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Enantioselective production of (*S*)-1-phenyl-1,2-ethanediol from dicarboxyesters by recombinant *Bacillus subtilis* esterase

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ABSTRACT

The whole cells of recombinant *Escherichia coli* BL21 overexpressing a *Bacillus subtilis* esterase (BsE) were utilized to sequentially hydrolyze the dicarboxyester of 1-phenyl-1,2-ethanediol for production of (*S*)-1-phenyl-1,2-ethanediol (PED), exhibiting high hydrolytic activity, excellent regio- and enantioselectivities towards the dicarboxyester of PED. Among the dicarboxyesters with different acyl chains (*e.g.*, acetyl, *n*-butyl, and *n*-hexyl), the best enantioselectivity ($E = 176$) was observed when PED diacetate was employed as the initial substrate. Various reaction conditions were systematically investigated for enantioselective hydrolysis of PED diacetate. Under the optimal reaction conditions, kinetic resolution of 100 mM PED diacetate resulted in 49% conversion within 1 h, affording (*S*)-PED in 96% *ee*. A 150-ml scale reaction was performed, affording (*S*)-PED in 49% yield and 95% *ee*. After recrystallization in chloroform, the optical purity of (*S*)-PED was improved up to >99% *ee*, with a total yield of 45%. These results imply that this recombinant esterase (BsE) is a potentially promising biocatalyst for bioproduction of (*S*)-PED, an important chiral building block with wide application in pharmaceutical industry and liquid-crystal display materials.

1. Introduction

Optically pure 1,2-diols are important precursors and intermediates for a variety of synthetic applications [1]. Among these 1,2-diols, enantiomerically pure 1-phenyl-1, 2-ethanediol (PED) is widely used in synthesis of chiral catalysts [2,3], macrocyclic polyether [4], pharmaceutically active compounds [5] and liquid crystals [6]. Thus, preparation of such chiral entities has triggered modern researchers to investigate various synthetic methods, including chemocatalysis and biocatalysis.

As PED has two hydroxyl groups, it needs selective protection of one hydroxyl to prepare optically pure PED with chemical methods, which takes more synthetic steps and leads to more pollution. Therefore, bioproduction of optically pure (*R*) or (*S*)-PED becomes more and more attractive. Biocatalytic methods, such as bioreduction of α-hydroxy ketones [7–10], hydrolysis of epoxides with epoxide hydrolases [11–13] and lipase-mediated kinetic resolution of vicinal diols or esters [14–19], have been applied for the synthesis of enantiomerically pure 1,2-diols. However, microbial reduction involves in complicated cofactors regeneration, since most of oxidoreductases are cofactor-dependent. Although epoxide hydrolases are cofactor-independent, they usually produce (*R*)-diols, which leads to less variety. Optically active (*S*)-PED can also be obtained by lipase-mediated transesterification in organic solvents, though direct transesterification of 1,2-diols is less effective in most organic solvents, giving the corresponding monoacetate with poor enantioselectivity in the case of a number of lipases tested [20–22].

Esterases (E.C. 3.1.1.1) and lipases (E.C. 3.1.1.3) are a class of versatile biocatalysts for the preparation of chiral compounds in industry as they are stable, frequently exhibit high activities as well as chemo-, regio- or stereoselectivity, and do not require expensive cofactors [23,24]. In the work described herein, whole cells of recombinant *Escherichia coli* BL21 overexpressing an esterase from *Bacillus subtilis* ECU0554 (BsE) were evaluated for (*S*)-PED formation in aqueous medium through sequential hydrolysis of corresponding dicarboxyester. The recombinant BsE showed high hydrolytic activity, excellent regio- and stereoselectivity for the production of optically pure (*S*)-PED. Some dicarboxyesters with different acyl chains (acetyl, *n*-butyl and *n*-hexyl) were employed as substrate. Various reaction conditions were systematically investigated for enantioselective hydrolysis of PED diacetate.

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2. Experimental

2.1. Microorganisms and materials

The wild-type strain, *B. subtilis* ECU0554, was isolated and identified in our previous work [25]. Recombinant *E. coli* BL21 over-expressing BsE was constructed as described previously [26].

