Engineering Microbiology 2 (2022) 100020

Contents lists available at ScienceDirect

Engineering Microbiology

journal homepage: www.elsevier.com/locate/engmic

Original Research Article

Biochemical characterization of a polyethylene terephthalate hydrolase and design of high-throughput screening for its directed evolution☆

Xiaotong Wang[a,b], Chaoyi Song[a,b], Qingsheng Qi[a], Youming Zhang[a,b,*], Ruijuan Li[a,b,*], Liujie Huo[a,b,*]

[a] State Key Laboratory of Microbial Technology, Shandong University, Qingdao 266237, China
[b] Helmholtz International Laboratory, Shandong University-Helmholtz Institute of Biotechnology, Qingdao 266237, China

ARTICLE INFO

Keywords:
PETase
Protein secretion
Directed evolution
DNA shuffling
High-throughput screening

ABSTRACT

Polyethylene terephthalate (PET), one of the most widely used plastics in the world, causes serious environmental pollution. Recently, researchers have focused their efforts on enzymatic degradation of PET, which is an attractive way of degrading and recycling PET. In this work, PET hydrolase *Sb*PETase from *Schlegelella brevitalea* sp. nov. was biochemically characterized, and rational design was performed based on its sequence similarity with the previously reported *Is*PETase from *Ideonella sakaiensis*, resulting in a triple mutant with increased activity. Furthermore, using a sec-dependent signal peptide PelB and colicin release protein Kil, we set up a high-efficiency secretion system of PETase in *Escherichia coli* BL21(DE3), enabling higher PETase secretion. Utilizing this secretion system, we established a high-throughput screening method named SecHTS (secretion-based high-throughput screening) and performed directed evolution of *Is*PETase and *Sb*PETase through DNA shuffling. Finally, we generated a mutant *Is*PETase^{S139T} with increased activity from the mutant library.

1. Introduction

Plastic is widely used around the world due to its insulating properties, corrosion resistance, and low price. Plastic has a wide range of applications in industry (wires, electrical enclosures), agriculture (agricultural tools), medical treatment (medical appliances, artificial blood vessels), and people's daily lives. In 2016, plastic production reached 335 million tons per annum, with Europe alone producing 60 million tons. Over the next 20 years, the amount of plastic produced is expected to double [1]. As a consequence, large amounts of plastic waste have accumulated in our environment. As of 2015, approximately 6,300 Mt of plastic waste had been generated, of which around 9% was recycled, 12% was incinerated, and 79% was accumulated in landfills or the natural environment [2]. Polyethylene terephthalate (PET), one of the most widely used polymeric plastic materials, is composed of terephthalic acid (TPA) and ethylene glycol (EG). It is a popular material used to produce beverage bottles and fabric because of its transparency, malleability, and resistance to natural degradation processes [3]. However, the extensive application of PET plastic has led to its accumulation in landfills and oceans, contaminating the soil and seawater. This has become a huge threat to the environment, and has become the focus of international research efforts.

Many methods are available to recycle PET wastes including physical and chemical processes. However, these methods have not been widely used due to their high temperature and high-pressure requirements, or due to the considerable amounts of solvents and degrading agents that are needed for de-polymerization, which create ecological and contamination issues [4]. However, it is possible to biodegrade PET at mild temperatures and pH conditions in the absence of hazardous chemicals [5]. Studies on enzymatic degradation of PET have been underway for a long time and are still ongoing. Müller et al. [6] purified the polyester hydrolase TfH from the actinomycete *Thermobifida fusca* DSM43793 and demonstrated for the first time that a commercial PET film (10% crystallinity) can be effectively hydrolyzed by an enzyme. According to the comparison of TfH with other lipases and esterases, Kleeberg et al. [7] named TfH as cutinase. Baker et al. [8] and Tokiwa and Calabia [9] have studied the degradation of various polyesters and found that cutinase functions directly in degrading synthetic polyesters. Subsequently, some PET hydrolases were isolated from actinomycetes [10] and other bacteria (Table S1) [11–14]. In ad-

☆ Given his role as Editor-in-Chief, Dr. Youming Zhang, had no involvement in the peer-review of this article and has no access to information regarding its peer-review. Full responsibility for the editorial process for this article was delegated to Dr. Jasmina Nikodinovic-Runic.

* Corresponding authors.
E-mail addresses: zhangyouming@sdu.edu.cn (Y. Zhang), liruijuan@sdu.edu.cn (R. Li), lhuo@sdu.edu.cn (L. Huo).

https://doi.org/10.1016/j.engmic.2022.100020
Received 5 January 2022; Received in revised form 14 April 2022; Accepted 14 April 2022
Available online 23 April 2022

dition, another LC-cutinase, which was isolated from leaf-branch compost using a metagenomic approach, also showed an ability to degrade PET [15].

Recently, Yoshida et al. [16] isolated a bacterium from a PET bottle recycling site, named *Ideonella sakaiensis* 201-F6, which can produce and secrete PET hydrolases (*Is*PETase). Several crystal structures of *Is*PETase have been determined independently by other research groups [19,18,17,20,21]. Some researchers have made great efforts to obtain mutants of *Is*PETase with improved hydrolytic activity through site-directed mutagenesis. Based on structural analyses, Joo et al. [20] found that the activity of the mutant *Is*PETase^{R280A} increased by 22.4% in 18 h by expanding the substrate binding site when PET film was used as a substrate. Austin et al. [19] produced a double mutant *Is*PETase$^{S238F/W159H}$ based on homology modeling, which was predicted to narrow the PETase active site and improve PET degradation activity. Other mutants with improved catalytic efficiency were also obtained through structure-guided mutagenesis by Liu et al. [21] and Ma et al. [22]. Furthermore, Son et al. [23] obtained a thermally stable and highly efficient PET degradation variant *Is*PETase$^{S121E/D186H/R280A}$ through a protein engineering strategy.

The mutants described above are all obtained from *Escherichia coli* by a conventional and time-consuming purification method. To reduce the time required for purification, researchers have recently developed extracellular production methods by utilizing various signal peptides in *E. coli* or *Yarrowia lipolytica* [26,25,24]. Although there are many examples of *Is*PETase site-directed mutation, there are no reports of DNA shuffling of PETase so far, which is a nonnegligible strategy that can improve the desired properties by modifying the target protein. However, a high-throughput method is required to screen the mutation library. A few methods have been reported to date, with several aimed at simplifying the methods for detecting the reaction products *in vitro* [27,28]. Heyde et al. [29] presented a method for surface display expression of PETase variants in *E. coli* BL21(DE3), which is suitable for integration into high throughput screening systems. But this method requires an extra TEV protease cleavage step due to potential steric hindrance of the large construct on the cell surface. Furthermore, the inefficiency of bacterial membrane translocation machinery also limits the amount of surface displayed enzyme [30].

