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Enzymatic resolution of a chiral chlorohydrin precursor for (*R*)-α-lipoic acid synthesis *via* lipase catalyzed enantioselective transacylation with vinyl acetate

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ABSTRACT

A new and efficient process was developed by lipase-catalyzed transacylation to resolve ethyl 8-chloro-6-hydroxy octanoate (ECHO) to produce an important chiral precursor for the synthesis of (*R*)-α-lipoic acid. After optimization of biocatalyst, solvent, acyl donor, temperature and enzyme loading, (*S*)-*O*-acetylated ECHO was achieved in 94% *ee*, 35% isolated yield and 38 g L^{-1} d^{-1} space-time yield using Novozym 435 as biocatalyst. Subsequently, the enzymatic resolution reaction was successfully repeated for 7 batches, retaining over 40% conversions.

1. Introduction

α-Lipoic acid, an excellent antioxidant widely used in pharmaceuticals, health products and skin conditioners, has been recognized as a B-family vitamin involved in the biochemical decarboxylation of α-keto acids and as a growth factor for certain microorganisms. Despite generally commercialized as racemate, the anti-oxidation activity of α-lipoic acid was evidenced to reside in its (*R*)-enantiomer [1] which occurs naturally in mitochondrial complexes. With the physiological function of regulating neuronal calcium homeostasis [2] and pro-inflammatory cytokines as well as altering the expression of "toxic genes", (*R*)-α-lipoic acid has been widely used to treat diabetes [3,4], liver diseases [5], radiation injury [6] and recommended as a "neuroprotective agent" [7].

Since the first isolation and identification of α-lipoic acid from natural sources, several routes for its synthesis by chemical methods have been developed, *e.g.*, from adipate, cyclohexanone and their derivants [8]. With the growing demand for enantiomerically pure active pharmaceutical ingredients, great attention has been paid to the stereoselective synthesis of optically pure (*R*) or (*S*)-enantiomer, usually via asymmetric synthesis from its precursor [9]. Enzyme-catalyzed organic reactions have provided a great impetus to organic synthesis these years [10–13] for several advantages, such as mild and environmentally benign reaction conditions and remarkable chemo-, regio-, and stereoselectivities. (*R*)-α-Lipoic acid has been obtained through kinetic resolution of racemate by enzymatic esterification of the carboxylic group that is 4 carbon atoms away from the stereogenic center [14,15]. However, enzymatic resolution involving a remote chiral center represents a tough task in practice with a poor enantioselectivity ($E < 5$).

In this study, we proposed an alternative route for enzymatic resolution of racemic ethyl 8-chloro-6-hydroxy octanoate (ECHO), a key chlorohydrin precursor for (*R*)-α-lipoic acid (Scheme 1) [16]. Lipases are well known for their low cost and great tolerance against various substrates and reaction solvents, and lipase-catalyzed acylation of the hydroxyl group represents an important class of enzymatic transformations in organic synthesis. Considering these merits, a new method for resolving racemic ECHO is reported herein through direct transacylation of the chiral hydroxyl group, instead of esterifying the remote carboxylic group of ECHO, using a commercially available immobilized lipase, Novozym 435, under the optimal conditions which are described in details.

