A Two-Step Enzymatic Resolution Process for Large-Scale Production of (*S*)- and (*R*)-Ethyl-3-Hydroxybutyrate

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Abstract: An efficient two-step enzymatic process for production of (*R*)- and (*S*)-ethyl-3-hydroxybutyrate (HEB), two important chiral intermediates for the pharmaceutical market, was developed and scaled-up to a multikilogram scale. Both enantiomers were obtained at 99% chemical purity and over 96% enantiomeric excess, with a total process yield of 73%. The first reaction involved a solvent-free acetylation of racemic HEB with vinylacetate for the production of (*S*)-HEB. In the second reaction, (*R*)-enriched ethyl-3-acetoxybutyrate (AEB) was subjected to alcoholysis with ethanol to derive optically pure (*R*)-HEB. Immobilized *Candida antarctica* lipase B (CALB) was employed in both stages, with high productivity and selectivity. The type of butyric acid ester influenced the enantioselectivity of the enzyme. Thus, extending the ester alkyl chain from ethyl to octyl resulted in a decrease in enantiomeric excess, whereas using bulky groups such as benzyl or *t*-butyl, improved the enantioselectivity of the enzyme. A stirred reactor was found unsuitable for large-scale production due to attrition of the enzyme particles and, therefore, a batchwise loop reactor system was used for bench-scale production. The immobilized enzyme was confined to a column and the reactants were circulated through the enzyme bed until the targeted conversion was reached. The desired products were separated from the reaction mixture in each of the two stages by fractional distillation. The main features of the process are the exclusion of solvent (thus ensuring high process throughput), and the use of the same enzyme for both the acetylation and the alcoholysis steps. Kilogram quantities of (*S*)-HEB and (*R*)-HEB were effectively prepared using this unit, which can be easily scaled-up to produce industrial quantities. *Biotechnol Bioeng* **74**: 256–263, 2001.
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INTRODUCTION

Both enantiomers of ethyl-3-hydroxybutyrate (HEB) are chiral starting materials for the production of numerous biologically active compounds of commercial interest. The (*R*)-enantiomer is an intermediate for an anti-glaucoma drug (Blacker and Holt, 1997) and (*S*)-HEB is used for synthesizing pheromones (Mori, 1989) and carbapenem antibiotics (Bucciarelli et al., 1999). Although the (*R*)-enantiomer can be obtained from hydrolysis of poly-3-hydroxybutyrate in ton quantities, the (*S*)-enantiomer is more difficult to prepare on a large scale. One possibility is asymmetric hydrogenation of ethylacetoacetate using the BINAP-coordinated Ru(II) complexes, which were developed by Noyori and coworkers (Blacker and Holt, 1997; Noyori et al., 1987). However, this method requires special equipment and harsh conditions (100 atmospheres) to achieve high yields and selectivity. Another option is the biological reduction of β-keto-esters using baker's yeast in aqueous (Bertau et al., 1998; Kometani et al., 1989; Wendhausen et al., 1998) or nonconventional media (North 1996; Rotthous et al., 1997). Despite the high enantioselectivity often achieved, long reaction times accompanied by low product yield are encountered.

Enzymatic resolution has also been described for preparation of (*S*)-HEB or similar β-hydroxyesters. Sugai and coworkers (Sugai and Ohta, 1989; Sugai et al., 1995) used vinylbutanoate as the acylating agent and porcine pancreatic lipase to increase the enantiomeric excess of (*S*)-HEB from 89% to 99.4%. In another approach, benzylamine was used as a nucleophile agent with *Candida antarctica* lipase in dioxane to yield (*R*)-*N*-benzyl-3-hydroxybutyramide at 99% ee (Garcia et al., 1992; Gotor, 1999). Several enzymes and acylating agents have been used to resolve long-chain 3-hydroxyesters (Bornscheuer et al., 1993). However, the best results obtained for (*S*)-3-hydroxyoctanoic acid methylester were 78% ee at 49% conversion.

The aforementioned enzymatic resolution procedures are inadequate for scale-up for several reasons: (i) the low enantiomeric excess achieved; (ii) the large ratio of enzyme to substrate used; and (iii) use of large amounts of solvents resulting in dilute product solutions. The consequences of using dilute solutions include low throughput, difficulty in recovering the product from solvents, and the need to re-

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cycle the solvent. The ability to use concentrated (solvent-free) reaction mixtures increases the volumetric productivity of the reactors and the distillation columns and, therefore, space–time yields are higher and investments in equipment are lower. Omission of solvent from the system is also beneficial from an environmental standpoint.

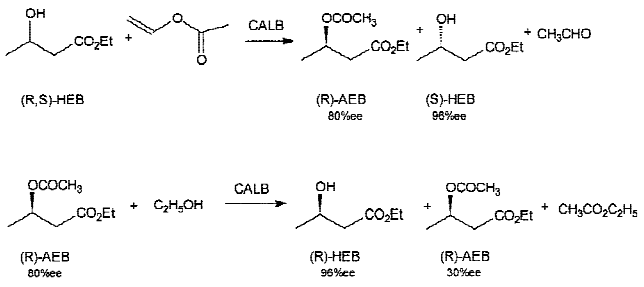
An inherent drawback of resolution processes is that the theoretical yield for a single enantiomer is 50%. To increase the process yield, the undesired enantiomer must be racemized and recycled. Racemization is not always chemically feasible, as in the case of β-hydroxyesters. A second option, where the other enantiomer also has a market, is to recover the opposite enantiomer by hydrolysis of the reacted ester. This is economically feasible only if the optical purity of this ester is high enough. With β-hydroxyesters, this route is problematic due to the difficulty in hydrolyzing selectively the acetoxy derivative without affecting the ester group. As a consequence, the only seemingly effective and selective method for obtaining the second enantiomer is by enzymatic catalysis.