Here, we conducted sequence alignment based on the amino acid sequence of *Is*PETase and found an uncharacterized protein from *Schlegelella brevitalea* sp. nov., which has been reported as PETase by Danso et al. [31]. After biochemical characterization, we found that its enzyme activity was lower than *Is*PETase and we named it *Sb*PETase. We subsequently executed site-directed mutagenesis in *Sb*PETase and employed *Is*PETase and *Sb*PETase sequences to perform random mutagenesis by DNA shuffling to obtain mutants with enhanced hydrolytic activity. Moreover, we established a high-efficiency secretion system in *E. coli* BL21(DE3) and constructed a Secretion-based High-Throughput Screening method (SecHTS), giving rise to a more convenient screening platform for PETase variants, as well as omitting the process of protein purification.

2. Materials and methods

2.1. Strains, plasmids, and reagents

Bacterial strains and plasmids used in this study are listed in Table 1, and primers used in this study are listed in Table 2. *E. coli* clones were grown in LB medium (1% tryptone, 0.5% yeast extract, and 1% NaCl), and if necessary, antibiotics (ampicillin, kanamycin) were added to the medium at 50 μg/ml. Agar (1.2%) was added to make solid medium. Antibiotics were purchased from Sangon Biotech (Shanghai, China); restriction enzymes were purchased from New England Biolabs (Beijing, China); PCR polymerases were purchased from TaKaRa (Beijing, China); the standards of bis(2-hydroxyethyl) terephthalic acid (BHET) and TPA were purchased from TCI (Shanghai, China); the standard of Mono(2-hydroxyethyl) terephthalic acid (MHET) was purchased from Macklin (Shanghai, China); and other reagents were purchased from Dingguo Changsheng Biotechnology (Beijing, China) unless noted otherwise.

Table 1
Bacterial strains and plasmids used in this work.

Strain/plasmid	Source
Strains	
E. coli BL21(DE3)	TransGen (Beijing, China)
E. coli DH5α	TransGen (Beijing, China)
Schlegelella brevitalea sp. nov	German Collection of Microorganisms and Cell Cultures GmbH, DSMZ (Braunschweig, Germany)
Plasmids	
pET22b	Lab Stock
pRSFduet-1	Lab Stock

Table 2
Primers used in this work.

Primer	Sequence (5′-3′)
Primers used in the construction of the expression vectors	
Is-F	AGCCGGCGATGGCCATGGATCAGACCAACCCCTACGCC
Is-R	TCAGTGGTGGTGGTGGTGGCTGCAGTTCGCGGTGCGGAAGTC
Sb-F	CAGCCGGCGATGGCCATGGATCAGACCAACCCCTACCAGCG
Sb-R	TCAGTGGTGGTGGTGGTGGTACGGGCAGCTCTCGCGGTA
Kil-F	ACTTTAATAAGGAGATATACATGAGGAAAAGATTTTTTG
Kil-R	GGTTTCTTTACCAGACTCGATTACTGAACCGCGATCCCCG
Is-F1	CAGACCAACCCCTACGCC
Is-R1	GCTGCAGTTCGCGGTGCGGAAGTC
Sb-F1	CAGACCAACCCCTACCAGCG
Sb-R1	GTACGGGCAGCTCTCGCGGTA
Primers used in the site-directed mutagenesis studies	
Y60A-F	GTGGTGCCCGGC**GCC**CTCGCCCGCCAGTCCAGCATC
Y60A-R	ACTGGCGGGCGAG**GGC**GCCGGGCACCACCGCCACCGCG
L61T-F	GTGCCCGGCTAC**ACC**GCCCGCCAGTCCAGCATCCGCT
L61T-R	TGGACTGGCGGGC**GGT**GTAGCCGGGCACCACCGCCAC
W132A-F	CGCGGTGATGGGC**GCG**TCGATGGGCGGAGGCGGCACGC
W132A-R	CTCCGCCCATCGA**CGC**GCCCATCACCGCGAGACGGTTG
W132H-F	CGCGGTGATGGGC**CAT**TCGATGGGCGGAGGCGGCACG
W132H-R	CTCCGCCCATCGA**ATG**GCCCATCACCGCGAGACGGTTG
V181I-F	GAAAACGACACC**ATC**GCCCCGATCTCGCGACATGCCT
V181I-R	GCGAGATCGGGGC**GAT**GGTGTCGTTTTCGCAAGCGATC
T212S-F	CAACGGCTCGCAC**TCC**TGCGCCAACACCGGCAACAGCA
T212S-R	GGTGTTGGCGCA**GGA**GTGCGAGCCGTTGTTGATCTC
T212F-F	CAACGGCTCGCAC**TTC**TGCGCCAACACCGGCAACAGCA
T212F-R	CGGTGTTGGCGCA**GAA**GTGCGAGCCGTTGTTGATCTCG
R259A-F	CTGCGCAGCAGC**GCC**CTCTCGGAGTACCGCGAGAGCT
R259A-R	GTACTCCGAGAG**GGC**GCTGCTGCGCAGGTCGGCCTGA

The mutation sites are indicated by bold letters.

2.2. Construction of the expression vectors

The genes encoding *Is*PETase (accession number WP_054022242) and Kil protein (Gene ID 2693958) were chemically synthesized (GENEWIZ, Suzhou, China) and respectively cloned into pET22b and pRSFduet-1 vectors. The gene encoding *Sb*PETase was amplified from the genome of *S. brevitalea* sp. nov. by PCR and cloned into the pET22b vector. The signal peptide gene sequences of *Is*PETase and *Sb*PETase were predicted using the SignalP 4.1 Server (http://www.cbs.dtu.dk/services/SignalP/) and then removed.

2.3. Expression and purification of the recombinant proteins

The recombinant protein was expressed in *E. coli* BL21(DE3) after being induced by 0.1 mM isopropyl β-D-thiogalactopyranoside (IPTG) at 18°C. After 18-22 hours, the cells were harvested by centrifugation at 4,200 rpm and 4°C for 15 min, and then resuspended in buffer A (50 mM Tris-HCl, 300 mM NaCl, 10 mM imidazole, pH 7.6). Sonication followed and the lysate was centrifuged at 20,000 rpm and 4°C for 25 min,

the supernatant was then collected and applied to a His-Select nickel column (Bestchrom, Shanghai, China). After washing the unbound proteins with buffer A, the target protein was eluted with buffer B (50 mM Tris-HCl, 300 mM NaCl, 250 mM imidazole, pH 7.6). PD-10 Desalting Columns (GE Healthcare Life Sciences) were used to change buffer, and the protein was stored in buffer C (50 mM Tris-HCl, 300 mM NaCl, 10% glycerol, pH 7.6). The concentration of purified proteins was determined using a NanoDrop One (Thermo Scientific, USA) at 280 nm wavelength and calculated by molar extinction coefficient.

