Supporting Information

Orderly cascade of immobilized-enzyme catalysis and photocatalysis for continuous-microflow production of 2-phenylbenzothiazole

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Abstract: Green synthesis technology of 2-phenylbenzothiazole (2-PBZ)—an important versatile scaffold—has been widely developed. However, its economic, efficient, and sustainable synthesis remains challenging. Here, for the first time, we propose to cascade immobilized enzyme catalysis and photocatalysis in a continuous-microflow manner to realize the online reuse of the enzyme, eliminate cross-inhibitions between the two-step reaction processes, provide stronger and more uniform illumination, and enhance process efficiency. This novel microflow system required only 4.6 min of the total residence time to achieve >99% product yield, and 93.7% of the total catalytic activity was retained after running for 300 min. This orderly cascaded continuous-flow microreactor system widens the limitations of photoenzymatic approaches and provides the potential foundation for the industrial production of 2-PBZ.

Experimental Procedures

1. Materials and chemicals

We purchased 2-phenylbenzothiazole (2-PBZ, 98%), benzaldehyde (BZD, 98%), and N^1-(3-trimethoxysilylpropyl) diethylenetriamine (95%) from Shanghai Meryer Chemical Technology Co., Ltd., and 2-aminothiophenol (2-AP, 97%) was purchased from Energy Chemical Technology (Shanghai) Co., Ltd. Trypsin from bovine pancreas (2500 USP), silica (300-400 mesh), and EY were purchased from Shanghai Aladdin Bio-Chem Technology Co., Ltd. Folin and Ciocalteu's phenol reagent was purchased from Sigma-Aldrich, Inc. N-(3-dimethylaminopropyl)-N-ethylcarbodiimide hydrochloride (99%), N, N-diisopropylethylamine (99.5%), and N-hydroxy succinimide (98%) were purchased from Shanghai Bide Pharmatech Ltd.

2. Immobilization and characterization of enzyme

A schematic diagram of the covalent immobilization of trypsin on the silica carrier is shown in Figure 3a. The first was to realize the amination of the surface of the silica particles using methods reported in the literature.[1] Silica (2 g), 8 mL N^1-(3-Trimethoxysilylpropyl) diethylenetriamine, and 2 mL N, N-diisopropylethylamine were added to 100 mL of ethanol. The solution was stirred at 220 rpm for 12 h at 25 °C. The solid particles obtained by centrifugation were immersed, washed three times with ethanol, and then three times with water. Finally, they were dried in an oven at 80 °C for 12 h to obtain amino-functionalized silica particles (silica-NH_2).

The second was the covalent attachment of trypsin to silica-NH_2, which was appropriately adjusted according to a previously reported method.[2] First, 160 mg trypsin was added to 60 mL of 50 mM phosphate buffer (pH = 6.8) for dissolution. Then, 2 g of amino-functionalized silica particles, 700 mg of N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride, and 150 mg of N-hydroxy succinimide were added to the enzyme solution, which was stirred at 220 rpm for 6 h at room temperature. The solid particles obtained after centrifugation were washed four times with a 70% (v/v) acetonitrile aqueous solution. Finally, the particles were dried under vacuum at 40°C for 2 h to obtain enzyme-immobilized silica particles (trypsin@silica).

A confocal laser microscope (Olympus, FV1200) was used to observe the fluorescence distribution of trypsin on the surface of the silica carrier. First, a small amount of the powder sample was added to 100 μL of FITC aqueous solution at a concentration of 10 μg/mL. The dyeing process was maintained in

the dark for 15 min. After washing three times with pure water, the sample was resuspended, and 10 μL of the resuspended solution was taken and dropped onto a glass slide. The sample was excited with a 488 nm laser, and the received light range was 500-600 nm. Then, the sample was observed under a laser confocal microscope. An intelligent Fourier infrared spectrometer (Nicolet 6700FTIR, Shanghai Thermoelectric Technology Instrument Co., Ltd.) was used for the FT-IR analysis.

3. Determination of loading amount of enzyme

The quantitative determination of the loading amount of trypsin was performed using Lowry's method.[3] First, 500 mg of the immobilized sample was added to 40 mL of 0.1 M NaOH solution, and the mixture was stirred at 220 rpm for 3 h. After that, 10 mL of the supernatant was collected. $CuSO_4$ solution (200 μL, 0.02 M) was added to the supernatant solution, was mixed well, and allowed to stand for 10 min. Then, 2 mL of Folin and Ciocalteu's phenol reagent were added to the solution, which was quickly mixed evenly within 2 s. The obtained solution was placed in a water bath at 40 °C for 10 min. After cooling to room temperature, the absorbance at 500 nm was measured using an ultraviolet-visible spectrophotometer (Shimadzu, UV-2450), and the results were compared with the standard curve measured by the standard protein solution. Each sample was measured three times, and the average value was recorded.