This article describes an efficient two-step process for large-scale production of (*S*) and (*R*)-HEB in high optical purity (>96% ee) (Scheme 1). In the first stage, the racemic HEB is acetylated with vinylacetate in the presence of lipase from *Candida antarctica* B (CALB) to yield 40% (*S*)-HEB. In the second stage, the (*R*)-enriched ethyl-3-acetoxybutyrate (AEB) is reacted with ethanol to give optically pure (*R*)-HEB using the same enzyme. The main features of the process are the exclusion of solvent and the use of the same enzyme (CALB) for both acetylation and alcoholysis steps.

MATERIALS AND METHODS

Enzymes

Lipases from *Burkholderia* sp. (L-1), *Candida rugosa* (L-2), *Pseudomonas* sp. (L-4), *Candida antarctica* A (L-5), *Candida antarctica* B (L-2), *Humicola* sp. (L-8), and pig liver esterase (E-1) were purchased from Roche Diagnostics (formerly Boehringer Mannheim). Liver acetone powders (porcine, bovine, sheep, horse, rabbit) and porcine pancreatic lipase (PPL) were purchased from Sigma. Lipases from *Alcaligenes* (QL and PL) and *Achromobacter sp*. were purchased from Meito Sangyo. Immobilized *Candida antarctica* lipase B (CALB) was purchased from Roche Diagnostics (L-2 c-f C2). The water content of the enzyme was 1% to 2% w/w according to the supplier. The screening kit for the acetylation of HEB with vinylacetate was purchased from Altus Biologics (ChiroScreen TE). All enzymes were used as supplied without further treatment.



Scheme 1. A two-step enzymatic resolution process for production of (*S*)- and (*R*)-HEB.

Reagents

Ethyl-3-hydroxybutyrate (HEB) was purchased from Frutarom (Israel) and contained 0.19% H_2O according to Karl Fischer analysis. Vinylacetate was purchased from Fluka (150 ppm H_2O). Sodium beads were purchased from Aldrich. Diisopropylether (Acros) contained 270 ppm H_2O. All other chemicals used in this work were obtained commercially and were of analytical grade unless otherwise stated.

Analytical Methods

The progress of reactions was followed by gas chromatography (GC) analyses using a Hewlett-Packard 5890 Series II gas chromatograph, equipped with a 15-m capillary column (ID = 0.25 mm) packed with Rtx-1. The temperature program was 60°C (5 min), 4°C min^{-1}, 80°C, 25°C min^{-1}, 300°C. Retention times were 7.7 min for HEB and 11.9 min for AEB.

Calibrated GC analyses were conducted on a Hewlett-Packard 5890 Series II gas chromatograph, using a 15-m Stabilwax column (ID = 0.53 mm). The temperature program was 40°C (8 min), 5°C min^{-1}, 200°C, 25°C min^{-1}, 250°C. Isoamyl alcohol was used as an internal standard. Under these conditions the retention times were 1.7, 7.3, 9.2, 18.8, and 20 min for vinylacetate, ethyl-*trans* crotonate, isoamyl alcohol, HEB, and AEB, respectively.

Gas chromatography–mass spectrometry (GC-MS) analyses were performed on a Hewlett-Packard 5890 Series II gas chromatograph fitted with an HP-5971A mass selective detector, under conditions similar to those of GC analyses.

The %ee of HEB and AEB was determined by high-performance liquid chromatography (HPLC) using a Hewlett-Packard 1050 series instrument equipped with an ultraviolet detector (Model 975, Jasco). The column used was a Chiracel OD from Daicel (10 μm, 4.6 × 250 mm), with λ = 215 nm and flow = 1 mL/min. Eluent composition was: hexane:isopropanol 98:2 (4 min); hexane:isopropanol 85:15 (14 min); hexane:isopropanol 98:2 (20 min). Under these conditions the retention times were: (*R*)-AEB, 8.1 min; (*S*)-AEB, 9.7 min; (*R*)-HEB, 12.6 min; and (*S*)-HEB, 14.9 min.

^{1}H-NMR spectra were recorded on a Varian 500-MHz spectrometer in $CDCl_3$. Optical rotation measurements were done using a digital polarimeter (Model DIP-370, Jasco).

Enzymatic Lab-Scale Reactions

Transesterification reactions of HEB with butanol were performed using 1.5 mmol HEB, 4.5 mmol butanol, 10 mL hexane, and 100 mg enzyme powder. The flasks were shaken at 140 rpm, 35°C, and samples were taken out periodically for analysis.

Screening for a suitable enzyme for the acetylation of HEB with VA was performed with the ChiroScreen-TE kit (Altus Biologics) at room temperature with 0.375 mmol HEB, 0.75 mmol VA, and 2 mL diisopropylether (DIPE). Optimization of parameters in the lab-scale reactions was performed using 10-mL vials (45°C), with magnetic stirring and varying the amounts of reagents. Specific conditions are given in what follows.

Enzymatic alcoholysis of AEB was performed in 10-mL vials containing 5.75 mmol AEB, 5.75 mmol ethanol, 0.25 mL hexane, and 200 mg CALB, with magnetic stirring (45°C), unless otherwise stated.

