Electronic Supplementary Material (ESI) for Reaction Chemistry & Engineering.

Supporting Information

Facile mussel-inspired polydopamine-coated 3D-printed bioreactors for continuous flow biocatalysis

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1. Materials and methods

1.1 Reagents and materials

*Gc*APRD wild type was prepared according to the previous procedure.[1] Chemicals for cell cultivation, enzyme purification, and immobilization were purchased from Nacalai Tesque (Japan), except for 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES) and dithiothreitol (DTT), which were purchased from Sigma Aldrich (USA) and Wako (Japan), respectively. Protein concentration measurement reagent was purchased from Bio-Rad (USA). Commercial grade solvents, acetophenone, and *rac*-1-phenylethanol were purchased from Nacalai Tesque (Japan). (*S*)-1-phenylethanol was a gift from Afifa Ayu Koesoema, Okayama University.

Dopamine (hydrochloride) was supplied by Merck & Co., Inc. (USA). Glutaraldehyde (ca. 50% in water, ca. 5.6 mol / L) was purchased from Tokyo Chemical Industry (Japan). Poly (ethyleneimine) solution, average Mn ~ 60,000 by GPC, average Mw ~ 750,000 by LS, 50 wt.% in H_2O was supplied by Sigma Aldrich (Japan).

1.2 3D-printed bioreactors fabrication

3D-printer (Value3D MagiX MF-2200D equipped with tip diameter 0.4 mm MF-2200D printhead), 3D-printing filament (Value3D MagiX material PP filament 1.75 mm 500 g reel), and 3D stage sealant ATP-101 were purchased from MUTOH shop (Japan). The 3D-printed bioreactors were designed by CAD programs, Autodesk Tinkercad and Autodesk Fusion 360, and were fabricated by the 3D-printer with nozzle temperature 235 ℃ and bed temperature 95-100 ℃.

1.3 3D-printed batch bioreactors surface functionalization and *Gc*APRD immobilization

3D-printed batch reactors surface treatment and enzyme immobilization procedure were modified from the literatures.[2,3] The 3D-printed bioreactors were washed with an excess of ethanol and acetone. Then, the bioreactors were treated with 1.0 mL of 2 mg/mL of dopamine (DA) in Tris-HCl buffer (10 mM), pH 8.5 for >20 h, and washed with excess distilled water to achieve polydopamine (PDA) layer. Next, the glutaraldehyde (GA) layer was grafted with 1.0 mL of GA in distilled water 25% v/v for 2 h. The polyethylenimine (PEI) layer was added with 1.0 mL of 5% w/v PEI for 2 h followed by the additional GA layer, 25% v/v, 1.0 mL overnight. All the surface functionalization procedure was conducted at room temperature. After that, *Gc*APRD was immobilized by incubating 1.0 mL enzyme solution with 0.25 mg/mL protein in the reactors at

4 ℃ overnight. The supernatant was removed and measured residual protein amount by Bradford method[4] using UV-1900-UV-Visible spectrophotometer from Shimadzu (Japan) to assess immobilization yield (**1**).

$$\text{Immobilization yield (\%)} = \left(\frac{[\text{Protein}]_I - [\text{Protein}]_R}{[\text{Protein}]_I} \right) \times 100\ (\%) \qquad (\mathbf{1})$$

$[\text{Protein}]_I$ = Initial protein concentration (mg/mL)

$[\text{Protein}]_R$ = Concentration of residual protein in the supernatant after the *Gc*APRD immobilization (mg/mL)

After removing the enzyme solution, the reactors were incubated with 0.85% NaCl at 4 ℃ for 2 h to remove the protein immobilized by non-specific interaction. The immobilized *Gc*APRD was used for a ketone reduction to assess reaction yield and enzyme activity.

