Supporting Information for:

Immobilization of Lipase from *Pseudomonas Fluorescens* on Porous Polyurea and Its Application in Kinetic Resolution of Racemic 1-Phenylethanol

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Part 1. Chemicals and Reagents.

The relative information is described in Part 1 in Supporting Information. Toluene diisocyanate (TDI, 80% of 2,4- and 20% of 2,6-TDI, industrial grade) was from Wanhua Chemicals, China. Lipase from *Pseudomonas fluorescens* (PFL) was purchased from Sigma-Aldrich, and contained 16.0 wt% lipase protein as determined in our Lab. 1-phenylethanol (PEOH, 97%) was from Sigma-Aldrich, and (S)-1-phenylethanol from Alfa Aesar. Potassium bromide (AR), hexane and isopropanol (both HPLC grade) were bought from Tianjin Kemiou Chemicals, China. All these chemicals were used as received. Water used throughout this work was double-distilled. Glutaraldehyde (GA, 50 wt% aqueous solution), *p*-nitrobenzaldehyde (NBA), *p*-nitrophenyl palmitate (NPP), *p*-nitrophenol (NP), ethyl aldehyde (≥99.5%, Standard for GC) and vinyl acetate (99.00%), all AR grade, were purchased from Aladdin.

Part 2. Determination of Amine Group on PPU

The density of amine groups on PPU was determined through spectrophotometery based on absorbance of NBA.[1,2] The process includes two steps (as schematized in **Fig. S1**): Step 1. Formation of imine by reacting PPU with *p*-nitrobenzaldehyde (NBA) as depicted by **Figure S1**.150 mg of PPU was added into a glass bottle containing 10 mL of anhydrous acetonitrile with 0.01 mL of acetic acid premixed (acetic acid 0.1%, v/v). After gentle shaking, 200 mg (1.323 mmol) of NBA was added. The bottle was capped, sonicated to make a good dispersion, and incubated for 4 h in a bath shaker at 50 ºC with shaking rate at 140 osc/min. At the end of reaction, the content of the bottle was centrifuged to separate from the supernatant, the solid product washed repeatedly using anhydrous acetonitrile (12×20 mL), and dried at 60 ºC under vacuum; Step 2. The powder PPU with the imine attached on the surface (Substance I) was redispersed into a mixture of anhydrous acetonitrile (10 mL) and water (10 mL) with 0.1% (v/v) of acetic acid (0.02 mL), the content was sonicated in a bath of 50 ºC with shaking for 1 h with purpose for the PPU-imine to hydrolyze back to its original PPU and NBA, as shown by the reversing reaction in **Figure S1.** After the reaction, solid PPU was separated from the supernatant, thoroughly rinsed with the mixture of acetonitrile-water, and the concentration of regenerated NBA was measured with a UV-vis spectrophotometer (Lambda 35, Perkin-Elmer, USA) at 265 nm, the maximal absorbance of NBA in the binary solvent of

acetonitrile-water. The density of surface amine groups was calculated based on the amount of NBA detected from the supernatant.

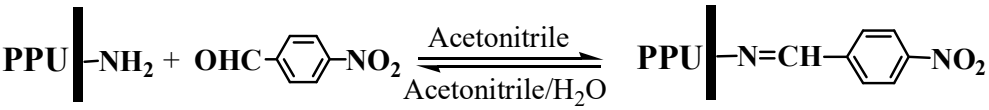

Figure S1. PPU iminization with aldehyde of *p*-nitrobenzaldehyde in acetonitrile and the hydrolysis in acetonitrile-water mixture for determination of amine amount

The results of amine amount versus the concentration of NBA solution are given in **Figure S2** (curve I), which indicates that amount of NBA reacted with amines on PPU was in constant increase with NBA concentration when it was inferior to 0.10 mol/L; whereas this amount was practically constant once NBA concentration reached 0.10 mol/L and thereafter.

The effect of reaction time was also studied with NBA concentration of 0.13 mol/L, which was slightly higher than the turning point of 0.10 mol/L, in order to have reproducible data on amine density as shown in Figure S2 (curve I). The result is also displayed in Figure S2 (curve II), which shows clearly that reacted NBA amount was increasing with reaction time up to 4 h, and it became constant afterwards. At this time, 0.157 mmol of NBA was reacted per gram of PPU, which is in good agreement with the value determined by variation of NBA concentration (0.153 mmol of NBA per gram of PPU, Curve I of Figure S2).