(*R*,*S*)-PED was purchased from Yueyang Yetop Fine Chemicals Co. (Hunan, China). Acetic anhydride, *n*-butyric anhydride and *n*-hexanoic anhydride were purchased from Alfa Aesar (Tianjin, China). All other chemicals were also commercially available, with purity of analytic grade.

2.2. General procedure for chemical synthesis of dicarboxyesters

To a certain anhydride (3 equiv.), was added 5.52 g (0.04 mol) of (*R*,*S*)-PED, and the mixture was refluxed. The reaction was stopped until (*R*,*S*)-PED was completely consumed as monitored by TLC (a mixed solvent of petroleum ether and ethyl acetate was employed as the mobile phase. The volume ratios of petroleum ether to ethyl acetate for **1a**, **2a** and **3a** were respectively 10:1, 15:1, 20:1, and their corresponding R_f values were 0.7, 0.85, 0.9).

The product was extracted into ethyl acetate (100 ml 3×), then washed with an aqueous solution of saturated sodium bicarbonate (100 ml 3×) and dried over anhydrous sodium sulfate. The organic solvent was evaporated under reduced pressure and the product was dried under vacuum.

*(R,S)-1-Phenyl-1,2-ethanediol diacetate (**1a**)*: ^{1}H NMR (300 MHz, $CDCl_3$), δ/ppm: 2.10 (s, 6H), 4.10 (dd, 1H, J = 11.89, 8.68 Hz), 4.20 (dd, 1H, J = 11.89, 3.39 Hz), 4.90 (dd, 1H, J = 8.25, 3.39 Hz), 7.2–7.4 (m, 5H).

*(R,S)-1-Phenyl-1,2-ethanediol butyrate (**2a**)*: ^{1}H NMR (300 MHz, $CDCl_3$), δ/ppm: 0.92 (t, J = 7.4 Hz, 3H), 0.94 (t, J = 7.3 Hz, 3H), 1.71–1.59 (m, 4H), 2.29 (t, J = 7.3 Hz, 2H), 2.36 (t, J = 7.4 Hz, 2H), 4.36–4.26 (m, 2H), 6.04 (dd, J = 7.5, 4.7 Hz, 1H), 7.38–7.27 (m, 5H).

*(R,S)-1-Phenyl-1,2-ethanediol hexanoate (**3a**)*: ^{1}H NMR (300 MHz, $CDCl_3$), δ/ppm: 0.88 (t, J = 6.6 Hz, 3H), 0.89 (t, J = 6.8 Hz, 3H), 1.34–1.26 (m, 8H), 1.67–1.56 (m, 4H), 2.29 (t, J = 7.6 Hz, 2H), 2.36 (t, J = 7.5 Hz, 2H), 4.35–4.27 (m, 2H), 6.02 (dd, J = 7.5, 4.2 Hz, 1H), 7.36–7.01 (m, 5H).

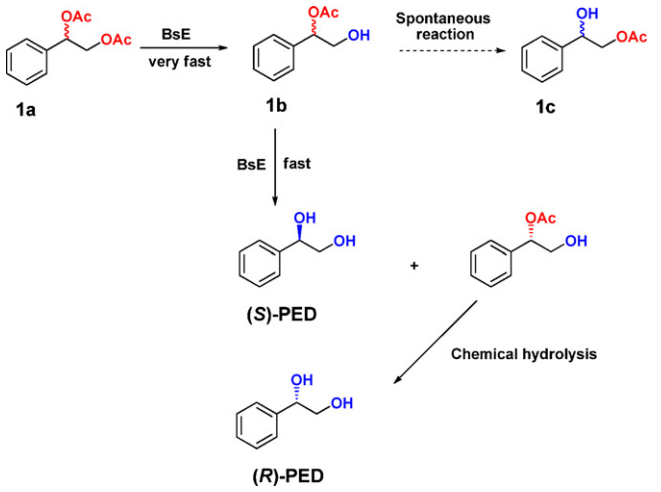
2.3. Recombinant E. coli cell production

Production of recombinant *E. coli* BL21 cells was performed as described previously [26]. The cells were harvested by centrifugation at 8500 × *g* for 10 min and washed twice with physiological saline and stored at 4 °C for further use. The recombinant cell can be stored at 4 °C for 2 weeks without any enzyme activity decrease, and still retains 96% of its initial activity for 2 months.