1.4 Ketone reduction by immobilized *Gc*APRD and enzyme activity

The reaction was conducted on a micromole scale. 1.0 mL of reaction mixture consisted of acetophenone (6.2 mM), 2-propanol (15% v/v), NAD^+ (0.20 mM), and HEPES-NaOH buffer (0.10 M, pH 7.2) was added to the 3D-printed batch bioreactors with immobilized *Gc*APRD. The reaction was conducted at 37 ℃ for 60 min. A portion of the mixture was extracted with diethyl ether to determine the reaction yield by chiral gas chromatography (GC) (GC-14B equipped with a flame ionization detector and a CP-Chirasil-Dex-CB column (Varian 0.32 mm x 0.25 μm x 50 m) using He carrier gas (5 mL/min, head pressure: 274 kPa, injector: 180 ℃, detector: 180 ℃) from Shimadzu, Japan), using 3-methyl-1-butanol as an internal standard. The GC analysis conditions were 40 ℃ 1 min, 1 ℃/min, 120 ℃ 10 min. The enantiomeric excess (*ee*) values were determined by GC analysis, and the absolute configurations of 1-phenylethanol were determined by comparing their GC retention times with the corresponding authentic samples prepared beforehand.[5] The retention times of internal standard, ketone, (*R*)-alcohol, and (*S*)-alcohol were 6.5, 10.0, 14.5, and 14.9 min, respectively.

The specific activity of the immobilized *Gc*APRD was calculated by equation (**2**).

$$\text{The specific activity of } Gc\text{APRD } (\mu\text{mol}/(\text{min}\cdot\text{mg protein})) = \frac{\text{Reaction yield } (\mu\text{mol})}{\text{reaction time (min)}\times\text{protein in reaction (mg)}} \quad (\mathbf{2})$$

1.5 Flow process establishment

3D-printed microfluidic bioreactors' surface was functionalized by PDA and GA following the procedure in **section 1.3**. Then, *Gc*APRD was immobilized onto the functionalized surface following the procedure in **section 1.3**. Afterward, the 3D-printed microfluidic bioreactor with immobilized *Gc*APRD was equipped with Swagelok tube fitting (Swagelok, Japan) and connected with a pump, Jasco Pu-2085 Plus Semi-Micro HPLC Pump, (Jasco, Japan).

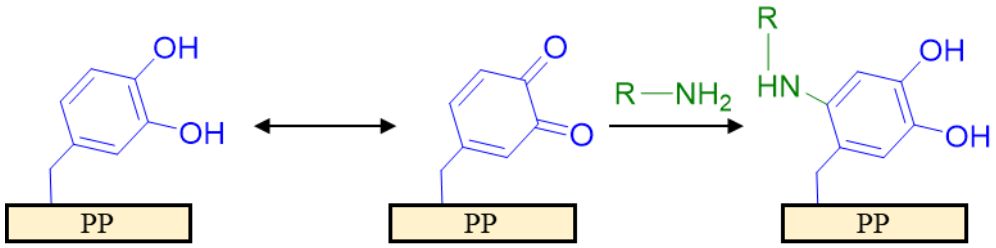
The reaction mixture consisted of acetophenone (5.0 mM), 2-propanol (15% v/v), NAD^+ (0.20 mM), and HEPES-NaOH buffer (0.10 M, pH 7.2) was fed through the pump and the installed 3D-printed bioreactor to proceed the reaction with adjusted flow rates at room temperature. The output was collected by test tubes and occasionally determined % yield and % *ee* by GC analysis.

The product's mixture was extracted by diethyl ether 3 times, dried over $MgSO_4$, filtered, and evaporated. The corresponding alcohol was purified by silica gel column chromatography (hexane:ethyl acetate, 3:1) to afford (*S*)-1-phenylethanol and characterized by ^{1}H-NMR analysis (Bruker Biospin AVANCE III 400 spectrometer at 400 MHz in $CDCl_3$). The ^{1}H-NMR spectra of alcohols were in agreement with that reported in the literature.[6]

(*S*)-1-Phenylethanol: ^{1}H-NMR (400 MHz, $CDCl_3$, 25 ˚C, TMS): δ=7.28-7.42 (m, 5H, Ph-H), 4.90-4.95 (m, 1H, -CH-), 1.78 (d, 1H, *J* = 2.8 Hz, -OH), 1.53 (d, 3H, *J* = 6.5 Hz, -CH_3).

