ChemPubSoc Europe

DOI: 10.1002/cctc.201500061

CHEMCATCHEM Full Papers

Efficient Biosynthesis of Ethyl (*R*)-3-Hydroxyglutarate through a One-Pot Bienzymatic Cascade of Halohydrin Dehalogenase and Nitrilase

Peiyuan Yao,[a] Lei Wang,[a, b] Jing Yuan,[a] Lihua Cheng,[c] Rongrong Jia,[c] Meixian Xie,[c] Jinhui Feng,[a] Min Wang,[b] Qiaqing Wu,*[a] and Dunming Zhu*[a]

An effective one-pot bienzymatic synthesis of ethyl (*R*)-3-hydroxyglutarate (EHG) from ethyl (*S*)-4-chloro-3-hydroxybutyrate (ECHB) was achieved by using recombinant *Escherichia coli* cells expressing separately or co-expressing a mutant halohydrin dehalogenase gene from *Agrobacterium radiobacter* AD1 and a nitrilase gene from *Arabidopsis thaliana*. The activity of nitrilase was inhibited by high concentration of ECHB and NaCN. Consequently, the one-pot one-step process was implemented by fed-batch of ECHB and NaCN with high accumulative product concentration (up to 0.9 mol L^{-1}). The biotransformation of ECHB to EHG was successfully achieved at 1.2 mol L^{-1} substrate concentration by a one-pot two-step process. As such, this one-pot bienzymatic transformation should be useful in synthesizing these important optical pure β-hydroxycarboxylic acids.

Introduction

Cascade reactions avoid time-consuming and yield-reducing isolation and purification of intermediates.[1] Compared to chemical reactions, biotransformations are more suitable for one-pot cascades because of the similar reaction conditions, and more attractive for nontoxicity and high chemo-, regio-, and enantioselectivity. A large number of important and versatile enzymes have been employed in multienzymatic cascade routes.[2] For example, biocatalytic cascade reactions have been developed for the synthesis of enantiopure epoxides, β-azidoalcohols or β-hydroxynitriles from prochiral α-haloketones by using alcohol dehydrogenase and halohydrin dehalogenase.[3] Chiral α-hydroxycarboxylic acids have been synthesized from aldehydes through biocatalytic cascade of hydroxynitrile lyase and nitrilase.[4] The asymmetric synthesis of chiral β-hydroxycarboxylic acids have also been reported with carbonyl reductase and nitrilase.[5] Therefore, cascade biotransformation offers a very appealing methodology for the synthesis of valuable organic compounds.

Ethyl (*R*)-3-hydroxyglutarate (EHG) is a key intermediate for the synthesis of rosuvastatin.[6] Recently, we reported an efficient enzymatic method for the synthesis of EHG from ethyl (*R*)-4-cyano-3-hydroxybutyate (HN) by using a nitrilase from *Arabidopsis thaliana* (AtNIT2).[7] Halohydrin dehalogenase (HHDH) can convert halohydrin to the corresponding epoxide followed by the epoxide ring opening reaction in the presence of nucleophiles.[8] Additionally, HN could be synthesized from ethyl (*S*)-4-chloro-3-hydroxybutyrate (ECHB) through the formation and ring-opening of the epoxide intermediate catalyzed by HHDH.[3e,9] Commercially available ECHB could be synthesized from (*S*)-epichlorohydrin or ethyl 4-chloroacetoacetate.[3e,10] Therefore, we envisioned that a reaction cascade with both HHDH and nitrilase would offer a highly efficient bioprocess for the synthesis of EHG from the readily available ECHB. As such, a novel bioconversion system, which involves a three-reaction, two-enzyme, one-pot process starting from ECHB to EHG (Scheme 1), was established by using recombinant *Escherichia coli* expressing separately or co-expressing genes of a HHDH and a nitrilase as the biocatalysts. The fed-batch (semi-batch operation) of EHCB and NaCN was investigated to achieve the high accumulative product concentration.

Results

A mutant halohydrin dehalogenase (Hhe) obtained by statistical analysis of protein sequence activity relationships

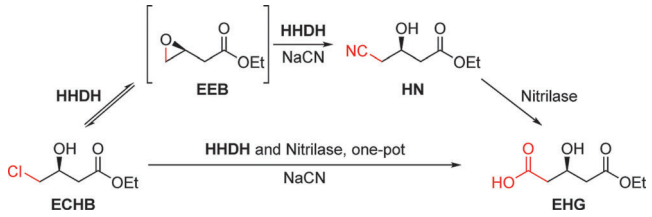
[a] Dr. P. Yao,[+] L. Wang,[+] J. Yuan, Prof. J. Feng, Prof. Q. Wu, Prof. D. Zhu
National Engineering Laboratory for Industrial Enzymes
Tianjin Engineering Center for Biocatalytic Technology
Tianjin Institute of Industrial Biotechnology
Chinese Academy of Sciences
32 Xi Qi Dao, Tianjin Airport Economic Area
Tianjin 300308 (P.R. China)
Fax: (+86) 22-24828703
E-mail: wu_qq@tib.cas.cn
zhu_dm@tib.cas.cn

[b] L. Wang,[+] Prof. M. Wang
Key Laboratory of Industrial Fermentation Microbiology
Ministry of Education, College of Biotechnology
Tianjin University of Science & Technology
No. 29, 13th. Avenue
Tianjin Economic and Technological Development Area
Tianjin 300457 (P.R. China)

[c] L. Cheng, R. Jia, M. Xie
Hebei Chengxin CO., LTD
South of Yuanzhao Rd., Yuanshi County
Shijiazhuang city, Heibei province 051130 (P. R. China)

[+] These authors contributed equally to this work.

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/cctc.201500061.



