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Optimization and Scale-up of a Bioreduction Process for Preparation of Ethyl (*S*)-4-Chloro-3-hydroxybutanoate

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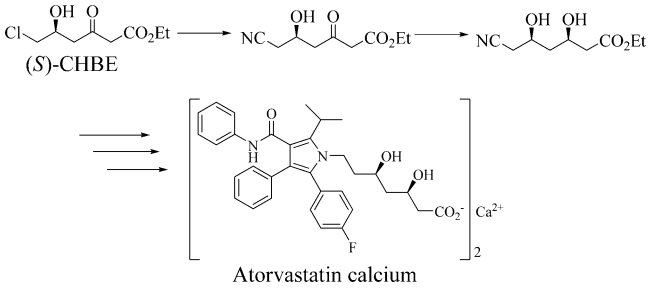
ABSTRACT: Ethyl 4-chloro-3-oxobutanoate (COBE) was asymmetrically reduced with *Escherichia coli* cells expressing a reductase (ScCR) from *Streptomyces coelicolor* to afford enantiopure ethyl (*S*)-4-chloro-3-hydroxybutanoate [(*S*)-CHBE], which is an important precursor for preparing the drug atorvastatin. The substrate load was fixed at 100 g/L, and the concentration of coenzyme NAD^+ was limited to 0.1 mM based on cost considerations. Under these conditions, the other reaction parameters were optimized as 25 °C and pH 6.5, with a biocatalyst dose of 10 kU/L in the presence of isopropanol (1.5 equiv of COBE), which acted as a cosubstrate for regenerating NADH. The reaction was performed in a toluene−aqueous biphasic system (1:1, v/v), with agitation at the maximal linear rate of 0.88 m/s. Finally, the bioreaction was performed on a pilot scale using a 50 L thermostated stirred-tank-reactor, affording (*S*)-CHBE in 85.4% yield and 99.9% ee, and a total turnover number (TTN) of 6060 for the cofactor NAD^+. The specific production was calculated to be 36.8 $g_{product}/g_{dcw}$, which is the highest value reported to date among the whole-cell-mediated processes for producing (*S*)-CHBE.

INTRODUCTION

Ethyl (*S*)-4-chloro-3-hydroxybutanoate [(*S*)-CHBE] is an important precursor for the production of the HMG-CoA reductase inhibitor atorvastatin, a cholesterol-lowering drug with annual sales exceeding 10 billion USD (Scheme 1).

Scheme 1. Synthetic route of atorvastatin calcium from (*S*)-CHBE



Enzymatic asymmetric reduction of ethyl 4-chloro-3-oxobutanoate (COBE) is an important method for preparing optically active (*S*)-CHBE. Many microorganisms can catalyze the COBE reduction to afford (*S*)-CHBE.[1] Some COBE reductases with high activities and stereoselectivities have been cloned. Yamamoto et al. used a homologue of *fabG* to catalyze the asymmetric reduction of COBE, yielding 48.7 g/L of the product (*S*)-CHBE.[2] Ye et al. employed *E. coli* cells coexpressing a carbonyl reductase gene from *Pichia stipitis* and a glucose dehydrogenase gene from *Bacillus megaterium* to catalyze the COBE reduction in a biphasic system with ethyl caprylate as the organic phase, yielding 1.26 M (*S*)-CHBE in the organic phase in >99% ee and 90% yield through batch feeding of COBE and glucose.[3] Codexis Incorporation established a green route for the synthesis of the atorvastatin precursor via asymmetric reduction of COBE using a ketoreductase combined with a glucose dehydrogenase coupled tandemly with a halohydrin dehalogenase to convert the produced chlorohydrin into cyanohydrin.[4] The two-step route employing three enzymes created a greener reaction process that was recognized with a 2006 American Presidential Green Chemistry Challenge Award.

