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Asymmetric hydrolysis of 2-hydroxy-carboxylic esters using recombinant *Escherichia coli*

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Abstract—Optically active 2-hydroxy-carboxylates are important compounds for their use as intermediates in the synthesis of pharmaceuticals and stereoblock polymers. *Enterobacter* sp. DS-S-75 and the recombinant *Escherichia coli* harbouring the 4-chloro-3-hydroxybutyrate (CHB) hydrolase gene from the strain DS-S-75 showed asymmetric hydrolytic activity towards 2-hydroxy-carboxylates, as well as towards CHB. It was discussed that the hydroxyl group in the substrate was particularly important for the asymmetric hydrolytic activity of the CHB hydrolase, and as such, it was re-designated to EnHCH (hydroxy-carboxylic ester hydrolase derived from *Enterobacter* sp.). Using the recombinant cell, both the reaction rate and the concentration of the substrates were significantly improved upon when compared to that of DS-S-75. Optically active 2-hydroxy-carboxylates could be synthesized on a practical basis for industrial production in this report.
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1. Introduction

Optically active 2-hydroxy-carboxylates, such as 2-hydroxybutyrate and lactate, are important building blocks for glycols,[1] halo esters[2] and epoxides;[3] compounds which are important intermediates of pharmaceuticals.[4] In particular, (*R*)-2-hydroxybutyrate has found application as antitumour antibiotics, while (*R*)-lactate [D-(+)-lactate] has been noted as a heat stabilizer of poly L-lactic acid by stereo-complex formation.[5–7] Therefore, several methods have been reported for the formation of these optically active 2-hydroxy-carboxylates. The chemical methods depend on the chiral precursors,[8,9] which is generally expensive. Although methods using common D-fructose have been reported,[10] they require extreme reaction conditions (−78 °C). The biocatalytic method, oxidative resolution of the racemate[11,12] and asymmetric reduction[13] of the corresponding β-ketoester have already been reported, which depend on an expensive electron donor as a coenzyme. (*R*)-Lactic acid has also been produced at fermentation from carbohydrates using the microorganism,[14,15] which use complex metabolic pathways. Since there are many concerning factors which influence the synthesis, controlling the reaction on an industrial scale is not easy.

In our earlier study, we isolated the original microorganism, *Enterobacter* sp. DS-S-75 (International deposition No. FERM BP-5494) from a soil sample.[16] Using the resting cells or the cell-free extract, (*S*)-4-chloro-3-hydroxybutyrate (CHB) in the racemate was converted to (*S*)-3-hydroxy-γ-butyrolactone (HL) via a one pot asymmetric hydrolysis and lactonization reaction, thereby releasing the chloride ion under simple and mild conditions (Scheme 1). More recently, it was reported that the catalyzing enzyme (designated as CHB hydrolase) could be purified, and that the gene encoding the enzyme could be isolated from strain DS-S-75 (GeneBank Accession No. AB236152).[17] This gene was constitutively and stably expressed using *E. coli* DH5α[18] as a host. The productivity of (*R*)-CHB and (*S*)-HL was notably improved by the use of the recombinant DH5α(pKK-E3) (FERM BP-08466) cells harbouring the CHB hydrolase gene. Moreover, studies on purified CHB hydrolase showed hydrolytic activity towards not only the non-chlorinated 3-hydroxy-carboxylic esters of CHB, but also the 2-hydroxy-carboxylic esters, which were converted to the corresponding carboxylic acids.[17]

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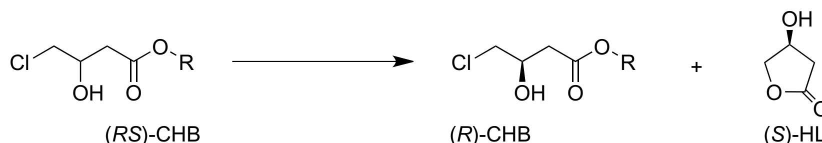


