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# Increased Catalyst Productivity in α-Hydroxy Acids Resolution by Esterase Mutation and Substrate Modification

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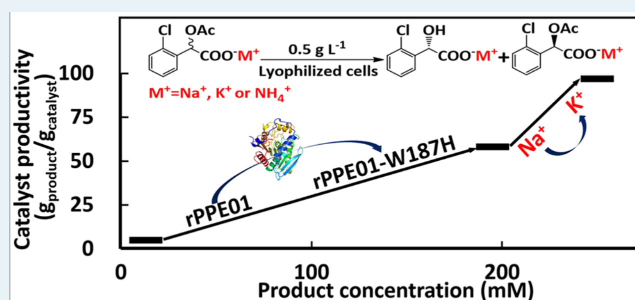
Supporting Information




**ABSTRACT:** Optically pure α-hydroxy acids and their derivatives are versatile chiral building blocks in the pharmaceutical industry. In this study, the potential of a recombinant *Pseudomonas putida* esterase (rPPE01) for the enzymatic resolution of α-acetoxy acids was significantly improved by combinatorial engineering of both the biocatalyst and substrate. Semirational design based on homologous modeling and molecular docking provided a single-point variant, W187H, whose $k_{cat}/K_M$ for sodium 2-acetoxy-2-(2′-chlorophenyl)acetate (Ac-CPA-Na) was increased 100-fold, from 0.0611 to 6.20 $mM^{-1}$ $s^{-1}$, while retaining its excellent enantioselectivity and broad substrate spectrum. Biocatalyst deactivation under the operating conditions was decreased by using the potassium salt of Ac-CPA instead of Ac-CPA-Na. With 0.5 g $L^{-1}$ of lyophilized cells containing rPPE01-W187H, 500 mM (*R*,*S*)-Ac-CPA-K was selectively deacylated with 49.9% conversion within 15 h, giving satisfactory enantiomeric excesses (ee) for both the *S* product (>99% ee) and the remaining *R* substrate (98.7% ee). Consequently, the amount of (*S*)-2-hydroxy-2-(2′-chlorophenyl)acetate prepared per unit weight of lyophilized cells was improved by a factor of 18.9 compared with the original productivity of the wild-type esterase. Further enzymatic resolution of other important hydroxy acids at the 100 mL scale demonstrated that the rPPE01-W187H-based bioprocess is versatile and practical for the large-scale preparation of chiral α-hydroxy acids.

**KEYWORDS:** *catalyst productivity, deacylation, enzymatic resolution, α-hydroxy acid, product:catalyst ratio, semirational design*

## ■ INTRODUCTION

α-Hydroxy acids are widely used as chiral synthons in the chemical and pharmaceutical industries.[1,2] Enantiopure 2-hydroxyphenylacetic acid and its derivatives represent the most important class of hydroxy acids because of their high commercial value.[3−5] (*R*)-2-Hydroxy-2-(2′-chlorophenyl)acetic acid is the key chiral intermediate in the synthesis of (*S*)-clopidogrel, a platelet aggregation inhibitor with a large market share, used for heart attack and stroke treatment. Various biocatalytic approaches to the synthesis of enantiopure α-hydroxy acids have been developed,[1] including reductase-catalyzed asymmetric reduction of the corresponding keto ester,[3,4,6] nitrilase-catalyzed enantioselective hydrolysis of mandelonitrile and its derivatives,[7,8] and esterase or lipase catalyzed resolution of the *O*-acetates or esters of hydroxy acids.[9,10] Among these pathways, the enzymatic resolution of *O*-acetylated hydroxy acids by esterase or lipase (Scheme 1) shows great potential for application due to their practical use in chemical industry[11] and has been extensively studied.[10,12−14] Several commercially available lipases [e.g., *Pseudomonas* sp. Lipase (Röhm GmbH) and Lipase PS (Amano)] have been used in such biotransformations. However, most of them show poor productivity, resulting in high costs in terms of catalyst consumption [0.98 g of product were obtained per gram of catalyst using the most efficient Lipase PS (Amano) to acetylate substituted mandelic acids],[12] which is unsuitable for industrial-scale applications.[15]