4. Experimental setup

In the experimental device (Figure 1), a methanol aqueous solution containing reactant 2-AP was used as stream 1, and a methanol aqueous solution containing reactant BZD was used as stream 2. These two streams were driven by two liquid chromatography pumps (Beijing Satellite Co., Ltd.) into micromixer 1 (the inner diameter of the mixing microchannel was 0.58 mm, and the material was 316L stainless steel) for rapid mixing at a flow rate of 0.25-2 mL/min. The mixed solution was first pumped into a temperature-controlled coil (2 m in length and 1.6 mm in inner diameter) and then pumped into a micro-packed bed reactor (4 mm inner diameter, 1 mm wall thickness, and 400 mm length). Both ends of the packed columns were plugged with quartz wool. After exiting the micro-packed bed reactor, the main stream was first mixed with EY solution pumped by a metering pump (Beijing Satellite Co., Ltd.) at a flow rate of 0.5-2 mL/min in micromixer 2 (0.58 mm in inner diameter). Then, the main stream was mixed with the gas coming from the laboratory gas path in micromixer 3 (inner diameter of 1 mm). The photocatalytic reaction tube was spirally wound around the periphery of the internal cylindrical lamp, and the spiral reaction tube was placed at the central axis of the external cylindrical lamp. The material

of the reaction tube was transparent polytetrafluoroethylene with an inner diameter of 0.58, 1, or 1.6 mm, and the length depended on the residence time requirement. There were uniform LED distributions on the cylindrical surfaces of both the internal and external lamps, with a total power of 20 W and emission of 440 nm blue light. The entire photocatalytic device was placed in a dark box, and radiators were configured to cool the lamps.

5. Sample analysis

For the batch reaction, the reaction solution was sampled directly using a syringe. For the flow reaction, after the system ran stably, samples were collected through a relevant sampling port. 10 μL solution from the obtained sample solution, and it was added to 990 μL of 70% (v/v) methanol aqueous solution and shaken well. After the sample was filtered with a 0.22 μm syringe filter, it was used for ultra-high-performance liquid chromatography (UPLC) analysis. Waters ACQUITY UPLC I-Class equipped with an ACQUITY T3 column (2.1 mm × 100 mm, 1.8 μm, Waters) and a PDA detector capable of full-wave scanning were employed for UPLC analysis, which was performed at 25 °C with a detection wavelength of 254 nm using methanol:water (7:3, v/v) as the eluent at a flow rate of 0.3 mL/min. The retention times of BZD, 2-AP, Int, and 2-PBZ were 1.2, 2.2, 3.3, and 5.8 min, respectively.

6. Illumination simulation

The ray optics module in COMSOL Multiphysics 5.6 was used to simulate the illuminance distribution in the reaction channel. A dielectric film with a thickness of 500 μm and refractive index of 1.37 was set at the boundary of the reaction channel. The LED was simplified as a point light source that emitted visible light with a wavelength of 440 nm in the spherical direction, and the number of rays in the wave vector space was set to 8000. The samples were randomly distributed. Through the ray tracing simulation, the light intensity value under the path of 10000 μm was calculated by the transient state, and the step length was 10 μm. Finally, the space-time function values of the light intensity on a certain cross section of the reaction channel were derived, and Origin 2018 was used for visualization. The light intensity values based on the spatial variable were converted into a three-dimensional matrix; the tolerance of the spatial variable was set to 0.01, and the repeated value was replaced with a summed value. The Renka Cline method was adopted for gridding the matrix transformation.

Supplementary tables and figures

Table S1. Examples of conventional methods for the synthesis of 2-phenylbenzothiazole via condensation of *o*-aminophenol derivatives with aldehydes.

Metal Catalyst	Conditions	Time	Yield (%)	Ref.
$RuCl_3$	80°C, Air, $[bmim]PF_6$	30 min	83	4
$Fe(NO_3)_3$	120°C, O_2, P(*t*-Bu)$_3$ · HBF_4	24 h	89	5
$[Cp^*IrI_2]_2$	80°C, Toluene	24 h	68	6
AgOTs	100°C, N_2	12 h	96	7

Table S2. Summarization of methods used for 2-PBZ production in literatures and this work.

Method	Conditions	Substrate concentration (mol · L^{-1})	Enzyme dosage (g · $mmol^{-1}$ 2-AP)	Reaction time	Synthesis mode	Yield (%)	Production (g · h^{-1} · L^{-1})	Ref.
Metal catalysis	$RuCl_3$, 80°C, Air, $[bmim]PF_6$	1	--	30 min	Batch	83	350.72	4
Metal catalysis	$Fe(NO_3)_3$, 120°C, O_2, P(*t*-Bu)$_3$ · HBF_4	0.5	--	24 h	Batch	89	3.92	5

Visible-light catalysis	Disulfide photosensitizes, Air, r.t.	0.1	--	4 h	Batch	92	4.86	8
Enzyme catalysis	Bakers' yeast, CH_2Cl_2, r.t.	0.8	0.25	24 h	Batch	75	5.28	9
Enzyme catalysis	Laccase, O_2, pH=4.0, r.t.	0.5	0.1	24 h	Batch	85	3.74	10
One-pot photo-enzyme catalysis	Trypsin, SR 43, Air, r.t.	0.1	0.05	10 min	Batch	96	121.70	11
Enzyme catalysis + photocatalysis	Immobilized-trypsin, EY, Air, r.t.	0.1	0.001[#]	4.6 min	Continuous	> 99	272.83	This work

[#] The substrate amount relative to the immobilized enzyme dosage is calculated according to the continuous running time of 300 min.