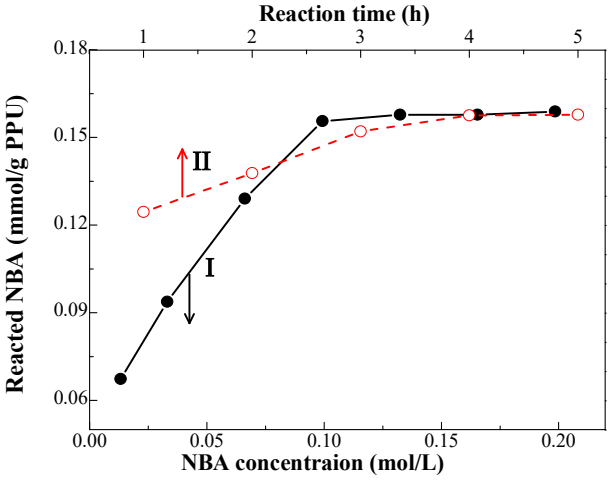

Figure S2. Effect of reaction time (○) and NBA concentration (●) on the reacted amount of NBA

Part 3. Reaction of PPU Amine with Glutaraldehyde and Lipase Immobilization

In order to accomplish the immobilization, PPU was first activated by reacting first with GA to attach an aldehyde functional group onto PPU surface. In doing so, 300 mg of PPU was added into 30 mL of GA aqueous solution (GA concentration varied from 0.0334 mol/L, 0.33 wt%, to 0.5008 mol/L, 5.01 wt%) in a bath shaker at 50 ºC and shaken at 140 osc./min for 5 h. The activated PPU (GA-PPU) was separated and rinsed with water to remove unreacted GA. The activation was carried out in deionized water and in PBS buffer solution of different pH. The effect of pH in PPU activation by GA were also studied with GA concentration fixed at 1.0 wt% (0.1 mol/L).

For subsequent PFL immobilization, 50 milligram of activated GA-PPU was added into 3 mL of PFL solution (maximal PFL immobilization, concentration of 2.56 mg/mL, pH 8.0), sonicated to make a homogeneous dispersion, and followed by shaking at 140 osc./min and 30 °C for 5 h. The solid was recovered by centrifugation and rinsed with a phosphate buffer solution three times to remove unimmobilized PFL in excess. The activated GA-PPU as-prepared was stored in a refrigerator at -20 °C prior to use. Under these conditions, 61.98% of PFL in the reaction system, i.e. (2.56 mg/mL×3×0.6198) 4.76 mg of PFL was immobilized onto 50 mg (0.05 g) of activated PPU, which gave 95.2 mg of PFL immobilized per gram of PPU (95.2 mg/g PPU) as the maximal immobilization.

The active content of PFL enzyme protein was estimated by Bradford method with Bovine serum albumin as the standard. The immobilized amount of PFL enzyme protein was determined by comparing the amount of protein added for the immobilization with that detected in the supernatant after immobilization. Effect of PFL amount added on lipase immobilization was also studied through the same measurements.

Part 4. Determination of PFL Activity and stability

Activity, thermal stabilities, reusability of the immobilized PFL (i.e. PFL-PPU) and effect of pH on its activity were tested through evaluation of the catalytic activity of the enzyme in hydrolysis of NPP (*p*-nitrophenyl palmitate) to yield NP (*p*-nitrophenol), a practice commonly used for estimation of enzyme activity. The activity of the enzyme is expressed by enzyme unit (U), which is defined as

the amount of PFL protein required to yield 1.0 μmol of NP per minute in the hydrolysis of NPP under specified conditions. This definition for enzyme unit was used to express the activity for both the immobilized and the free enzyme. For the effects of pH, the tests were done at 50 °C from pH 7.5 to 9.0. These same tests were also done in parallel for the free lipase, the results compared with those obtained from the immobilized PFL.

In a typical hydrolysis of NPP to yield NP, 2.0 mL of phosphate buffer solution (PBS, pH 8.0) containing 3.0×10^{-3} mg of free lipase (4.8×10^{-4} mg of the protein) was added to a glass tube of 25 mL, and heated to 50 °C, followed by addition of 0.67 mg of NPP dissolved in 1.0 mL of ethanol by one shot. The reaction was allowed to proceed for 2 min and stopped by moving the glass tube to an ice-water bath. Water was then added to dilute the reaction solution to 10 mL, followed by a filtration. The concentration of generated NP in the filtrate was analyzed using a UV-vis spectrophotometer at 410 nm, based on a previously established calibration curve from NP standard solutions.[3] For the parallel test done with the immobilized PFL (PFL-PPU), 5.5×10^{-3} mg of PFL-PPU was used (which contained the same amount of protein as the free lipase), with the rest being kept unchanged. For activity determination at different pH, it referred to the pH of the initial lipase solution before heating up to 50 °C and the addition of NPP solution in ethanol. The rest of the process was strictly followed. For the thermal stability, the tests were conducted at pH 8.0 from 30 °C to 60 °C. The reusability of the immobilized PFL was measured at 30 °C.