In our previous work, an esterase producer, *Pseudomonas putida* ECU1011, was isolated from soil for the enantioselective deacylation of (*S*)-2-acetoxy-2-(2′-chlorophenyl) acetate [(*S*)-Ac-CPA] from its racemate.[16] Subsequently, the corresponding esterase (rPPE01) was successfully cloned and heterogeneously expressed in *Escherichia coli*.[14] The excellent thermostability and high enantioselectivity (*E* > 200) of rPPE01 toward α-acetoxycarboxylates indicated its great potential in industrial applications. However, practical applications of rPPE01-mediated bioprocesses were impeded by its relatively low enzymatic activity and poor operational stability. Further enhancement of the activity and stability are therefore highly desirable to achieve industrial applications of rPPE01 in the enzymatic resolution of α-hydroxy acids.

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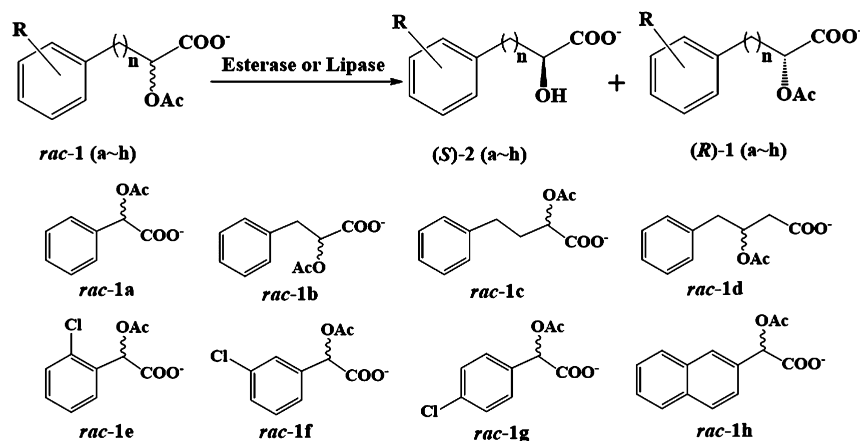
Scheme 1. Esterase or Lipase Catalyzed Resolution of Racemic α-Acetoxycarboxylates




Protein engineering is frequently used to tailor native enzymes for industrial purposes, and great progress has been made in recent decades.[17] Two different and complementary strategies have been developed for protein engineering: directed evolution followed by high-throughput screening of the numerous variants[18] and rational design based on detailed information on the three-dimensional structure and structure–function relationship of the enzyme.[19] Recently, with the rapid increase in the number of enzyme structures available from the Protein Data Bank (PDB) and highly developed bioinformatic tools, rational design has become an effective strategy for designing desired enzymes. A BLAST search of the PDB revealed that three crystal structures from a hormone-sensitive lipase family showed considerable sequence identities (>40%) with rPPE01, which made it possible to predict the three-dimensional structure of rPPE01 by homology modeling. Because it is a member of the $\alpha/\beta$-hydrolase fold family, another advantage of the rational design of rPPE01 was the in-depth insights available into its reaction mechanism.[20] Rational design of the "hot spots" that might potentially affect the enzymatic activity would significantly increase the efficiency of desired mutant hits. A semirational design based on the homology-modeled structure of rPPE01 was therefore chosen to engineer rPPE01, i.e., to identify hot spots for subsequent mutations.

The poor operational stability of biocatalysts is a common restriction in the industrialization of enzymatic processes. Several strategies have been developed to stabilize enzymes for industrial use, such as immobilization and use of additives.[21,22] Dissolved salts are important additives for enzyme stabilization. However, the effects of stabilization with different salts may differ.[23] The stabilization effects of various cations and anions at moderate or high concentrations (ionic strength >0.01 mol $kg^{-1}$) are ranked qualitatively in the Hofmeister series.[24] The substrate Ac-CPA exists in the ionic form in our study,[14] and a high substrate concentration would result in a high ionic strength in the reaction system. Cations at such a high salt concentration might therefore significantly affect the biocatalyst in this study. This might provide a simple and direct method of relieving biocatalyst deactivation by choosing a suitable substrate counterion (cation).

