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## COMMUNICATION

## Synthesis of ethyl (*R*)-4-cyano-3-hydroxybutyrate at high concentration by using a novel halohydrin dehalogenase HHDH-PL from *Parvibaculum lavamentivorans* DS-1

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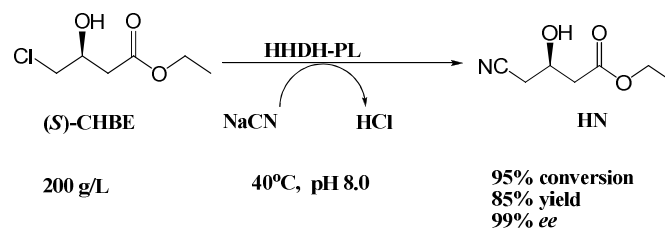
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We identified and characterized a novel halohydrin dehalogenase HHDH-PL from *Parvibaculum lavamentivorans* DS-1. Substrate specificity study indicated that HHDH-PL possessed a high activity toward ethyl (*S*)-4-chloro-3-hydroxybutanoate ((*S*)-CHBE). After optimization of the pH and temperature, the whole cell catalysis of HHDH-PL was applied into the synthesis of ethyl (*R*)-4-cyano-3-hydroxybutyrate (HN) at 200 g·L<sup>-1</sup> of (*S*)-CHBE, which generated 95% conversion and 85% yield in 14 h.

Atorvastatin calcium (Lipitor) is an important member of the statin family and widely used as an inhibitor of HMG-CoA reductase for the treatment of hypocholesterolemia and atherosclerosis. HN is the key precursor of the side chains of atorvastatin with the annual demands more than 100 tons. Many disadvantages emerge in the chemical routes for HN, including formation of extensive by-product and the usage of toxic catalyst. Recently, two green chemo-enzymatic processes for manufacture of HN have been established to reduce the wastes, hazards and costing from the chemical routes. One of the process was developed by DeSantis et al., which used a highly enantioselective nitrilase to catalyze asymmetric hydrolysis of 3-hydroxyglutaryl nitrile.<sup>1</sup> The other route to prepare HN was via biotransformation of (*S*)-CHBE using a halohydrin dehalogenase (HHDH), which met a substrate concentration of 140 g·L<sup>-1</sup> and gave 93% yield in 18 h.<sup>2</sup> This commercial HHDH was engineered by Richard. J. Fox et al. based on the HHDH HheC from *Agrobacterium radiobacter* AD1. They employed the ProSAR-

driven evolution method with 2500-fold improvement in the volumetric productivity for HN.<sup>3,4</sup>

HHDHs (EC 4.5.1.X) with the promiscuity activity can not only convert the vicinal halohydrins to corresponding epoxides, but also produced β-substituted alcohols undergo ring opening of epoxides in the presence of nucleophiles (NO<sub>2</sub><sup>-</sup>, CN<sup>-</sup>, N<sub>3</sub><sup>-</sup>, SCN<sup>-</sup>, HCOO<sup>-</sup>, OCN<sup>-</sup> and X<sup>-</sup>).<sup>3,5</sup> The best studied HheC exhibited a high enantioselectivity and regioselectivity to many halohydrins and epoxides, and was applied in the formation of optically active chloroalcohols, epoxides and β-functionalized alcohols.<sup>6-9</sup> So far only a limited number of HHDHs have been cloned and characterized biochemically. These HHDHs showed low activities toward (*S*)-CHBE which limited the application for preparation of HN. Herein, we reported a new HHDH called HHDH-PL from *P. lavamentivorans* DS-1 which could tolerate a high concentration of (*S*)-CHBE. Sequence alignment and substrate specificity studies indicated that HHDH-PL came from a new group of HHDH family. After process optimization, biotransformation of (*S*)-CHBE at 200 g·L<sup>-1</sup> with 40 g·L<sup>-1</sup> (dry cell weight) of HHDH-PL were performed and generated 95% conversion and 85% yield in 14 h (Scheme 1).



Scheme 1. Synthesis of HN from (*S*)-CHBE by HHDH-PL.