Cofactor regeneration is very important for the efficient reduction of COBE. Glucose dehydrogenase[2,5] is an efficient cofactor-regenerating enzyme because of its higher activity and better stability as compared with formate dehydrogenases.[6] Although good results were obtained by using glucose dehydrogenase as the regenerating enzyme, the atom economy of this process was as low as 43.3%, necessitating the use of additional glucose dehydrogenase. In addition, gluconate was formed as a coproduct, making it necessary to add a basic solution in order to control the pH of the reaction. Substrate coupled cofactor regeneration is a promising method in bioreduction without any need to add external enzymes. Alcohol dehydrogenase-catalyzed oxidation of isopropanol is a good alternative for cofactor regeneration because of the higher atom economy (74.2%) in asymmetric reduction of COBE and no requirement for pH control.[1a,7] Some sugar alcohols were also used as cosubstrates for substrate coupled cofactor regeneration in the bioreduction of COBE.[8] In our laboratory, an NADH-dependent carbonyl reductase (ScCR) was identified from *Streptomyces coelicolor* with high activity and stereoselectivity toward the asymmetric reduction of COBE. It also showed high activity for isopropanol dehydrogenation so that the in situ regeneration of the cofactor NADH could be realized by a simple method called "substrate-coupled cofactor regeneration".[9] In this work, a pilot scale reaction was performed in a 50 L thermostatted stirred-tank-reactor (STR)

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for the production of (*S*)-CHBE using *E. coli* cells expressing recombinant ScCR as the biocatalyst for both COBE reduction and isopropanol oxidation after optimization of the reaction parameters.

RESULTS AND DISCUSSION

Determination of the Optimum Reaction pH. COBE and (*S*)-CHBE were not very stable in the high pH environment because of the presence of an active ester bond.[10] The stabilities of COBE and (*S*)-CHBE were investigated from pH 6.0 to 8.0. As shown in Table 1, the decomposition of COBE happened spontaneously at pH 6−8. The product (*S*)-CHBE was relatively stable and did not undergo any detectable hydrolysis at the slightly acidic pH values of 6.0−6.5. However, when the pH was increased to 7.0 or above, (*S*)-CHBE underwent hydrolysis. Therefore, to avoid the decomposition of product, the ideal reaction pH should be lower than 7.0. The maximum activity of ScCR was observed at pH 6.5, which was then chosen as the reaction pH.

Table 1. Spontaneous decomposition of COBE and (*S*)-CHBE under different pH conditions[a]

	spontaneous decomposition (mmol/L·h)	
pH	COBE	(*S*)-CHBE
6.0	4.1	N.D.
6.5	6.7	N.D.
7.0	23.2	5.3
8.0	98.8	19.8

[a]The reactions were performed by mixing 2 g of COBE or (*S*)-CHBE with 10 mL of a buffer at the indicated pH values. A constant pH was maintained by the addition of 1 M NaOH, and the spontaneous decompositions of COBE and (*S*)-CHBE were calculated according to the consumption of NaOH. N.D.: not detected.

Optimization of Reaction Parameters. The reaction optimization was performed in a toluene-buffer biphasic system using whole cells of *E. coli* expressing recombinant ScCR as the biocatalyst. Although as high as 600 g/L of COBE (in the organic phase) could be reduced as we reported previously,[9] a high load of the biocatalyst (20 g/L dry cells in the aqueous phase) was required, resulting in obvious emulsification, which is not favored for industrial production because of complicated downstream processing. Therefore, to simplify the product extraction, the substrate load was decreased and fixed at 100 g/L, and the concentration of externally supplemented cofactor NAD^+ was fixed at 0.1 mM. The reaction results are summarized in Table 2.

1. Biocatalyst Loading. Biocatalyst loading is a key parameter that must be optimized for biotransformation processes. As shown in entries 1 and 2 of Table 2, the conversion of substrate reached 97.9% when 20 kU/L of ScCR (in the aqueous phase, ca. 3.73 g/L of lyophilized cells) was employed. When the enzyme load was increased to 40 kU/L, the substrate COBE was completely converted. However, taking the production cost into consideration, and to avoid emulsification caused by protein and cell overloading, 20 kU/L of biocatalyst was chosen as the dosage for further investigation.

2. Agitation Rate. The reduction of COBE catalyzed by recombinant ScCR was performed in a toluene−water biphasic system. The agitation rate had a significant effect on the mass transfer. The stability of the reductase decreased when the propeller tip speed was too high.[11] As shown in Table 2

Table 2. Asymmetric reduction of COBE at various reaction parameters

entry	ScCR load[a] (kU/L)	temp. (°C)	*i*-PrOH/COBE (mol/mol)	agitation rate (rpm)	tol./aq.[b] (v/v)	conv. (%)
1	40	30	1.5	200	5:5	100
2	20	30	1.5	200	5:5	97.9
3	20	30	1.05	200	5:5	91.1
4	20	30	1.05	300	5:5	92.5
5	20	25	1.05	200	5:5	97.9
6	20	25	1.05	200	1:5	86.1
7	20	25	1.5	200	5:5	99.1

[a]Lyophilized cells of recombinant *E. coli* producing ScCR were used as the biocatalyst (cells were loaded in the aqueous phase). [b]Tol./aq.: volume ratio of toluene to aqueous phase.