In light of the above analysis, the semirational design of an esterase and substrate–cation optimization were used to improve the enzymatic activity and operational stability, respectively. On the basis of our smart design, a single-point variant with superior activity was obtained and used to reduce the biocatalyst load. The productivity of the engineered biocatalyst in the reaction process was further improved by the stabilizing effect on the enzyme obtained by switching the substrate counterion. An rPPE01-W187H-based bioprocess with significantly increased catalyst productivity, defined as the weight gain ratio of the product to the catalyst ($P/C$), was achieved in this study.

## ■ EXPERIMENTAL SECTION

**Chemicals.** Racemic 2-hydroxy-3-phenylpropanoic acid was purchased from the Yurlic Chemical S&T Co., Ltd. (Shanghai, China). Racemic 2-bromonaphthalene was purchased from the Kangtuo Chemical Co., Ltd. (Shanghai, China). Racemic 2-hydroxy-2-phenylacetic acid, 2-hydroxy-2-(2′-chlorophenyl)-acetic acid, 2-hydroxy-2-(3′-chlorophenyl)acetic acid, and 2-hydroxy-2-(4′-chlorophenyl)acetic acid were obtained from the Guangde Chemical Co., Ltd. (Anhui, China). Unless otherwise stated, all other chemicals and reagents were obtained commercially. The synthesis of 2-hydroxy-4-phenylbutyrate and 3-hydroxy-4-phenylbutyrate as well as the further acetylation of the hydroxy acids were conducted according to previously published protocols.[14,25]

**Homology Modeling and Molecular Docking.** The crystal structures in the PDB showing high sequence identities with rPPE01 (>40%) were selected as templates. The rPPE01 structure was modeled with a multiple-template-based homology modeling method, using the program Modeller 9.11, aided by the graphical interface EasyModeller.[26] After further energy minimization using the same software package, the quality of the modeled structure was assessed using the Procheck program (http://nihserver.mbi.ucla.edu/SAVES/). Docking experiments were performed using the program AutoDock Vina, and the images were created using PyMOL.[27]

**Mutant Construction and Esterase Expression.** Mutations were introduced using a Mut Express II Fast Mutagenesis Kit (Vazyme Biotech Co., Ltd.), according to the manufacturer's instructions and confirmed by sequencing. The primer sequences are listed in the Supporting Information (SI). The plasmids with specific mutations were then transformed into *E. coli* BL21 (DE3) for esterase expression. Cultivation of the recombinant *E. coli* cells expressing rPPE01 or its mutants and enzyme purification using $Ni^{2+}$ affinity chromatography were performed as described previously.[14]

**Enzymatic Activity and Enantioselectivity Assay.** The enzymatic activity, conversion, and enantioselectivity toward acetoxy acids were analyzed using chiral HPLC, as described previously.[14] One unit of enzyme activity was defined as the amount of enzyme releasing 1.0 $\mu$mol of hydroxy acid per minute under the assay conditions. The residual activity was analyzed spectrophotometrically by measuring the hydrolysis rate of 1 mM *p*-nitrophenyl acetate to *p*-nitrophenol[28] and expressed as a percentage of the initial activity measured at 0 h.

**Characterization of Esterase Variant rPPE01-W187H.** The initial hydrolysis rates of various $\alpha$-acetoxycarboxylates by purified rPPE01-W187H were measured using the same method as that used with rPPE01-WT.[14] The initial rates of Ac-CPA-Na deacylation under various reaction conditions were measured to evaluate the effects of temperature and pH on the enzymatic activity. The thermostabilities of rPPE01-W187H and its wild type were investigated by measuring the residual activities after incubation of the purified esterase (0.40 mg $mL^{-1}$) at different temperatures (30, 40, and 50 °C) for 12 h.

**Enzymatic Resolution of $\alpha$-Acetoxy Acid by rPPE01-W187H or its Wild Type.** The reaction, using 50 mM phosphate buffer (pH 6.5), lyophilized *E. coli* cells expressing rPPE01-W187H or its wild type, Ac-CPA (300 mM), and 0.5 equiv of $Na_2CO_3$, was performed using a total volume of 10 mL at 30 °C. The pH of the reaction mixture was automatically maintained at 6.5 by titrating with 0.5 M $Na_2CO_3$ during the process.