1.6 Scanning electron microscopy (SEM) and energy-dispersive X-ray spectroscopy (EDX) analysis

PP, PP-PDA, PP-PDA-GA-PEI-GA'-*Gc*APRD, were characterized by SEM and EDX analysis (Bench-top Scanning Electron Microscope (SEM) proX supplied by Phenom-World, Netherlands).



Scheme S1 Schiff base reaction or Michael addition.[7,8]

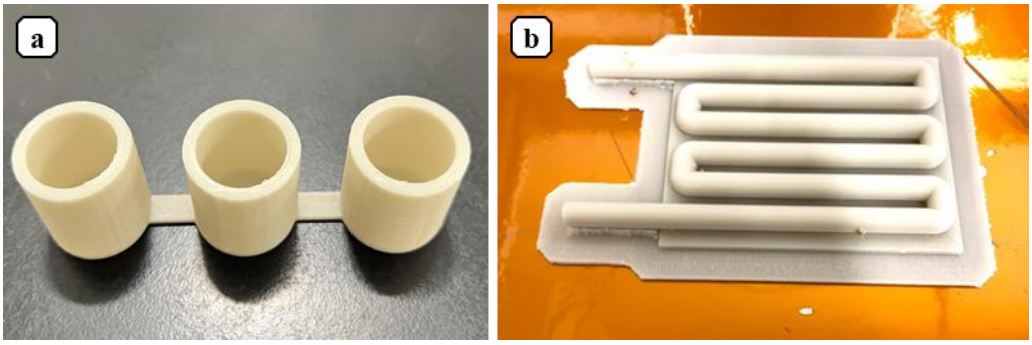


Figure S1 3D-printed bioreactors. a) Batch bioreactors and b) microfluidic bioreactor.

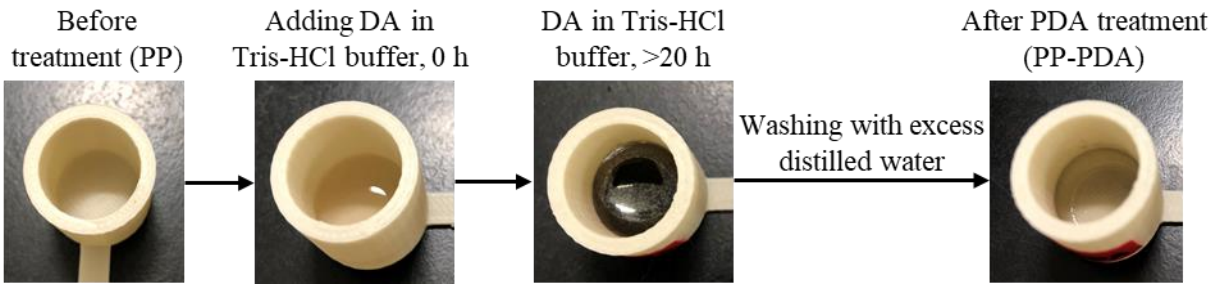


Figure S2 3D-printed batch bioreactor treated by PDA. The bioreactor was filled with 1.0 mL of 2 mg/mL of DA in Tris-HCl buffer (10 mM), pH 8.5, and incubated at room temperature for >20 h.