2.4. Enzymatic activity assays

Purified protein (50 nM) was incubated in 500 μl buffer (50 mM Na_2HPO_4-HCl, pH 7.0) that contained 4 mM BHET for 18 hours at 30°C, or in 500 μl buffer (50 mM Na_2HPO_4-HCl, pH 8.0) that contained a piece of PET film (φ 5 mm, commercial beverage bottle) for 24 hours at 30°C. The reaction was stopped by heat treatment (80°C, 10 min) and the liquid was filtered using a 0.22 μm filter and then analyzed by High Performance Liquid Chromatography (HPLC). All samples were processed in triplicate and the reaction mixtures with boiled proteins were used as the negative control.

2.5. HPLC analysis

HPLC was performed on an LC-16 system (Shimadzu with SPD 16 detector) equipped with an Agilent Eclipse XDB-C18 analytical column (4.6 × 250 mm). The mobile phase was 20 mM phosphate buffer (pH 2.5, A)/methanol (B). The gradient elution program was as follows: 0 to 25 min, 30-65% (v/v) methanol; 25-35 min, 65-100% methanol; and the flow rate was 1.0 mL/min. Each sample was injected at a volume of 20 μl, and the column temperature was kept at 30°C. The effluent was detected at a wavelength of 240 nm.

2.6. Effects of pH and temperature on enzyme activity

The *Sb*PETase activity was assayed at different pH values (6.0-10.0) and temperatures (25°C-42°C). Purified protein was incubated in 500 μl buffer (for pH 6.0-8.0, 50 mM Na_2HPO_4-HCl; for pH 9.0-10.0, 50 mM glycine-NaOH) that contained 4 mM BHET for 18 h. The reaction was stopped by heat treatment (80°C, 10 min) and the liquid was filtered using a 0.22 μm filter and then analyzed by HPLC. For *Is*PETase, because its optimum pH on BHET and commercial PET film is different [21], we also assayed the optimum pH of *Sb*PETase on commercial PET film.

2.7. Site-directed mutagenesis of SbPETase

Site-directed mutagenesis was designed and performed to obtain mutants with increased activity. All mutants were created by the Quikchange method [32], and the primers used to construct these mutants are listed in Table 2.

2.8. Secretion in E. coli BL21(DE3)

The vectors of recombinant protein were individually co-electroporated with pRSFduet-kil into *E. coli* BL21(DE3). After induced by 0.1 mM IPTG at 28°C for 20 hours, samples were centrifuged at 4,200 rpm and 4°C for 15 min and the supernatant was collected. Then nickel affinity chromatography was used to purify the secreted protein. For the enzyme activity assay in fermentation broth, sterilized substrate (BHET or PET film) was added to the culture medium at the time of induction. After culturing at 28°C for 20 hours, the supernatant was collected and analyzed by HPLC.

2.9. Plate clearing assay

In order to establish a high-throughput screening method, we performed BHET plate clearing assays. The BHET plates are LB agar plates containing 50 μg/ml kanamycin, 50 μg/ml ampicillin, 0.1 mM IPTG, and 4 mM BHET powder. The *E. coli* BL21(DE3) harboring plasmids pRSFduet-kil and corresponding PETase expression vectors were inoculated on BHET plates and incubated at 30°C for up to 48 hours, producing and secreting PETase. Zones of clearing around the colonies were used as a criterion for the BHET/PET-degrading activity.

2.10. DNA shuffling

DNA shuffling was used to generate libraries by random fragmentation of one PETase gene or a pool of PETase homologous genes, followed by reassembly of the fragments in a self-priming PCR reaction. JBS DNA shuffling kit (# PP-103, Germany) was used for DNA shuffling experiments.

Taking *Is*PETase and *Sb*PETase as the starting pool of genes, DNA shuffling consisted of the following steps. (i) Preparation of genes to be shuffled: genes for *Is*PETase and *Sb*PETase were obtained by PCR from pET22b-*Is*PETase and pET22b-*Sb*PETase, respectively, and purified by agarose gel purification. (ii) Fragmentation with DNase I: the PCR products were digested into fragments (approximately 50 bp) by DNase I at 37°C for 4 min and purified by agarose gel purification. (iii) Self-priming reassembly: take the purified DNA fragments of *Is*PETase and *Sb*PETase in 50 μl PCR mixture to a final concentration of 10-20 ng/μl and reassembled by thermocycling in the presence of Taq DNA polymerase. (iv) Reassembled products were amplified by conventional PCR: the second PCR was performed with specific primers, which carry the homologous arm of the cloning site of the pET22b vector (Table 1). After agarose gel purification of the target band, the amplified library was assembled into the pET22b vector and electroporated into *E. coli* BL21(DE3), which already contained pRSFduet-Kil, then plated on LB agar plates with 50 μg/ml kanamycin and 50 μg/ml ampicillin. After overnight culture at 37°C, the transformants were regarded as a mutant library.

2.11. High-throughput screening method

The high-throughput screening method contains three steps: preliminary screening, secondary screening, and enzyme activity quantification. Preliminary screening, also named qualitative screening, involves picking clones from the mutant library, then inoculating them on BHET plates. Clones that can produce cleared zones are considered capable of degrading BHET. Clones from preliminary screening are then applied to secondary screening. These clones were inoculated in 1 ml LB (unless otherwise specified, LB used in this method contains 50 μg/ml kanamycin, 50 μg/ml ampicillin) and incubated at 37°C overnight, then 40 μl of the overnight culture was inoculated into 1 ml LB, and incubated at 37°C for 2.5 h. At this stage, OD_{600} should be between 0.8 and 1.0. After OD_{600} was normalised to 0.8, the culture was inoculated on BHET plates for 1 μl each, and all clones were tested in triplicate. Clones with a clearing zone diameter bigger than wild type were selected, and the mutants from the supernatant of the culture medium were purified and the enzyme activity was quantified (for protein purification, enzymatic assays, and HPLC analysis methods see Sections 2.3–2.5).