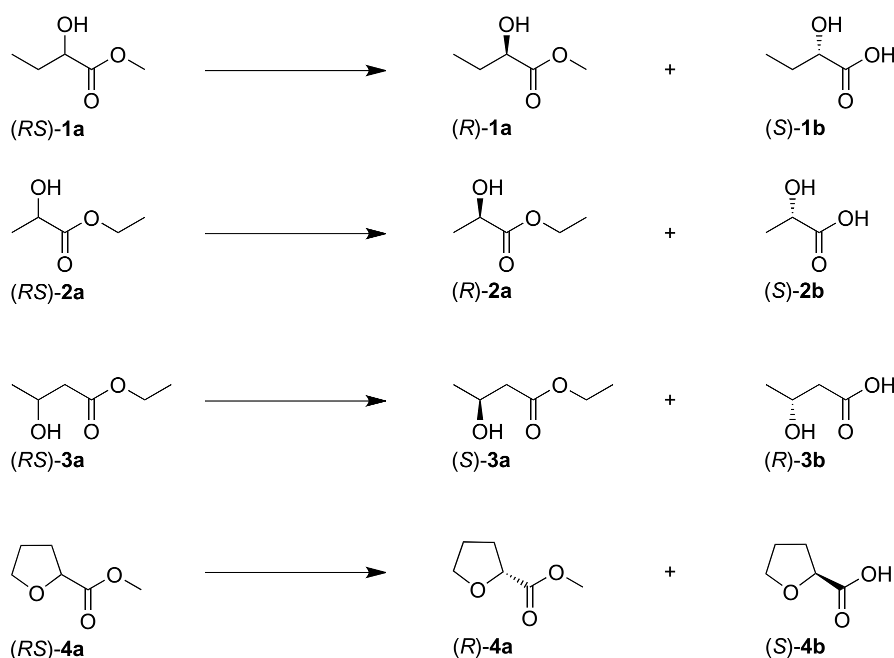
Scheme 1. Asymmetric conversion of CHB to HL using CHB hydrolase.

Herein we report, the enantioselectivity of strains DS-S-75 and DH5α(pKK-E3) towards the aforementioned 2-hydroxy-carboxylic esters. In addition, the application of DH5α(pKK-E3) for the industrial production of 2-hydroxy-carboxylates, such as methyl (*R*)-2-hydroxybutyrate **1a**, (*S*)-2-hydroxybutyric acid **1b** and (*R*)-lactic ester **2a**, showed significant improvements when compared to conventional methods.

2. Results and discussion

The strain DS-S-75 was cultured on a 100 mL scale and the reaction using the culture broth was performed with (*RS*)-**1a** and (*RS*)-**2a** as the substrates (Scheme 2). As a result, strain DS-S-75 showed asymmetric hydrolytic activity with high enantioselectivity towards these substrates. In addition, this activity was also shown for ethyl (*RS*)-3-hydroxybutyrate **3a** and methyl (*RS*)-tetrahydrofuran-2-carboxylate **4a**. Especially, as for **1a** and **3a**, the conversion ratio was ideal so that the corresponding remaining esters (*R*)-**1a** and (*S*)-**3a**, and carboxylic acids formed (*S*)-**1b** and (*R*)-**3b**, were obtained with excellent enantiomeric excesses (>98% ee) (Table 1). However, the esters did not achieve excellent enantiomeric excesses when the substrate concentration was higher. The reactions using the culture broth of DH5α(pKK-E3) were also performed by the same method as that of strain DS-S-75. As a result, DH5α(pKK-E3) showed the same enantioselectivity as that of strain DS-S-75. It was discussed that the CHB hydrolase showed asymmetric hydrolytic activity not only for CHB with dehalogenation, but also for the aforementioned 2- and 3-hydroxy-carboxylic esters. However, this enzyme did not show any degrading activity towards 4-chloro-3-hydroxybutyronitrile, diethyl malate and 3-chloro-1,2-propanediol.[17] Also, the degrading activity was not shown towards methyl 2-amino-butyrate and methyl 2,3-dichloropropionate (data not shown). It was discussed that this enzyme showed asymmetric hydrolytic activity towards carboxylic monoesters with hydroxyl groups. From these discussions, we re-designated the CHB hydrolase to EnHCH (hydroxy-carboxylic ester hydrolase derived from *Enterobacter* sp. DS-S-75). This enzyme also showed high enantioselectivities towards butyric esters. *E*-values were 458, 553; **1a** and 1190, 1330; **3a**.