Scheme 1. Access to EHG from ECHB by using a one-pot bienzymatic cascade of HHDH and nitrilase. EEB = Ethyl (*S*)-3,4-epoxybutanoate.

(ProSAR),[11] was produced by overexpressing the gene in *E. coli* BL21(DE3) (Figure S1 in the Supporting Information). The recombinant whole cells were used as the catalyst for the transformation of ECHB to HN with NaCN as the base and source of CN^-. With 10 wt% wet cell loading, ECHB was completely converted to HN in 12 h at the substrate concentration up to 500 mmol L^{-1} (Table S1 in the Supporting Information). To avoid the strong alkaline condition and yellow precipitant at high CN^- concentration, a 30% NaCN solution was automatically added into the reaction mixture to control the pH in the range of 8.0–9.0, and the reaction was performed at different substrate concentrations. As shown in Figure S5, ECHB at a concentration of 1.2 mol L^{-1} was completely converted to HN within 3 h (>99% conversion) with same wet cell loading, and the conversion reached 98% at the substrate concentration of 1.5 mol L^{-1}. The results revealed that the cyanization of ECHB catalyzed by Hhe was rapid and highly efficient.

In our previous report, the *E. coli* BL21(DE3) whole cells expressing a nitrilase gene from *A. thaliana* (AtNIT2) effectively catalyzed the biotransformation of HN to EHG, and the substrate at the concentration of 1.5 mol L^{-1} could be completely converted to EHG within 4.5 h with 6.0 wt% wet-cell loading of the biocatalyst.[7] Therefore, it would be very interesting to develop a highly efficient bienzymatic cascade process for the synthesis of EHG from ECHB by combining these two effective biotransformations. As such, the plasmid pETDuet-AtNIT2–Hhe was constructed and transferred into *E. coli* BL21 (DE3) to simultaneously express the genes encoding Hhe and AtNIT2 (Figure S3). SDS PAGE suggested that both Hhe and AtNIT2 were expressed, but the protein productivity of Hhe in this system was perceptibly better than that of AtNIT2 (Figure S4).

The halohydrin dehalogenase and nitrilase activities of the recombinant whole cells were evaluated (Table S2 in the Supporting Information). The results show that the whole cells co-expressing both genes had comparable halohydrin dehalogenase activity to the cells only expressing Hhe gene, but co-expression obviously reduced the nitrilase activity of the whole cells.

Temperature and pH are important factors that greatly affect the activity and stability of enzymes. Hence the effects of pH and temperature on the Hhe and AtNIT2 activities were investigated by using the *E. coli* cells separately expressing the Hhe and AtNIT2 genes. The optimal pH values for Hhe and AtNIT2 activities were 9.0 and 8.0, respectively (Figure S6 A). The Hhe and AtNIT2 showed the maximum activities at 50 °C and 40 °C, respectively (Figure S6 B in the Supporting Information). The thermal stability studies illustrated that the whole cells retained almost 100% activity of Hhe and AtNIT2 at 30 °C for 6 h. However, the activities declined if they were incubated at 40 °C and 50 °C, especially the activity of AtNIT2 dropped rapidly (Figure S7).

Therefore, the one-pot biotransformation of ECHB to EHG was performed at various initial substrate concentrations by using 10.0 wt% wet-cell loading of pETDuet–Hhe–AtNIT2/*E. coli* BL21 whole cells as the catalyst. The pH of reaction system was maintained at pH 8.0 by adding NaCN as the base and source of CN^-, and the reaction temperature was kept at 30 °C. The results showed that the whole cell catalyst was able to completely convert ECHB at a concentration of 100 mmol L^{-1} to EHG within 60 min (Figure 1 A), and at a concentration of 300 mmol L^{-1} within 100 min (Figure 1 B) without intermediate residue as evidenced by GC analysis. Nevertheless, ECHB at a concentration of 600 mmol L^{-1} was converted to the intermediate HN within 5 h, but only approximately 20% of EHG was formed (Figure S8). The results suggested that the nitrilase activity was inhibited by high concentrations of ECHB.

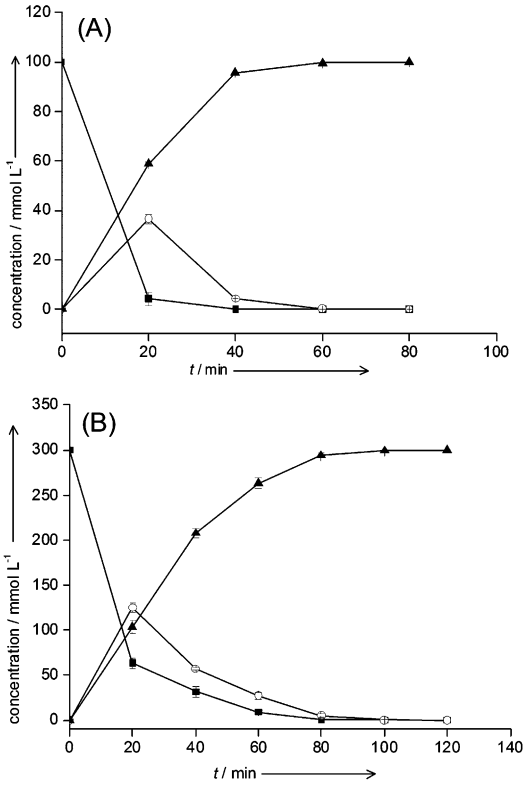


Figure 1. Biotransformation of ECHB to EHG with pETDuet–AtNIT2–Hhe/*E. coli* BL21 whole cells as the biocatalyst by automatically adding 30% NaCN to keep the pH at 8.0. The reaction mixtures contained sodium phosphate buffer (50 mL, 50 mmol L^{-1}, pH 8.0), 10.0 wt% wet-cells loading and the substrate at concentrations of A) 100 mmol L^{-1} and B) 300 mmol L^{-1}. At the indicated time intervals, aliquots (200 μL each) were taken, and 30% H_2O_2 (20 μL) and 6 mol L^{-1} HCl (20 μL) were then added. The concentrations of ECHB (■), HN (○), and EHG (▲) were determined by GC analysis.