3. Results

3.1. Establishment of the efficient secretion system for PET degradation enzymes in E. coli BL21(DE3)

To construct a high efficiency secretion system in *E. coli*, we developed a method that combines signal peptide PelB (pectate lyase B

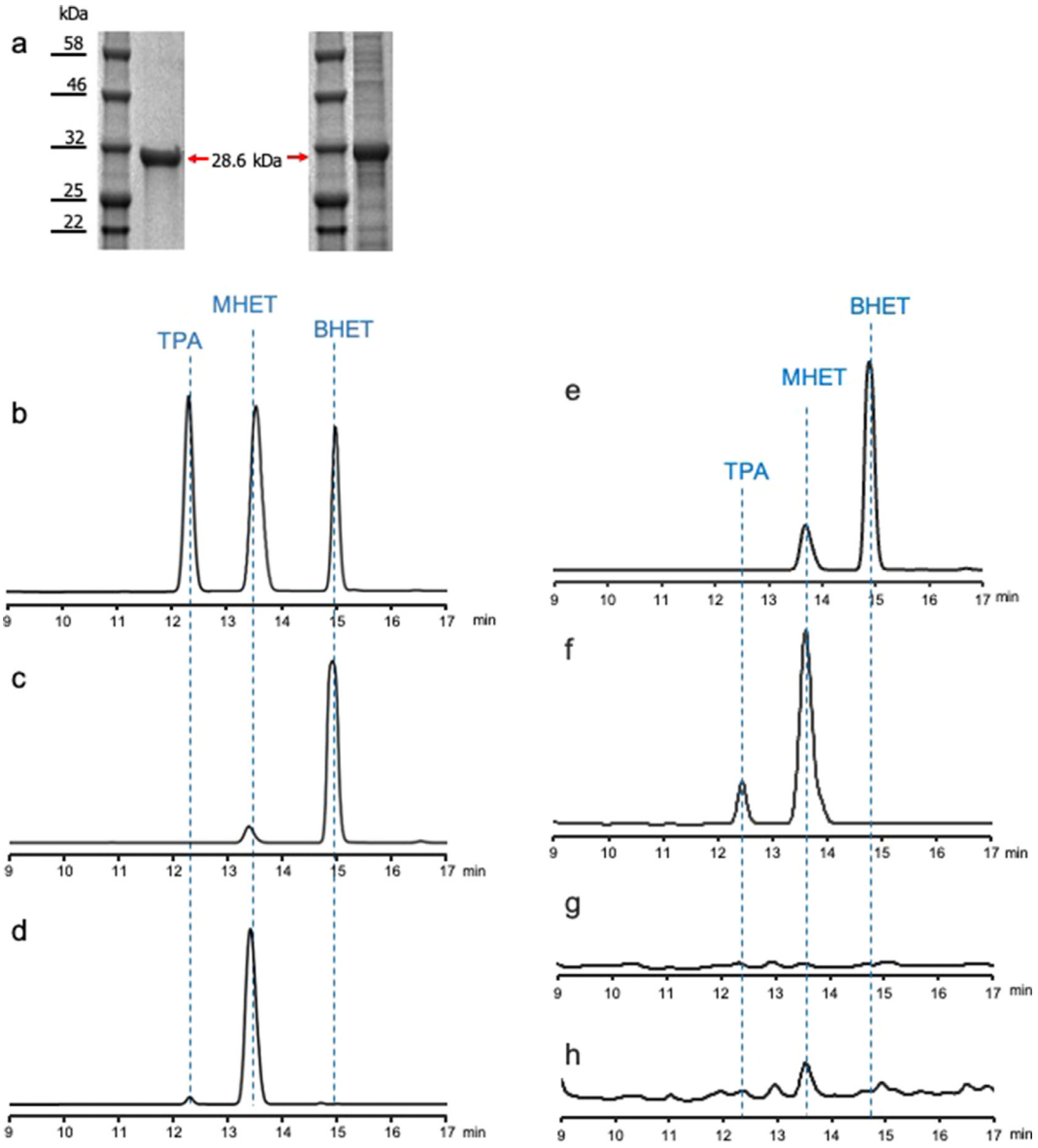

Fig. 1. a) SDS-PAGE of *Is*PETase purified from fermentation supernatant (left) and cytosol (right). HPLC analysis of reaction products of secreted *Is*PETase activity assay are shown in b-h. b) The standards of TPA, MHET, and BHET. c) High temperature treated *Is*PETase in BHET assay buffer; d) purified *Is*PETase in BHET assay buffer. e) BHET powder in the culture medium supernatant of *E. coli* BL21 harboring empty vectors. f) BHET powder in the culture medium supernatant of BL_{SPIs}. g) PET film in the culture medium supernatant of *E. coli* BL21 harboring empty vectors. h) PET film in the culture medium supernatant of BL_{SPIs}.

signal peptide) and colicin release protein Kil. The recombinant protein containing the signal peptide PelB will be targeted to *E. coli* periplasm via the sec-dependent transport pathway, where the signal peptide is removed by a signal peptidase [33]. The colicin release protein Kil releases periplasmic protein by inducing membrane solubilization [34]; as a result, the recombinant proteins can be efficiently secreted into the culture medium when co-expressed with Kil protein. Using *Is*PETase as a model, we cloned the gene fragment encoding *Is*PETase into the expression vector pET22b, which contains sec-dependent signal peptide PelB upstream of the multiple cloning site, and named it pET22b-*Is*PETase. The gene fragment encoding Kil was then cloned into the vector pRSFduet-1 and named pRSFduet-Kil. *E. coli* BL21(DE3) was co-transformed with pET22b-*Is*PETase and pRSFduet-Kil, and we named the co-expression strain BL_{SPIs}.

After fermentation of BL_{SPIs}, we purified the secreted protein by Ni affinity chromatography and observed a band of ~30 kDa by SDS-PAGE. Compared with intracellularly purified protein, the protein purified directly from the culture medium contains almost no impurity, so no further purification was required (Fig. 1a). We purified approximately 12 mg secreted protein from one liter of fermentation supernatant, resulting in a yield approximately 5-fold higher than the strain that was not co-expressed with Kil protein (data not shown).

To measure the activity of the secreted *Is*PETase, an *in vitro* assay was performed utilizing BHET as a model substrate. The results showed that secreted *Is*PETase exhibited obvious BHET degradation activity (Fig. 1b-d). As shown in Fig. 1e-h, we also detected the degradation productsMHET and/or TPA) in BL_{SPIs} fermentation broth, which were hydrolyzed from BHET powder or commercial PET films, demonstrating that secreted *Is*PETase had activity in culture medium.