**Optimization of Substrate Counterions.** To identify the best cation for the substrate in the enzymatic resolution of Ac-CPA by rPPE01-W187H, various alkalis ($Na_2CO_3$, $K_2CO_3$, or $NH_3{\cdot}H_2O$) were added to neutralize the substrate and acetic acid produced in the reaction. The enzymatic reactions were carried out at a substrate concentration of 500 mM, as described above. Samples were taken periodically to measure the conversions and residual activities.

**Preparation of Enantiopure Hydroxy Acids.** For larger-scale preparation, $\alpha$-acetoxy acid (20 or 50 mmol) and 0.5 equiv of $K_2CO_3$ (10 or 25 mmol) were dissolved in 100 mL of phosphate buffer (50 mM, pH 6.5). Lyophilized recombinant *E. coli* cells (0.05 or 0.2 g) were added to initiate the reaction at 30 °C. When the conversion reached ~50%, the reaction mixture was acidified with 2 M $H_2SO_4$ and extracted twice with ethyl acetate. Then the combined extracts were dried over anhydrous sodium sulfate for product isolation. Enantiopure (*R*)-$\alpha$-acetoxy acids and (*S*)-$\alpha$-hydroxy acids were obtained after subsequent chromatographic separation, and the (*R*)-$\alpha$-acetoxy acids were chemically deacylated in aqueous NaOH solution (20%, w/v) at room temperature. After complete hydrolysis, the products were acidified again and extracted as described above. The conversion and ee values for the substrates and products during the process were analyzed using chiral HPLC as described in the SI. The isolated products were validated based on specific rotation and $^1$H NMR spectra, and the data are also given in the SI.

## RESULTS AND DISCUSSION

On the basis of the PDB BLAST search, three crystal structures showing high sequence identities with rPPE01, from *Alicyclobacillus acidocaldarius* (PDB entry: 2HM7, 42% identity), archaeon *Pyrobaculum calidifontis* VA1 (PDB entry: 2YH2, 42% identity), and a metagenomic library (PDB entry: 2C7B, 41% identity), were selected as templates. The multiple-template-based homology-modeled structure of rPPE01 predicted by Modeller was validated using Procheck to ensure its reliability. The rPPE01 model consists of a core domain, belonging to the canonical $\alpha/\beta$-hydrolase topological fold, and two separate helical regions.[29] A detailed inspection of the solvent-accessible surface around the conserved catalytic triad

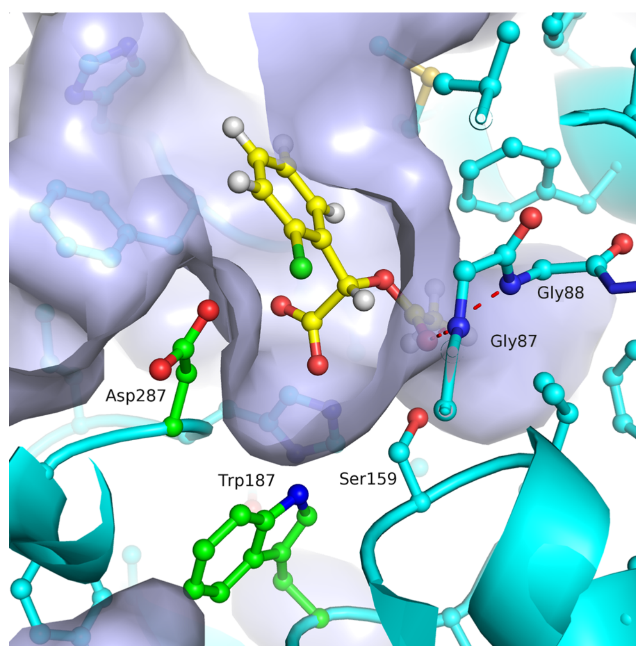



**Figure 1.** Close-up view of rPPE01 active site in complex with the most favorable docked position of (*S*)-Ac-CPA (yellow sticks). The predicted hot spots, W187 and D287, are highlighted as green sticks.