Part 5. Kinetic Resolution of (R,S)-1-Phenylethanol

The immobilized enzyme on PPU was finally used as enantioselective catalyst in kinetic resolution of racemic 1-phenylethanol, through its esterification with vinyl acetate as depicted by **Fig. S3**.

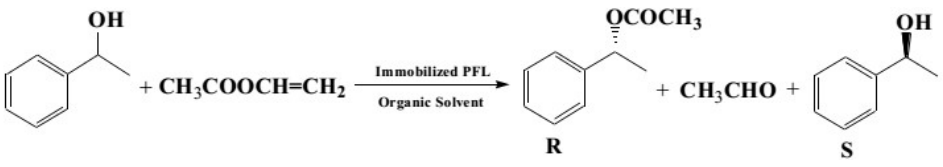

Figure S3. Kinetic resolution of racemic 1-phenylethanol through its reaction with vinyl acetate PFL as the enantioselective catalyst

In a typical experiment of kinetic resolution, (R,S)-1-phenylethanol (PEOH, 0.5 mmol) and vinyl acetate (VA, 1.0 mmol) were dispersed in isooctane or octane (4.0 mL), shaken at 40 °C shortly, followed by addition of 0.43 mg of protein (2.7 mg of free PFL powder, or 5.0 mg of the immobilized PFL, i.e. PFL-PPU) to start the reaction. 0.2 mL of the liquid sample was withdrawn at different reaction time, diluted with hexane to 10 mL, and filtered for analysis. The conversion of racemic PEOH and its enantiomeric excess (ee) were determined using HPLC (Agilent 1200) with a Chiracel OD-H column (4.6 mm × 250 mm, 5 μm). The samples were analyzed with a mixture of hexane/2-propanol (9/1 by volume) as eluent at 0.8 mL/min for 9 min at 254 nm. The conversion of the racemate (C), the enantionmeric excess of the substrate (ee_s), the enantionmeric excess of the product (ee_p) and the enantiomeric ratio of the reaction (E) were defined as following:[4,5]

$$C(\%) = (1 - \frac{C_2}{C_1}) \times 100 \qquad ee_s(\%) = \frac{C_S - C_R}{C_S + C_R} \times 100$$

$$ee_p(\%) = \frac{C_{PR} - C_{PS}}{C_{PS} + C_{PR}} \times 100 \qquad E = \frac{\ln [1 - C(1 + ee_p)]}{\ln [1 - C(1 - ee_p)]}$$

Where, C_1 and C_2 are the concentrations of (R,S)-1-phenylethanol before and after the reaction, respectively; C_S and C_R the concentration of (S)- and (R)- PEOH after the reaction; C_{PS} and C_{PR} the concentrations of the resolution products (S)-1- and (R)-1-phenylethyl acetate, respectively.

The influence of temperature on the resolution of PEOH was investigated at (10 °C, 20 °C, 30 °C and 40 °C). With isooctane replaced by octane, the reaction was also carried out at 10 °C with the other conditions kept unchanged. As for the reusability of the immobilized PFL on PPU, the solid PPU-PFL in the sample was collected from the reaction system by centrifugation after each run. After rinsing with octane and vacuum dried at 20 °C, it was subjected for the subsequent test.

Part 6. Morphology and porous properties of PPU before and after PFL immobilization.

The surface morphology of PPU and that of PPU after PFL immobilization (PFL-PPU) were examined using scanning electron microscopy (SEM, Hitachi S4800). For visual illustration, the corresponding SEM pictures are shown in **Fig. S4**.