3.2. Biochemical characterization of SbPETase

Currently known PET degradation enzymes were mainly found in Actinobacteria, Proteobacteria, and several fungi, and were divided into two types (Type I and Type II) [35]. To discover and characterize

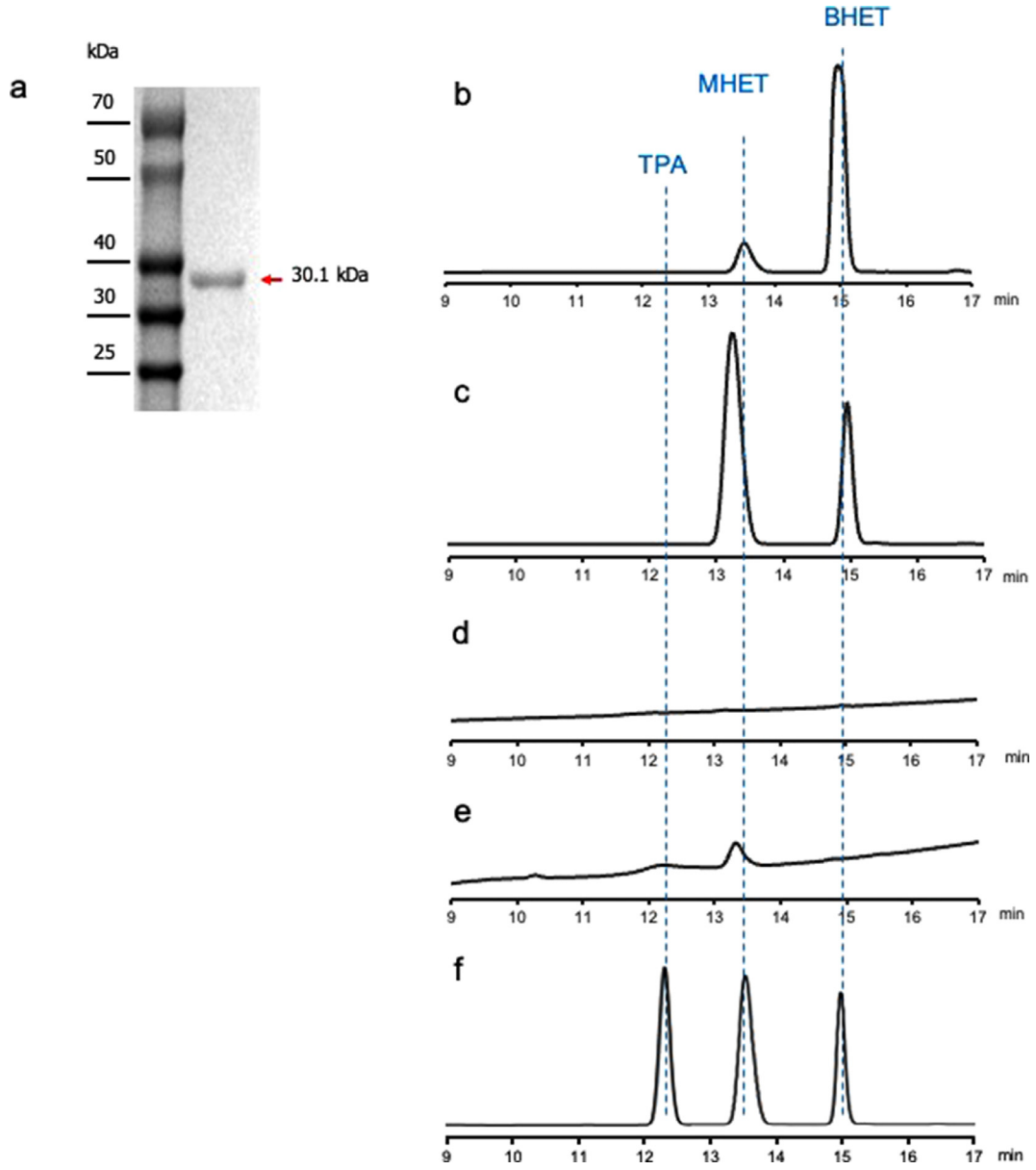

Fig. 2. a) SDS-PAGE of *Sb*PETase purified from fermentation supernatant. HPLC analysis of reaction products of *Sb*PETase activity assays are shown in b-e. b) High temperature treated *Sb*PETase in BHET assay buffer; c) purified *Sb*PETase in BHET assay buffer; d) high temperature treated *Sb*PETase in PET film assay buffer; e) purified *Sb*PETase in PET film assay buffer. f) Standards of TPA, MHET, and BHET.

more PET hydrolases, protein sequences alignment was performed using *Is*PETase sequence. This alignment identified an α/β hydrolase from *S. brevitalea* sp. nov., which has been reported but not characterized [31], that we named *Sb*PETase. The amino acid sequence of *Sb*PETase shares 91% coverage and 67% identity with *Is*PETase and the catalytic amino acids are conserved, including Ser-Asp-His catalytic triad (with the conserved serine hydrolase Gly–x1–Ser–x2–Gly motif) and the two cysteines that form the disulfide bond. The *Sb*PETase sequence is also highly conserved among most of the substrate binding sites identified by Joo et al. [20]. However, the counterparts of Ile208 in subsite I and Thr88 in subsite II of *Is*PETase are mutated to Val and Leu, respectively, in the sequence of *Sb*PETase (Val181 and Leu61) (Fig. S1). Phylogenetic analysis indicated that *Sb*PETase and *Is*PETase share a close genetic relationship (Fig. S4).

To perform the biochemical characterization of *Sb*PETase, we constructed the secretory expression vector pET22b-*Sb*PETase, and co-transformed it with pRSFduet-Kil plasmid into *E. coli* BL21(DE3) to construct the secretory strain BL_{SPSb}. After fermentation of BL_{SPSb}, *Sb*PETase was purified from the fermentation supernatant (Fig. 2a). Using the *in vitro* activity assay, we demonstrated that *Sb*PETase could degrade PET and BHET into MHET and a small quantity of TPA just like *Is*PETase (Fig. 2b-e), but the activity was only half (BHET as substrate) or one eighth (PET film as substrate) that of *Is*PETase (Fig. S2). The optimum conditions showed that at 30°C, pH 7.0 (BHET as substrate) or pH 8.0 (PET film as substrate), *Sb*PETase showed the highest activity (Fig. S3).