**Table 1** Summary of sequence information and activity assay of SDR enzymes

SDR	Plasmids enzyme site	Genebank accession	Activity assay
SDR1	pET20b(+) <i>NdeI</i> , <i>XhoI</i>	ABS62758.1	- <sup>a)</sup>
SDR2	pET20b(+) <i>NdeI</i> , <i>XhoI</i>	ABS62418.1	- <sup>a)</sup>
SDR3	pET28a(+) <i>NcoI</i> , <i>XhoI</i>	ABS61994.1	- <sup>a)</sup>
SDR4	pET20b(+) <i>NcoI</i> , <i>XhoI</i>	AAS42725.1	- <sup>a)</sup>
SDR5	pET28a(+) <i>NcoI</i> , <i>XhoI</i>	ACJ78562.1	- <sup>a)</sup>
SDR6	pET28a(+) <i>NcoI</i> , <i>XhoI</i>	ABS64560.1	- <sup>b)</sup>
SDR6	pET20b(+) <i>NcoI</i> , <i>XhoI</i>	ABS64560.1	- <sup>c)</sup>
SDR6	pET32a(+) <i>NcoI</i> , <i>XhoI</i>	ABS64560.1	+ <sup>d)</sup>

<sup>a)</sup> expressed successfully and tested having no HHDH activity. <sup>b)</sup> expressed unsuccessfully and tested having no HHDH activity. <sup>c)</sup> barely expressed in soluble and tested having no HHDH activity. <sup>d)</sup> expressed successfully and tested having HHDH activity.

**Table 2.** Pairwise sequence identities of HHDHs

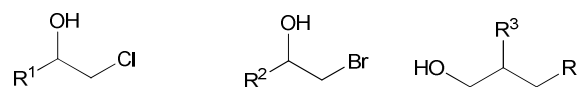
Group	HHDHs	Organisms	Identity (%)							
			HheA <sub>AD2</sub>	HheA	HheA <sub>Am</sub>	HheB <sub>GP1</sub>	HheB	HheC	HalB	HHDH-PL
A	HheA <sub>AD2</sub>	<i>Arthrobacter</i> sp. strain AD2	100	97.0	97.13	18.3	18.3	33.2	30.0	32.8
	HheA	<i>Corynebacterium</i> sp. strain N-1074		100	97.13	18.7	18.7	33.2	30.0	32.8
	HheA <sub>Am</sub> <sup>10</sup>	<i>Agromyces mediolanus</i> strain ZJB120203			100	18.3	18.3	33.2	30.0	33.6
B	HheB <sub>GP1</sub>	<i>Mycobacterium</i> sp. strain GP1				100	98.3	23.0	17.8	28.1
	HheB	<i>Corynebacterium</i> sp. strain N-1074					100	22.6	17.4	28.5
C	HheC	<i>A. radiobacter</i> AD1						100	91.3	34.8
	HalB	<i>A. tumefaciens</i>							100	31.3
This work	HHD-PL	<i>P. lavamentivorans</i> DS-1								100

Biochemical and structural investigations have shown HHDHs were structurally and mechanistically related to short-chain dehydrogenases/reductases (SDR) enzyme superfamily.<sup>3</sup> In order to discover new HHDH genes, we used three kinds of HHDH amino acid sequences (HheA<sub>AD2</sub>, HheB<sub>GP1</sub> and HheC) as queries for PSI-BLAST search against the nr database of NCBI. More than 1,200 amino acid sequences were collected for multiple sequence alignment with the three HHDH sequences by ClustalW2<sup>11</sup> software in tens. We distinguished SDR sequences with the following standards: the number of amino acids was 220-260; the conserved catalytic triad was Ser-Tyr-Arg (Lys). There were six SDR enzymes sequences from *Bacillus cereus* ATCC 10876 and *P. lavamentivorans* DS-1 were collected after 120 times iterations alignment. These SDR genes were cloned and expressed in *Escherichia coli* BL21 (DE3) for HHDH activity assay (Table 1). The SDR6 gene (HHDH-PL) expressed in *E. coli* BL21 (DE3) using pET32a (+) as the expression plasmids was identified having HHDH activity. However, no HHDH activities were obtained when it expressed in the plasmids of pET28a (+) or pET20b (+). The other SDRs were tested had no HHDH activity, even after changing the