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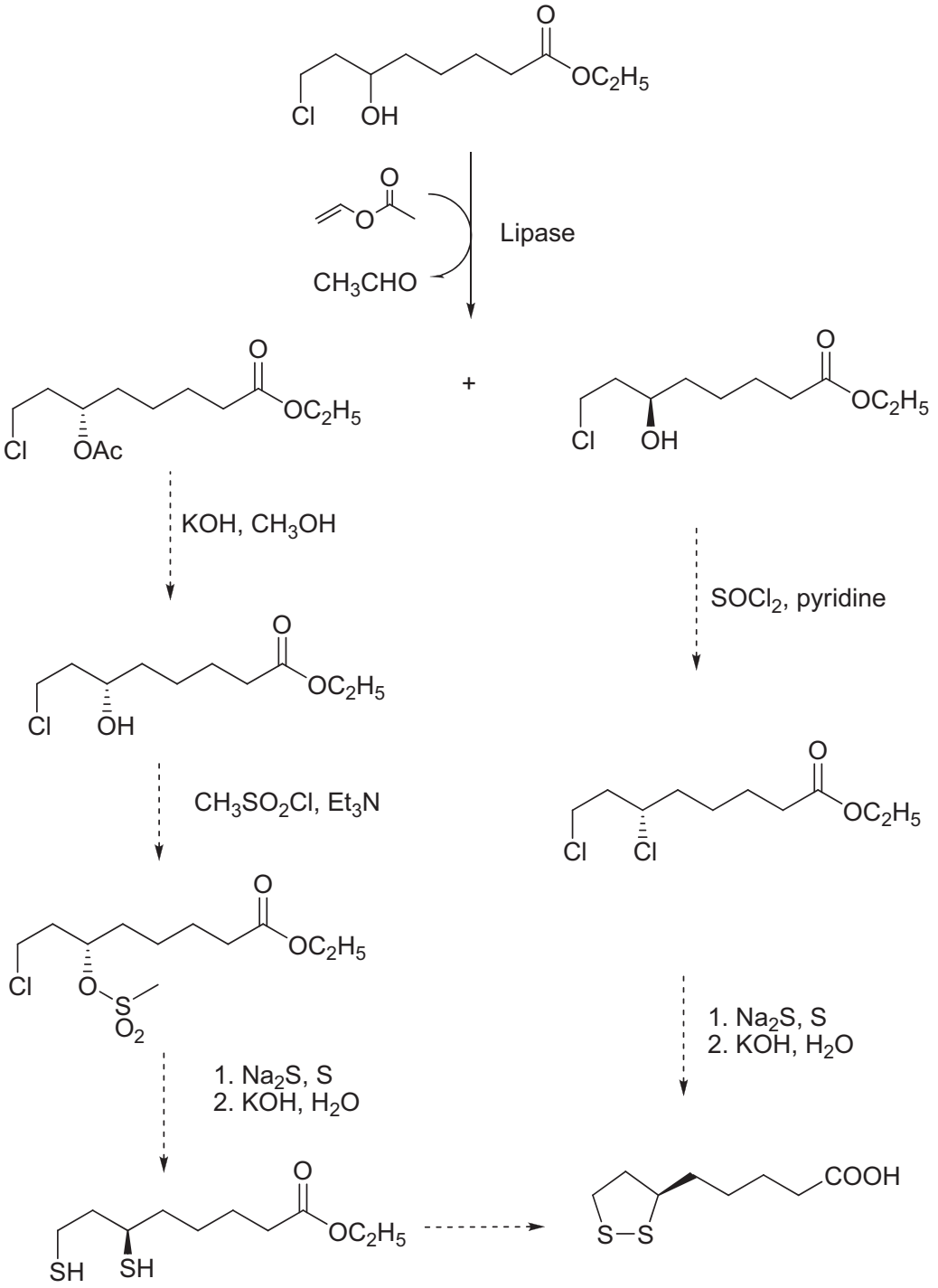

Scheme 1. The synthetic route of (*R*)-α-lipoic acid from racemic ethyl 8-chloro-6-hydroxy octanoate.

2. Experimental

2.1. Enzymes and reagents

Novozym 435 (lipase B from *Candida antarctica*) and Lipozym TLIM (from *Thermomyceslanuginosus*) were purchased from Novozymes Co. (Denmark). Lipase CRL (from *Candida rugosa*) and lipase PPL (from porcine pancreas, Type II) were purchased from Sigma (St. Louis, MO). Lipase AYS, lipase AH, lipase PS-C, lipase PS-D, lipase AK, lipase AS, lipase D and lipase OF were purchased from Amano Enzymes Co. (Japan). Lipases from *Pseudomonas fluorescens*, *Pseudomonas putida* and *Bacillus subtilis* were obtained from our laboratory. Racemic ethyl 8-chloro-6-hydroxyoctanoate was kindly provided by Fushilai Medicine & Chemical Co., Ltd. (Changshu, Jiangsu, China). All other chemicals used in this work were of analytical grade from local supplies, and were dried with molecular sieve 3 Å before using.