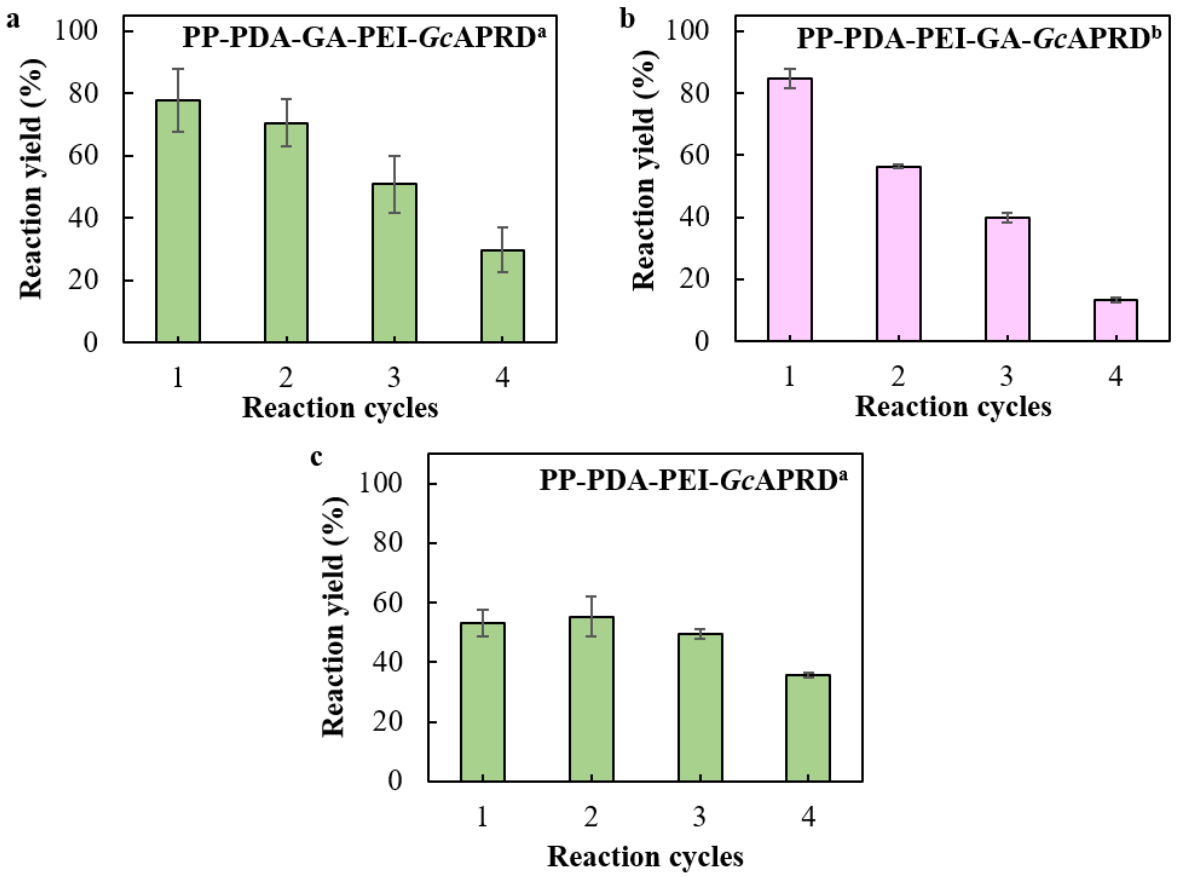


Figure S3 Recyclability of *Gc*APRD immobilized on (a), (c) functionalized 3D-printed bioreactors and (b) functionalized PP filaments. *Ee* of the product was >99% (*S*) for all reactions. [a] Recyclability of the immobilized *Gc*APRD was assessed in 3D-printed batch bioreactors. The reaction mixture consisted of acetophenone (6.2 mM), 2-propanol (15% v/v), NAD^+ (0.20 mM), and HEPES-NaOH buffer (0.10 M, pH 7.2) up to 1.0 mL. The reaction in each cycle was conducted at 37 °C, for 60 min. Afterward, the supernatant was removed, extracted with diethyl ether, and analyzed by GC. The reactors were washed by the reaction mixture without NAD^+ and proceeded to the next reaction cycle.

