in front of *cr1* was identified to possess better catalytic efficiency. These two enzymes, along with the endogenous cofactor pool in *E. coli*, formed an *in situ*-cofactor regenerating system for the biosynthesis of *t*-butyl-6-cyano-(3*R*, 5*R*)-dihydroxyhexanoate, a key intermediate for the synthesis of atorvastatin. After symmetric optimization, the bioconversion reached completion with a productivity of 120 g l^{-1} day^{-1} at a substrate concentration of 35 g/L after 7 h at 0.5 L scale. The unique features of this process, particularly without addition of exogenous cofactor, would be highly beneficial for industrial applications. Thus, the present work not only demonstrates the feasibility of using whole-cell biocatalysis approach to prepare **2**, but also expands the utility of biocatalysis in the preparation of various chiral drugs or intermediates.

Acknowledgements

This work was supported by the grants from the "111" Project from the Ministry of Education of China, and the State Administration of Foreign Experts Affairs of China (No: 111-2-07), National Key Project of Science and Technology (2014ZX09201004) and the Priority Academic Program Development of Jiangsu Higher Education Institutions.

References

[1] Corsini A, Maggi FM, Catapano AL. Pharmacology of competitive inhibitors of HMG-CoA reductase. Pharmacol Res 1995;31:9–27.
[2] Istvan ES, Deisenhofer J. Structural mechanism for statin inhibition of HMG-CoA reductase. Science 2001;292:1160–4.
[3] Kathawala FG. HMG-CoA reductase inhibitors: an exciting development in the treatment of hyperlipoproteinemia. Med Res Rev 1991;11:121–46.
[4] Hollingsworth RI, Wang G. 4-Cyano-3-hydroxybutanoyl hydrazines, derivatives and process for the preparation thereof. US Patent. US 6114566. 00.09.05.
[5] Scheffer JL, Bette V, Mortreux A, Nowogrocki G, Carpentier J-F. Preparation and stereoselective hydrogenation of chiral (4-hydroxy-tetrafuranylidene) carboxylates: a new formal entry to functional anti- and *syn*-3,5-dihydroxyesters. Tetrahedron Lett 2002;43:2679–82.
[6] Everaere K, Franceschini N, Mortreux A, Carpentier J-F. Diastereoselective synthesis of *syn*-3,5-dihydroxyesters via ruthenium-catalyzed asymmetric transfer hydrogenation. Tetrahedron Lett 2002;43:2569–71.
[7] Liljeblad A, Kallinen A, Kanerva LT. Biocatalysis in the preparation of the statin side chain. Curr Org Synth 2009;6:362–79.
[8] Müller M. Chemoenzymatic synthesis of building blocks for statin side chains. Angew Chem Int Ed 2005;44:362–5.
[9] Chen YJ, Chen C, Wu XR. Dicarbonyl reduction by single enzyme for the preparation of chiral diols. Chem Soc Rev 2012;41:1742–53.
[10] Patel RN. Synthesis of chiral pharmaceutical intermediates by biocatalysis. Coord Chem Rev 2008;252:659–701.

Please cite this article in press as: Wu X, et al. Enzymatic preparation of *t*-butyl-6-cyano-(3*R*, 5*R*)-dihydroxyhexanoate by a whole-cell biocatalyst co-expressing carbonyl reductase and glucose dehydrogenase. Process Biochem (2014), http://dx.doi.org/10.1016/j.procbio.2014.10.023