2.2. General procedure for lipase-catalyzed transacylation

To optimize the reaction conditions, enzymatic reactions were usually performed in shake flasks on a shaker. In a typical reaction,

a certain amount of (*R*,*S*)-ECHO and proportional amount of vinyl acetate were added into 10 ml solvent contained in a 100-ml shake flask. After the enzyme was added, the mixture was sealed and shaken at 200 rpm in an incubator. The detailed conditions were listed in the caption of figures or footnote of tables.

2.3. Repeated batch reactions

Racemic ECHO (50 mM), vinyl acetate (60 mM) and diisopropyl ether (DIPE, 50 ml) were added into a 100-ml three-necked flask, and then preheated to 40 °C. After 0.5 g of carrier-bound lipase (Novozym 435) was added, the mixture was mechanically stirred (100 rpm) at 40 °C. The reaction was terminated at about 42% conversion by filtration of the immobilized enzyme. The filtered enzyme was washed with DIPE (25 ml × 2) after each use, and then added into a fresh reaction mixture for the next batch reaction.

2.4. Enzymatic transacylation in a preparative scale

A mixture of ECHO (50 mM), vinyl acetate (60 mM) and DIPE (1 L) was added into a 2-L three-necked flask fitted with reflux condenser and mechanical stirrer (100 rpm), and then preheated to 40°C with a temperature-controlled water bath. The reaction was initiated by addition of 10 g Novozym 435 and incubation at 40 °C. The reaction was terminated when the conversion reached 42%. After removing the enzyme from the reaction mixture by filtration, substrate and product were separated by column chromatography on silica gel using petroleum ether/ethyl acetate (10:1, v/v) as eluent, and recovered by removal of the solvent under reduced pressure and dried under vacuum. The product was characterized by ^{1}H NMR, ^{13}C NMR and mass spectrum. The optical purity of product was determined by chiral gas chromatography and polarimetry.

2.5. Analysis methods

Enantiomeric excess of substrate (ee_s) was measured by gas chromatography with chiral column after pre-derivation as described below. The pre-derivation of samples (500 μl) was performed by evaporating the solvent (DIPE) and then reacting with 100 μl propionic andydride and 20 μl pyridine for 2 h in boiling water bath. The mixture subsequently was washed with 2 M H_2SO_4 (300 μl) and water (200 μl × 3), diluted with 400 μl ethyl acetate, followed by drying over anhydrous Na_2SO_4. Enantiomeric excess of product (ee_p) and ee_s were analyzed by gas chromatography with chiral column directly, and conversion of substrate was calculated with ee_s and ee_p.

Gas chromatography was performed on Shimadzu GC-14C (Kyoto, Japan), equipped with FID detector and high-resolution CP-ChiralSil-DEX CB chiral column (Varian, 25 m × 0.25 mm × 0.25 μm) using N_2 as carrier gas. The column, injector and detector temperatures were set at 155, 280 and 280 °C. Retention times: (*R*)-*O*-acetylated ECHO, 16.7 min; (*S*)-*O*-acetylated ECHO, 17.4 min; (*R*)-*O*-propionylated ECHO, 24.9 min; (*S*)-*O*-propionylated ECHO, 25.7 min.

3. Results and discussion

3.1. Screening of biocatalysts

During the last few decades, lipases have been applied as powerful tools in numerous of organic synthesis because of their excellent activity and robustness. More importantly, they could smoothly catalyze reactions without addition of expensive cofactor and could be reused after immobilization, which makes them commercially available at competitive prices. Initially, several lipases were therefore investigated for the resolution of racemic ECHO using vinyl acetate as both acyl donor and reaction medium (Table 1). Among the lipases tested, Novozym 435 exhibited relatively high transacylation ability and the highest enantioselectivity (E = 34) towards the *rac*-ECHO, and the E value of the reaction under arbitrarily chosen reaction conditions was much higher compared to previous reports about direct resolution of α-lipoic acid *via* esterification with alcohol (E < 5) [15]. Thus, Novozym 435, a commercial immobilized lipase, was chosen as the preferable catalyst for further study.