The resolution of 2-and 3-hydroxy-carboxylic esters using EnHCH could be performed under milder conditions (temperature, pH and pressure). This composition was simpler with a single enzyme and inexpensive racemates, without expensive cofactor or coenzyme for asymmetric oxidation and reduction reactions by biocatalysts. It was suggested that the EnHCH enzyme would be useful for an industrial reaction. Similar resolution methods towards the above mentioned esters have been reported.[19–21] However, these reactions have not shown high enantioselectivity and pro-



Scheme 2. Asymmetric hydrolysis of racemic hydroxy-carboxylic esters.

Table 1. The comparison of DH5α(pKK-E3) and strain DS-S-75 in asymmetric hydrolysis

Substrate[a]	Strain	Esters			Acid			E[c]	Time (h)
		Residual ratio[b]	(%)	ee[b] (% ee)	Molar conv.[b]	(%)	ee[b] (% ee)		
(*RS*)-**1a**			(*R*)-**1a**			(*S*)-**1b**			
	DS-S-75	50.0		98.0	50.6		98.0	458	36
	DH5α(pKK-E3)	49.8		98.5	50.3		98.1	553	1.5
(*RS*)-**2a**			(*R*)-**2a**			(*S*)-**2b**			
	DS-S-75	39.5		98.5	60.0		65.8	22.3	40
	DH5α(pKK-E3)	39.3		98.5	60.5		65.2	21.8	1
(*RS*)-**3a**			(*S*)-**3a**			(*R*)-**3b**			
	DS-S-75	49.7		99.5	50.1		99.0	1190	6
	DH5α(pKK-E3)	49.8		99.5	50.2		99.1	1330	0.25
(*RS*)-**4a**			(*R*)-**4a**			(*S*)-**4b**			
	DS-S-75	32.5		98.5	64.7		53.2	14.5	20
	DH5α(pKK-E3)	32.6		98.5	63.5		53.3	14.5	0.5

[a] Each 8 g of **1a** or **3a**, and 2 g of **2a** or **4a** was added to 100 mL of culture broth.
[b] Quantitative analysis and enantiomeric excess were determined by GC.
[c] E value was calculated from enantiomeric excess of substrate (ee_s) and product (ee_p) using the formula: $E = \mathrm{Ln}[(1 - ee_s) * (ee_p/(ee_s + ee_p))]/\mathrm{Ln}[(1 + ee_s) * (ee_p/(ee_s + ee_p))]$.

ductivity towards the aforementioned compounds. In particular, the culture broth of DH5α(pKK-E3) has been shown to have approximately 20–25-fold higher activity than that of the strain DS-S-75 towards CHB and *p*-nitrophenyl butyrate because expression per cell is high in previous reports.[17] High activity was also seen towards 2- and 3-hydroxy-carboxylic esters. In addition, the supernatant after centrifugation showed almost no activity as well as that of strain DS-S-75, such that this control of the reaction step made it easier to maintain the high yields of chiral products.

The practical reactions for the industrial production of optically active 2-hydroxy-carboxylates were then performed with a jar-fermenter. Only 300 mL of the culture broth of DH5α(pKK-E3) was needed in a 3.0-L scale reaction, since the recombinant cells had high activity. The culture broth was diluted with water up to 3.0 L. This small scale culture was not only economical, but also allowed the easy isolation of the products because of the high product yields and small amounts of cells and cell medium. For 8% (w/v) of each (*RS*)-**1a** and **2a**, both reactions were completed within 20 h and 72 h, respectively. Although the culture broth of strain DS-S-75 could not be diluted, (*R*)-**1a** was obtained in 98% ee after 36 h, while (*R*)-**2a** did not achieve 98% ee. In spite of the dilution, DH5α(pKK-E3) was superior to strain DS-S-75 in both reaction rate and concentration of each substrate.