2.4. Enantioselective hydrolysis of dicarboxyesters

The resting cells of recombinant *E. coli* BL21 were resuspended in 0.9 ml KPB (potassium phosphate buffer, 200 mM, pH 7.0). The enantioselective hydrolysis reaction was initiated by adding racemic dicarboxyester dissolved in 0.1 ml ethanol into a shaking incubator to give a final substrate concentration of 100 mM at 30 °C, 180 rpm. Samples (250 μl each) were withdrawn at different time intervals and immediately extracted with ethyl acetate (500 μl). After centrifugation (8500 × *g*, 5 min), the samples were analyzed by HPLC. Data are means of at least three parallel reactions.

Enantiomeric excess of PED (ee_p) and that of corresponding secondary ester (ee_s) were determined by HPLC. Conversion of corresponding secondary ester (c) was calculated as $c\,(\%) = ee_s/(ee_s + ee_p) \times 100$. Enantioselectivity ($E$) was calculated as $E = \ln[(1-c)(1-ee_s)]/\ln[(1-c)(1+ee_s)]$.



Scheme 1. Sequential hydrolysis process of 1-phenyl-1,2-ethanediol diacetate by recombinant BsE. The dotted line indicates spontaneous reaction.

2.5. Analytical methods

Samples (250 μl each) were withdrawn at different time intervals and immediately extracted with ethyl acetate (500 μl). After centrifugation (8500 × *g*, 5 min), the samples were dried over anhydrous sodium sulfate for 12 h and filtered with 0.45 μm membrane filter.

After filtration, samples from the hydrolysis reaction mixture were analyzed on a HPLC (Agilent-1100) equipped with a chiral column Chiracel OB-H (Φ 0.46 cm × 25 cm, Daicel, Japan), and were isocratic eluted with hexane:isopropanol = 90:10 (v/v) at a flow rate of 1 ml/min and detected at 210 nm. 20 μl of each sample was injected at room temperature. The retention times for compounds were shown as follows: (*R*)-PED, 7.0 min; (*S*)-PED, 8.8 min; (*R*,*S*)-**1a**, 11.2 min; (*S*)-**1b**, 10.3 min; (*R*)-**1b**, 14.5 min; (*R*,*S*)-**2a**, 43.6 min; (*S*)-**2b**, 20.7 min; (*R*)-**2b**, 29.1 min; (*R*,*S*)-**3a**, 71.2 min; (*S*)-**3b**, 42.8 min; (*R*)-**3b**, 55.4 min.

3. Results and discussion

3.1. Sequential hydrolysis of diacetate for (S)-PED formation

The BsE in whole-cell system was more stable than the isolated enzyme. The half-time of the esterase in whole-cell system was 11-fold higher than that of the isolated enzyme [26]. We therefore employed recombinant whole cell of *E. coli* BL21 as biocatalyst for production of (*S*)-PED.

The catalytic performance of biocatalyst was studied using (*R*,*S*)-**1a** as a model substrate. As shown in Scheme 1, during the hydrolysis process of diacetate, a secondary ester **1b** was first detected. It was liberated in a highly regioselective but non-enantioselective manner, occurring far from the stereocentre. This process proceeded very fast and the initial reaction rate of the first step is 36.8 mM/min, and **1b** was formed without any enatioselectivity. Then **1b** was transformed to (*S*)-PED by further enzymatic hydrolysis, and the amount of (*S*)-PED increased with the reaction time. Compared with the first step, the initial reaction rate of the second step was 6.2 mM/min, which is much slower, but the overall reaction could also complete within 1 h (Fig. 1). (*S*)-PED was obtained in 49% yield and 96% *ee* (Table 2, entry 1). As we know, the use of (*R*,*S*)-**1b** as substrate is a chemical way to force the hydrolysis to occur at merely the stereocentre rather than elsewhere in the molecule of (*R*,*S*)-**1a**. However, the chemical synthesis of **1b** needs protecting groups which increases synthetic steps and for