Preparation of Various β-Hydroxybutyric Acid Esters

Hexyl-, isopropyl-, octyl-, and benyzyl-3-hydroxybutyrate were prepared via a transesterification reaction on the racemic ethyl-3-hydroxybutyrate (HEB), according to the following general procedure.

In a round-bottomed flask were placed HEB (1 mmol), alcohol (5 mmol) and *p*-TsOH · H_2O (0.005 mmol). The reaction mixture was magnetically stirred under reflux. Samples were withdrawn periodically and analyzed by GC. At the end of the reaction (disappearance of HEB), the mixture was cooled to room temperature and evaporated to dryness. The products were purified over a silica gel column (eluent hexane:ethylacetate 7:3) to afford the desired ester, and the identity was verified by NMR and GC-MS.

Synthesis of (*R,S*)-*Tert*-Butyl-3-Hydroxybutyrate

In a 100-mL round-bottomed flask, *tert*-butyl-3-oxobutyrate (9.54 g, 60.4 mmol; Aldrich), was dissolved in MeOH (50 mL) under nitrogen atmosphere with stirring. The reaction mixture was cooled to 0°C in an ice-water bath. $NaBH_4$ (2.74 g, 72.5 mmol) was added in five portions. After the addition was completed the temperature was increased to room temperature. Samples were withdrawn periodically and analyzed by HPLC. At the end of the reaction, the mixture was evaporated to dryness. The residues were dissolved in ethylacetate and washed three times with water. The organic phase was dried over $MgSO_4$, filtered, and evaporated to dryness to afford the product 9.17 g (94.9%).

Enzymatic Acetylation Reactions in a Stirred Enzymatic Reactor

The reactions were performed in a jacketed glass reactor (100 mL) with a sinter (no.1) at the bottom. A glass mechanical stirrer with three blades was used at 300 rpm. Each batch contained 0.23 mol HEB, 0.23 mol VA, and 2.15 g CALB. Each cycle lasted 1.3 h at 45°C. The mixture was filtered through the sinter, and fresh reagents were added to the enzyme.

Enzymatic Bench-Scale Reactions

Bench-scale experiments were carried out in the loop reactor system shown in Figure 1. The bench-scale unit included: (1) a jacketed glass biocatalytic reactor (2-cm internal diameter, 20-cm height) with an internal sintered glass membrane covered by an 8-mm layer of glass beads (425 to 600 μm; Sigma), and the height of the enzyme layer was 14 cm (wetted by vinylacetate); (2) a circulating piston pump (Model QB 108355, Fluid Metering, Inc.); and (3) a 1-L stirred jacketed glass vessel serving as a heater, circulation, storage, and drainage tank. All tubing was Teflon.

Experiments were carried out at 45°C. For the preliminary acetylation experiments (four consecutive cycles) the quantities used were: 4 mol HEB, 4 mol VA, and 38 g CALB. In all other experiments, 6 mol each of HEB and VA were used per cycle.

Enzymatic alcoholysis was performed with 2.7 mol AEB, 2.7 mol ethanol, 116 mL hexane, and 38 g CALB.

Distillation

The distillation of the product obtained from the HEB production unit was performed in a Normschliff distillation column (diameter 80 mm) containing Sulzer CY packing (Hastelloy C-22). According to the manufacturer's data the column is equivalent to ca. 20 theoretical plates.

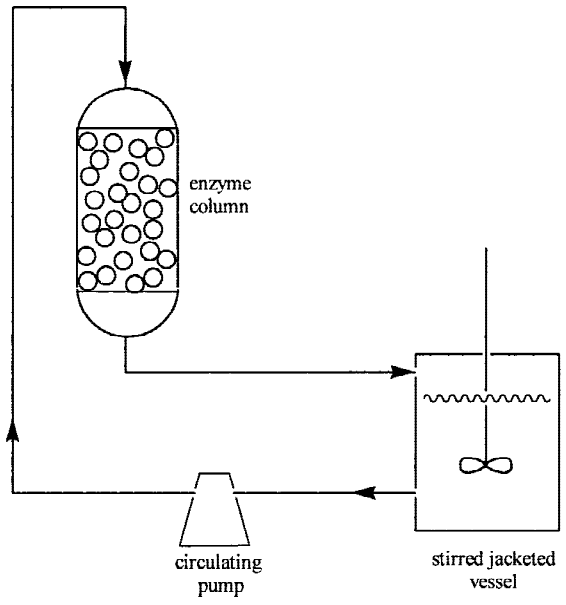


Figure 1. Schematic diagram of the bench-scale unit.

Chemical Hydrolysis

Basic Hydrolysis with SodiumEthoxide

A solution of 2 *M* sodium ethoxide was prepared by dissolving 2.3 g sodium beads in 50 mL ethanol under nitrogen. *R*-enriched AEB (1.5 g) was mixed (magnetic stirring) with 20 mL ethanol in a three-neck round-bottomed flask at room temperature. Sodium ethoxide (4 mL) was added dropwise through a funnel within a period of 4 h. Samples (400 μL) were withdrawn, the ethanol was evaporated, and extraction was performed with 1 mL diethylether and 0.5 mL water. The organic phase (analyzed by GC) contained 35% ethyl *trans*-crotonate (ETC) and 65% HEB.