**Table 1. Steady-State Kinetic Parameters and Enantioselectivities of rPPE01-WT and Variants for Substrate Ac-CPA-Na**

| enzyme | $K_M$ (mM) | $k_{cat}$ ($s^{-1}$) | $k_{cat}/K_M$ ($mM^{-1}$ $s^{-1}$) | $E^c$ |
|---|---|---|---|---|
| rPPE01-WT[a] | 146 | 8.90 | 0.0611 | >200 |
| rPPE01-W187H[b] | 19.9 | 123 | 6.20 | >200 |
| rPPE01-D287A[a] | 4.30 | 21.6 | 5.01 | 8.7 |

[a]The kinetic parameters were determined at its optimal pH (pH 6.2, 50 mM sodium phosphate buffer) and temperature (50 °C). [b]The kinetic parameters were determined at its optimal pH (pH 6.5, 50 mM sodium phosphate buffer) and temperature (40 °C). [c]The enzymes are selective toward the *S* substrate, producing the (*S*)-2-hydroxy-2-(2′-chlorophenyl) acetic acid in large excess.

**Table 2. Substrate Specificity of rPPE01-W187H and Its Wild Type**

| substrate | initial rate ($\mu$mol $min^{-1}$ mg $protein^{-1}$) | |
|---|---|---|
| | rPPE01-WT[a] | rPPE01-W187H[b] |
| *rac*-**1a** | 1.37 ± 0.09 | 76.7 ± 3.1 |
| *rac*-**1b** | 1.17 ± 0.06 | 15.4 ± 0.1 |
| *rac*-**1c** | 1.47 ± 0.20 | 1.34 ± 0.10 |
| *rac*-**1d** | n.d.[c] | n.d.[c] |
| *rac*-**1e** | 2.09 ± 0.01 | 75.2 ± 0.6 |
| *rac*-**1f** | 1.06 ± 0.10 | 51.7 ± 4.3 |
| *rac*-**1g** | 1.44 ± 0.06 | 40.2 ± 0.3 |
| *rac*-**1h** | 0.40 ± 0.02 | 3.72 ± 0.20 |

[a]Data cited from reference 14. [b]The initial rates were measured using purified enzyme under its optimal reaction condition. Values represent the mean of three replicates ± standard deviation. [c]n.d.: not detectable.

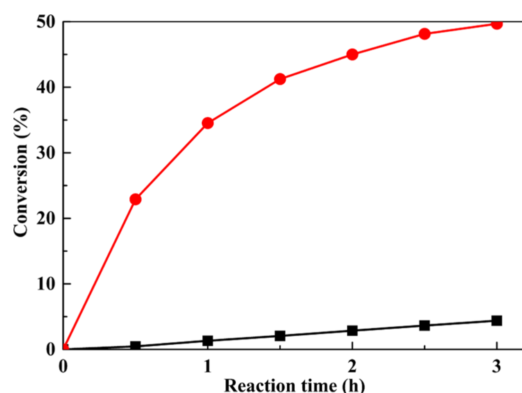



**Figure 2.** Bioresolution of Ac-CPA-Na at 300 mM by rPPE01-W187H (red dot) and rPPE01-WT (black solid square) at a catalyst load of 0.5 g $L^{-1}$ of lyophilized cells. The reaction was conducted at 30 °C, and the pH was maintained at 6.5 by automatically titrating 0.5 M $Na_2CO_3$.

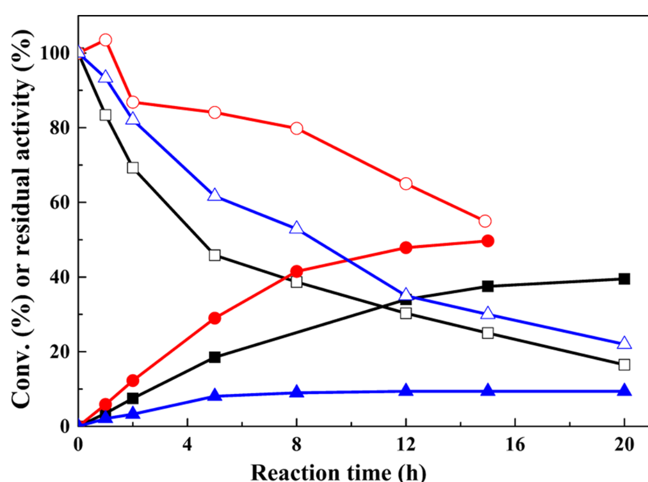



**Figure 3.** Enzymatic resolution of Ac-CPA at 500 mM, with different salts, by rPPE01-W187H. Shown are conversions of substrate in the form of sodium salt (black solid square), ammonium salt (blue solid triangle), or potassium salt substrate (red dot) and the residual enzyme activity with the substrate of sodium salt (black open square), ammonium salt (blue open triangle), or potassium salt (red circle).