3.3. Rational design of SbPETase by site-direct mutagenesis

Due to the differences in substrate binding sites between *Sb*PETase and *Is*PETase (Fig. S1), and since reported *Is*PETase mutants showed improved catalytic efficiency [19,20], we performed the following site-directed mutagenesis: Y60A, L61T, W132H, W132A, V181I, T212F, T212S, and R259A. All *Sb*PETase mutants were obtained from culture medium directly and an *in vitro* activity assay was performed, which

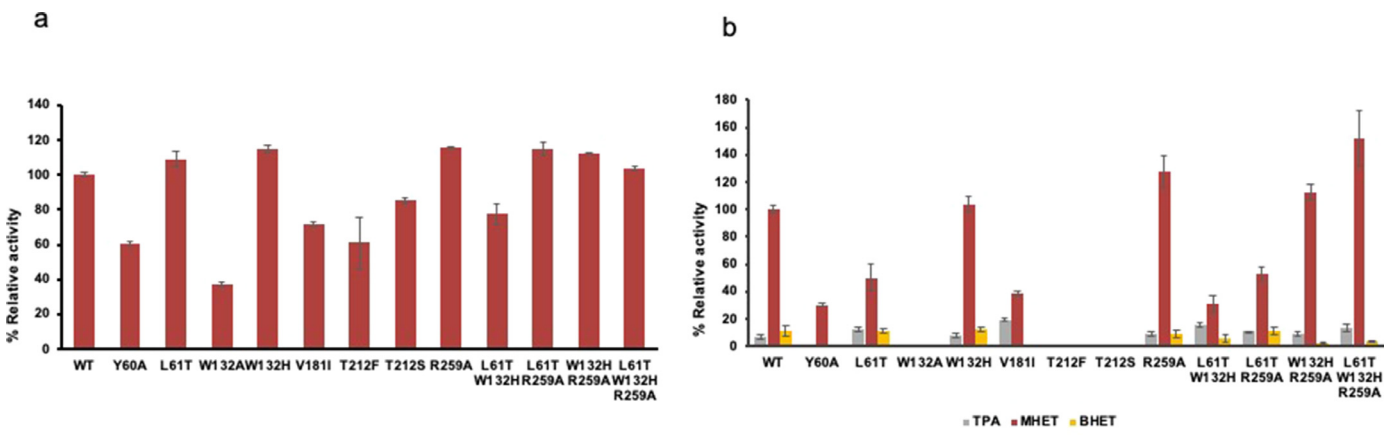

Fig. 3. Comparison of degradation efficiencies of the variants of SbPETase. a) BHET powder as substrate, b) PET film (φ 0.5cm) as substrate. The production levels of BHET, MHET, and TPA by each protein are presented as percentages of the wild type, the amount of MHET produced by wild type was defined as 100%.

generated two mutants (*Sb*PETase^{W132H}, *Sb*PETase^{R259A}) with improved catalytic efficiency (Fig. 3). The activity of another mutant *Sb*PETase^{L61T} was only improved towards BHET (Fig. 3). We then combined these three mutants (*Sb*PETase^{W132H}, *Sb*PETase^{R259A}, *Sb*PETase^{L61T}), to generate three double mutants and one triple mutant. An *in vitro* activity assay showed that the triple mutant had the highest catalytic efficiency towards PET film (Fig. 3). However, its activity was still much lower than that of *Is*PETase.

3.4. Directed evolution of SbPETase and IsPETase and the establishment of a high-throughput screening method

Since a high-efficiency secretion system has been established, in order to obtain PETase mutants with increased catalytic activity, we decided to perform directed evolution of *Is*PETase by DNA shuffling. However, due to the disadvantages of the traditional screening method, which is laborious and time-consuming, we needed to establish a new approach for screening the mutants rapidly and easily. On account of the water-insoluble property of BHET, we utilized BHET as a model substrate for PET degradation. The secretory strain BL_{SPIs} and BL_{SPSb} were employed to perform BHET plate clearing assay. To analyze the feasibility of this method, we inoculated the same amount of BL_{SPIs} and BL_{SPSb} growing in log phase on the BHET plate. After 36 h of incubation at 30°C, both colonies generated clear circles and the circle around BL_{SPIs} was larger than that around BL_{SPSb}; the cleared zone diameter roughly reflects the level of enzyme activity. So, we set up a screening method taking advantage of the BHET plate clearing assay and named it Secretion-based High-Throughput Screening method (SecHTS). The screening process is shown in Fig. 4b.

Since there is high homology between *Is*PETase and *Sb*PETase, we firstly performed DNA shuffling [36] of these two genes (Fig. 4a). Then we cloned the DNA shuffling products into pET22b. After transforming these vectors into *E. coli* BL21(DE3) harboring pRSFduet-Kil, we obtained a mutant library containing approximately 10,000 clones (Fig. 4b). Next, we screened this random mutant library using the SecHTS method.

Firstly, we picked 1,000 random clones from the library, and after preliminary screening, 734 clones that did not generate cleared zones were excluded. Then we performed secondary screening in the remaining 266 clones to pick out 14 clones with bigger cleared zones and four clones containing the same amino acid sequences were removed (Fig. 4c). Ten variants were further purified from the fermentation supernatant, and their activities were quantified *in vitro*. We obtained a mutant, 765, with higher PET film degradation activity than *Is*PETase, in which the amount of degradation product TPA was 8% higher than that of *Is*PETase (Fig. 5). Sequence analyses of the mutant showed that the Ser139 residue mutated to threonine, and it was named *Is*PETase^{S139T}. Ser139 is located near substrate binding subsite II [20], so we suspected that the mutated residue of Ser139 to Thr may affect the binding of the substrate, which benefits the catalytic activity of this mutant.

To enlarge the starting pool genes, we selected another four PETases (TfH, TfCut2, Est119, and Cut190) with higher similarity to *Is*PETase from currently known PET degradation enzymes to perform DNA shuffling with *Is*PETase and *Sb*PETase. But unfortunately, we were unable to obtain target fragments after self-priming reassembly and second PCR steps.

4. Discussion

Although multiple *Is*PETase mutants with improved hydrolytic activity have been obtained through site-directed mutagenesis [19,38,23,37], there have been no attempts at random mutations and directed evolution of PETases. The main goal of our work was thus to establish a high-throughput screening method and apply it to the directed evolution of PET degrading enzymes.

In this study, we established a secretion system in *E. coli* BL21(DE3), and showed that co-expression of the Kil protein greatly improved the secretion efficiency. Then we biochemically characterized *Sb*PETase from *Schlegelella brevitalea* sp. nov. According to the differences in substrate binding sites between *Sb*PETase and *Is*PETase mutants, we constructed several *Sb*PETase mutants, some of which showed improved catalytic activity. A few mutants (*e.g.,* *Sb*PETase^{L61T}) could degrade BHET with high efficiency, however, there was no improvement in the degradation activity of PET. Therefore, large polymer segments may be unable to fit into a binding cleft that is not suitable [37].