As such, the inhibitory effects of ECHB or NaCN on the AtNIT2 activity were investigated. The nitrilase activity of the whole cells of pET32a(+)–AtNIT2/*E. coli* BL21(DE3) was measured at different concentrations of ECHB and NaCN by using the standard activity assay method. The results showed that ECHB had a clear inhibitory effect on the nitrilase activity above a concentration of 300 mmol L^{-1} (Figure 2A), and NaCN inhibited the nitrilase activity at concentrations above 200 mmol L^{-1} (Figure 2B).

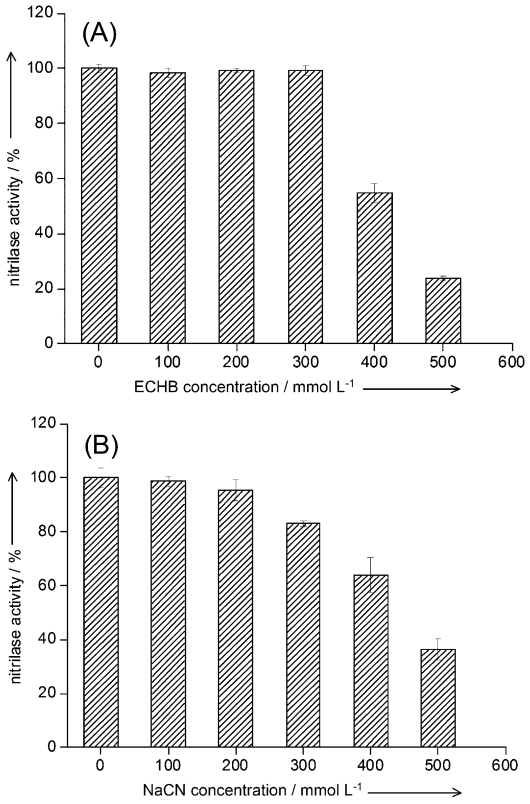


Figure 2. Effects of the concentrations of A) ECHB and B) NaCN on the nitrilase activity of pET32a(+)–AtNIT2/*E. coli* BL21(DE3). The nitrilase activity (in 1 mL volume) was determined according to the standard activity assay method. The reaction mixtures with indicated concentrations of ECHB and NaCN were prepared from a 0.5 mol L^{-1} stock solution in sodium phosphate buffer, and brought to pH 8.0 by the addition of phosphate acid.

To achieve the biotransformation of ECHB to EHG at high substrate concentration, batch feeding of substrate was adopted to avoid the inhibition of ECHB on the AtNIT2 activity. The inhibitory effect of NaCN on the AtNIT2 activity was prevented by automatically adding a 30% NaCN solution to keep the pH at 8.0 by a pH/ORP controller. As such, the substrate concentration of every batch feeding was set to 300 mmol L^{-1}. As the activities of Hhe and AtNIT2 began to decline if the whole cells were incubated for 6 h at 30 °C, the fed-batch of substrate was tested by using 10.0 wt% wet-cell loading of pETDuet-AtNIT2-Hhe/*E. coli* BL21 to shorten the reaction time for allowing multiple batches feeding. It was found that ECHB at a concentration of 600 mmol L^{-1} was completely converted to the final product within 6 h through batch feeding twice (Figure 3).

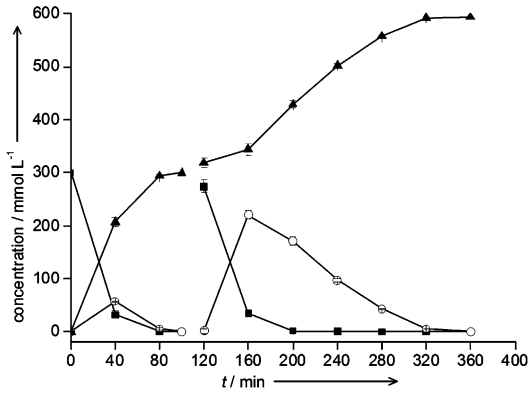


Figure 3. Biotransformation of ECHB to EHG through batch feeding of the substrate using pETDuet–Hhe–AtNIT2/*E. coli* BL21. The reaction mixtures contained sodium phosphate buffer (50 mmol L^{-1}, pH 8.0) and 10.0 wt% wet cells loading in a total volume of 50 mL, substrate concentration of every batch feeding was 300 mmol L^{-1}, and the second batch feeding was added at 100 min, 30% NaCN was automatically added to keep the pH at 8.0. At the indicated time intervals, aliquots (200 μL each) were taken, and 30% H_2O_2 (20 μL) and 6 mol L^{-1} HCl (20 μL) were added to quench and acidify the reaction mixture. The concentrations of ECHB (■), HN (○), and EHG (▲) were determined by GC analysis.

After centrifugation, the reaction was quenched by addition of 30% H_2O_2, the solution was acidified and then extracted with ethyl acetate. Removal of the solvent gave the desired product EHG in 82.7% yield. However, the intermediate HN was not completely converted to the final product upon feeding of a third batch of ECHB. An amount of 5% of the intermediate HN was not converted if the third batch was fed at a substrate concentration of 120 mmol L^{-1} (Figure S9A), and an amount of 35% of HN was left if the third batch was fed at an ECHB concentration of 300 mmol L^{-1} (Figure S9B).