Table 1
Screening of enzymes for transacylation of racemic ECHO with vinyl acetate.[a]

Entry	Enzyme	Time [h]	Conv. (%)	ee_p (%)	E[d]
1	Novozym 435	48	49	86 (*S*)	34
2	Lipase MY	72	30	66 (*R*)	6
3	Lipase CRL	72	26	67 (*R*)	6
4	Lipase AYS	72	22	59 (*R*)	5
5	Lipase AK	24	48	44 (*S*)	4
6	Lipozym TLIM	72	28	29 (*S*)	2
7	Lipase PS-C	12	54	19 (*S*)	2
8	Lipase PS-D	48	48	20 (*S*)	2
9	Lipase AH	72	12	15 (*R*)	1
10	Lipase AS	72	n.a.[b]	n.d.[c]	n.d.
11	Lipase D	72	n.a.	n.d.	n.d.
12	Lipase OF	72	n.a.	n.d.	n.d.
13	*P. putida* lipase	72	8	59 (*S*)	4
14	*B. subtilis* lipB	72	13	49 (*S*)	3
15	*P. fluorescens* lipase	72	33	29 (*S*)	2

[a] Reactions were performed at 30 °C with 10 mM racemic ECHO, 50 mg enzymes in 10 ml vinyl acetate.
[b] No activity.
[c] Not determined.
[d] Enantioselectivity (E) = ln[1 − c(1 + ee_p)]/ln[1 − c(1-ee_p)], which was calculated according to Chen et al. [17].

3.2. Effect of acyl donor

It has been demonstrated that acyl donor has great influence on both enantioselectivity and reaction rate [18]. Preliminary experiment showed that no esterification products were detected when acetic acid was used as an acyl donor. Therefore, a variety of acyl donors including esters and anhydrides were examined and summarized in Table 2. Obviously, transacylation of ECHO using esters as acyl donor gave higher conversion and better stability than anhydrides. The highest reactivity and stability were achieved in the transacylation of ECHO using vinyl acetate as acyl donor. In case of enol ester as an acyl donor, the lipase-catalyzed transacylation with a secondary alcohol proceeded more easily, affording the corresponding ester of the chiral alcohol and vinyl alcohol. The latter can escape from the reaction system in the form of acetaldehyde after isomerization, therefore making the reaction irreversible. Although 8-chloro-6-hydroxy octanoate has an acyl group as well as a hydroxyl group, no side reaction, such as intramolecular cyclization and polymerization, was observed in all the cases, based on the GC spectra of reaction samples. Hence, vinyl acetate was used as the acyl donor for the subsequent experiments

3.3. Effect of solvent

For enzymatic resolution of racemic ECHO in an organic solvent system, a careful investigation on the reaction medium was conducted since it has been demonstrated that the activity and enantioselectivity of lipases are greatly affected by the nature of the non-aqueous solvents used when catalysis occurs in a nearly anhydrous environment [19]. In this work, effect on the reaction of six hydrophobic solvents was evaluated, and the highest conversion (43%) was observed in DIPE (Table 3). The poor solubility of ECHO in isooctane and heptane was suspected to account for the moderate conversion and enantioselectivity. A suitable organic

Table 2
Effect of acyl donor on enzymatic transacylation of ECHO.[a]

Acyl donor		1st batch		2nd batch		Stability (%)[b]
		Conv. (%)	ee_p (%)	Conv. (%)	ee_p (%)	
Ester	Vinyl acetate	43	94	41	95	95
	Vinyl propionate	29	96	25	96	86
	Vinyl *n*-butyrate	34	97	24	98	71
Anhydride	Acetic anhydride	19	94	15	93	79
	Propionic andydride	18	81	9	59	50
	Butyric anhydride	25	99	14	>99	56
	Isobutyric anhydride	17	>99	9	>99	53
	Pentanoic anhydride	13	>99	12	>99	92

[a] Reactions were performed at 40 °C for 7 h with 50 mM racemic ECHO, 60 mM acyl donors and 50 mg Novozym 435 in 10 ml DIPE.
[b] Ratio of ECHO conversion in the 2nd batch reaction to that in the 1st batch.