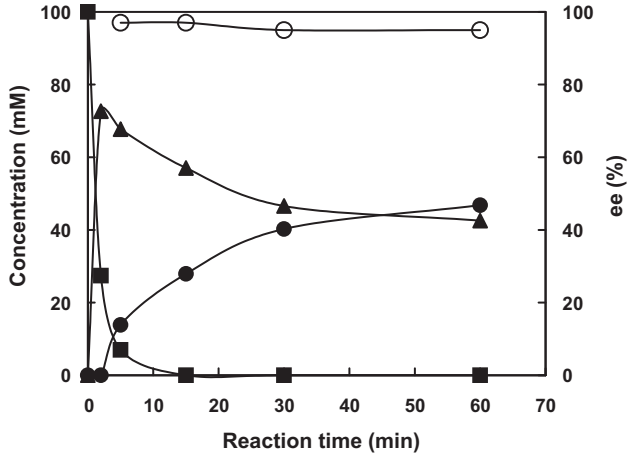


Fig. 1. Time course of *rac*-**1a** sequential hydrolysis catalyzed by recombinant *E. coli* BL21 overexpressing BsE. Reaction conditions: 0.9 ml KPB buffer (200 mM, pH 7.5), 0.1 ml ethanol dissolving 1 M *rac*-**1a**, wet cells of 10 mg ml^{-1}, 35 °C, 180 rpm. (■) *rac*-**1a**; (▲) **1b**; (●) PED; (○) *ee* of PED.

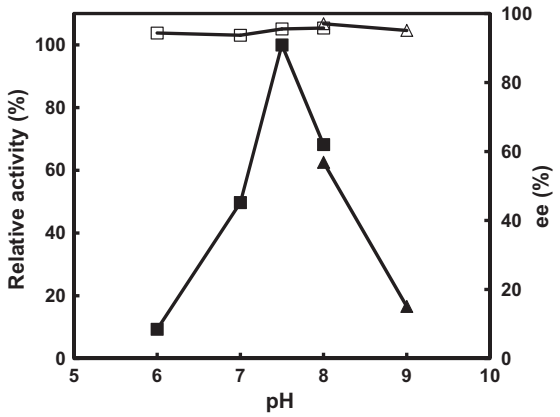


Fig. 2. Effects of pH on *rac*-**1a** sequential hydrolysis catalyzed by recombinant *E. coli* BL21 overexpressing BsE. Reaction condition: 4.5 ml buffer with pH ranging from 6.0 to 9.0 (200 mM KPB, pH 6.0~8.0 and 200 mM glycine–NaOH, pH 8.0~9.0), 0.5 ml ethanol dissolving 1 M *rac*-**1a**, wet cells of 10 mg ml^{-1}, 30 °C, 180 rpm. (■) Relative activity in KPB buffer; (▲) relative activity in glycine–NaOH buffer; (□) *ee* in KPB buffer; (△) *ee* in glycine–NaOH buffer.

this reason it should be avoided when other possibilities are at hand. Therefore, direct hydrolysis of PED diacetate (**1a**), instead of PED monoacetate (**1b**), seems more reasonable both technically and economically.

3.2. Optimization of reaction conditions for (S)-PED bioproduction

It is well known that the buffer pH plays an important role in enzymatic reaction. The variation of pH will alter the ionic state of the substrate and the stereochemical configuration of enzyme in the neighborhood of active sites, which in turn influence the enzymatic activity and enantioselectivity. In order to determine the optimum pH for the hydrolysis of **1a**, the reaction was carried out at different pH ranging from 6.0 to 9.0 at 30 °C. As shown in Fig. 2, the optimum pH was around 7.5, which was similar to the optimum pH (7.4) of the esterase from *B. subtilis* (BS2) and its double mutant E188W/M193C used for removal of carboxyl protecting groups of glutamate diesters [27]. Moreover, the *ee* of PED was not sensitive to pH changes and high *ee* values could be obtained under all conditions, being kept at >95%.

Effect of temperatures on the biocatalytic resolution of **1a** was also studied. The *ee* of PED and relative activities at different temperatures (20–50 °C) are shown in Fig. 3. The relative activity of the enzyme increased when the temperature was raised from 20 °C to 35 °C. But at higher temperatures, activities of the enzyme decreased dramatically. Only very slight changes in the *ee* of (*S*)-PED were observed as the temperature changed. It declined only slightly to 94.5% *ee* at 50 °C. Therefore, based on the results obtained above, pH 7.5 and 35 °C were chosen as the optimal reaction conditions and were used in the subsequent experiments.