**Table 3.** Substrates range and steady-state kinetic parameters of HHDH-PL

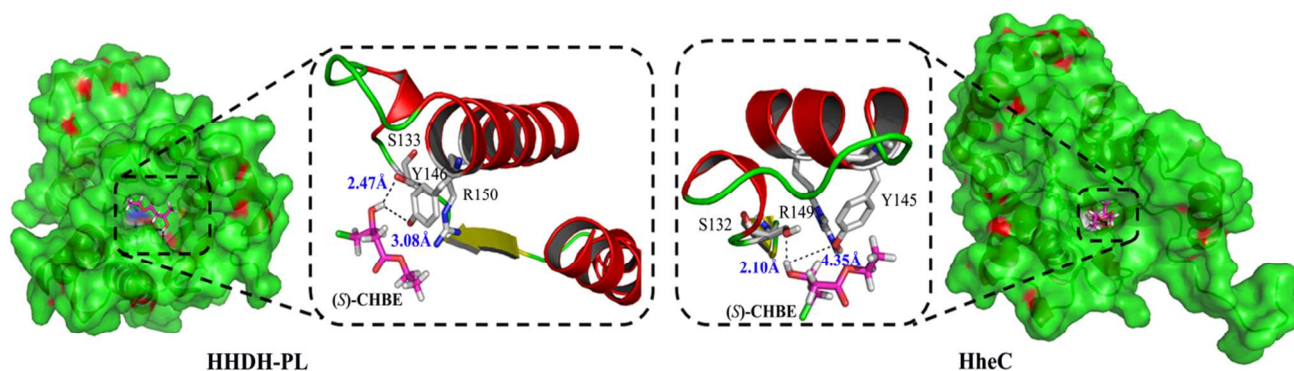
Substrate	Relative activity (%)	$k_{cat}$ (s <sup>-1</sup> )	$K_m$ (mM)	$k_{cat}/K_m$ (s <sup>-1</sup> mM <sup>-1</sup> )
<b>1</b>	100.0 <sup>a)</sup>	4651±8.2	30.5±1.12	153±6.3
( <i>R,S</i> )- <b>2</b>	0.9±0.23	ND <sup>b)</sup>	ND	ND
( <i>R,S</i> )- <b>3</b>	5.4±0.71	ND	ND	ND
( <i>S</i> )- <b>4</b>	82.4±0.86	519±11.2	4.1±0.3	127±5.9
( <i>S</i> )- <b>5</b>	112.9±3.45	2935±9.6	9.9±1.4	296±35.8
( <i>R,S</i> )- <b>6</b>	37.6±1.23	1534±21.3	32.0±1.9	47.9±3.7
<b>7</b>	125.7±3.12	8015±13.5	43.9±1.5	182±5.7
( <i>R,S</i> )- <b>8</b>	127.2±4.85	5243±9.9	31.2±2.2	168±10.7
( <i>R,S</i> )- <b>9</b>	194.1±6.32	4070±32.2	14.5±0.4	280±4.8
( <i>R,S</i> )- <b>10</b>	76.7±4.61	4255±21.5	36.3±0.6	117±0.7
( <i>R,S</i> )- <b>11</b>	1.5±0.37	ND	ND	ND

<sup>a)</sup> The activity to substrate **1** was selected as comparison (100%). <sup>b)</sup> ND: the activities toward the substrates were too low to determine the kinetic parameters.