[b] Recyclability of the immobilized *Gc*APRD was assessed using PP filaments as support. PP filaments was treated with DA, GA, PEI, GA, and *Gc*APRD was immobilized. The reaction mixture consisted of acetophenone (5.0 mM), 2-propanol (15% v/v), NAD^+ (0.20 mM), and HEPES-NaOH buffer (0.10 M, pH 7.2) up to 1.5 mL. The reaction in each cycle was conducted at 30 ˚C, 200 rpm for 180 min. Afterward, the supernatant was removed, extracted with diethyl ether, and analyzed by GC. The PP filaments were washed by distilled water and the reaction mixture without NAD^+ and proceeded to the next reaction cycle.

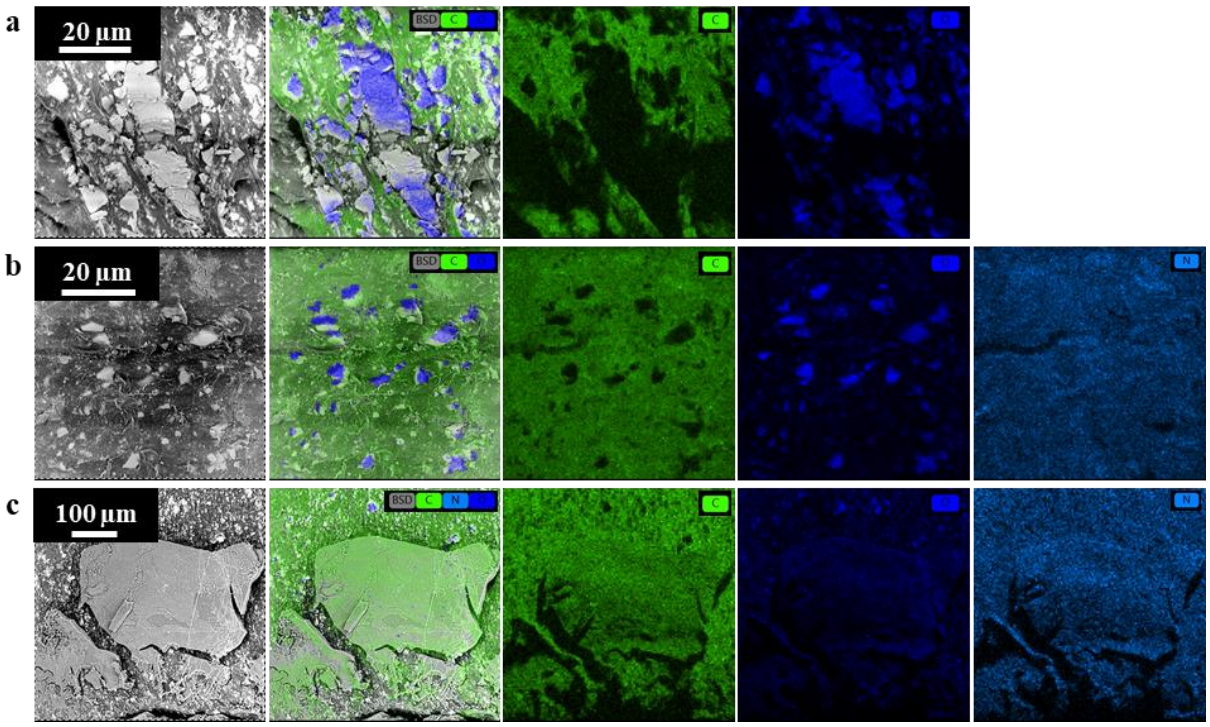


Figure S4 Elemental mapping images of a) PP, b) PP-PDA, and c) PP-PDA-GA-PEI-GA'-*Gc*APRD.