The production of optically active (*S*)-**3a**, (*R*)-**3b** and (*R*)-**4a** was also carried out. These chiral compounds are also important as intermediates in the syntheses of pharmaceuticals. In particular, (*R*)-**3b** is used as an anti-glaucoma drug,[22] while (*R*)-**4a** is used as a β-lactam antibiotic.[23] Therefore, several methods for the production of these optically active intermediates have been reported.[13,19,24–26] Using the same diluted culture broth of DH5α(pKK-E3) as described above, the enantiomeric purity of residual (*S*)-**3a** achieved from (*RS*)-**3a** of 15% (w/v) was 98% ee within 6 h. From (*RS*)-**4a** of 8% (w/v), (*R*)-**4a** was obtained with 98% ee within 20 h. Although the culture broth of strain DS-S-75 was not diluted, (*S*)-**3a** and (*R*)-**4a** did not achieve 98% ee in the above mentioned substrate concentration.

After the reaction, the recombinant cells were removed and the residual esters and corresponding formed acids were purified. (*R*)-**1a**, (*R*)-**2a**, (*S*)-**3a** and (*R*)-**4a** could be purified by distillation without a decrease in enantiomeric purities. The overall yields of these compounds from the corresponding racemates were 32.4%, 34.5%, 36.4% and 23.4%, where (*S*)-**1b** and (*R*)-**3b** were crystallized as sodium salts with overall yields of 16.7% and 15.5%, respectively.

3. Conclusions

We have developed inexpensive procedures for the production of optically active 2-hydroxy-carboxylates as well as 3-hydroxy-carboxylates with high concentration and enantiomeric purity from inexpensive and common racemates using a diluted culture broth of recombinant *E. coli* DH5α(pKK-E3) under mild reaction conditions and simple composition. Our method would be useful for the industrial production.

4. Experimental

4.1. Growth of microorganism

E. coli DH5α(pKK-E3)[17] was cultured in 500 mL Erlenmeyer flasks containing 100 mL of a nutrient medium A (peptone 20 g L^{-1}, yeast extract 10 g L^{-1}, glycerol 5 g L^{-1} and ampicillin sodium 100 mg L^{-1}, pH 6.8) for 20 h at 30 °C on a rotary shaker (130 rpm) after inoculation. *Enterobacter* sp. DS-S-75[16] was cultured in nutrient medium B (Polypeptone 10 g L^{-1}, yeast extract 10 g L^{-1}, glycerol 10 g L^{-1}, pH 6.8) for 24 h under the same conditions as that of DH5α(pKK-E3).

4.2. Chemicals

The chemicals used in this study were of reagent grade. (*RS*)-**1a**–**3a** and **1b**–**4b** were purchased from Tokyo Kasei (Tokyo, Japan). (*RS*)-**4a** was prepared in our laboratory as follows. Sulfuric acid (6 mL) was slowly added to a stirred solution of (*RS*)-**4b** (150 g) and methanol (418 mL). After reflux for 10 h at 70 °C, the crude product was extracted with 550 mL of dichloromethane. The organic layer was washed with 5% $NaHCO_3$ solution until the pH of the water layer reached 7.0. The organic layer was washed with water, and dried over anhydrous Na_2SO_4. The crude product was then purified (81% yield) by distillation under vacuum condition (bp 80 °C/20 mmHg); ^{1}H NMR δ 1.88–2.09 (m, 3H), 2.22–2.32 (m, 1H), 3.74 (s, 3H), 3.90–4.06 (m, 2H), 4.47 (dd, $J = 5.1$, 8.4 Hz, 1H).