The cost of biocatalyst is also a limitation for practice. To find the most economical cell concentration for the biotransformation, we examined the effect of cell concentration on the kinetic resolution of **1a**. With the decrease of cell concentration, the reaction time increased. Unexpectedly, the *ee* of PED also declined correspondingly, as shown in Table 1. When the reaction lasted for a longer time, **1c** had been detected. Further study showed that **1b** was not stable in aqueous medium, if the reaction was hold for a longer period, a part of *rac*-**1b** could be transformed into *rac*-**1c**, which was hydrolyzed without stereoselectivity, thus leading to the decrease of *ee* value. Nevertheless, due to the high activity of recombinant BsE towards **1a**, the enzymatic hydrolysis can completely finish within 1 h. During this period, the acyl transfer from the secondary position to the primary position was not detected. When the cell concentration was increased up to 20 mg (wet cell) ml^{-1}, the reaction rate did not change so much. So 10 mg ml^{-1} was selected as the favorable cell concentration for kinetic resolution. In initial explorative experiments, more than 100 wild-type strains were isolated from the soil. Unfortunately, the results were not so satisfactory,

Table 1
Effect of cell concentration on sequential hydrolysis of *rac*-**1a** catalyzed by recombinant *E. coli* BL21 overexpressing BsE.[a]

Cell concentration (mg ml^{-1})	*t* (min)	ee_s (%)[b]	ee_p (%)[b]	*c* (%)[c]
2	300	75	72	51
5	120	77	87	47
10	60	96	96	50
20	45	97	97	50

[a] Reactions were carried out in 0.9 ml KPB buffer (200 mM, pH 7.5) with 0.1 ml ethanol dissolving 100 mM rac-**1a** and different concentration of wet cells at 35 °C, 180 rpm.
[b] *ee* of **1b** (ee_s) and *ee* of PED (ee_p) were determined by HPLC.
[c] Conversion of rac-**1b** (*c*) was calculated as $c\,(\%) = ee_s/(ee_s + ee_p) \times 100$.

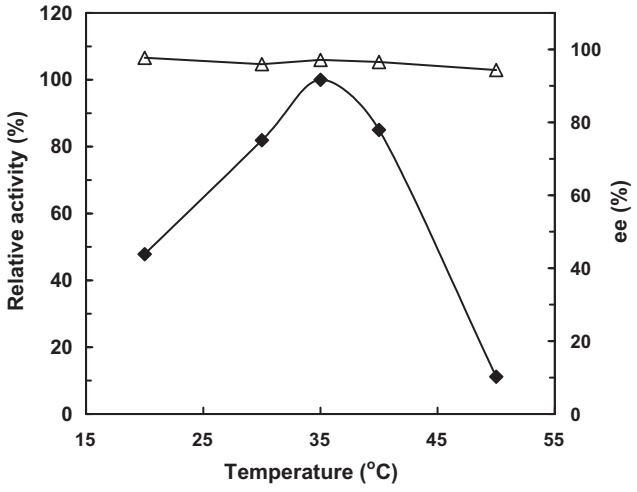


Fig. 3. Effects of temperature on *rac*-**1a** sequential hydrolysis catalyzed by recombinant *E. coli* BL21 overexpressing BsE. Reaction condition: 4.5 ml KPB buffer (200 mM, pH 7.5), 0.5 ml ethanol dissolving 1 M *rac*-**1a**, wet cells of 10 mg ml^{-1}, temperature ranging from 30 °C to 50 °C, 180 rpm. (♦) Relative activity, (△) *ee* of PED.

Table 2
Enantioselectivity of BsE-producing recombinant *E. coli* BL21in kinetic resolution of **1a**–**3a**.

Substrate	*t* (min)	ee_s (%)[a]	ee_p (%)[a]	*c* (%)[b]	*E*[c]
rac-**1a**	60	96	96	50	176
rac-**2a**	60	44	95	32	65
rac-**3a**	160	20	91	18	26

Reactions were carried out in 0.9 ml KPB buffer (200 mM, pH 7.5) with 0.1 ml ethanol dissolving 100 mM different substrates and 10 mg ml^{-1} of wet cells at 35 °C, 180 rpm.