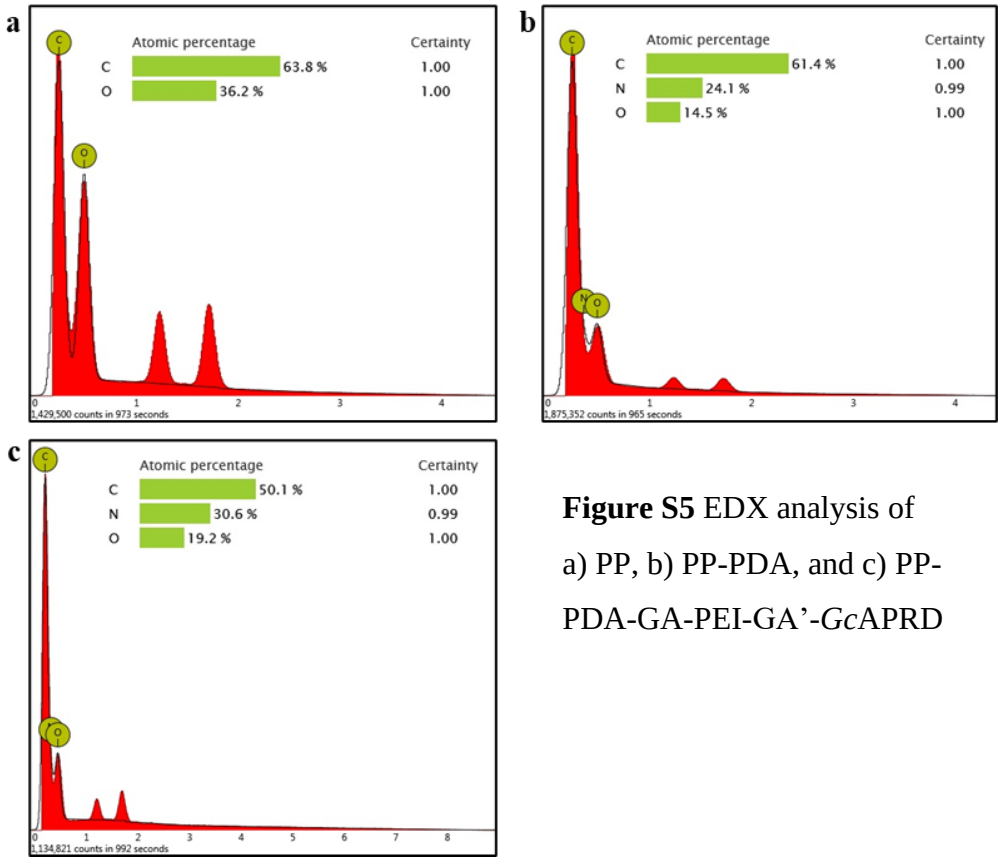


Figure S5 EDX analysis of a) PP, b) PP-PDA, and c) PP-PDA-GA-PEI-GA'-*Gc*APRD

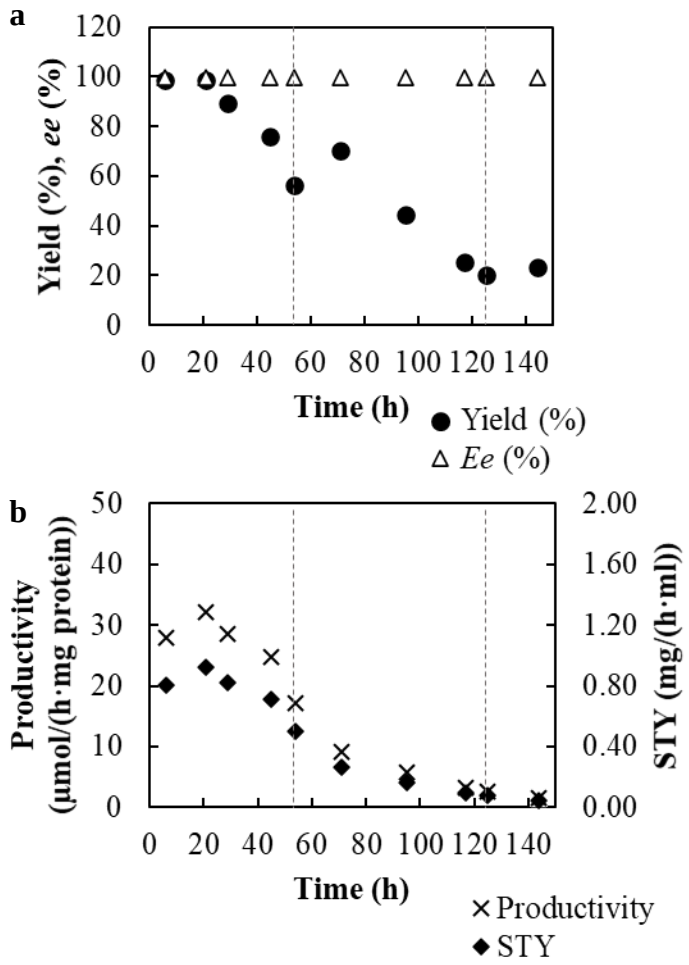


Figure S6 Flow process establishment utilizing 3D-printed microfluidic bioreactor with immobilized *Gc*APRD (PP-PDA-GA-*Gc*APRD), a) analytical yield (%) and *ee* (%, *S*), and b) productivity (μmol/(h·mg protein)) and STY (mg/(h·ml of bioreactor volume)). The reaction conditions are described in **section 1.5** in the supporting information. The reaction mixture was fed thru the pump and 3D-printed microfluidic bioreactor with flow rate 25 μl/min at 0-54 h, 10 μl/min at 54-125 h, and 5 μl/min at 125-144 h. As in **Figure S6a**, the reaction yield was about 99% up to 21 h and dropped to 89%, 76%, and 56% at 29 h, 45 h, and 54 h, respectively. Then, we slowed down the flow rate to increase retention time, and the yield was increased to 70% at 71 h. After that, the reaction yield was dropped to 20% at 125 h. We decreased the flow rate to be 5 μl/min, but the yield was not noticeably changed (23% at 144 h). The *ee* of the product was >99% (*S*) through the whole process. For productivity and STY (**Figure S6b**) at 0-45 h, we obtained productivity 24.7-32.1 μmol/(h·mg protein) or STY 0.71-0.92 mg/(h·ml). Subsequently, up to 54 h, productivity and STY were gradually dropped to 17.2 μmol/(h·mg protein) and 0.50 mg/(h·ml), respectively. The productivity and STY were continuously decreased and retained 1.5 μmol/(h·mg protein) productivity and 0.04 mg/(h·ml) STY at 144 h.