The above results showed that ECHB was completely converted to the intermediate HN, but the hydrolysis of HN to the final product EHG was incomplete, suggesting that the AtNIT2 activity was not high enough to completely hydrolyze HN. Moreover, the AtNIT2 protein productivity in pETDuet-AtNIT2-Hhe/*E. coli* BL21 was relatively low compared to that of Hhe (Figure S3), and the AtNIT2 activity obviously declined in the co-expression whole cells (Table S2). Therefore, the whole cell biocatalysts separately expressing the Hhe and AtNIT2 genes were used and the cell loading was adjusted to have comparable Hhe and AtNIT2 activities. To achieve the complete conversion of substrate to the final product EHG, 6.0 wt% wet-cell loading of pET32a(+)–AtNIT2/*E. coli* BL21(DE3) and 10.0 wt% of pET32a(+)–Hhe/*E. coli* BL21(DE3) were used in the batch-fed reaction, and the other reaction conditions were the same as described above. As shown in Figure 4, 900 mmol L^{-1} of the substrate ECHB was completely converted to the final product EHG in 6 h, and the isolated yield was 84.3%.

The above results showed that the efficiency of the one-pot one-step biotransformation of ECHB to EHG was severely affected by the inhibition of ECHB on the AtNIT2 activity. To eliminate this inhibitory effect, the biotransformation of ECHB to EHG was performed in a bienzymatic two-step mode by using the whole cells separately expressing the Hhe and

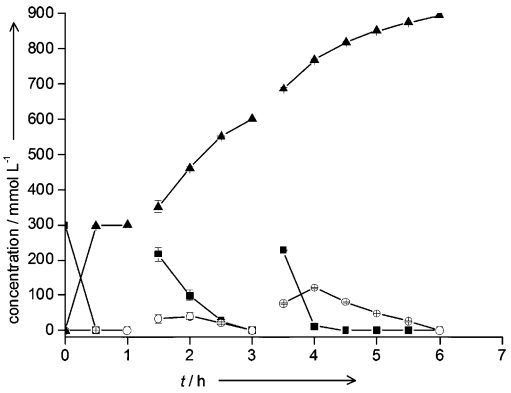


Figure 4. Biotransformation of ECHB to EHG through batch feeding of the substrate using pET32a(+)–AtNIT2/*E. coli* BL21(DE3) and pET32a(+)–Hhe/*E. coli* BL21(DE3). The reaction mixtures contained sodium phosphate buffer (50 mmol L^{-1}, pH 8.0) and 16.0 wt% wet-cells loading in a total volume of 50 mL, substrate concentration of every batch feeding was 300 mmol L^{-1}, 30% NaCN was automatically added to keep the pH at 8.0. At the indicated time intervals, aliquots (200 μL each) were taken, then 30% H_2O_2 (20 μL) and 6 mol L^{-1} HCl (20 μL) were added. The concentrations of ECHB (■), HN (○), and EHG (▲) were determined by GC analysis.

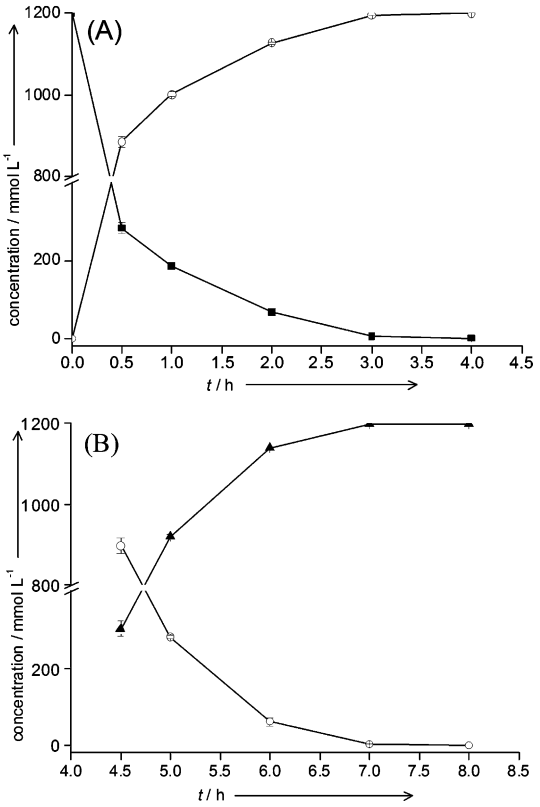


Figure 5. Bienzymatic two-step biotransformation of ECHB to EHG. The reaction mixture contained Tris–H_2SO_4 buffer (50 mmol L^{-1}, pH 9.0), 10.0 wt% pET32a(+)–Hhe/*E. coli* BL21(DE3) wet-cells loading in a total volume of 50 mL. A) The initial substrate concentration was 1.2 mol L^{-1}, 30% NaCN was automatically added to keep the pH at approximately 8.0–9.0. B) After the substrate was converted to HN, the pH of the reaction system was adjusted to 8.0 by using 1 mol L^{-1} H_2SO_4, and 6.0 wt% pET32a(+)–AtNIT2/*E. coli* BL21(DE3) wet cells were added. At the indicated time intervals, aliquots (200 μL each) were taken, and after quenching, acidification, extraction with ethyl acetate, the concentrations of ECHB (■), HN (○), and EHG (▲) were determined by GC analysis.

AtNIT2 genes. It was found that ECHB at a concentration of 1.2 mol L^{-1} was completely converted to HN within 4 h in the first step (Figure 5A). After the conversion of ECHB to HN was complete, the pH of the reaction system was adjusted to 8.0 and 6.0 wt% AtNIT2 wet cells were added to hydrolyze the intermediate HN. As shown in Figure 5B, HN was completely hydrolyzed to the desired product EHG within 4 h, which was isolated in 86.7% yield.