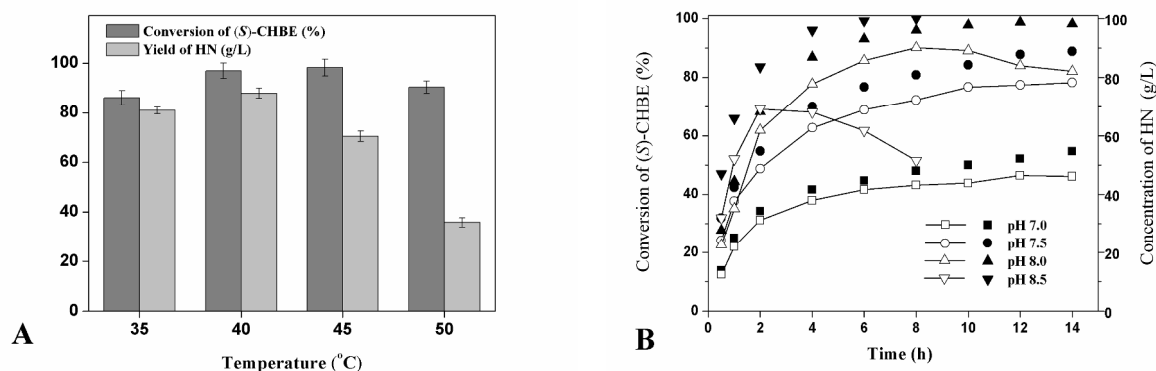


$R^1 = \text{CH}_2\text{Cl}$  (1);  $\text{CH}_3$  (2);  $\text{CH}_2\text{OH}$  (3);  $R^2 = \text{CH}_2\text{Br}$  (7);  $\text{CH}_3$  (8);  $R^3 = \text{Br}$  (10);  $\text{Cl}$  (11)  
 $\text{CH}_2\text{CN}$  (4);  $\text{CH}_2\text{COOEt}$  (5); Phenyl (6) H (9)

<sup>a)</sup> The activity to substrate **1** was selected as comparison (100%). <sup>b)</sup> ND: the activities toward the substrates were too low to determine the kinetic parameters.



**Fig 1.** Three-dimensional representation of (*S*)-CHBE in the active sites of HDDH-PL and HheC by docking. The substrates and catalytic triad residues were described in sticks.

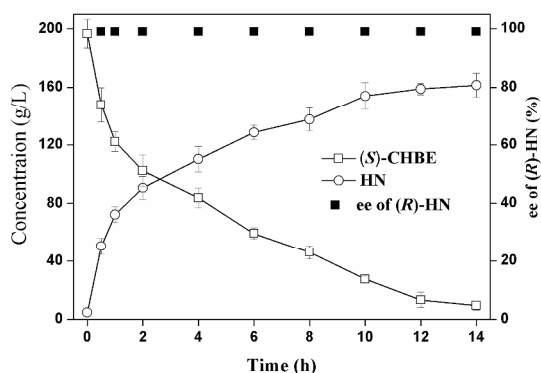


**Fig 2.** A: effects of temperatures on the formation of HN; B: effect of pHs on the formation of HN. Symbols: the conversions of (*S*)-CHBE at the different pHs: 7.0 (■), 7.5 (●), 8.0 (▲), 8.5 (▼); the concentrations of HN at the different pHs: 7.0 (□), 7.5 (○), 8.0 (△), 8.5 (▽).

HDDHs.<sup>12</sup> The  $k_{cat}$  and  $K_m$  of (*S*)-CHBE (**5**) were  $2935\text{ s}^{-1}$  and  $9.9\text{ mM}$  respectively. These results demonstrated that HDDH-PL possessed high activities on both short carbon-chain (C2-C3) and long carbon-chain (C4-C6) halohydrins. Surprisingly, HDDH-PL showed significantly higher activity in conversion of (*S*)-CHBE to HN as compared to HheA<sub>AD2</sub>, HheB<sub>GP1</sub> and HheC (Figure S5).

For further insight into the excellent activity of HDDH-PL on (*S*)-CHBE based on the structure information, the docking studies were performed by AutoDock software<sup>13</sup>. The generated model structure of HDDH-PL and the crystal structure of HheC (PDB code: 1PWX)<sup>14</sup> were docked with (*S*)-CHBE for comparison. The catalytic triad of HDDHs was composed of Ser-Tyr-Arg, in which Ser was involved in substrate binding and Arg acquired a proton from OH of Tyr to lower its pKa. Tyr then acted as a catalytic base seized a proton from the OH of (*S*)-CHBE, subsequently oxygen anion disrupted the carbon-halogen bond to form corresponding epoxide. Docking results in Fig. 1 revealed that HDDH-PL owned a more open substrate-binding pocket than HheC, which was propitious for accepting and transporting of (*S*)-CHBE. In addition, the distance of the substrate OH between Tyr146 was  $3.08\text{ Å}$  for HDDH-PL, while it was  $4.35\text{ Å}$  for HheC. We could draw a conclusion that it was more difficult for HheC to acquire a proton from the OH of (*S*)-CHBE than HDDH-PL, which resulted in a lower activity to (*S*)-CHBE.