(entries 3 and 4), when the agitation rate was increased from 200 to 300 rpm, the final conversion was not obviously increased, indicating that in this reaction system 200 rpm was sufficient to achieve phase mixing. At this agitation rate, the tip speed was 0.88 m/s, which was set as the maximal linear rate in further studies.

3. Reaction Temperature. Reaction temperature is a very important factor in bioreduction. Although the reaction rate can be accelerated by using relatively high temperatures, the poor stability of many reductases results in rapid enzyme inactivation, leading to earlier termination of the reaction and relatively low conversion. Therefore, an appropriate decrease of the reaction temperature can improve the stability of the enzyme and increase the product yield.[12]

As shown in Figure 1, when the reaction was performed at 25 °C, although the reaction rate was relatively slow as compared with that at 30 °C, the final conversion at 25 °C reached >99%, higher than the 97.9% conversion obtained at 30 °C. As shown in Figure 2, the residual activities of ScCR in the aqueous phase increased during the early stage, peaked at about 6 h, and then decreased gradually. The activity increase may be due to the disruption of cells and release of the intracellular ScCR. When the reaction was performed at 25 °C, deactivation of the recombinant ScCR was relatively slow. Although its activity decreased quickly during the first 10 h, it was stabilized in the later stage of the reaction when very little COBE was remaining. After 23 h reaction at 25 °C, the residual activity was still higher than 70%. However, when the reaction was performed at 30 °C, the ScCR was deactivated, with only 21% residual activity after 10 h, and no activity was detected at 23 h.

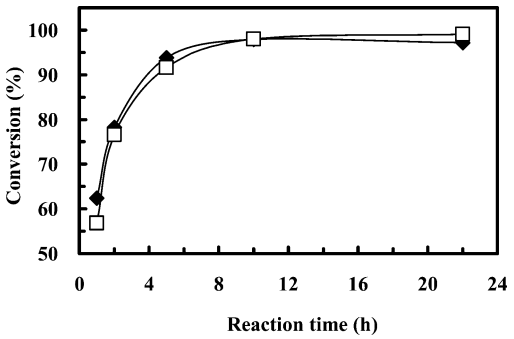

Figure 1. Progress curves of COBE bioreduction at 25 °C (squares) and 30 °C (diamonds).

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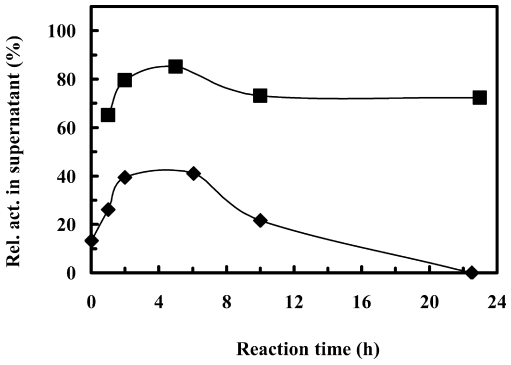

Figure 2. Activities of ScCR detected in the aqueous phase during the bioreductive reaction performed at 25 °C (squares) and 30 °C (diamonds). The initial ScCR activity of the lyophilized cells was defined as 100%.

Therefore, 25 °C was chosen as the optimal reaction temperature.

4. Phase Ratio. An organic−aqueous biphasic system was selected for the bioreduction of COBE because of the poor solubility and spontaneous hydrolysis of COBE in water. In addition, toluene was successfully employed as an organic solvent for this system previously.[9,13] The phase ratio is an important parameter to be carefully considered in a biphasic reaction system.[14] As shown in entries 5 and 6 of Table 2, when the volume ratio of toluene to water was decreased from 5:5 to 1:5, the conversion significantly decreased from 98% to 86%. Obviously, a decrease in the proportion of the organic phase to aqueous phase was not favorable for the bioconversion, and thus, the phase ratio of 5:5 was adopted.

5. Isopropanol Concentration. Cofactor regeneration is an important parameter for bioreduction reactions. In addition to its ability to catalyze the reduction of COBE, recombinant ScCR can also catalyze the oxidation of isopropanol, thus enabling the simple in situ regeneration of the cofactor NADH from NAD^+. However, because the reaction is reversible, excess isopropanol was necessary to drive the reaction forward. As shown in entries 5 and 7 of Table 2, when a relatively high molar ratio of isopropanol (1.5 equiv to COBE) was employed, a nearly complete (>99%) conversion could be achieved at 25 °C, which simplifies the product isolation. The total turnover number (TTN) of NADH was calculated to be 6006.