(Ser159, Asp256, and His286) and oxyanion hole shows two regions of substrate binding sites, one for the acyl group and the other for the alcohol group of the ester substrate. (*S*)-Ac-CPA was docked into the binding pocket, and the most favorable substrate-binding position was analyzed to identify potential hot spots, as shown in Figure 1. The residues W187 and D287 were chosen as potential mutation sites on the basis of speculation that the high $K_M$ value (146 mM) and low $k_{cat}$ value (8.90 $s^{-1}$) of rPPE01 were caused by inappropriate accommodation of the negatively charged substrate. It was observed that the side chain of D287 partially occupied the alcohol-group-binding pocket, and its substitution by alanine was designed to enlarge the binding pocket and to eliminate possible repulsion between the carboxyl group of aspartic acid and the hydrophobic phenyl moiety or the negatively charged carboxyl group of the substrate. The other residue, W187, was located at the bottom of the alcohol-binding pocket, toward the negatively charged carboxyl group of the substrate. The substitution of W187 by the polar and smaller histidine was designed to increase the local polarity and to expand the binding pocket for facile accommodation of the carboxyl group from the substrate. Two designed mutants (D287A and W187H) were constructed and subjected to subsequent enzymatic activity and enantioselectivity analysis.

The steady-state kinetic parameters for the Ac-CPA substrate (shown in Table 1) indicated that the catalytic efficiencies of both the constructed mutants were significantly higher than that of wild-type rPPE01. For the W187H variant, a 7.4-fold decrease in $K_M$ combined with a 13.9-fold increase in $k_{cat}$ contributed to a total 100-fold increase in $k_{cat}/K_M$. For the D287A mutant, the 81.6-fold increase in $k_{cat}/K_M$ was ascribed to the 34.0-fold decrease in $K_M$ and the 2.4-fold increase in $k_{cat}$. The W187H variant retained excellent enantioselectivity ($E$ > 200). However, the enantioselectivity of the D287A variant decreased significantly (the $E$ value decreased from >200 to 8.7); this might be explained by excessive enlargement of the binding pocket. The W187H variant exhibited significantly increased catalytic efficiency and retained excellent enantioselectivity toward *rac*-Ac-CPA-Na. The potential application of the W187H variant to the production of chiral hydroxy acids was therefore further investigated.

The initial reaction rates of rPPE01-W187H and rPPE01-WT toward a series of $\alpha$-acetoxycarboxylates (shown in Scheme 1) revealed that the mutant showed increased activities toward most of the substrates (as shown in Table 2), with excellent enantioselectivities ($E$ > 200, data not shown). The influence on the activity of the distance between the phenyl ring and the acetoxy group was more significant for rPPE01-W187H than for the wild type. The enzymatic activity of rPPE01-W187H decreased sharply with increasing distance (**1a** < **1b** < **1c**), but the influence of distance was negligible for the wild type. The excellent enzymatic activity and enantioselectivity of rPPE01-W187H toward a series of $\alpha$-acetoxycarboxylates indicated that rPPE01-W187H could act as a versatile biocatalyst for the production of enantiopure $\alpha$-hydroxy acids.