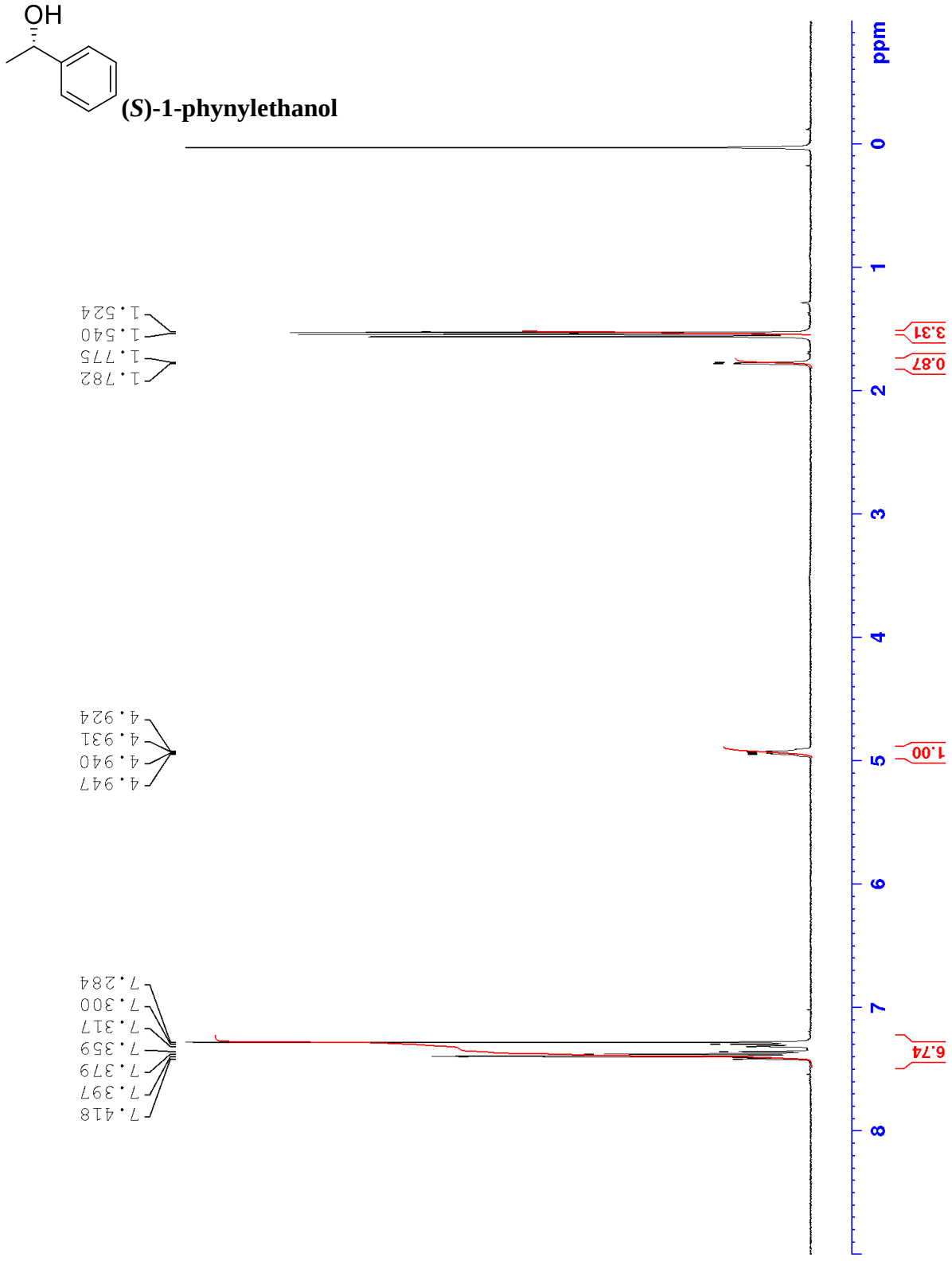
Table S1 Comparison of acetophenone reduction by the immobilized *Gc*APRD in this study and free *Gc*APRD from the previous study.

	***Gc*APRD immobilized on 3D-printed microfluidic bioreactor in this study**	**Free *Gc*APRD in millimole scale from the previous study[9]**
Process	Continuous flow	Batch
Units of enzyme in the reaction	12.5	50-250
Yield	43.2 mg analytical yield, 32.5 mg isolated yield	98 mg isolated yield
Product (mg) per 1 unit of enzyme	3.5 mg analytical yield, 2.6 mg isolated yield	0.4-2.0 mg isolated yield

Table S2 General information of 3D-printed microfluidic reactors.

3D-printed microfluidic bioreactor (one unit)	
PP used (g)	36.5 ± 4.0
PP price (USD)	6.3 ± 0.7
Printing time	6-7 h

^{1}H NMR spectrum of (*S*)-1-phynylethanol



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