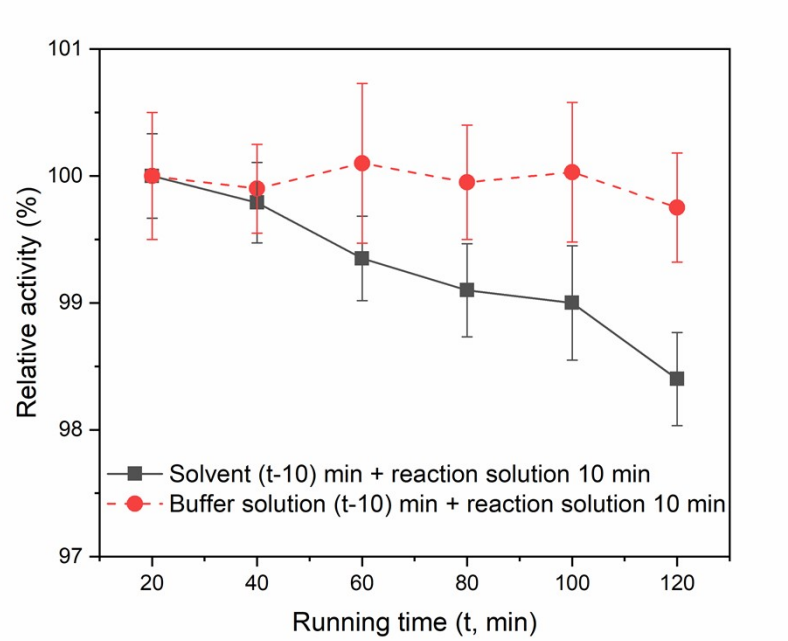

Figure S1. The effect of the solvent of the reaction solution on the activity of immobilized enzyme. For the black line, the solvent (70% MeOH) is first pumped into μ-PBR for running (t-10) min, and then reaction solution for 10 min. For the red line, phosphate buffer (50 mM, pH = 6.8) is first pumped into μ-PBR for running (t-10) min, and then reaction solution for 10 min. All flow rates are 2 mL/min. Relative activity refers to the ratio of the yield of t min to that of t = 20 min.

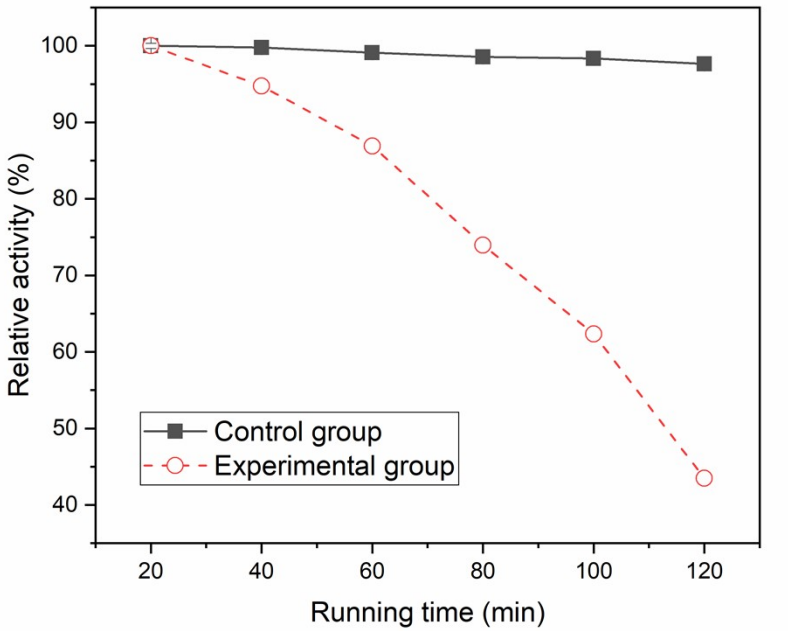

Figure S2. The effect of inhibition of int on the activity of immobilized enzyme. For the control group, the fresh reaction solution (the concentration of both reactants is 0.1 M) is continuously pumped into μ-PBR at a flow rate of 2 mL/min. For the experimental group, 20 min is taken as an operation stage. At

the beginning, 40 mL of fresh reaction solution (the concentration of both reactants is 0.1 M) is pumped into μ-PBR at a flow rate of 2 mL/min. 20 min later, additional substrate is added to the solution coming out of the reactor so that the concentration of both reactants is 0.1 M, and then the solution is pumped into μ-PBR again for the second operation stage. By analogy, six operation stages are completed. The relative activity refers to the ratio of the yield measured in the outlet solution after a certain running time to the yield measured when the running time is 20 min.

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