**Table 3. Preparation of Important Enantiopure $\alpha$-Hydroxy Acids with rPPE01-W187H[a]**

| substrate | concn (mM, g $L^{-1}$) | cell load (g $L^{-1}$) | $t$ (h) | conv (%) | (*S*)-hydroxy acid yield (%), ee (%) | (*R*)-hydroxy acid[b] yield (%), ee (%) |
|---|---|---|---|---|---|---|
| *rac*-**1a** | 500, 97.4 | 0.5 | 15 | 50.0 | 43.1, 98.9 | 41.1, 97.8 |
| *rac*-**1b** | 500, 104 | 2.0 | 10 | 49.5 | 44.5, >99.0 | 42.5, 97.7 |
| *rac*-**1e** | 500, 114 | 0.5 | 15 | 49.5 | 44.1, 98.8 | 42.4, 97.0 |
| *rac*-**1f**[c] | 200, 45.6 | 0.5 | 8 | 49.7 | 43.4, >99.0 | 41.2, 97.8 |
| *rac*-**1g**[c] | 200, 45.6 | 0.5 | 12 | 49.4 | 39.3, >99.0 | 40.1, 97.7 |

[a]The enzymatic resolutions were conducted at a substrate concentration of 200−500 mM in the 50 mM KPB buffer at 100 mL scale. The reaction mixtures were stirred by magnetic agitation, and the pH was automatically maintained at 6.5 by titrating 0.5 M $K_2CO_3$. The conversion and ee values were analyzed by HPLC as described in the Experimental Section. [b]The (*R*)-hydroxy acids were obtained after alkaline hydrolysis of the residual (*R*)-*O*-acetate conducted in aqueous NaOH solution (20%, wt%) at room temperature. [c]The reactions were conducted at 200 mM substrate concentration owing to substrate inhibition.


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**Table 4. Comparison of Esterase rPPE01-W187H with Other Reported Counterparts for Preparation of (*R*)-2-Hydroxy-2-(2′-chlorophenyl)acetic acid**

| catalyst | substrate | substrate concn (g $L^{-1}$) | catalyst load(g $L^{-1}$) | conv (%) | ee (%) | P/C (g/g) | ref |
|---|---|---|---|---|---|---|---|
| CgKR1 | *o*-chlorobenzoyl formate | 300 | 100[a] | >99 | 98.7 | 15[c] | 4 |
| SCR | *o*-chlorobenzoyl formate | 198 | 200[a] | 98 | >99 | 5.0[c] | 3 |
| YtbE | *o*-chlorobenzoyl formate | 500 | 50[b] | >99 | >99 | 10 | 8 |
| LaN | *o*-chloromandelonitrile | 50.3 | 52[b] | 94.5 | 96.0 | 1.0 | 7 |
| rPPE01 | Ac-CPA-Na | 68.4 | 0.5[b] | 4.4 | >99 | 5.1 | this study |
| rPPE01-W187H | Ac-CPA-K | 114 | 0.5[b] | 49.5 | 98.8 | 96 | this study |

[a]The wet cells were used as catalyst. [b]The lyophilized cells were used as catalyst. [c]The weight of the wet cells was divided by 5 to estimate the dry weight for calculating the P/C ratio.

The catalytic properties of rPPE01-W187H were investigated to explore its potential as a candidate biocatalyst for the up-scaled enzymatic resolution of $\alpha$-hydroxy acids. Both the pH−activity and temperature−activity profiles of rPPE01-W187H were different from those of rPPE01-WT. The optimal pH for W187H was around 6.5, a slight shift from the pH of 6.2 for the wild-type enzyme (SI Figure S1A). The mutant W187H showed maximum activity at 40 °C and retained >92% of its maximum activity over a broad temperature range from 30 to 50 °C (SI Figure S1B), whereas the wild-type rPPE01 showed maximum activity at 50 °C, with a narrow optimum temperature range. The broad temperature profile of rPPE01-W187H allows enzymatic resolution to be conducted at 30 °C (exhibiting 94.6% of its maximum activity at 40 °C), avoiding enzyme deactivation as a result of high concentrations of substrate and/or product at high temperature, as observed in our previous study.[14] rPPE01-W187H lost the majority of its activity at 50 °C for 12 h, whereas its wild type retained 96% of the initial activity under the same conditions (SI Figure S2), However, no obvious inactivation was observed for either the mutant or the wild type after incubations at 30 and 40 °C for 12 h.