Discussion

A single vector system for co-expression is easy to manipulate the expression strains and ensures that the component proteins are in the same host cell. In contrast, the multiple vector strategies in *E. coli* may require the vectors compatible for expression of the component genes and double or triple selection with antibiotics, which may affect cell viability.[12] Thus we used the commercial vector pETDuet-1 from Novagen for co-expression of halohydrin dehalogenase and nitrilase genes. The results demonstrated that it was possible to co-express a bacterial halohydrin dehalogense and a plant-derived nitrilase in *E. coli* and that the recombinant pETDuet–ATNIT2–Hhe/*E. coli* BL21 whole cells were able to implement the biosynthesis of EHG from ECHB. The comparably lower expression of the nitrilase gene might have been caused by problems in the transcription/translation of the plant-derived nitrilase gene.

HHDH-catalyzed dehalogenation of vicinal halohydrins proceeds under alkaline conditions (pH > 7), and the reaction releases the H^+ and Cl^-, leading to a decrease of the pH of the reaction system.[8b] Cyanide is usually added in excess to drive the equilibrium to complete conversion in HHDH-catalyzed dehalogenation through ring opening by the cyanide. Nevertheless, with the increase of substrate concentration, the addition of increasing amount of NaCN results in a significant alkalization of the reaction media, which probably causes the loss of the enzyme activity and the incomplete conversion of the substrate. Moreover, the buffer solution with high CN^- concentration is not stable (precipitation of a brown solid occurred within hours).[13] The 30% NaCN solution has pH value of approximately 13–14, and the dehalogenation of ECHB results in a decrease of the pH. As such, a 30% NaCN solution was automatically added to keep the pH of the reaction system at 8.0.

The substrate ECHB and intermediate HN are base-sensitive compounds[3e] and may undergo various reactions if they are kept in strong alkaline conditions for a prolonged time, resulting in extensive byproduct formation and low reaction yield. Thus the biosynthesis of EHG from ECHB was performed at an appropriate pH within the shortest time possible.

Multienzymatic biocatalytic processes have been receiving increasing attention in recent years. To the best of our knowledge, there is no example in the literature for the bienzymatic cascade or co-expression of HHDH and nitrilase in one host cell. In the current study, we established a novel one-pot bienzymatic cascade using recombinant *E. coli* whole cells separately expressing or co-expressing the HHDH and nitrilase genes as

the biocatalyst for the efficient synthesis of EHG from ECHB and sodium cyanide. Furthermore, this bienzymatic process should find a wide range of possible applications because both enzymes are able to convert a wide range of aromatic and aliphatic substrates.

The inhibition of the ATNIT2 activity by ECHB limited the one-pot one-step biotransformation of ECHB to EHG at higher substrate concentration. Although this inhibitory effect was minimized by batch feeding of ECHB, further work will focus on protein engineering to obtain mutant ATNIT2 the activity of which is not inhibited by high concentrations of ECHB, and the immobilization of the whole cells or enzymes of halohydrin dehalogense and nitrilase to enhance the substrate tolerance, stability, and reusability.

Conclusions

An efficient recombinant whole-cell catalyst combining halohydrin dehalogenase (HHDH) and nitrilase was successfully developed by separately expressing or co-expressing these two genes in *Escherichia coli* cells, and applied to achieve the biosynthesis of ethyl (*R*)-3-hydroxyglutarate (EHG), a key intermediate for the synthesis of rosuvastatin.[6] High concentration of ethyl (*S*)-4-chloro-3-hydroxybutyrate (ECHB) and NaCN showed inhibitory effect on the nitrilase activity of the recombinant *E. coli* cells. Fed-batch addition of 30% NaCN solution into the reaction system was adopted to minimize the inhibitory effect and to control the pH of the reaction mixture. Batch feeding of ECHB at a concentration of 300 mmol L^{-1} reduced its inhibition on the nitrilase activity and 0.9 mol L^{-1} accumulative product concentration was obtained in the one-pot one-step process. The one-pot two-step process with the *E. coli* cells separately expressing these two genes as the biocatalyst prevented the inhibitory effect of ECHB and resulted in the complete transformation of ECHB at a concentration of 1.2 mol L^{-1} to EHG without residual intermediate ethyl (*R*)-4-cyano-3-hydroxybutyate. These results suggest that this bienzymatic cascade of HHDH and nitrilase should be a very promising approach for the synthesis of EHG and its β-hydroxycarboxylic acid analogs.

Experimental Section

Materials

The ECHB and HN were purchased from Hubei Tuochukangyuan Pharm & Chem. Ethyl (*S*)-3,4-epoxybutanoate (EEB) was purchased from Nanjing Chemlin Chemical Industry Co., Ltd. NaCN was supplied by Hebei Chengxin CO., Ltd. Tris(hydroxymethyl)-aminomethane (Tris) was purchased from Affymetrix, Inc. Difco™ LB Broth, Miller (Luria-Bertani) was purchased from Becton Dickinson and Company. Ampicilin was purchased from Beijing Probe Bioscience Co., Ltd. Isopropyl β-D-1-thiogalactopyranoside was purchased from AMRESCO Inc. All other chemicals were purchased from Sinopharm Chemical Reagent Co., Ltd. The restriction enzymes and other reagents for molecular biology were supplied by Fermentas (Germany) and TaKaRa (Japan). The GC analysis was performed on an Agilent 7890A GC system. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker Avance III 400 MHz NMR spectrometer. High-resolution MS was recorded on a Bruker micrOTOF-QII.