Table 3
Effect of solvent on the enzymatic transacylation of ECHO with vinyl acetate.[a]

Solvent	1st batch		2nd batch		Stability (%)[b]
	Conv. (%)	ee_p (%)	Conv. (%)	ee_p (%)	
Diisopropyl ether	43	94	41	95	95
Methyl tertiary butyl ether	38	97	34	99	89
Isooctane	35	98	22	98	63
n-Heptane	28	93	19	93	68
Toluene	15	99	13	97	87
Butyl acetate	11	94	10	91	91

[a] Reactions were performed at 40 °C for 7 h with 50 mM *rac*-ECHO, 60 mM vinyl acetate and 50 mg Novozym 435 in 10 ml various solvents.
[b] Ratio of ECHO conversion in the 2nd batch reaction to that of the 1st batch.

solvent should dissolve enough substrates for the lipase-catalyzed transacylation, and the solvent should not affect the lipase activity and stability significantly. The results showed that Novozym 435 generally exhibited high conversion in non-polar solvents but no conversion was observed in hydrophilic solvents such as acetonitrile, DMSO and alcohols (data not shown) which might alter or denature enzymes by stripping off the so-called "essential water" from the enzyme [20], and alter the native conformation of the lipase by disrupting hydrogen bonding and hydrophobic interactions, thereby leading to very low activity which greatly limits the application of enzymatic procedures in this area. The reaction gave higher ee_p in MTBE than DIPE, but lower conversion and worse stability. DIPE therefore was finally considered as the best medium for the biocatalytic resolution of racemic ECHO, with an increased *E* value of this reaction from 35 to 69.

3.4. Effect of reaction temperature

Temperature is one of the important factors which influence enzyme activity. The effect of reaction temperature on the enzymatic transacylation of ECHO was investigated over a range from 20 °C to 50 °C. As shown in Table 4, it is demonstrated that the reaction rate increased with the rising of temperature while enantioselectivity varied slightly. However, higher temperature (over 40 °C) would result in a relatively rapid loss of enzyme stability. Indeed, above a certain temperature, enzyme inactivation occurs, and the stability decreases. Comparing the results of 40 and 50 °C, we found that the conversion at 50 °C was higher than that at 40 °C, however, after 2 batches use, its relative conversion attained 95% at 40°C, while 73% at 50 °C. This was because high temperature (50 °C) could deactivate the Novozym 435. Considering the activity and stability of Novozym 435, we adopted 40 °C for further experiments in this work.

3.5. Effect of enzyme loading

The amount of enzyme loading is a crucial economical factor for successful practical application. Therefore, the effect of enzyme loading on the initial rate of transacylation was examined with varied amounts of enzyme ranging from 1 to 25 g L^{-1} using DIPE as the reaction medium at 40 °C. As shown in Fig. 1, the initial rates of enzymatic transacylation of ECHO depended linearly on the amount of enzyme applied, but further increase of enzyme loading from 10 g L^{-1} to 25 g L^{-1} resulted in only slight improvement. It demonstrated the saturation of the combination between the enzyme and substrate, and extra addition of enzyme will not lead to notable acceleration of reaction further. Due to the obvious adsorption of substrate by the immobilization

Table 4
Effect of temperature on enzymatic transacylation of ECHO.[a]

Temperature (°C)	1st batch		2nd batch		Stability (%)[b]
	Conv. (%)	ee_p (%)	Conv. (%)	ee_p (%)	
20	24	98	22	98	92
30	28	97	25	94	89
40	43	94	41	95	95
50	60	67	44	96	73

[a] Reactions were performed at different temperatures for 7 h with 50 mM *rac*-ECHO, 60 mM vinyl acetate and 50 mg Novozym 435 in 10 ml DIPE.
[b] Ratio of ECHO conversion in the 2nd batch reaction to that of the 1st batch.

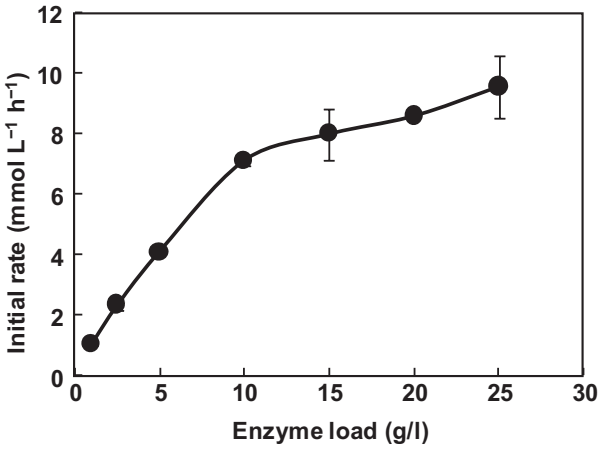

Fig. 1. Initial rate of the enzymatic transacylation of ECHO with vinyl acetate at varied enzyme loads. Reactions were performed at 40° C for 0.5 h with 50 mM racemic ECHO, 60 mM vinyl acetate and Novozym 435 of varied loadings in 10 ml DIPE.