To verify the improvements offered by the mutant W187H in practical applications, biocatalytic resolution of Ac-CPA-Na (300 mM) with either mutant or wild-type rPPE01 was conducted with a catalyst loading of 0.5 g $L^{-1}$ of lyophilized cells. As shown in Figure 2, the reaction catalyzed by the mutant W187H reached 49.6% conversion after 3 h, whereas the conversion in the case of the wild type was only 4.4% after 3 h. Obviously, the mutant W187H was much more active than rPPE01-WT, and its enantioselectivity was similar to that of the parent (>99% $ee_p$) during the whole reaction process. These results are in accordance with the kinetic data obtained, further demonstrating that rPPE01-W187H is a more competent biocatalyst for large-scale production of enantiopure $\alpha$-hydroxy acids.

The substrate concentration was then increased to 500 mM at the same catalyst loading to further improve the substrate or product loads and catalyst productivity, with a view to industrial applications.[15] A higher substrate or product concentration would reduce the cost of product isolation to a large extent, and a high catalyst productivity can reduce the cost of enzyme consumption. A lower conversion (39.0%) was observed even when the reaction time was prolonged to 20 h (as shown in Figure 3); this was ascribed to poor substrate tolerance of the esterase. A similar phenomenon was also observed for an rPPE01-WT-catalyzed resolution reaction at a high substrate concentration.[14] In our study, the high concentration (500 mM) of substrate in the ionic form would result in a high ionic strength (∼0.5 mol $kg^{-1}$). We suspect that various substrate counterions might affect the stability and activity of rPPE01-W187H, and the optimum cation might serve as an inherent additive, stabilizing the enzyme against substrate/product deactivation. On the basis of the Hofmeister series[22] of cations and anions, we chose the potassium and ammonium salts to provide potential substrate counterions instead of the sodium salt previously used. Conversion of the substrate with potassium ions reached ∼50% after 15 h, probably as a result of decreased deactivation during the catalytic process, as confirmed by the higher residual activity (Figure 3). Ammonium cations reduced deactivation slightly, but conversion of the substrate reached only 9.4% after 20 h. This phenomenon might be explained by possible activity inhibition by the ammonium salt in the reaction system. Generally, by simply switching the substrate counterion from $Na^+$ to $K^+$, a 500 mM substrate (Ac-CPA-K) could be easily deacetylated with excellent $ee_p$ (>99%) and $ee_s$ (98.7%) within 15 h, affording satisfactory results at high substrate concentration.

To confirm the feasibility of the biotransformation process on a large scale, several important $\alpha$-hydroxy acids, that is, 2-hydroxy-3-phenylpropanoic acid, 2-hydroxyphenylacetic acid, and their derivatives, were prepared at the 100 mL scale under the optimized reaction conditions. After the conversion reached ∼50%, the reaction mixture was acidified and extracted using the normal workup; chromatographic separation gave enantiopure (*R*)-2-acetoxy acids and (*S*)-2-hydroxy acids. Finally, both enantiomers of the hydroxy acids were obtained with high ee values after further alkaline hydrolysis of the resultant (*R*)-2-acetoxy compounds (Table 3).

## CONCLUSION

In summary, combinatorial engineering approaches to the biocatalyst and substrate counterion were successfully used to tailor-make the "best" biocatalyst and substrate form for the economical production of enantiopure $\alpha$-hydroxy acids. A semirational design based on analysis of structure−activity relationships was effective in significantly improving the esterase activity, providing a superior mutant with a 100-fold increase in $k_{cat}/K_M$ for Ac-CPA. Potassium ions as an inherent additive to stabilize the enzyme against deactivation were found to be the best substrate counterion for improving enzyme use. Compared with other reported bioprocesses aimed at producing optically active 2-hydroxy-2-(2′-chlorophenyl)acetic acid (listed in Table 4), rPPE01-W187H-mediated enzymatic resolution gave significantly higher catalyst productivity. Accordingly, the cost of enzyme consumption could be greatly reduced, and the emulsification caused by biocatalyst overload could be avoided. The economically feasible and technologically efficient bioprocess described in this study demonstrates


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the possibility of establishing an ideal bioprocess and solving specific problems using goal-oriented engineering strategies.

## ■ ASSOCIATED CONTENT

### Supporting Information

Additional experimental results including the characterization of the rPPE01-W187, $^{1}$H NMR spectra, and other data. This material is available free of charge via the Internet at http://pubs.acs.org.

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### Notes

The authors declare no competing financial interest.

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