Supplementary information

A fluorescence-activated droplet sorting (FADS) assay for ultra-high-throughput screening of PET hydrolases based on a pH indicator

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1. Materials

All chemicals were of analytical-reagent grade and were purchased from Sigma-Aldrich, Aladdin, unless specified. All enzymes were purchased from New England Biolabs, TransGen Biotech, unless specified. Oligonucleotide synthesis and DNA sequencing were carried out by Genewiz (China). Polydimethylsiloxane (PDMS) prepolymer Sylgard 184 was purchased from Dow Corning, QX200 Droplet Generation Oil was purchased from Bio-Rad, and Invitrogen™ SNARF™-4F 5-(and-6)-Carboxylic Acid (C-SNARF-4F) from ThermoFisher. Goodfellow PET film was purchased from Goodfellow GmbH (UK).

2. Methods

2.1 Experimental setup and microfluidic chip fabrication

The optical setup for FADS screening includes a high-speed camera mounted on an inverted microscope (IX81, Olympus, Japan), a high-voltage amplifier, an excitation laser and photomultiplier tubes (PMTs) for fluorescence detection. For device fabrication, three microfluidics chips were used for cell encapsulation, substrate and probe picoinjection, and sorting, respectively. The microchips were fabricated using standard soft lithography techniques[1, 2]. PDMS pre-polymer and its curing agent were mixed thoroughly at a 10:1 weight ratio and degassed for 30 minutes. The mixture was then poured into a silicon wafer master mold containing different microchannel designs and cured at 80°C for 2 h. Upon cooling, the PDMS slabs were removed from the mold and holes were bored as inlets and outlets for the dispersed and continuous phases. The PDMS slabs were then exposed to oxygen plasma and sealed onto glass slides. For the picoinjection and sorting devices, the electrode channels were filled with low-melting-temperature solder wire (Indium Co., USA). Aquapel was injected into the microchannel to render it hydrophobic and subsequently flushed with pressurized air. Teflon tubes were attached to the inlets and outlets of the microchips, and fluids were pumped into the microchips by syringe pumps (Harvard Apparatus, USA).

2.2 Development of pH-based FADS system

The screening assay utilized bis(2-hydroxyethyl) terephthalate (BHET) as the model substrate and C-SNARF-4F as the pH-sensitive fluorescent indicator. Full-spectrum scans were conducted to determine the optimal excitation and emission wavelengths of the probe in a CoStar 96-well black polystyrene plate using Tecan Infinite M200 Pro microtiter plate (MTP) reader (Zurich, Switzerland). A preliminary validation was performed using the C-SNARF-4F probe with buffers of different pHs (4.6 to 9.0) to ascertain the working range of the fluoroprobe. A model experiment was then performed to sort a premixed library containing a 1:9 ratio of active (DepoPETase β) to inactive (pET 22b vector) cells. Overnight cultures of the two strains were prepared in 5 mL LB containing 100 μg/mL ampicillin, incubated at 37 °C with shaking at 220 rpm. OD600 measurements were taken, and the cells were mixed at a ratio of 1:9 active to inactive cells. The cell mixture was then diluted in the induction medium

(LB containing 100 μg/mL ampicillin, 100 mM Isopropyl β-d-1-thiogalactopyranoside (IPTG)) to an appropriate concentration before encapsulation. Monodispersed droplets were generated using the drop-making chip at a flow rate of 160 μL/h diluted cell culture and 300 μL/h QX200 oil. The droplets were collected and incubated in a 1 mL syringe at 20°C for 48 hours for expression. A 10 mM BHET solution was prepared by mixing the BHET powder with 0.1 M glycine-NaOH buffer (pH 9.0) and sonicated till completely dissolved. C-SNARF-4F probe was added to the substrate solution to a final concentration of 50 μM. Prior to picoinjection, the droplets were subjected to a heat-treatment at 50°C for 20 min and subsequently cooled to room temperature. For picoinjection, the droplets were re-injected into a picoinjection microchip. By applying a voltage of 100 V at a frequency of 25 kHz, the droplets were injected with the substrate-dye mixture and collected in a 1 mL syringe. For sorting, the droplets were reintroduced into the sorting chip and passed through an excitation beam; the resulting fluorescence signals were detected by PMTs. Sorting was performed based on a predefined fluorescence threshold, whereby droplets exceeding the threshold were deflected by high-voltage pulses of 600 V and 25 kHz. The sorted cells were recovered and assessed to determine the sorting efficiency.