[11] Huang Y, Liu N, Wu XR, Chen YJ. Dehydrogenases/reductases for the synthesis of chiral pharmaceutical intermediates. Curr Org Chem 2010;14:1447–60.
[12] Yasohara Y, Kizaki N, Hasegawa J, Wada M, Kataoka M, Shimizu S. Stereoselective reduction of alkyl 3-oxobutanoate by carbonyl reductase from *Candida magnoliae*. Tetrahedron: Asymmetry 2001;12:1713–8.
[13] Öhrlein R, Baisch G. Chemo-enzymatic approach to statin side-chain building blocks. Adv Synth Catal 2003;345:713–5.
[14] DeSantis G, Wong K, Farwell B, Chatman K, Zhu ZL, Tomlinson G, et al. Creation of a productive, highly enantioselective nitrilase through gene site saturation mutagenesis. J Am Chem Soc 2003;125:11476–7.
[15] Wu XR, Liu N, He YM, Chen YJ. Cloning, expression, and characterization of a novel diketoreductase from *Acinetobacter baylyi*. Acta Biochem Biophys Sin 2009;41:163–70.
[16] Greenberg WA, Varvak A, Hanson SR, Wong K, Huang HJ, Chen P, et al. Development of an efficient, scalable, aldolase-catalyzed process for enantioselective synthesis of statin intermediates. Proc Natl Acad Sci USA 2004;101:5788–93.
[17] Wu XR, Wang LL, Wang SZ, Chen YJ. Stereoselective introduction of two chiral centers by a single diketoreductase: an efficient biocatalytic route for the synthesis of statin side chains. Amino Acids 2010;39:305–8.
[18] Ishihara K, Yamamoto H, Mitsuhashi K, Nishikawa K, Tsuboi S, Tsuji H, et al. Purification and characterization of α-keto amide reductase from *Saccharomyces cerevisiae*. Biosci Biotechnol Biochem 2004;68:2306–12.
[19] Chang Q, Griest TA, Harter TM, Petrash JM. Functional studies of aldo-keto reductases in *Saccharomyces cerevisiae*. Biochim Biophys Acta 2007;1773:321–9.
[20] Giver LJ, Newman LM, Mundorff E, Huisman GW, Jenne SJ, Zhu J, et al. Compositions and methods for producing stereoisomerically pure statins and synthetic intermediates therefor. US Patent. US 20080248539. 08.10.09.
[21] Richter N, Neumann M, Liese A, Wohlgemuth R, Weckbecker A, Eggert T, et al. Characterization of a whole-cell catalyst co-expressing glycerol dehydrogenase and glucose dehydrogenase and its application in the synthesis of L-glyceraldehyde. Biotechnol Bioeng 2010;106:541–52.
[22] Jiang JP, Wu XR, Chen YJ. Strategy to solve cofactor issues in oxidoreductase catalyzed biocatalytic applications. Chin J Biotechnol 2012;28:410–9.
[23] Rodriguez C, Lavandera I, Gotor V. Recent advances in cofactor regeneration systems applied to biocatalyzed oxidative processes. Curr Org Chem 2012;16:2525–41.
[24] Wu XR, Jiang JP, Chen YJ. Correlation between intracellular cofactor concentrations and biocatalytic efficiency: coexpression of diketoreductase and glucose dehydrogenase for the preparation of chiral diol for statin drugs. ACS Catal 2011;1:1661–4.
[25] Liang B, Lang QL, Tang XJ, Liu AH. Simultaneously improving stability and specificity of cell surface displayed glucose dehydrogenase mutants to construct whole-cell biocatalyst for glucose biosensor application. Bioresour Technol 2013;147:492–8.
[26] Endo T, Koizumi S. Microbial conversion with cofactor regeneration using engineered bacteria. Adv Synth Catal 2001;343:521–6.
[27] Galkin A, Kulakova L, Yoshimura T, Soda K, Esaki N. Synthesis of optically active amino acids from alpha-keto acids with *Escherichia coli* cells expressing heterologous genes. Appl Environ Microbiol 1997;63:4651–6.
[28] Weckbecker A, Hummel W. Improved synthesis of chiral alcohols with *Escherichia coli* cells co-expressing pyridine nucleotide transhydrogenase, $NADP^+$-dependent alcohol dehydrogenase and NAD^+-dependent formate dehydrogenase. Biotechnol Lett 2004;26:1739–44.
[29] Volontè F, Marinelli F, Gastaldo L, Sacchi S, Pilone MS, Pollegioni L, et al. Optimization of glutaryl-7-aminocephalosporanic acid acylase expression in *E. coli*. Protein Expr Purif 2008;61:131–7.
[30] Zhang JL, Tao SS, Zhang BJ, Wu XR, Chen YJ. Microparticle-based strategy for controlled release of substrate for the biocatalytic preparation of L-homophenylalanine. ACS Catal 2014;4:1584–7.

Please cite this article in press as: Wu X, et al. Enzymatic preparation of *t*-butyl-6-cyano-(3*R*, 5*R*)-dihydroxyhexanoate by a whole-cell biocatalyst co-expressing carbonyl reductase and glucose dehydrogenase. Process Biochem (2014), http://dx.doi.org/10.1016/j.procbio.2014.10.023