Based on the high efficiency of HDDH-PL for preparation of HN by conversion of (*S*)-CHBE, we systematically investigated the optimal temperature and pH of this process. Fig. 2 summarized the effects of temperature (ranged from  $35\text{ °C}$  to  $50\text{ °C}$ ) and pH (7.0-8.5) on the formation of HN. The highest yield of  $87\text{ g}\cdot\text{L}^{-1}$  of HN was found at  $40\text{ °C}$ . With the highest conversion of 97%, the yield of HN was just  $70\text{ g}\cdot\text{L}^{-1}$  at  $45\text{ °C}$ . HDDH-PL showed a low activity at  $35\text{ °C}$  which gave 85% conversion and  $78\text{ g}\cdot\text{L}^{-1}$  of HN. When the reaction was performed at  $50\text{ °C}$ , the yield of HN decreased to  $36\text{ g}\cdot\text{L}^{-1}$ . From the results it could be concluded that  $40\text{ °C}$  was the optimal temperature for production of HN. The lower temperature ( $35\text{ °C}$ ) lowered the catalytic activity of HDDH-PL, and the higher temperature ( $45$  and  $50\text{ °C}$ ) accelerated the hydrolysis of HN. The optimisation of pH was investigated at  $40\text{ °C}$  and showed in Fig. 2B. At pH 8.5, the conversion was 100% in 6 h with  $50\text{ g}\cdot\text{L}^{-1}$  of HN yield. In this case, the yield was reduced extremely after 2 h which was due to the formation rate of HN was lower than the hydrolysis rate. At the neutral pH 7.0, the lowest conversion and yield were obtained. The yield of HN reached  $85\text{ g}\cdot\text{L}^{-1}$  at pH 8.0 in 8 h and decreased slowly in the following 4 h. Several conclusions could be drawn from the results: pH 8.0 was the optimal pH for synthesis of HN, even if it had a slight effect on the hydrolysis of HN; the high pH of 8.5 improved the activity of HDDH-PL but accelerated the hydrolysis of HN; the pH of 7.0 and 7.5 were unfavourable for the conversion of (*S*)-CHBN.



**Fig 3.** The process of the formation of HN by biotransformation of (S)-CHBE at 200 g·L<sup>-1</sup> using the whole cells of HHDH-PL. Symbols: concentration of (S)-CHBE (□), concentration of HN (○), and ee of (R)-HN (■).

At last we scaled up the process by biotransformation of 200 g·L<sup>-1</sup> of (S)-CHBE under the optimal conditions (40 °C and pH 8.0) (Fig. 3). The reaction was carried out in a 1 L jacket three-neck bottom containing 400 mL 100 mM PBS buffer (pH 7.5). To it 50% sulfuric acid was added to adjust pH to 4.0 and then 30% of (wt/vol) sodium cyanide solution was added to pH 7.5. The mixture temperature was heated to 40 °C and followed by adding 20 g lyophilized cells of HHDH-PL and 100 g (S)-CHBE (0.6 mol). Subsequently, a pH stat was used to control the pH at 8.0±0.05 by adding 30% of sodium cyanide solution. The reaction process was monitored by taking samples from the mixture for GC analysis. The reaction was completed after 14 h when the conversion of (S)-CHBE reached 95%. To the mixture 8 g CaCl<sub>2</sub> was added to stir for 1 h and the cells were removed through filtration. The separated mixture was extracted three times with ethyl acetate (300 mL). The organic extracts were combined and dried over anhydrous sodium sulphate. The ethyl acetate was removed by evaporation under vacuum and remained 80.68 g (0.513 mol and yield 85%) yellow liquid.