Acidic Hydrolysis with Hydrogen Chloride

In a 100-mL round-bottomed flask *R*-enriched AEB (5.08 g, 29.16 mmol) was cooled to 0°C in an ice-water bath and 30 mL of 3N HCl was added with stirring. At the beginning, AEB did not dissolve in the aqueous layer; however, dissolution occurred during the course of the reaction. Samples were withdrawn from the reaction mixture, extracted with ethylacetate, and analyzed by GC. After 3 h, the reaction mixture contained 38% HEB (partial hydrolysis), 56% 3-hydroxybutyric acid (full hydrolysis), and 6% AEB. This composition did not change over an additional 48 h.

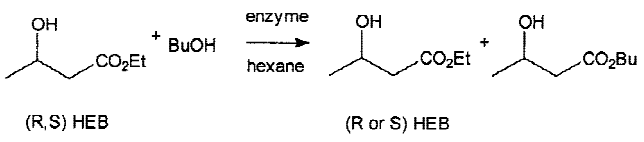
RESULTS AND DISCUSSION

Transesterification with Butanol

The first route to be examined for the resolution of HEB was transesterification with butanol in hexane as a solvent (Scheme 2).

Various enzymes were screened, but none exhibited promising enantioselectivity (Table I). All of the enzymes showed preference toward the (*R*)-enantiomer, except for *Alcaligenes* lipase and *Achromobacter* lipase.

It is likely that two factors contributed to the lack of selectivity: the distance from the chiral center to the ester group, and the ester being a primary functional group. It is well established that enzymes are more selective when acting upon a secondary functional group (Tuomi and Kazlauskas, 1999). Therefore, a different route was considered, namely acetylation of the alcohol moiety with vinylacetate (VA).



Scheme 2. Transesterification of HEB with butanol.

Table I. Screening of enzymes in the transesterification reaction of HEB and butanol.

Enzyme	Conversion[a] (%)	ee[b] (% *R*-butylester)
Burkhoderia sp. lipase	76	0
Candida rugosa lipase	34.1	27
Pseudomonas sp. lipase	38	12
Candida antarctica A lipase	2	
Porcine pancreatic lipase	38.4	8.3
Candida antarctica B lipase	51	31
Humicola sp. lipase	0	
Pig liver esterase	11.3	0
Porcine liver acetone powder	56.3	31
Bovine liver acetone powder	10.5	21
Sheep liver acetone powder	15.4	0
Horse liver acetone powder	39	24
Rabbit liver acetone powder	20.5	59
Alcaligenes lipase-QL	54	22 (*S*-enantiomer)
Achromobacter sp. lipase	7.2	51 (*S*-enantiomer)
Alcaligenes lipase-PL	13.8	43 (*S*-enantiomer)

Reaction conditions: 1.5 mmol HEB, 4.5 mmol butanol, 10 mL hexane, 100 mg enzyme (35°C, 140 rpm).

[a]Determined from GC chromatogram after 44 h. [b]Determined from a chiral HPLC chromatogram.

Acetylation with Vinylacetate

Twenty-five enzymes were screened for their selectivity in the acetylation reaction presented in Scheme 1 (Table II).

Protease from *Bacillus lichenformis* (ChiroCLEC BL) acetylated the (*S*)-enantiomer with good selectivity (66% conversion, 86% ee of R-HEB), and two lipases were found to be selective toward the (*R*)-enantiomer: porcine pancreatic lipase (PPL) and *Candida antarctica* B lipase (CALB). The latter enzyme was much more active (initial specific activities of CALB and PPL were 16.5 and 0.1 μmol/mg · h, respectively) and could be obtained commercially in immobilized form (Roche Diagnostics). Therefore, all subsequent studies were performed with CALB.

Type of Ester

It was of interest to examine the influence of the type of ester on the enantioselectivity of the enzyme. X-ray crystallographic studies indicate that CALB has a limited amount of available space in the active site pocket, and is therefore expected to exhibit a high degree of enantioselectivity (Anderson et al., 1998; Uppenberg et al., 1995). Uppenberg and coworkers, used 1-phenylethanol octanoate to show that only the *R*-enantiomer could form a tetrahedral intermediate allowing for catalysis. Accordingly, CALB acetylated the *R*-enantiomer of all of the esters described in Table III. However, extending the alkyl chain from ethyl to octyl resulted in a decrease in ee, whereas using bulky groups, such as benzyl or *t*-butyl, improved the enantioselectivity of the enzyme.

These results can be rationalized considering the molecular recognition model described for CALB by Rotticci et al.

Table II. Screening of enzymes in the acetylation reaction of HEB and VA.

Enzyme	Conversion[a] (%)	ee[b] (% AEB)
Pig liver esterase	12.5	66 (*R*)
Pseudomonas burkholderia lipase	47.2	58 (*R*)
Candida antarctica A lipase	35	64 (*R*)
Porcine pancreatic lipase	43.5	85 (*R*)
***Candida antarctica* B lipase**	**58**	**87 (*R*)[c]**
Humicola lanuginosa lipase	24	0
Candida rugosa lipase	23	41 (*R*)
α-Chymotrypsin	7.2	26 (*R*)
Penicillin acylase	0	
Aspergillus niger lipase	0	
Mucor meihei lipase	47	78 (*R*)
Candida lypolytica lipase	9	53 (*R*)
Bacillus sp. protease	17.5	58 (*S*)
ChiroCLEC CR	80	0
ChiroCLEC BL	66	71 (*S*)[d]
ChiroCLEC PC	96	0
Rhizopus delemar lipase	63	0
Rhizopus niveus lipase	5	
Rhizopus oryzae lipase	17	39 (*S*)
Geotricum candidum lipase	36	0
Mucor javanicus lipase	4	
Chromobacterium viscosum lipase	7.6	
Alcaligenes sp. lipase	69	0
Aspergillus oryzae protease	7	
Candida rugosa esterase	6	

Reaction conditions: 0.375 mmol HEB, 0.75 mmol butanol, 2 mL DIPE; enzymes from Altus ChiroScreen Kit used as supplied, at room temperature.