4.3. The reaction of microbial resolution

For the examination of the hydrolytic reaction, 5 g of $CaCO_3$ as a pH neutralizer was added to 100 mL of culture broth in 500 mL Erlenmeyer flasks. The hydrolytic reactions were started by the addition of 8 g of **1a** or **3a**, and 2 g of **2a** or **4a** to the substrate at 30 °C on a rotary shaker (130 rpm), respectively. After the cells were removed by centrifugation, the amount and the enantiomeric purity of the residual carboxylic esters and carboxylic acids formed were analyzed by gas chromatography, as described below.

4.4. The production of carboxylic esters and acids

For the production, 300 mL of culture broth was added to a 5-L jar fermenter Model KMJ-5B (Mitsuwa Rikagaku, Osaka, Japan) with 2.7 L of distilled water. Each 529 g (15%) of (*RS*)-**3a** or each 261 g (8%) of the other racemate was added as a substrate. The reaction was performed under the following conditions: agitation, 500 rpm; temperature, 30 °C; the pH was controlled at 6.9 with 25% (w/v) NaOH as for **1a** and **3a** or 14% (w/w) ammonium aqueous solution as for **2a** and **4a**. The reaction solution was analyzed as described in the analytical methods.

After the reaction using DH5α(pKK-E3), the cells were removed using a UF membrane (MiniKros Tangential Flow Separation Module PS/50K 8000 cm^2, SPECTRUMLABS, CA, USA). Compounds **1a**, **3a** and **4a** were extracted three times with equal volumes of ethyl acetate. As for the extraction of **2a**, dichloromethane was used. Each organic layer was evaporated and the crude esters remaining distilled under the following conditions: **1a** or **3a**, bp 60 °C/15 mmHg: **2a**, bp 40 °C/20 mmHg: **4a**, bp 80 °C/20 mmHg. The aqueous layer including **1b** or **3b** was washed twice with ethyl acetate and evaporated. The first syrup obtained was dissolved in 2.3 L of methanol and filtered under reduced pressure. The solution was then evaporated. The second syrup was resolved in 1 L of 2-propanol and left at 4 ° for 24 h. The resulting precipitate was harvested as a sodium salt (**1c**,**3c**) of either **1b** or **3b** by filtration under educed pressure and freeze dried. These specific rotations were: **1a**, $[\alpha]_D^{20} = +2.45$ (neat): **2a**, $[\alpha]_D^{20} = +11.2$ (neat): **3a**, $[\alpha]_D^{20} = +18.0$ (neat): **4a**, $[\alpha]_D^{20} = -17.7$ (neat): **1c**, $[\alpha]_D^{20} = -10.4$ (*c* 10, H_2O): **3c**, $[\alpha]_D^{20} = -14.1$ (*c* 10, H_2O).

4.5. Analytical methods

The concentrations and enantiomeric purities of the carboxylic esters and the corresponding carboxylic acids were determined by gas chromatography (Model GC-14A, Shimadzu, Kyoto, Japan). For analysis of the concentration, the GC was equipped with a PEG 20M-HP (5%, 60/80 mesh, GL Science, Tokyo, Japan) column (3.2 mm diameter and 1 m length). The pH values of the carboxylic acid samples were adjusted to 4.0 with 80% phosphoric acid. The conditions were as follows: sample size, 1 μL; injection and detector temperature, 240 °C; flow rate, 50 mL min^{-1}; detector, a hydrogen flame ionization detector (FID). The column temperature programme was as follows: 5 min at 150 °C, increased to 200 °C over 10 min. For analysis of the enantiomeric purity, each carboxylic ester sample (with the exception of **2a** and **3a**) was subjected to a capillary column CHIRALDEX^R-G-TA (0.25 mm diameter, 30 m length, Astec Inc., NJ). Compounds **2a** and **3a** were trifluorinated by treatment with 5% (v/v) trifluoroacetic anhydride in dichloromethane. The evaporated syrups were dissolved in ethanol before injection. The carboxylic acids **1b**–**4b** were converted to the corresponding esters with 4-dimethylaminopyridine (DMAP). The conditions were as follows: sample size, 0.2 μL; injection and detector temperature, 200 °C; column temperature, 90°; flow rate, 50 mL min^{-1}; split ratio, 1:100; detector, FID.

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