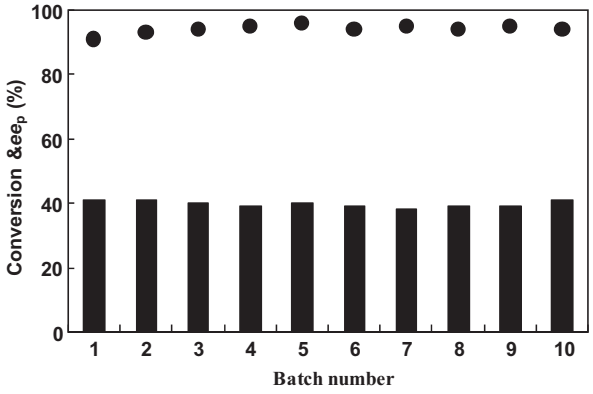

Fig. 2. Repeated batch reactions of enzymatic transacylation of ECHO. Reactions were performed at 40° C with 50 mM racemic ECHO, 60 mM vinyl acetate and 0.5 g Novozym 435 in 50 ml DIPE until the conversion reached 40%. Symbols: (solid bar) conversion (solid circle) ee_p.

carriers of enzyme in the presence of over-dose of Novozym 435 (data not shown) and from the economic point of view, an enzyme loading of 10 g L^{-1} was adopted in the following repeated-batch reactions.

3.6. Gram scale synthesis of (S)-O-acetylated ECHO

The enzymatic transacylation of ECHO (50 mM) was preliminarily scaled up by 100-fold to a reaction volume of 1 L in a 2-L three-necked flask with mechanic agitation. The reaction was monitored by GC analysis, and stopped at 3.5 h with a conversion of 42%. The space-time yield of (*S*)-acetylated ECHO was 38 g L^{-1} d^{-1}. After isolation and purification by silica gel column and normal work-up, 4.9 g of product was obtained with 94% *ee* and 35% isolated yield; $[a]_D^{25}$: +21.7 (*c*1.01, $CHCl_3$) [16]. ^{1}H NMR (500 MHz, $CDCl_3$) δ 5.04-4.99 (m, 1H),δ 4.14-4.08 (q, 2H, *J* = 7.1Hz), δ 3.56-3.47 (m, 2H), δ 2.30-2.27 (t, 2H, 7.4Hz), δ 2.04 (s, 3H), δ 2.02-1.96 (m, 2H), δ 1.69-1.52 (m, 4H), δ 1.40-1.29 (m, 2H), δ 1.26-1.23 (t, 3H, 7.2 Hz); ^{13}C NMR (125 MHz, $CDCl_3$) δ = 174.09 (C═O), 171.26 (C═O), 72.04 (CH), 60.93 (CH_2), 41.41 (CH_2), 37.79 (CH_2), 34.78 (CH_2), 34.45 (CH_2), 25.38 (CH_2), 25.33 (CH_2), 21.75 (CH_3), 14.91 (CH_3); MS (ESI): *m*/*z* = 265.12 (M^++1), 207.09, 205.09, 169.12.

3.7. Operational stability

One of the most important characteristics to evaluate feasibility of an immobilized enzyme is its operational stability and reusability over an extended period of time in practical application. From the viewpoint of process economics, the more the number of batches an enzyme is used, the more economical the process is. An optimal solvent system for lipase-catalyzed transacylation should not only increase the lipase activity toward ECHO but also not impair the stability of lipase during repeated use. Experiments were performed to examine the stability of Novozym 435. Therefore, the operational stability of Novozym 435 was evaluated by reusing in the transacylation of ECHO with vinyl acetate. As shown in Fig. 2, the enzyme retained its high activity for 7 batches of transacylation at an ECHO concentration of 50 mM. Although the conversion still kept 40%, the reaction time was lengthened after 8 batches reaction, indicating that the enzyme activity started to decline. During the 10 batches of reaction, the conversions were kept over 40% and (*S*)-*O*-acetylated ECHO was produced with an improved ee_p from 91% to 96%. To the best of our knowledge, this is the first time that bioresolution reaction of α-lipoic acid precursor was achieved in repeated operation.