[a]Determined from HPLC chromatogram at 72 h. [b]Determined from HPLC chromatogram. [c]Remaining S-HEB >96% ee. [d]Remaining R-HEB 86% ee.

(1998), who investigated the enantioselectivity of this enzyme by performing structure–activity studies as well as molecular modeling. According to their model, the fast-reacting enantiomers bind with the medium-sized substituent (*M*) to a small pocket situated at the bottom of the active site, and with the large-sized substituent (*L*) pointing out toward the surface of the enzyme. In the case of 3-hydroxybutyric acid esters, the (*R*)-enantiomer is the fast-reacting enantiomer. The *M* substituent is the methyl group, and the *L* substituent is the alkyl- or aralkyloxycabonyl methylene group. In a fashion similar to that described by Rotticci et al., variation of the *L* group, from ethoxycarbonyl methylene (in HEB) through *i*-propoxy-, *t*-butoxy-, to benzyloxycarbonyl methylene, did not detract from the high stereoselectivity and reaction rates observed for HEB itself. However, when the alkoxy group was a long-chain hydrocarbon (*n*-hexyloxy- and *n*-octyloxy-), the stereoselectivity (and reaction rate) fell significantly (Table III). Apparently, a long alkyl chain is too large to enable a good fit into the *L* binding pocket, resulting in poor enantioselectivity. The results indicate that the commercially available ethylester is a highly suitable substrate.

Characterization of Reaction Conditions

Various reaction parameters were examined in order to establish the best conditions for the acetylation reaction. The aim was to reach a conversion slightly above 50% with essentially no residual (*R*)-HEB remaining and, consequently, to obtain (*S*)-HEB with high optical purity.

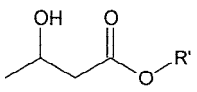
The influence of the solvent type was examined by comparing the rate and enantioselectivity of the enzyme in hexane, diisopropylether (DIPE), and in a neat solution. The results in Table IV show that there was no significant influence of solvent on the reaction, with the reaction rates being similar and, after 3 h, the ee reached >95%. THF and CCl_4 gave similar results. Considering that only four solvents were evaluated in this reaction, a more general conclusion regarding the influence of solvent type on the selectivity of CALB cannot be drawn.

Because the two reactants, HEB and vinylacetate, form a homogeneous nonviscous solution, the addition of a solvent was not necessary, and the high selectivity of the enzyme was maintained in the neat system. The advantages of a solvent-free system are obvious.

The ratio of VA/HEB was also evaluated and the results are presented in Table V. The stoichiometric amount of 0.5 mol VA per 1 mol HEB was adequate to reach a 34% conversion with formation of byproducts. Increasing the molar ratio from 0.75 to 2 produced similar results with the optimum, in terms of activity (60% conversion at 2 h), at a molar ratio of 1. A further increase in the VA:HEB ratio slowed the reaction rate. Consequently, equimolar amounts of VA and HEB, with no added solvent, were used for further work.

As to the level of the enzyme used, the desired time per

Table III. Dependence of CALB enantioselectivity on the ester type.



R′	Ethyl	Isopropyl	Benzyl	*t*-Butyl	Hexyl	Octyl
Conversion[a] (%)	60	65	64	64	63	65
Time (h)	3	6	5	5	8	12
ee[b] (% *S*-hydroxybutyric acid ester)	96	98	100	100	72	30

Reaction conditions: 5 mmol 3-hydroxybutyric acid ester, 5 mmol VA, 50 mg CALB (35°C).
[a]Determined from GC chromatogram. [b]Determined from chiral HPLC chromatogram.

Table IV. Influence of solvents on acetylation of HEB with vinylacetate using CALB.

	No solvent		DIPE		Hexane	
Time (h)	% C[a]	% ee (*S*)[b]	C[a]	% ee (*S*)[b]	C[a]	% ee (*S*)[b]
0.5	31	ND	30	ND	29	ND
1	45.7	ND	45.8	ND	44	ND
2	52	88	49	86	55	95
3	65	>98	57	95	61	97

Reaction conditions: 10 mmol HEB, 10 mmol vinylacetate, 10 mL solvent, 60 mg CALB (45°C). ND, not determined.

[a]Conversion was determined from chiral HPLC chromatogram. [b]Enantiomeric excess (ee) of remaining HEB determined from chiral HPLC chromatogram.

reaction cycle dictated the ratio of enzyme to HEB. An amount of 0.072 g CALB per gram HEB was chosen so that, after 1.3 h, the conversion would reach 60%. A typical reaction profile based on the optimal conditions is presented in Figure 2.

The stability of the enzyme under the reaction conditions was evaluated via its recycle in successive batches. Between cycles, the enzyme was washed with vinylacetate and kept at 4°C. The duration of each cycle was 1.3 h. The enzyme retained its activity during the first 20 cycles. A conversion of 60% was reached with >96% ee of (*S*)-HEB in each batch. At higher conversion rates (>70%) the ee reached >99%. These results were found to be promising for further scale-up.