In conclusion, a new HHDH gene HHDH-PL was identified and heterologously overexpressed in *E. coli* in this study. The sequence alignment assay and substrate specificity study indicated that HHDH-PL possessed different properties from the previously characterized HHDHs. In addition, HHDH-PL was applied in biotransformation of (S)-CHBE at a concentration of 200 g·L<sup>-1</sup>, which generate 95% conversion and 85% yield. To the best of our knowledge, this was the first report to prepare HN by using a natural HHDH at a high substrate concentration. The high substrate tolerance to (S)-CHBE suggested HHDH-PL was a promising biocatalyst for commercially preparation of HN, as well as for academic research. However, the substrate-to-catalyst ration was high and the catalytic efficiency was inferior to the method reported by Steven K. Ma.<sup>2</sup> The large amount of biocatalysts in the process will have an effect on both cost and the product separation. Hence, in our future work we will try to engineer the catalytic activity, stability and enantioselectivity of HHDH-PL for its broader practical application including preparation of HN and the other optically active secondary alcohols and epoxides.

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## Notes and references

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†Electronic Supplementary Information (ESI) available: [Experimental and <sup>1</sup>H and <sup>13</sup>C NMR spectra]. See DOI: 10.1039/c000000x/

- G. DeSantis, K. Wong, B. Farwell, K. Chatman, Z. L. Zhu, G. Tomlinson, H. J. Huang, X. Q. Tan, L. Bibbs, P. Chen, K. Kretz and M. J. Burk, *J Am Chem Soc*, 2003, **125**, 11476-11477.
- S. K. Ma, J. Gruber, C. Davis, L. Newman, D. Gray, A. Wang, J. Grate, G. W. Huisman and R. A. Sheldon, *Green Chem*, 2010, **12**, 81-86.
- J. E. T. V. Vlieg, L. X. Tang, J. H. L. Spelberg, T. Smilda, G. J. Poelarends, T. Bosma, A. E. J. van Merode, M. W. Fraaije and D. B. Janssen, *J Bacteriol*, 2001, **183**, 5058-5066.
- 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 and G. W. Huisman, *Nat Biotechnol*, 2007, **25**, 338-344.
- G. Hasnaoui-Dijoux, M. M. Elenkov, J. H. L. Spelberg, B. Hauer and D. B. Janssen, *Chembiochem*, 2008, **9**, 1048-1051.
- R. M. Haak, C. Tarabiono, D. B. Janssen, A. J. Minnaard, J. G. de Vries and B. L. Feringa, *Org Biomol Chem*, 2007, **5**, 318-323.
- M. M. Elenkov, I. Primozic, T. Hrenar, A. Smolko, I. Dokli, B. Salopek-Sondi and L. X. Tang, *Org Biomol Chem*, 2012, **10**, 5063-5072.
- L. S. Campbell-Verduyn, W. Szymanski, C. P. Postema, R. A. Dierckx, P. H. Elsinga, D. B. Janssen and B. L. Feringa, *Chem Commun*, 2010, **46**, 898-900.
- M. M. Elenkov, H. W. Hoeffken, L. Tang, B. Hauer and D. B. Janssen, *Adv Synth Catal*, 2007, **349**, 2279-2285.
- F. Xue, Z. Q. Liu, N. W. Wan and Y. G. Zheng, *Appl Biochem Biotechnol*, 2014, **174**, 352-364.
- M. A. Larkin, G. Blackshields, N. P. Brown, R. Chenna, P. A. McGettigan, H. McWilliam, F. Valentin, I. M. Wallace, A. Wilm, R. Lopez, J. D. Thompson, T. J. Gibson and D. G. Higgins, *Bioinformatics*, 2007, **23**, 2947-2948.
- Z. Y. You, Z. Q. Liu and Y. G. Zheng, *Appl Microbiol Biotechnol* 2013, **97**, 9-21.
- G. M. Morris, R. Huey, W. Lindstrom, M. F. Sanner, R. K. Belew, D. S. Goodsell and A. J. Olson, *J Comput Chem*, 2009, **30**, 2785-2791.
- R. M. de Jong, J. J. W. Tiesinga, H. J. Rozeboom, K. H. Kalk, L. Tang, D. B. Janssen and B. W. Dijkstra, *Embo Journal*, 2003, **22**, 4933-4944.