2.3 Gene mining and identification of new PET hydrolases

Using the amino acid sequence of the leaf-branch compost cutinase (LCC) (UniProt: G9BY57) as a template, a BLAST search was performed against the NCBI (https://www.ncbi.nlm.nih.gov/) and UniProtKB (https://www.uniprot.org/) databases, followed by redundancy reduction using CD-HIT. The Enzyme Similarity Tool from the Enzyme Function Initiative (EFI) was employed to compare all retrieved sequences by applying an alignment score threshold, leading to the construction of a sequence similarity network (SSN). The SSN was visualized using Cytoscape v.3.10.3. From the cluster containing LCC, 100 sequences were selected for multiple sequence alignment using MEGA software, during which divergent sequences were removed. A phylogenetic tree was constructed from the remaining sequences using the neighbor-joining method. Based on factors including phylogenetic branch, sequence similarity, and microbial origin, eight candidate sequences were selected for protein expression and activity characterization.

2.4 Construction, expression and activity characterization of putative PET hydrolases

Synthesis and insertion of the genes of the selected candidates into pET22b vector (with their native signal peptides) were carried out by Genewiz (Suzhou, China). The plasmids were transformed into *E. coli* BL21(DE3) cells and cultivated on an LB agar plate overnight at 37 °C. A single colony was inoculated into 20 mL of LB medium containing 100 μg/mL ampicillin and cultured at 37 °C with shaking at 220 rpm for 12–16 h. This pre-culture was used to inoculate 1 L of LB medium (100 μg/mL ampicillin) at a 1% inoculum volume, followed by incubation at 37 °C and 220 rpm until the OD600 reached 0.6–0.8. IPTG was then added to a final concentration of 0.1 mM, and induction was continued at 20°C for 24 h. The cells were

harvested by centrifugation at 5000 rpm for 50 min at 4 °C, and both the cell pellet and the supernatant containing the crude enzyme extract were collected. The activities of the putative PET hydrolases were evaluated using the fluorescent pH assay in a CoStar 96-well black polystyrene plate, and measurements were taken using a TECAN microplate reader. Reactions in each well consisted of 100 µL of 5 mM BHET supplemented with 50 µM C-SNARF-4F pH indicator and 100 µL of crude enzyme solution. PET hydrolase with the highest activity was selected for subsequent engineering for enhanced activity and thermostability.

2.5 Construction of DepoPETase β and *Sd*PETase mutagenesis libraries

The random mutagenesis libraries were generated using the standard error-prone PCR (epPCR) protocol reported previously with pET22b-DepoPETase β and pET22b-*Sd*PETase genes as templates. For the mutagenic PCR (95°C for 2 min, 1 cycle; 95°C, 20 s/63°C, 20 s/72°C, 30 s, 25 cycles; 72°C for 6 min, 1 cycle), EasyTaq DNA polymerase (5 U), dNTP mix (0.20 mM), template (pET22b-PETase), varied concentrations of $MnCl_2$, and primers were used. The PCR products were purified by using a TransGen PCR purification kit (TransGen Biotech, Beijing, China). The PCR products were cloned into the expression plasmid pET22b and transformed into E. coli BL21-Gold (DE3) cells for further expression and screening.