Stirred Reactor

An obvious and typical technical solution for many biocatalytic processes is a stirred batch reactor. In the case of a suspended immobilized enzyme, the procedure includes filtration of the catalyst and its subsequent recycling for the next batch.

The enzymatic reaction was evaluated in a 100-mL stirred reactor. The process parameters were those used for the laboratory-scale experiments (Fig. 2), with quantities scaled-up by a factor of 10. Using the stirred batch reactor, the reaction was successfully reproduced in the first cycle; that is, it achieved an identical time–conversion profile, and the desired ee of 96% at 60% conversion. Yet, the conversion and ee decreased with the cycle number (55, 50% conversion in the second and third cycles, and 92, 81.5% ee, respectively), most likely because of physical loss of enzyme during filtration and exchange of batches. Furthermore, we observed attrition of the enzyme particles due to the stirring, and large amounts of fines were observed toward the end of the second cycle. Attrition may result in physical loss of enzyme, loss of biocatalytic activity, and complication of the enzyme filtration. Based on these observations, it was decided to examine a batch loop reactor in which the reaction mixture is circulated through a column packed with the immobilized enzyme.

Loop Reactor

The feasibility of carrying out the process in a batchwise manner using a fixed-bed loop reactor (Fig. 1) was tested in a series of four consecutive runs with a fixed amount of enzyme confined to the bioreactor column. The process was scaled-up by a factor of 200 (20 mmol to 4 mol). The reactants were circulated at a rate of 470 mL/min through the enzyme bed. Once every hour, the direction of flow was reversed upward to enable backwashing. No loss of catalyst was observed during backwashing. The volume of the enzyme bed remained constant throughout the experiment. The reaction profile was very similar to that of the laboratory-scale reactions, achieving 60% conversion with 96% ee. However, the time needed to reach that level of conversion was 50% longer, namely 2 h. The enzyme retained its activity during the four consecutive cycles.

Bench-Scale Production

The objective of the bench-scale experiments was to prepare kilogram quantities of (*S*)-HEB, as a scale-up step, and to evaluate the recycling of the enzyme on a minipilot level. The performance of the enzyme using a large scale was comparable with that using the laboratory scale. There was, however, less consistency between the cycles. In the laboratory experiments (20 mmol) the enzyme activity in the first 20 cycles was constant, but, at bench scale, the time to reach 60% conversion gradually increased with the cycle number: 3 h for the first cycle compared with 6.5 h for the

Table V. Influence of VA/HEB molar ratio on acetylation reaction.

	VA/HEB (mol)									
	0.5		0.75		1		2		25[a]	
Time (h)	% C[b]	% ee (*S*)[c]	% C[b]	% ee (*S*)[c]	% C[b]	% ee (*S*)[c]	% C[b]	% ee (*S*)[c]	% C[b]	% ee (*S*)[c]
1	33	46	39.2	63	47.8	80	44.6	70.8	45	70
2	34	46	55	90	60	96	56	90.7	51.7	75
4	34	46	62	97	68	100	58	95	66	>98
6	34	46	65	100	70	100	72	100	70	100

Reaction conditions: 2.25 mmol HEB, 1.13 to 2.25 mmol vinylacetate, 5 mL DIPE, 50 mg CALB (35°C).

[a]VA served as a solvent, no DIPE added. [b]Conversion determined from chiral HPLC chromatogram. [c]Enantiomeric excess (ee) of the remaining HEB determined from chiral HPLC chromatogram.

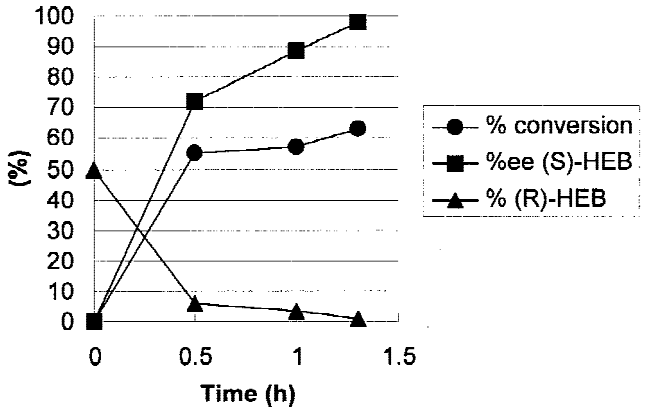


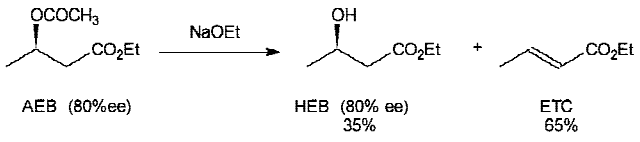
Figure 2. Typical results of an enzymatic acetylation reaction under optimized conditions. The reaction flask contained 20 mmol HEB 20 mmol VA, and 190 mg CALB. The reaction was performed at 45°C.

20th cycle. This may have been be due to longer overall reaction and handling times of the enzymatic reactor, with the latter being left at room temperature between cycles, as compared with more efficient handling in the small-scale studies wherein the enzyme was kept at 4°C between cycles. Thus, with one to two cycles per day, the enzyme was kept at room temperature for about 15 days. Nonetheless, the enzyme was still active after 20 successive cycles and could be used further. Based on the results obtained in the bench-scale experiment, the contribution of the enzyme to the cost of the product is estimated to be, at most, $9/kg product. This represents <5% of the value of the optically pure product.