Product Extraction. Because of the high polarity of (*S*)-CHBE, it has a high partition coefficient in water. At the end of the reaction, there was about 22% of the product (*S*)-CHBE remaining in the aqueous phase, and thus, extraction of (*S*)-CHBE from the aqueous phase was necessary. Three solvents, toluene, ethyl acetate, and butyl acetate, were investigated as extractants. As listed in Table 3, greater than 99% extraction yields were obtained after extracting twice with one-half volume of ethyl acetate or butyl acetate. When toluene was used as the extractant, three equal volume extractions were needed in order to obtain the same result.

Although the extraction efficiency of ethyl acetate was the highest among the solvents tested, its high water solubility (8.1% w/v, 25 °C) suggests that it is not a good extractant. Although butyl acetate had a high extraction efficiency and low water solubility (0.7% w/v, 20 °C), the use of butyl acetate would result in the formation of a binary mixture of solvents due to the presence of residual toluene in the aqueous phase of the biphasic reaction system, therefore increasing the cost of solvent recovery and separation. Thus, toluene was chosen as the most suitable solvent for the reaction and extraction because of its low water solubility (0.053% w/v, 25 °C), moderate boiling point (111 °C), and easy recovery (by vacuum distillation) after reaction or extraction.

Table 3. Comparison of the efficiency of (*S*)-CHBE extraction from the aqueous phase in the presence of different solvents

	extraction once		extraction twice	extraction triple
	extraction yield[a] (%)	partition coeff.[b] ($P_{o/w}$)	extraction yield[a] (%)	extraction yield[a] (%)
reaction toluene	78.6	3.7		
toluene (equal volume)	93.0	2.0	97.5	99.3
ethyl acetate (half volume)	96.4	6.4	99.6	
butyl acetate (half volume)	95.6	5.7	99.5	

[a]Defined as the ratio of the total (*S*)-CHBE extracted to the total (*S*)-CHBE produced. [b]Defined as the ratio of (*S*)-CHBE concentration in organic solvent to that in aqueous solution.

Scale-up. Production of recombinant ScCR by *E. coli* BL21/pET28a-ScCR was performed in a 30 L fermentor. The highest titer reached 60 kU/L, with a specific activity of 849 U/g wet cells (ca. 4.25 kU/g DCW). A pilot scale reaction for production of (*S*)-CHBE was performed in a 50 L thermostated stirred tank reactor using the optimized reaction parameters and fresh *E. coli* cells containing the recombinant ScCR. During the reaction process, the monitored pH was found to be stable with no decrease, implying that no spontaneous hydrolysis of COBE or (*S*)-CHBE occurred. When the reaction was terminated, the aqueous phase was separated and extracted with the same volume of toluene three times. The resultant organic solutions from either the reaction or extraction were combined and evaporated under vacuum. As the conversion was higher than 99%, the product (*S*)-CHBE was obtained by simple distillation under vacuum, without any protein-related impurities detected in the distilled product. In total, 3.456 kg of (*S*)-CHBE was obtained with a purity of 98.4% without future purification. The optical purity was >99.9% ee, and the specific rotation of neat product was calculated as −14.68°. The details are illustrated in Scheme 2. The quality of the resultant (*S*)-CHBE meets the required standards of (*S*)-CHBE (>98% GC purity and >99.5% ee)[4] and should be adequate for the preparation of atorvastatin. The isolation yield of the product was 85.4% with a specific production of approximately 36.8 g/g_{dcw}, which is, to the best of

Scheme 2. Asymmetric reduction of COBE catalyzed by recombinant ScCR-producing *E. coli* cells, with isopropanol-coupled NADH regeneration for preparation of (*S*)-CHBE