Analytical methods

The EEB, ECHB, HN, and EHG were analyzed by GC analysis with an Agilent 19091 J-413 HP-5 5% phenyl methyl siloxane column (30 m × 0.32 mm × 0.25 μm). The injector and detector temperatures were set at 220 °C and the oven temperature was programmed as follows: hold at 80 °C for 3 min, 20 °C min^{-1} to 150 °C, hold at 150 °C for 2 min, 20 °C min^{-1} to 220 °C, hold at 220 °C for 3 min. The split ratio was set to 1:20. 1 μL aliquots of each sample were injected. Under these conditions, retention times for EEB, ECHB, HN, and EHG were 2.2, 4.3, 5.6 and 6.7 min, respectively.

Genes and plasmids

Halohydrin dehalogenase (Hhe) gene from *Agrobacterium radiobacter* AD1 (GenBank accession number GP571591.1) and nitrilase (AtNIT2) gene from *Arabidopsis thaliana* (GenBank accession number CAA68934.3) were synthesized by Shanghai Xuguan Biotechnological Development Co., Ltd. (China). Plasmid pET32a(+) was used to separately express the halohydrin dehalogenase and nitrilase genes, and the vector pETDuet-1 was used for co-expression of these genes.

Bacterial strains and culture conditions

The plasmids constructed in the above section were transformed into *E. coli* BL21(DE3) cells. The resultant strains were routinely cultured in Luria–Bertani medium (containing 100 μg mL^{-1} ampicillin) at 37 °C, and induced by adding of 0.1 mmol L^{-1} isopropyl β-D-1-thiogalactopyranoside for approximately 6–8 h at 30 °C or approximately 10–12 h at 25 °C until the optical density at 600 nm (OD_{600}) was 0.6–0.8. The cells were harvested by centrifugation, washed once with sodium phosphate buffer (50 mmol L^{-1}, pH 8.0) and cryopreserved at −20 °C without loss of activity within 2 weeks.

Enzyme assays

The Hhe activity was determined in reaction mixtures (1.0 mL) containing 50 mmol L^{-1} sodium phosphate buffer (pH 8.0), 50 mmol L^{-1} ECHB and a suitable amount of wet cells. The nitrilase activity was determined in reaction mixtures (1.0 mL) containing 50 mmol L^{-1} sodium phosphate buffer (pH 8.0), 50 mmol L^{-1} HN and an appropriate amount of wet cells. The reaction mixtures were incubated at 30 °C for 10 min. Aliquots (200 μL each) were taken, 30% H_2O_2 (20 μL) and 6 mol L^{-1} HCl (20 μL) were added to quench and acidify the reaction mixture. The samples were extracted with ethyl acetate. The supernatants were analyzed by GC analysis after drying over sodium sulfate. One unit of enzyme activity was defined as the amount of enzyme that converted 1 μmol of substrate per min.

Effects of pH and temperature on Hhe and AtNIT2 activity

The Hhe and AtNIT2 activity of recombinant *E. coli* strain were investigated under different conditions according to the standard enzyme activity assay. For the investigation of the effects of pH on the enzyme activity, Na_2HPO_4–NaH_2PO_4 (pH 6.0–8.0), Tris–H_2SO_4 (pH 8.0–9.0), glycine–NaOH (pH 9.0–10.0) were used. To investigate the effect of reaction temperature on the enzyme activity, the reac-

tion was performed in the temperature range of 20–80 °C with 10 °C intervals.

One-pot biosynthesis of EHG by batch feeding of the substrate

The pETDuet–AtNIT2–Hhe/*E. coli* BL21 wet cells (5.0 g) were added into the solution of ECHB (2.5 g, 15 mmol) in 50 mmol L^{-1} sodium phosphate buffer (47 mL, pH 8.0). A 30% NaCN solution (≈3 mL) was automatically added by means of a pH/ORP controller to keep the pH at 8.0. The reaction was performed in a water bath at 30 °C. After all ECHB was completely converted to EHG as monitored by GC analysis, the second batch of ECHB (2.5 g, 15 mmol) was added to the reaction mixture. The reaction was stirred for another 260 min until no ECHB and HN were detected. The reaction mixture was centrifuged, 30% H_2O_2 was added to remove the residual cyanide, and the solution was acidified with 6 mol L^{-1} HCl to reach pH 1–2. The resulting solution was extracted with ethyl acetate (3×50 mL). The combined organic extracts were dried over anhydrous sodium sulfate, filtered and evaporated in vacuo to afford EHG (4.37 g, 82.7%) as a yellow oil.

The pET32a(+)–AtNIT2/*E. coli* BL21(DE3) wet cells (3.0 g) and the pET32a(+)–Hhe/*E. coli* BL21(DE3) wet cells (5.0 g) were added into the solution of ECHB (2.5 g, 15 mmol) in 50 mmol L^{-1} sodium phosphate buffer (45 mL, pH 8.0). A 30% NaCN solution (≈5 mL) was automatically added by means of a pH/ORP controller to keep the pH at 8.0. The reaction was performed in a water bath at 30 °C. After all ECHB was completely converted to EHG monitored by GC analysis, the second batch of ECHB (2.5 g, 15 mmol) was added. The reaction was stirred for another 120 min until no residual ECHB and HN were detected. The third batch of ECHB (2.5 g, 15 mmol) was added. Then the reaction mixture was stirred for another 180 min until no residual ECHB and HN were detected. The reaction mixture was centrifuged and 30% H_2O_2 was added to the supernatant to remove the residual cyanide, and the solution was acidified with 6 mol L^{-1} HCl to reach pH 1–2. The resulting solution was extracted with ethyl acetate (3×50 mL). The combined organic extracts were dried over anhydrous sodium sulfate, filtered, and evaporated in vacuo to afford EHG (6.68 g, 84.3%) as a yellow oil.