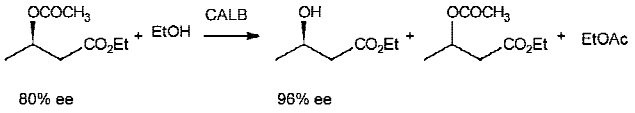
The product was separated from the (*R*)-enriched ester by fractional distillation. Ethyl-*trans* crotonate (ETC), which was present in the starting material (1%), was also separated from the product during the distillation process. The first fraction contained the "lights": excess vinylacetate and acetaldehyde. The next two small fractions contained mainly ETC, followed by the main fraction containing >99% (*S*)-HEB. Following a small mixed fraction (with 55% HEB), AEB was collected for recycling. The yield of the distillation was 75% and the overall yield for production of S-HEB was 33%. This could be expected to reach 40% when the mixed fractions recovered from the distillation column are recycled.

Recycling of (*R*)-AEB

At the end of the acetylation reaction, 60% of the racemic HEB substrate was converted to the acetoxy derivative (Scheme 1). The AEB was enriched with the *R*-enantiomer



Scheme 3. Chemical alcoholysis of AEB.



Scheme 4. Production of (*R*)-HEB via enzymatic alcoholysis of AEB.

(80% ee) and, therefore, there are two possibilities for recycling of this compound:

1. Chemical hydrolysis of the acetate with in situ racemization to obtain racemic HEB.
2. Selective enzymatic hydrolysis to obtain optically pure (*R*)-HEB.

1.*Chemical hydrolysis.* The reactions were performed with sodium ethoxide to avoid hydrolysis of the ethylester group. The main product was the elimination product ETC (65%), whereas HEB was not racemized during the reaction (Scheme 3).

Hydrolysis with hydrogen chloride resulted in formation of the fully hydrolyzed β-hydroxy acid. Thus, it was decided to evaluate the enzymatic route.

2. Enzymatic alcoholysis. To avoid hydrolysis of the ethylester group, alcoholysis in an organic solvent was chosen as the preferred method (Scheme 4). Ethanol was used as the nucleophile, because employment of other alcohols, such as butanol, resulted in transesterification of the ethylester, in addition to the desired alcoholysis of the acetate. CALB was found suitable for this reaction.

An excess of ethanol caused inhibition of the enzyme and a decrease in the reaction rate. Equimolar quantities were optimal. The use of a solvent-free mixture was possible, but extended use of the enzyme in the mixture resulted in degeneration of the enzyme matrix. The addition of solvent to the mixture improved the stability of the enzyme. Hexane was chosen due to the high reaction rate achieved. A volume ratio of 0.25 (hexane/AEB) was sufficient to achieve good results. Under optimal conditions, 55% conversion was

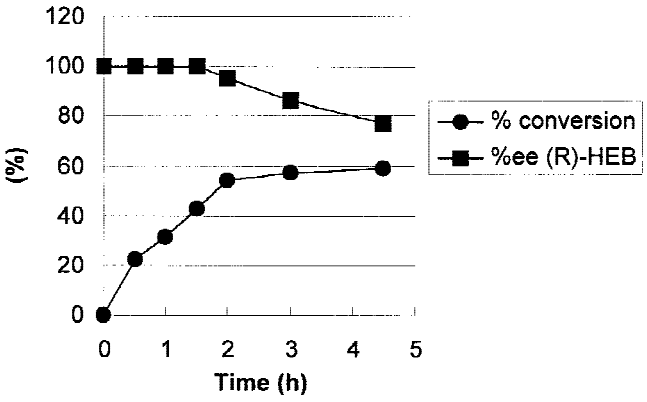


Figure 3. Reaction profile of the enzymatic hydrolysis of AEB. The reaction flask contained 11.5 mmol AEB, 11.5 mmol ethanol, 0.5 mL hexane, and 300 mg CALB. The reaction was performed at 45°C.

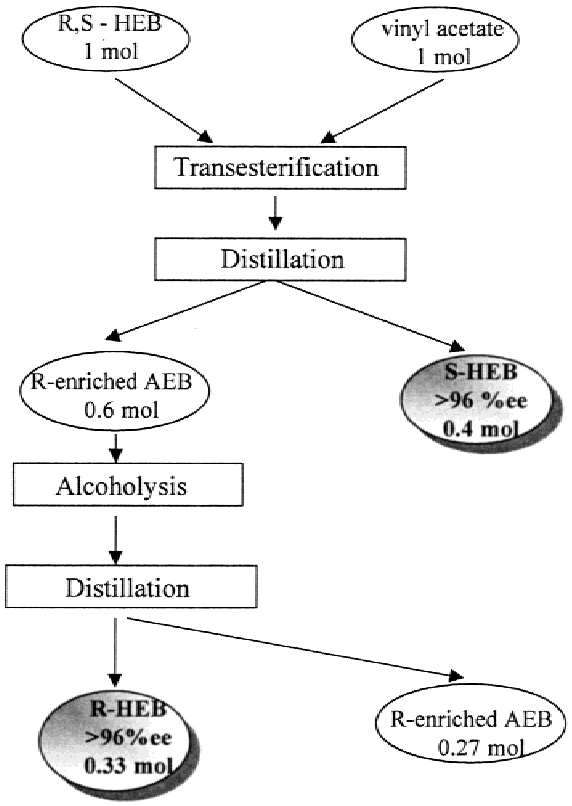


Figure 4. Outline of the process developed for the production of (*S*)- and (*R*)-HEB.

reached in 2 h to give (*R*)-HEB with an ee of 96%. A typical reaction profile is shown in Figure 3.