2.6 FADS Screening of the DepoPETase β and *Sd*PETase variants

Overnight culture was prepared by inoculating 10 µL of glycerol stock into 5mL of LB medium, followed by incubation at 37°C. The overnight culture was diluted with expression medium containing 100 µg/mL ampicillin and 0.1 mM IPTG to an appropriate concentration before encapsulation. The procedures for the generation of monodisperse droplets, substrate and probe mixture picoinjection and sorting of droplets were as detailed above. Before the addition of substrate and probe mixture, the droplets were incubated at 50°C for 30 minutes. The droplets were then reintroduced into a pico-injection microchip, and a mixture of 10 mM BHET and 50 µM C-SNARF-4F probe solution was injected into the droplets at a rate of 5 µL/h. The droplets were then collected in a syringe and subsequently reinjected into a sorting device at a flow rate of 30 µL/h. As the droplets passed through the excitation spot, they emitted fluorescence signals that were detected by the PMT and sorted according to the predefined sorting threshold. The collected droplets were then poured onto an LB agar plate to recover the cells.

2.7 Rescreening of PET hydrolases variants in a 96-well MTP

Colonies grown on LB agar plates were cultivated in flat-bottomed 96-well MTPs containing LB medium overnight at 37°C. Subsequently, replications were made from the stock plates into V-bottomed MTPs containing LB medium (100 µg/mL ampicillin) for expression. The clones in the second set of plates were cultivated in the microtiter plate shaker at 800 rpm under 37°C for 3 h, then induced with 50 µL LB containing 0.4 mM IPTG and further incubated at 20°C for 24 h. After the expression, the supernatants were collected by centrifugation and then used for screening.

2.8 Rescreening for improved hydrolytic activity and thermal stability using MTP-based pH assay

Re-screening of the FADS-sorted library in the 96-well MTP was performed using the pH-based assay with C-SNARF-4F as the fluorescent pH indicator. In a 96-well black MTP, 50 µL of the expression supernatant was added to 100 µL glycine-NaOH buffer (pH 9.0, 0.1 M). Following that, 50 µL of 10 mM BHET solution was added to initiate the enzymatic reaction. Fluorescence intensity was monitored using the Tecan Infinite M200 Pro MTP reader at 488 nm excitation and 590 nm emission, at 30°C for 1 h. For screening thermally stable strains of the *Sd*PETase, the expression supernatant was incubated at 45°C or 50°C for 10 min, then cooled to room temperature. A residual activity test was subsequently carried out using the procedure mentioned above.

2.9 Expression and purification of improved variants

Starter cultures were prepared by inoculating glycerol stocks into 5 mL LB culture medium (100 µg/mL ampicillin) and incubated overnight at 37°C. The cultures were then transferred into fresh 500 mL LB media at a 1:100 ratio and incubated at 37°C with shaking until OD600 reached 0.6-0.8. The cultures were induced with 0.1 mM IPTG and further incubated at 20°C for 24 h. Crude enzymes were collected via centrifugation, filtered through a hollow fiber microfiltration membrane (0.45 µm, Shandong Bona Biological Technology Group Co., Ltd., China) and concentrated five-fold using a hollow fiber ultrafiltration membrane (5000 NMWC, Shandong Bona Biological Technology Group Co., Ltd., China). The samples were purified using a 5 mL HiTrap HP column (GE Healthcare, USA). The loaded samples were washed with 30 mM imidazole to remove unbound protein, and the target protein was eluted using a 30-250 mM imidazole gradient. For desalting, a 50 mL HiPrep 26/10 Desalting column (GE Healthcare, USA) was used with 0.1 M sodium phosphate buffer (pH 8.0) as the exchange buffer. Using the BCA protein assay kit (Genstar, China), the concentration of the purified enzymes was determined, and for later use in PET hydrolysis, the enzymes were quantified to 0.5 mg/mL.