Biosynthesis of EHG through Hhe–AtNIT2 bienzymatic two-step cascade

The pET32a(+)–Hhe/*E. coli* BL21(DE3) wet cells (5.0 g) were added into a solution of ECHB (10.0 g, 60 mmol) in 50 mmol L^{-1} Tris–H_2SO_4 buffer (44 mL, pH 9.0). A 30% NaCN solution (≈6 mL) was automatically added by means of a pH/ORP controller to keep the pH at approximately 8.0–9.0. The reaction was performed in a water bath at 30 °C. After the substrate was completely converted to HN as monitored by GC analysis, the pH was adjusted to 8.0 by using 1 mol L^{-1} H_2SO_4, and the pET32a(+)–AtNIT2/*E. coli* BL21(DE3) wet cells (3.0 g) were added. After HN was not detected, the reaction mixture was centrifuged and 30% H_2O_2 was added to the supernatant to remove the residual cyanide, and the solution was acidified with 6 mol L^{-1} HCl to reach pH 1–2. The resulting solution was extracted with ethyl acetate (3×50 mL). The combined organic extracts were dried over anhydrous sodium sulfate, filtered, and evaporated in vacuo to afford EHG (9.16 g, 86.7%) as a yellow oil. ^{1}H NMR (400 MHz, [D$_6$]DMSO): δ = 4.16–4.25 (m, 1H, –C*H*OH), 4.03 (q, J = 7.0 Hz, 2H, –CH_2CH$_3$), 2.25–2.51 (m, 4H, –CH_2CO$_2$–), 1.16 ppm (t, J = 7.2 Hz, 3H, –CH_3); ^{13}C NMR (100 MHz, [D$_6$]DMSO): δ = 172.94, 171.37, 64.90, 60.23, 42.34, 14.52 ppm; high-resolution MS (ESI): *m/z*: calcd for $C_7H_{12}O_5Na^+$: 199.0613 $[M+Na]^+$, found: 199.0592.

Acknowledgements

This work was financially supported by the Key deployment project of Chinese Academy of Sciences (No. KSED-EW-Z-015).

Keywords: biotransformations · carboxylic acids · cyanides · enzyme catalysis · gene expression

[1] a) A. Bruggink, R. Schoevaart, T. Kieboom, *Org. Process Res. Dev.* **2003**, *7*, 622–640; b) D. E. Fogg, E. N. dos Santos, *Coord. Chem. Rev.* **2004**, *248*, 2365–2379; c) F. Lopez-Gallego, C. Schmidt-Dannert, *Curr. Opin. Chem. Biol.* **2010**, *14*, 174–183; d) P. A. Santacoloma, G. Sin, K. V. Gernaey, J. M. Woodley, *Org. Process Res. Dev.* **2011**, *15*, 203–212; e) E. Ricca, B. Brucher, J. H. Schrittwieser, *Adv. Synth. Catal.* **2011**, *353*, 2239–2262; f) I. Oroz-Guinea, E. Garcia-Junceda, *Curr. Opin. Chem. Biol.* **2013**, *17*, 236–249; g) H. Gröger, W. Hummel, *Curr. Opin. Chem. Biol.* **2014**, *19*, 171–179; h) J.-C. Wasilke, S. J. Obrey, R. T. Baker, G. C. Bazan, *Chem. Rev.* **2005**, *105*, 1001–1020.

[2] a) E. Brenna, F. G. Gatti, D. Monti, F. Parmeggiani, A. Sacchetti, *ChemCatChem* **2012**, *4*, 653–659; b) M. Fuchs, K. Tauber, J. Sattler, H. Lechner, J. Pfeffer, W. Kroutil, K. Faber, *RSC Adv.* **2012**, *2*, 6262–6265; c) S. Krauser, P. Kiefer, E. Heinzle, *ChemCatChem* **2012**, *4*, 786–788; d) T. Sehl, H. C. Hailes, J. M. Ward, R. Wardenga, E. von Lieres, H. Offermann, R. Westphal, M. Pohl, D. Rother, *Angew. Chem. Int. Ed.* **2013**, *52*, 6772–6775; *Angew. Chem.* **2013**, *125*, 6904–6908; e) S. Wu, Y. Chen, Y. Xu, A. Li, Q. Xu, A. Glieder, Z. Li, *ACS Catal.* **2014**, *4*, 409–420; f) T. Classen, M. Korpak, M. Scholzel, J. Pietruszka, *ACS Catal.* **2014**, *4*, 1321–1331; g) E. O'Reilly, C. Iglesias, D. Ghislieri, J. Hopwood, J. L. Galman, R. C. Lloyd, N. J. Turner, *Angew. Chem. Int. Ed.* **2014**, *53*, 2447–2450; *Angew. Chem.* **2014**, *126*, 2479–2482; h) M. Oslaj, J. Cluzeau, D. Orkić, G. Kopitar, P. Mrak, Z. Časar, *PLoS One* **2013**, *8*, e62250; i) T. Vajdič, M. Ošlaj, G. Kopitar, P. Mrak, *Metab. Eng.* **2014**, *24*, 160–172.

[3] a) B. Seisser, I. Lavandera, K. Faber, J. H. L. Spelberg, W. Kroutil, *Adv. Synth. Catal.* **2007**, *349*, 1399–1404; b) J. H. Schrittwieser, I. Lavandera, B. Seisser, B. Mautner, W. Kroutil, *Eur. J. Org. Chem.* **2009**, 2293–2298; c) W. Szymanski, C. P. Postema, C. Tarabiono, F. Berthiol, L. Campbell-Verduyn, S. de Wildeman, J. G. de Vries, B. L. Feringa, D. B. Janssen, *Adv. Synth. Catal.* **2010**, *352*, 2111–2115; d) S.-Y. Chen, C.-X. Yang, J.-P. Wu, G. Xu, L.-R. Yang, *Adv. Synth. Catal.* **2013**, *355*, 3179–3190; e) S. K. Ma, J. Gruber, C. Davis, L. Newman, D. Gray, A. Wang, J. Grate, G. W. Huisman, R. A. Sheldon, *Green Chem.* **2010**, *12*, 81–86.