Based on these results, a bench-scale experiment was carried out using the loop reactor system described previously. Four consecutive runs with 2.7 mol AEB were accomplished, with results comparable to those achieved on a laboratory scale (5.75 mmol AEB). Assuming a similar stability of the enzyme in both acetylation and alcoholysis stages, the overall yield of the process was found to be 73%, as seen in Figure 4.

CONCLUSIONS

An efficient two-step enzymatic resolution process for production of both enantiomers of HEB was developed and scaled-up to the multikilogram scale (Fig. 4). The first reaction involved solvent-free acetylation of HEB with vinylacetate, to produce (*S*)-HEB. In the second reaction, (*R*)-enriched AEB was subjected to alcoholysis with ethanol to afford the optically pure (*R*)-HEB. CALB was utilized in both stages with high productivity and selectivity. Kilogram quantities were prepared using a loop reactor system in which the enzyme was confined to a column reactor and the reagents circulated through the enzyme. This bench-scale unit can be easily scaled-up to produce industrial quantities. The advantages of the process are simplicity, minimal number of reagents, high throughput, mild conditions, high enzyme stability, and productivity, all of which point to economical feasibility.

References

Anderson EM, Larsson KM, Kirk O. 1998. One biocatalyst — many applications: The use of *Candida antarctica* B-lipase in organic synthesis. Biocatal Biotransform 16:181–204.

Bertau M, Burli M, Hingerbuhler E. 1998. Biotechnological pathways to enantiomerically pure compounds on an industrial scale. Chim Oggi 9/10:58–61.

Blacker AJ, Holt RA. 1997. Development of a multi-stage chemical and biological process for an optically active intermediate for an anti-glaucoma drug. In: Collins AN, Sheldrake GN, Crosby J, editors. Chirality in industry II. Chichester, UK: Wiley. p 245–262.

Bornscheuer U, Herar A, Kreye L, Wendel V, Capewell A, Meyer H, Scheper T, Kolisis F. 1993. Factors affecting the lipase catalyzed transesterification reactions of 3-hydroxyesters in organic solvents. Tetrahed Asymm 4:1007–1016.

Bucciarelli M, Davoli P, Forni A, Moretti I, Prati F. 1999. Enantioselective lipase-catalyzed acetylation of β-lactam precursors of carbapenem antibiotics. J Chem Soc Perkin Trans 1:2489–2494.

Garcia MJ, Rebolledo F, Gotor V. 1992. Enzymatic synthesis of 3-hydroxybutyramides and their conversion to optically active 1,3-aminoalchohols. Tetrahed Asymm 3:1519–1522.

Gotor V. 1999. Non-conventional hydrolase chemistry: Amide and carbamate bond formation catalyzed by lipases. Bioorg Med Chem 7: 2189–2197.

Kometani T, Kitatsuji E, Matsuno R. 1989. Baker's yeast mediated bioreduction. A new procedure using ethanol as an energy source. Chem Lett 1465–1466.

Mori K. 1989. Synthesis of optically active pheromones. Tetrahedron 45: 3233–3298.

North M. 1996. Baker's yeast reduction of β-keto esters in petrol. Tetrahed Lett 37:1699–1702.

Noyori R, Ohkuma T, Kitamura M. 1987. Asymmetric hydrogenation of β-keto carboxylic esters. A practical, purely chemical access to β-hydroxy esters in high enantiomeric purity. J Am Chem Soc 109: 5856–5858.

Rotthous O, Krueger D, Demuth M, Schaffiner K. 1997. Reductions of keto esters with baker's yeast in organic solvents — a comparison with the results in water. Tetrahedron 53:935–938.

Rotticci D, Haffner F, Orrenius C, Norin T, Hult K. 1998. Molecular recognition of *sec*-alcohol enantiomers by *Candida antarctica* lipase B. J Mol Catal B Enzymes 5:267–272.

Sugai T, Ohta H. 1989. Enzymatic preparation of ethyl (*S*)-3-hydroxybutanoate with high enantiomeric excess. Agric Biol Chem 53:2009–2010.

Sugai T, Tsuchiya S, Mochizuki N, Ohta H. 1995. Biocatalysis-mediated preparation of optically active hydroxy acids. Yuki Gosei Kagaku Kyokaishi 51:32–42.

Tuomi W, Kazlauskas R. 1999. Molecular basis for enantioselectivity of lipase from *Pseudomonas cepacia* toward primary alcohols. Modeling, kinetics, and chemical modification of Tyr29 to increase or decrease enantioselectivity. J Org Chem 64:2638–2647.

Uppenberg J, Ohrner N, Norin M, Hult K, Kleywegt GJ, Patkar S, Waagen V, Anthonsen T, Jones A. 1995. Crystallographic and molecular-modeling studies of lipase B from *Candida antarctica* reveal a stereospecific pocket for secondary alcohols. Biochemistry 34: 16838–16851.

Wendhausen R, Moran PJS, Joekes I, Rodrigues AR. 1998. Continuous process for large-scale preparation of chiral alcohols with baker's yeast immobilized on crysotile fibers. J Mol Catal B Enzymes 5:69–73.