2.10 Depolymerization of amorphous Goodfellow PET (Gf-PET) film and PET powder

For depolymerization reaction using purified enzyme, a round Gf-PET film of diameter 6 mm was incubated in 940 µL Glycine-NaOH (pH 9.0, 0.1 M) buffer with 60 µL purified enzyme (0.5 mg/mL) at 50°C. For depolymerization reaction using crude enzyme, Gf-PET film was incubated in 200 µL crude enzyme supplemented with Glycine-NaOH buffer (pH 9.0, 0.1 M). Aliquots of 50 µL were taken at different time intervals. The samples were heated at 100°C for 10 minutes to terminate the enzyme activity. A similar procedure was employed for the depolymerization of 20 mg PET powder. The resulting hydrolysis products were quantified via HPLC analysis.

3. Supporting Figure

3.1 Stability of BHET substrate for the pH-based fluorescence assay

(a) Validation of the effect of BHET self-hydrolysis using pH-sensitive fluorescent probe

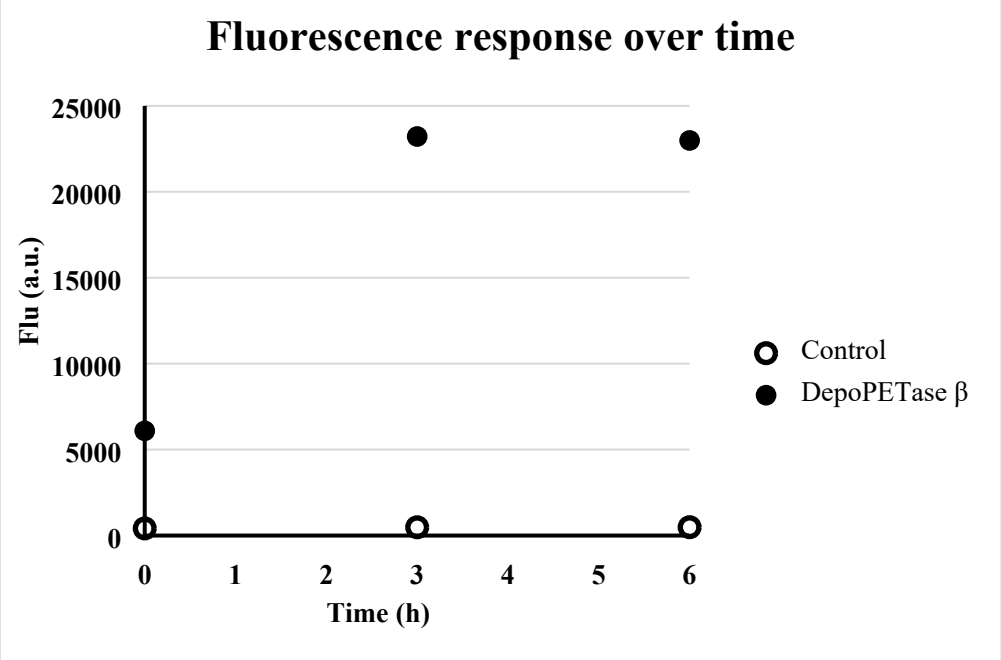

Figure S1 Validation of the pH-based fluorescence assay for enzymatic BHET hydrolysis. Each microwell contained a total of 200 µL reaction volume, consisting of 20 µL 0.5 mM C-SNARF-4F and 160 µL 10 mM BHET solution. 20 µL of crude enzyme DepoPETase β was added to initiate the BHET enzymatic hydrolysis; while for the control reaction, 20 µL of buffer solution was added in place of the enzyme. The open circles (○) represent the control measurements, while the solid circles (●) represent the fluorescent signals from the enzyme-induced reaction at 30°C over 6 h. No significant change in fluorescence was observed in the control group, confirming that the self-hydrolysis of BHET is negligible under these experimental conditions.

(b) Monitoring of BHET solution pH change with time

To assess the stability of the substrate, a 10 mM BHET solution was prepared and maintained at room temperature for 24 h. The pH of the solution was measured at 0 h (8.86) and 24 h (8.46). The resulting pH shift by 0.4 unit after 24 h confirmed that while BHET has a tendency to self-hydrolyze, the rate is sufficiently slow, and has negligible effect on the proposed pH-based detection assay during the standard measurement period.

References

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