Although the space-time yield of (*S*)-*O*-acetylated ECHO decreased slightly to 23.3 g L^{-1} d^{-1} because of the enzyme inactivation during the last 3 batches, the ratio of substrate to catalyst (S/C, g/g) was markedly enhanced from 1 to 11.

4. Conclusions

A new route for synthesis of a chiral chlorohydrin precursor for (*R*)-α-lipoic acid was established by enzymatic enantioselective transacylation of ECHO. After determining Novozym 435 as the most suitable catalyst, the biocatalytic reaction system was carefully optimized for optical resolution, resulting in vinyl acetate as acyl donor, DIPE as solvent, reaction temperature of 40 °C and enzyme loading of 10 g L^{-1}. The space-time yield of (*S*)-*O*-acetylated ECHO reached 38 g L^{-1} d^{-1} with ee_p of 94% for the gram-scale synthesis. Under the optimized conditions, Novozym 435 could be reused for 7 batches with conversions of over 40% which led to an increase of S/C from 1 to 11. This study suggests the potential applicability of enzymatic resolution for the preparation of (*R*)-α-lipoic acid precursor in terms of environmental friendliness and operational simplicity in a technical scale.

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References

[1] I. Gunsalus, L.S. Barton, W. Gruber, J. Am. Chem. Soc. 78 (8) (1956) 1763–1766.
[2] L. Packer, H.J. Tritschler, K. Wessel, Free Radic. Biol. Med. 22 (1) (1997) 359–378.
[3] A. Faust, V. Burkart, H. Ulrich, C.H. Weischer, H. Kolb, Int. J. Immunopharmacol. 16 (1) (1994) 61–66.
[4] P. Ou, H.J. Tritschler, S.P. Wolff, Biochem. Pharmacol. 50 (1) (1995) 123–126.
[5] A. Marshall, R. Graul, M.Y. Morgan, S. Sherlock, Gut 23 (12) (1982) 1088–1093.
[6] N. Ramakrishnan, W.W. Wolfe, G.N. Catravas, Radiat. Res. 130 (3) (1992) 360–365.
[7] S. Stoll, H. Hartmann, S. Cohen, W. Müller, Pharmacol. Biochem. Behav. 46 (4) (1993) 799–805.
[8] D.S. Acker. (1957) US Patent 2792406.
[9] R. Zimmer, U. Hain, M. Berndt, R. Gewald, H.U. Reissig, Tetrahedron: Asymm. 11 (4) (2000) 879–887.
[10] A.M. Klibanov, Nature 409 (6817) (2001) 241–246.

[11] K. Griebenow, A.M. Klibanov, J. Am. Chem. Soc. 118 (47) (1996) 11695–11700.
[12] A.M. Klibanov, Trends Biochem. Sci. 14 (4) (1989) 141–144.
[13] A.M. Klibanov, Trends Biotechnol. 15 (3) (1997) 97–101.
[14] N. Fadnavis, K. Koteshwar, Tetrahedron: Asymm. 8 (2) (1997) 337–339.
[15] H. Yan, Z. Wang, L. Chen, J. Ind. Microbiol. Biotechnol. 36 (5) (2009) 643–648.
[16] G. Rainer, L. Gunter, B. Thomas. (1998) US5731448.
[17] C.S. Chen, Y.F. Fujimoto, G. Girdaukas, C.J. Sih, J. Am. Chem. Soc 104 (1982) 7294–7299.
[18] J. Xu, T. Kawamoto, A. Tanaka, Appl. Microbiol. Biotechnol. 43 (3) (1995) 402–407.
[19] S. Tawaki, A.M. Klibanov, J. Am. Chem. Soc. 114 (5) (1992) 1882–1884.
[20] A. Zaks, A.M. Klibanov, J. Biol. Chem. 263 (7) (1988) 3194–3201.