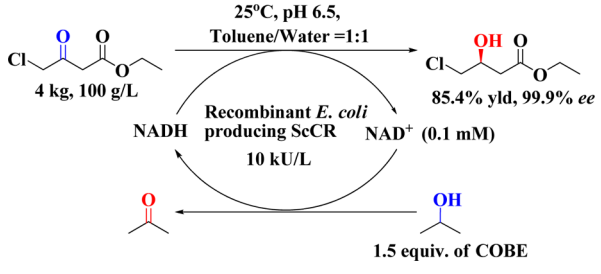

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Table 4. Summary of the reaction parameters for various bioprocesses reported for (*S*)-CHBE production[a]

entry	enzyme resource	cosubstrate	subs./prod. (g/L)	rxn. time (h)	TTN[e]	S.T.Y.[f] (g/L·d)	spec. prodn.[g] (g/g dcw)	lit.
1	*L. kefir*	*i*-PrOH	200[b]	14		363	14.3	1a
2	*C. parapsilosis*	glucose	230[b]	12		460	1.15	1e
3	*C. magnoliae* + GDH	glucose	208[b]	34	21 600	146		5a
4	*C. magnoliae* + GDH	glucose	430[c]	34	16 200	151		5a
5	*P. Stipitis* + GDH	glucose	233[c]	24	13 980	>116	4.66	5b
6	*S. coelicolor*	*i*-PrOH	(600)[c]	22	12 100	307	28.2	7
7	KRED + GDH	glucose	(160)[d]	8	11 780	480	178 g/g enzyme	4
8	*S. coelicolor*	*i*-PrOH	(100)[d]	24	6060	86.4	36.8	this work

[a]GDH: glucose dehydrogenase. [b]In an aqueous monophase system. [c]In an organic−aqueous biphasic system. Substrate loading and product concentration in the organic phase are listed. [d]In an organic−aqueous biphasic system. Substrate loading for the whole system is listed. [e]TTN: total turnover number of cofactor. [f]S.T.Y.: space-time yield. [g]dcw: dry-cell weight.

our knowledge, the highest among the known whole-cell-mediated processes for production of (*S*)-CHBE (Table 4). The space-time yield was calculated as 86.4 $g/L_{total}·d$.

(*S*)-CHBE: 98.4% purity, >99.9% ee, $[\alpha]_{neat}^{25}$ −14.68°, ^1H NMR (400 MHz, $CDCl_3$), δ/ppm: 1.29 (t, 3H), 2.65 (d, 2H), 3.60 (d, 2H), 4.19 (q, 2H), 4.27 (m, 1H).

The Green Features of This Bioprocess. Compared with the cofactor regeneration system using glucose as the cosubstrate, the bioreduction process using isopropanol as the cosubstrate is greener, with an increase in atom economy from 43.3% to 74.2%. In this work, the substrate COBE was completely converted, and the product was refined by a simple extraction/distillation process to afford product with high purity (≥98.4%). The reaction and extraction solvent (toluene) was recycled with a loss of only 4.1%. The *E* factor (kg waste per kg product) for the process was determined as 1.8 if the process water was excluded (Table 5), which was much lower than that (2.3) obtained from the process using isolated ketoreductase, glucose dehydrogenase as the biocatalyst for cofactor regeneration, and glucose as the cosubstrate.[4] The main contributors to the low *E* factor were the loss of the solvent toluene (46.1%), the use of excessive isopropanol, and the formation of coproduct acetone (combined ca. 35%). If water was also included, then the *E* factor would be 13.4.

Table 5. Consumption of raw materials in the bioproduction of (*S*)-CHBE

raw material	quantity [kg per kg (*S*)-CHBE]
COBE	1.16
toluene	0.83
i-PrOH	0.63
whole cells (wet)	0.14
NAD^+	0.00077
$MgSO_4$	0.0028
$K_2HPO_4·3H_2O$	0.0087
KH_2PO_4	0.011
water	11.6
E factor[a] (excluding water)	1.78
E factor (including water)	13.4

[a]The *E* factor was calculated as the mass of the total raw materials/mass of (*S*)-CHBE − 1.

EXPERIMENTAL SECTION

COBE was provided by Nantong Chengxin Amino Acid Co., Ltd. (Jiangsu Province, China). Peptone was obtained from Zhejiang Huzhou Huihe Biotechnology Co. (Zhejiang Province, China), and yeast extract was the product of Anqi Yeast Co. (Hubei Province, China). All other reagents were obtained commercially and were of the highest purity available.