[a] *ee* of PED (ee_s) and ee_p (*ee* of corresponding secondary ester) were determined by HPLC.

[b] Conversion of corresponding secondary ester (*c*) was calculated as $c\,(\%) = ee_s/(ee_s + ee_p) \times 100$.

[c] Enantioselectivity (*E*) was calculated as $E = \ln[(1-c)(1-ee_s)]/\ln[(1-c)(1+ee_s)]$.

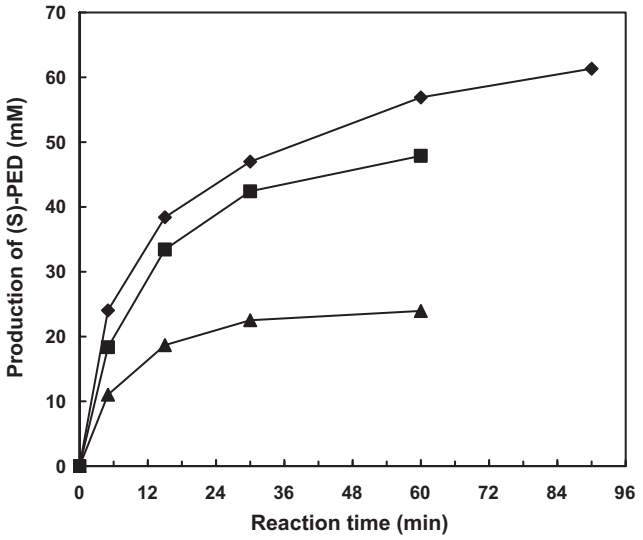


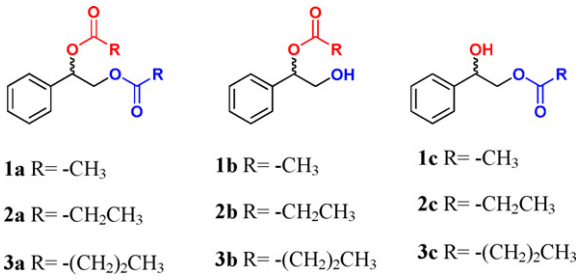
Fig. 4. Time courses of *rac*-**1a** hydrolysis at different concentrations catalyzed by recombinant *E. coli* BL21 overexpressing BsE. Reaction conditions: 0.9 ml KPB buffer (200 mM, pH 7.5), 0.1 ml ethanol dissolving *rac*-**1a**, wet cells of 10 mg ml^{-1}, 35 °C, 180 rpm, final concentration of *rac*-**1a**: (♦) 150 mM; (■) 100 mM; (▲) 50 mM.

and only moderate yield and *ee* were obtained. The activities of these isolated strains from the soil were not as high as that of recombinant BsE so that the hydrolysis of **1a** proceeded for more than 10 h. In a putative experiment, during such a period (10 h), about 60% of **1b** was transformed into **1c**. This is one possibility to explain why the *ee* of PED is always low.

Substrate concentration affects not only the reaction rate but also the enantioselectivity due to the possible existence of substrate inhibition. The effect of substrate concentration of **1a** on the product formation was investigated at a fixed ratio of substrate to catalyst (S/C). Different substrate concentrations ranging from 50 mM to 150 mM were employed for the kinetic resolution under the optimal conditions obtained above. As shown in Fig. 4, when the substrate concentration was increased from 50 mM to 100 mM, the rate of (*S*)-PED formation increased accordingly. When the substrate concentration increased from 100 mM to 150 mM, a longer reaction time was needed to reach 50% substrate conversion. Moreover, it also caused the problem of acyl migration which will reduce the *ee* value of PED to 90.7%. Therefore, 100 mM was selected as the optimal substrate concentration of reaction.

3.3. Effect of acyl groups with varied carbon chain length

Three dicarboxyesters with varied acyl chains **1a**–**3a** (acetyl, *n*-butyl, *n*-hexyl) were prepared (Scheme 2). The hydrolysis of these dicarboxyesters by recombinant *E. coli* cells was performed and compared. Conversion of substrate (*c*), *ee* value of PED (ee_p) and



Scheme 2. Dicarboxyesters of PED and their correponding primary monoesters and secondary monoesters.