Because the activity of *Sb*PETase mutants is still much lower than that of *Is*PETase, we performed directed evolution of *Sb*PETase and *Is*PETase through DNA shuffling. Additionally, we set up a high-throughput screening platform based on the secretion system in *E. coli* BL21(DE3) and named it SecHTS. In this way, the screening process becomes visualization, such that mutants can be first screened depending on the sizes of cleared zones on the plate clearing assay. This dramatically reduced the sample amount with almost no consumption of time and effort, allowing target mutants to be selected quickly from the library with a limited number of samples required for quantitative analysis. Using this method, we successfully obtained the mutant *Is*PETase^{S139T} with enhanced activity. Austin et al. [19] found that narrowing the binding cleft around the active site via mutation improved the degradation activity of PETase. Ser139 is not included in the characterized substrate binding site, but is located near binding subsite II

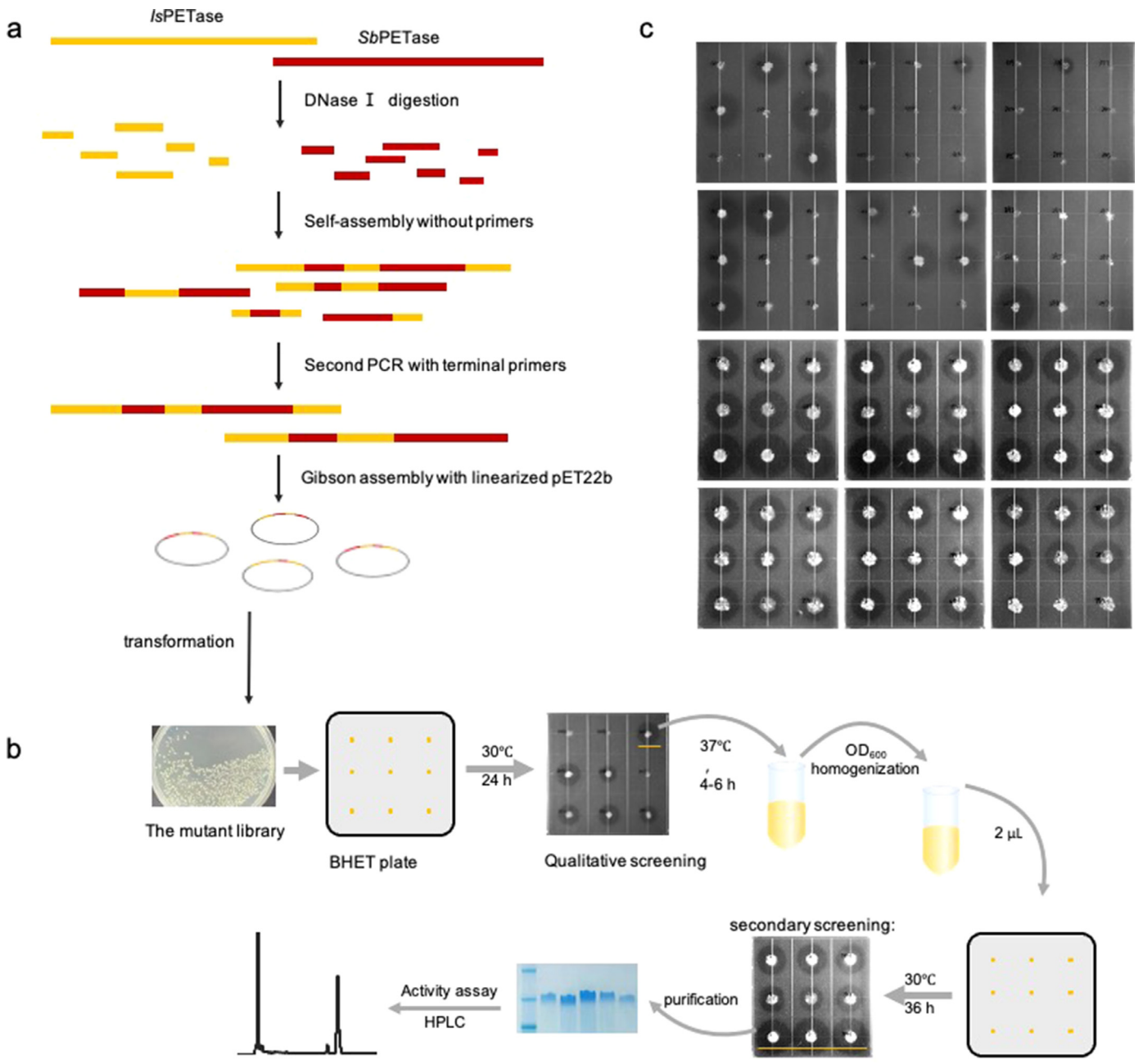

Fig. 4. a) The flow chart of construction of PETase mutant library using DNA shuffling. b) The screening process of SecHTS (Secretion-based High-Throughput Screening method). c) Several representative results of the BHET plate clearing assay.

[18,20]. The mutated residue at Ser139 to Thr may narrow the binding cleft due to larger steric hindrance of Thr, resulting in increased activity of this mutant. Furthermore, we utilized the optimum temperature and pH conditions of *Is*PETase to screen mutants, but the optimum conditions for some mutants may have changed, hampering our screening efforts.

Because of the individual differences between secretion strains harboring different PETase mutants, the quantity of enzymes secreted in the plate clearing assay could not be accurately controlled. Thus, the size of the cleared zone cannot be used for accurate quantification and time-consuming HPLC is still needed for final quantification. To solve this problem, fluorometric detection dependent high-throughput analysis methods could be applied to replace HPLC in the future [27,28]. Furthermore, DNA shuffling requires that genes in the starting pool contain regions of relatively high sequence homology, limiting the number of genes in the starting pools. In the future, some *in vitro* homology-independent techniques may potentially be used such as incremental truncation for the creation of hybrid enzymes (ITCHY) [39].

5. Conclusions

We biochemically characterized a PET-hydrolyzing *Sb*PETase from *Schlegelella brevitalea* sp. nov. using a high-efficiency secretion system. In addition, we established a SecHTS (secretion-based high-throughput screening) platform enabling rapid PETase library screening and feasibility verification. Overall, our study provides a foundation for accelerating the discovery of novel PETases as well as PETase variant screening for industrial applications.