Microorganism and Fermentation. *Escherichia coli* BL21/pET28a-ScCR expressing ScCR was constructed in our laboratory[9] and preserved at −80 °C in 25% (w/v) glycerol. The production of recombinant ScCR was performed in a 30 L fermenter. For seed cultivation, the preserved strain was inoculated into 3 mL of LB medium containing 50 μg/mL of kanamycin and cultured at 37 °C for 2 h, then transferred into 100 mL of LB medium containing 50 μg/mL of kanamycin. When the OD_{600} of the seed culture reached 0.6 at 37 °C, 1.0 L of culture was inoculated into a 30 L fermenter containing 20 L of fermentation medium (5 g of peptone, 5 g of yeast extract, 5 g of glycerol, 9 g of Na_2HPO_4, 3.4 g of K_2HPO_4, 0.7 g of Na_2SO_4, 0.25 g of $MgSO_4$, and 2.7 g of NH_4Cl per liter of tap water at a pH of 7.0). The fermentation was initiated at 37 °C, and then the dissolved oxygen was maintained higher than 20% by adjusting the agitation rate. When the dissolved oxygen significantly increased, a mixture of 250 g/L glycerol, 60 g/L yeast extract, and 60 g/L peptone was fed to the reactor. When the OD_{600} increased higher than 6.0, the temperature was decreased to 25 °C, and isopropylthio-β-*D*-galactoside (IPTG) was added to a final concentration of 0.2 mM. The activities of ScCR were determined periodically, and the fermentation was terminated when no significant increase of activity was observed. After centrifugation and washing with 0.9% (w/v) NaCl, the obtained cells was preserved at 4 °C or lyophilized for further use.

Activity Assay. An appropriate amount of cells was suspended in 5 mL of potassium phosphate buffer (KPB, 100 mM, pH 6.5) and disrupted using an ultrasonic oscillator. After centrifugation at 10 000 × *g*, 10 μL of supernatant was added into 970 μL of KPB (100 mM, pH 6.5) and incubated at 30 °C for 3 min. The reaction was started by the addition of 10 μL of NADH solution (10 mM) and 10 μL of COBE solution (200 mM, in dimethyl sulfoxide), and the change of absorbance at 340 nm was recorded for purposes of determining enzyme activity. One unit of enzyme activity was defined as the amount of enzyme that catalyzes the oxidation of 1.0 μmol NADH per minute under the described conditions.

Optimization of Reaction Parameters. The reactions were performed in a 500 mL three-necked flask. The reaction mixture comprised 150 mL of KPB (20 mM, pH 6.5), 30 g of COBE, 20 mg of NAD^+ (30 μmol), 150 mg of $MgSO_4·7H_2O$, and a predetermined amount of toluene, isopropanol, and lyophilized whole cells. The reactions were carried out at constant temperature with mechanic agitation. Samples were

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taken periodically and centrifuged, and the isolated organic phase was dried over anhydrous Na_2SO_4 and then assayed by gas chromatography using a CP-Chirasil-DEX CB column (Varian, USA).

Pilot Scale Reaction. The pilot reaction was performed in a 50 L thermostatted glass reactor containing 20 L of KPB (20 mM, pH 6.5), 20 L of toluene, 4.0 kg of COBE, 2.188 kg of isopropanol, 2.65 g of NAD^+, 9.6 g of $MgSO_4$, and 400 kU (0.47 kg) of wet cells at 25 °C with an agitation rate of 120 rpm for 24 h. When the reaction was terminated, the two phases were separated. The aqueous phase was heated to 60 °C for 30 min, and after centrifugation, the supernatant was extracted three times with the same volume of toluene. All of the resultant organic phases were combined and concentrated under vacuum. The crude product was refined by distillation under a reduced pressure, and the product was collected within a boiling point range of 84−90 °C under about 200 Pa, to yield 3.456 kg of (*S*)-CHBE.

■ CONCLUSIONS

Asymmetric bioreduction of COBE by recombinant *E. coli* whole cells expressing carbonyl reductase ScCR was investigated, and the main reaction parameters were optimized. A pilot scale reaction for the reduction of 4.0 kg of COBE was performed in a 50 L thermostated stirred tank reactor using the fresh wet cells of recombinant *E. coli* as a catalyst to provide enantiopure (*S*)-CHBE, an important chiral precursor of atorvastatin, in an isolated yield of 85.4% with >98% ee. The specific production of the bioprocess was calculated to be as high as 36.8 g/g dcw, which is the highest value known among the whole-cell-catalyzed production of (*S*)-CHBE reported thus far. The *E* factor of this bioprocess was calculated as only 1.78, lower than that of the production process reported by Codexis Inc. Moreover, this green-by-design process would be a good candidate for the manufacture of the key intermediate of atorvastatin.

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Notes

The authors declare no competing financial interest.

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