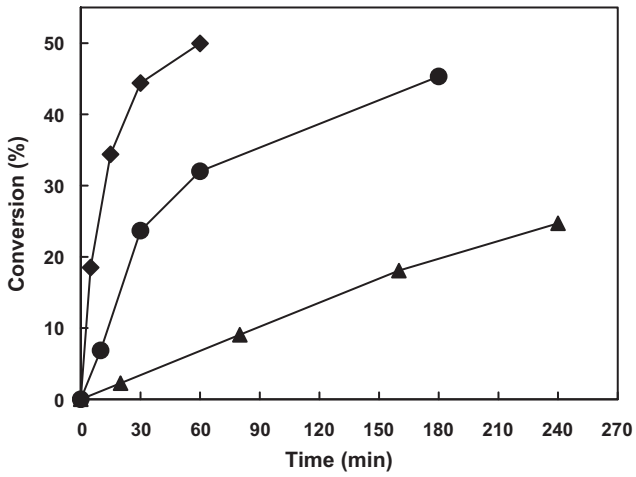


Fig. 5. Time courses of sequential hydrolysis of dicarboxyesters with increasing linear acyl chains catalyzed by recombinant *E. coli* BL21 overexpressing BsE. Reaction conditions: 0.9 ml KPB buffer (200 mM, pH 7.5), 0.1 ml ethanol dissolving 1 M different dicarboxyesters, wet cells of 10 mg ml^{-1}, 35 °C, 180 rpm, different substrate: (♦) *rac*-**1a**; (•) *rac*-**2a**; (▲) *rac*-**3a**.

enantiomeric ratio (*E*) were determined, as listed in Table 2. The recombinant *E. coli* cells exhibited the highest hydrolytic activity and most satisfactory enantioselectivity (*E* = 176) towards **1a** among the three substrates tested. As compared with **1a**, longer reaction time was needed for **2a** and **3a** to achieve an ideal conversion of 49%, especially for **3a** (Fig. 5). The reason might be attributed to the longer chain length of acyl group which may hinder the movement of substrates into active site of the enzyme [27]. What was worse, the *ee* of (*S*)-PED decreased to 78% when **3a** was used as substrate. Therefore, **1a** was selected as the best substrate and was used for the biopreparation of (*S*)-PED.

3.4. Kinetic resolution of *1a* in preparative scale

To further evaluate the potential of whole cells of recombinant *E. coli* BL21 for practical use, a preparative scale resolution of **1a** was conducted. A total amount of 1.8 g wet cells of recombinant *E. coli* BL21 and 3.3 g substrate (100 mM) were employed in a volume of 150 ml. The reaction was monitored by HPLC analysis, and it was stopped at 1 h. After extraction and normal work-up, 0.98 g of (*S*)-PED was obtained (49% yield, 95% *ee*). After simple recrystallization in $CHCl_3$, the *ee* of (*S*)-PED was enhanced up to >99%, with a total yield of 45%.

4. Conclusions

The preparation of (*S*)-PED by kinetic resolution of dicarboxyesters with the whole cells of recombinant *E. coli* BL21 overexpressing BsE was investigated. The recombinant strain exhibits high

esterase activity and excellent regio-, enantioselectivity towards dicarboxyesters, meanwhile, the best enantioselectivity ($E = 176$) was observed for diacetate among three dicarboxyesters with varied acyl chains (acetyl, *n*-butyl, *n*-hexyl). Then the reaction conditions were optimized in aqueous system, giving the optimal conditions as follows: pH 7.5, 35 °C, 10 mg ml^{-1} of cell concentration and 100 mM substrate. In this work, (*S*)-PED can be obtained through direct hydrolysis of *rac*-**1a** instead of *rac*-**1b** which needs protecting groups in its chemical synthesis process. Decrease of synthetic steps of substrate makes the process more reasonable both technically and economically. The preparative reaction (150-ml scale) was successfully performed, indicating a potentially promising biocatalyst for practical production of (*S*)-PED in the future.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.molcatb.2011.07.022.

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