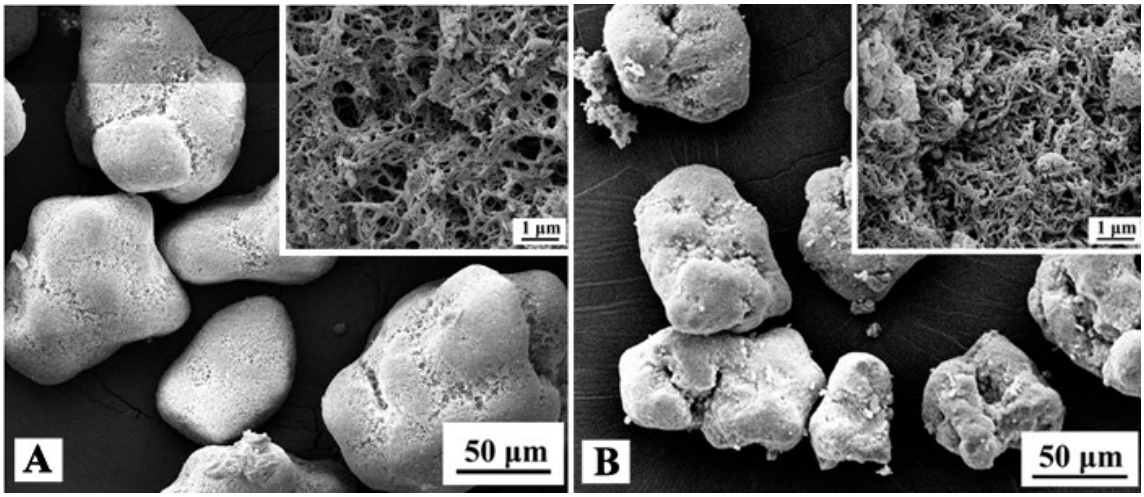

Figure S4. SEM pictures of (A) PPU and (B) PPU after PFL immobilization (PFL-PPU)

Porous properties of PPU, its derivative after reaction with GA (GA-PPU) and that after PFL immobilization (PFL-PPU) were characterized through Hg intrusion, using Micromeritics porosimeter (Autopore IV 9500) with hydraulic oil provided by the instrument manufacturer to achieve desired pressure.

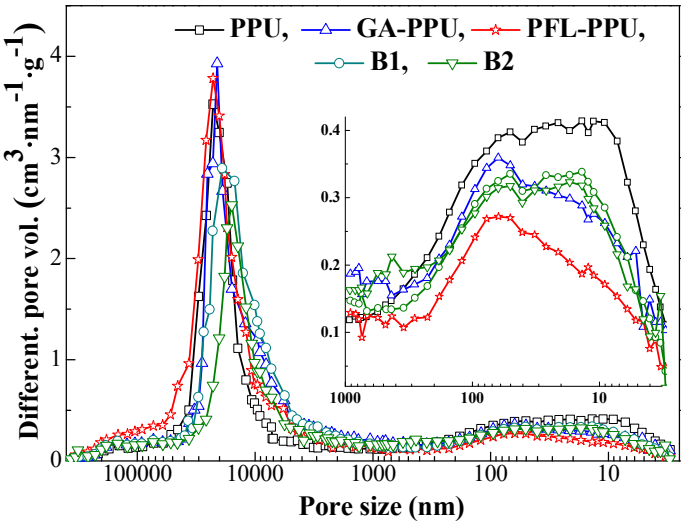

Figure S5. Pore size distribution of PPU, GA-PPU (PPU after GA activation), reference B1 for GA activation (prepared following exactly the same protocol as GA-PPU but in the absence of GA), PFL-PPU (PPU after PFL immobilization following GA activation) and reference B2 for PFL immobilization (prepared following exactly the same protocol as PFL-PPU in the absence of PFL)

Part 7. Thermostability of the free and immobilized PFL and reusability of immobilized PFL

Table S1. Thermostability of the free and immobilized PFL

Enzyme	Temperature (°C)	Incubation (h)		
		0	1	3
Free PFL	30	100	81	55
	40	100	70	42
Immobilized PFL	30	100	84	80
	40	100	79	71

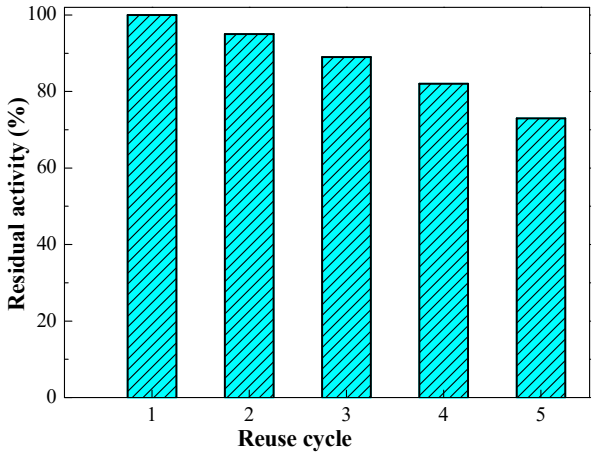

Figure S6. Residual activity of the immobilized PFL in repeated use for NPP hydrolysis

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