[4] a) C. Mateo, A. Chmura, S. Rustler, F. van Rantwijk, A. Stolz, R. A. Sheldon, *Tetrahedron: Asymmetry* **2006**, *17*, 320–323; b) S. Rustler, H. Motejadded, J. Altenbuchner, A. Stolz, *Appl. Microbiol. Biotechnol.* **2008**, *80*, 87–97; c) O. Sosedov, K. Matzer, S. Buerger, C. Kiziak, S. Baum, J. Altenbuchner, A. Chmura, F. van Rantwijk, A. Stolz, *Adv. Synth. Catal.* **2009**, *351*, 1531–1538; d) S. Baum, F. van Rantwijk, A. Stolz, *Adv. Synth. Catal.* **2012**, *354*, 113–122.

[5] a) H. Ankati, D. Zhu, Y. Yang, E. R. Biehl, L. Hua, *J. Org. Chem.* **2009**, *74*, 1658–1662; b) D. Zhu, H. Ankati, C. Mukherjee, Y. Yang, E. R. Biehl, L. Hua, *Org. Lett.* **2007**, *9*, 2561–2563.

[6] a) T. Konoike, Y. Araki, *J. Org. Chem.* **1994**, *59*, 7849–7854; b) M. Watanabe, H. Koike, T. Ishiba, T. Okada, S. Seo, K. Hirai, *Bioorg. Med. Chem.* **1997**, *5*, 437–444; c) N. Andrushko, V. Andrushko, G. König, A. Spannenberg, A. Börner, *Eur. J. Org. Chem.* **2008**, 847–853; d) Z. Časar, *Curr. Org. Chem.* **2010**, *14*, 816–845; e) Z. Časar, M. Steinbücher, J. Kosmrlj, *J. Org. Chem.* **2010**, *75*, 6681–6684; f) X. Chen, F. Xiong, C. Zheng, J. Li, F. Chen, *Tetrahedron* **2014**, *70*, 5794–5799; g) J. Fabris, Z. Časar, I. G. Smilović, M. Črnugelj, *Synthesis* **2014**, *46*, 2333–2346.

[7] P. Yao, J. Li, J. Yuan, C. Han, X. Liu, J. Feng, Q. Wu, D. Zhu, *ChemCatChem* **2015**, *7*, 271–275.

[8] a) A. Archelas, R. Furstoss, *Annu. Rev. Microbiol.* **1997**, *51*, 491–525; b) Z. Y. You, Z. Q. Liu, Y. G. Zheng, *Appl. Microbiol. Biotechnol.* **2013**, *97*,

9–21; c) E. J. de Vries, D. B. Janssen, *Curr. Opin. Biotechnol.* **2003**, *14*, 414–420.

[9] a) L. X. Tang, D. E. T. Pazmino, M. W. Fraaije, R. M. de Jong, B. W. Dijkstra, D. B. Janssen, *Biochemistry* **2005**, *44*, 6609–6618; b) M. Schallmey, R. J. Floor, B. Hauer, M. Breuer, P. A. Jekel, H. J. Wijma, B. W. Dijkstra, D. B. Janssen, *ChemBioChem* **2013**, *14*, 870–881.

[10] a) Z.-Y. You, Z.-Q. Liu, Y.-G. Zheng, *Appl. Microbiol. Biotechnol.* **2014**, *98*, 11–21; b) C. Jiang, H. Hong, *Lett. Org. Chem.* **2012**, *9*, 520–521.

[11] R. J. Fox, S. C. Davis, E. C. Mundorff, L. M. Newman, V. Gavrilovic, S. K. Ma, L. M. Chung, C. Ching, S. Tam, S. Muley, J. Grate, J. Gruber, J. C. Whitman, R. A. Sheldon, G. W. Huisman, *Nat. Biotechnol.* **2007**, *25*, 338–344.

[12] J. J. Kerrigan, Q. Xie, R. S. Ames, Q. Lu, *Protein Expression Purif.* **2011**, *75*, 1–14.

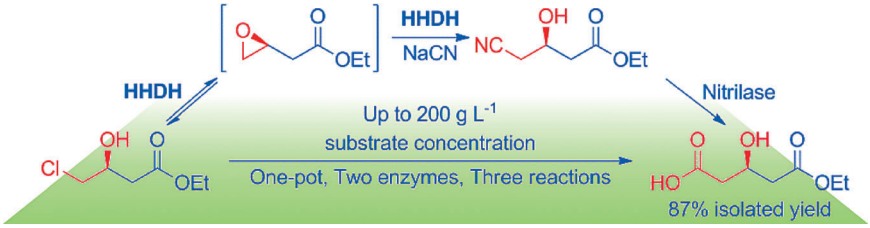
[13] Y.-X. Li, A. J. J. Straathof, U. Hanefeld, *Tetrahedron: Asymmetry* **2002**, *13*, 739–743.

Received: January 22, 2015
Published online on ■■ ▮▮, 0000

P. Yao, L. Wang, J. Yuan, L. Cheng, R. Jia, M. Xie, J. Feng, M. Wang, Q. Wu, D. Zhu**

■■ – ■■

Efficient Biosynthesis of Ethyl (*R*)-3-Hydroxyglutarate through a One-Pot Bienzymatic Cascade of Halohydrin Dehalogenase and Nitrilase



Team-up bonus: A novel one-pot bienzymatic cascade combining halohydrin dehalogenase (HHDH) and nitrilase for the synthesis of ethyl (*R*)-3-hydroxyglutarate from ethyl (*S*)-4-chloro-3-hydroxybutyrate is performed at high substrate concentration, and the product was obtained in high yield without separation and purification of the intermediate.