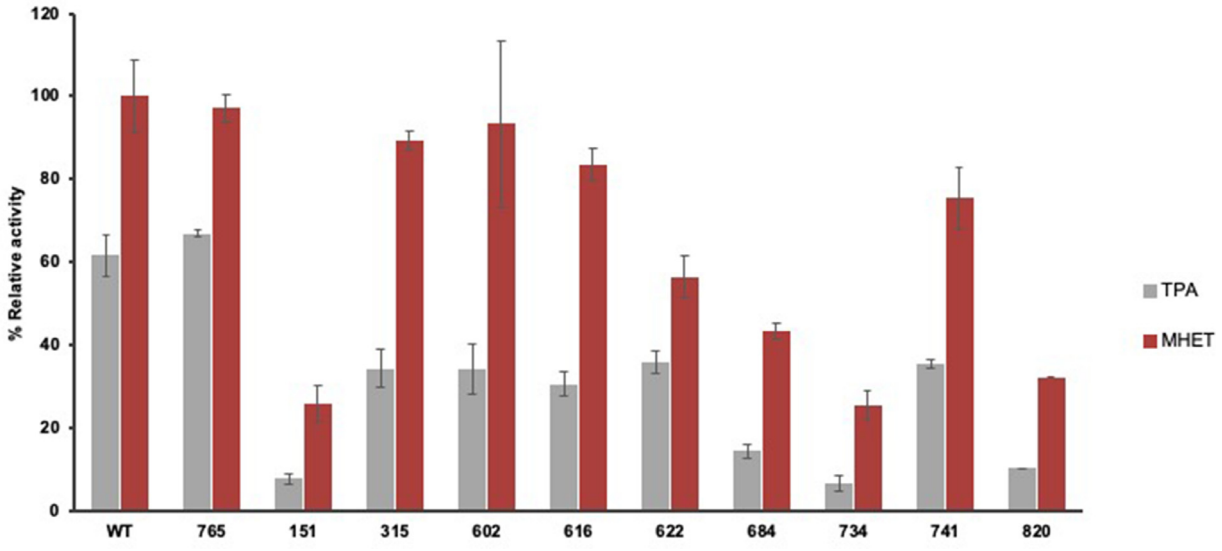

Fig. 5. Comparison of degradation efficiency of the variants of *Is*PETase and wild-type *Is*PETase (PET film as substrate).

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgment

We thank Drs. Jingyao Qu, Zhifeng Li, Jing Zhu and Guannan Lin (Core Facilities for Life and Environmental Sciences, State Key laboratory of Microbial Technology, Shandong University) for the detection and characterization by HPLC. This study was financially supported by the Qilu Youth Scholar Startup Funding of Shandong University (L. H.), National Natural Science Foundation of China (32170038) as well as the Sino-German mobility programme (M-0348).

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.engmic.2022.100020.

References

[1] O. Drzyzga, A. Prieto, Plastic waste management, a matter for the 'community', Microb. Biotechnol. 12 (1) (2019) 66–68.
[2] R. Geyer, J.R. Jambeck, K.L. Law, Production, use, and fate of all plastics ever made, Sci. Adv. 3 (7) (2017) e1700782.
[3] C.C. Chen, et al., Structural studies reveal the molecular mechanism of PETase, FEBS J. 285 (20) (2018) 3717–3723.
[4] B. Geyer, G. Lorenz, A. Kandelbauer, Recycling of poly(ethylene terephthalate) – a review focusing on chemical methods, Express Polym. Lett. 10 (2016) 559–586.
[5] R. Wei, W. Zimmermann, Biocatalysis as a green route for recycling the recalcitrant plastic polyethylene terephthalate, Microb. Biotechnol. 10 (6) (2017) 1302–1307.
[6] R.-J. Müller, et al., Enzymatic Degradation of Poly(ethylene terephthalate): Rapid Hydrolyse using a Hydrolase fromT. fusca, Macromol. Rapid Commun. 26 (17) (2005) 1400–1405.
[7] I. Kleeberg, et al., Characterization of a new extracellular hydrolase from Thermobifida fusca degrading aliphatic-aromatic copolyesters, Biomacromolecules 6 (1) (2005) 262–270.
[8] P.J. Baker, et al., Identification and comparison of cutinases for synthetic polyester degradation, Appl. Microbiol. Biotechnol. 93 (1) (2012) 229–240.
[9] Y. Tokiwa, Buenaventurada Calabia, Biodegradability and Biodegradation of Polyesters, J. Polym. Environ. 15 (2007) 259–267.
[10] E. Herrero Acero, et al., Enzymatic Surface Hydrolysis of PET: Effect of Structural Diversity on Kinetic Properties of Cutinases from Thermobifida, Macromolecules 44 (12) (2011) 4632–4640.
[11] T. Nimchua, H. Punnapayak, W. Zimmermann, Comparison of the hydrolysis of polyethylene terephthalate fibers by a hydrolase from Fusarium oxysporum LCH I and Fusarium solani f. sp. pisi, Biotechnol. J. 2 (3) (2007) 361–364.
[12] Å.M. Ronkvist, et al., Cutinase-Catalyzed Hydrolysis of Poly(ethylene terephthalate), Macromolecules 42 (14) (2009) 5128–5138.
[13] A. Bollinger, et al., A novel polyester hydrolase from the marine bacterium pseudomonas aestusnigri - structural and functional insights, Front. Microbiol. 11 (2020) 114.
[14] F. Kawai, et al., A novel Ca^{2+}-activated, thermostabilized polyesterase capable of hydrolyzing polyethylene terephthalate from Saccharomonospora viridis AHK190, Appl. Microbiol. Biotechnol. 98 (24) (2014) 10053–10064.
[15] S. Sulaiman, et al., Isolation of a novel cutinase homolog with polyethylene terephthalate-degrading activity from leaf-branch compost by using a metagenomic approach, Appl. Environ. Microbiol. 78 (5) (2012) 1556–1562.
[16] S. Yoshida, et al., A bacterium that degrades and assimilates poly(ethylene terephthalate), Science 351 (6278) (2016) 1196–1199.
[17] X. Han, et al., Structural insight into catalytic mechanism of PET hydrolase, Nat. Commun. 8 (1) (2017) 2106.
[18] T. Fecker, et al., Active Site Flexibility as a Hallmark for Efficient PET Degradation by I. sakaiensis PETase, Biophys. J. 114 (6) (2018) 1302–1312.
[19] H.P. Austin, et al., Characterization and engineering of a plastic-degrading aromatic polyesterase, Proc. Natl. Acad. Sci. U. S. A. 115 (19) (2018) E4350–e4357.
[20] S. Joo, et al., Structural insight into molecular mechanism of poly(ethylene terephthalate) degradation, Nat. Commun. 9 (1) (2018) 382.
[21] Liu, B., et al. 2018 Protein Crystallography and Site-Direct Mutagenesis Analysis of the Poly(ethylene terephthalate) Hydrolase PETase from Ideonella sakaiensis. Chembiochem. 2018. **19**(14): p. 1471-1475.
[22] Yuan Ma, et al., Enhanced Poly(ethylene terephthalate) Hydrolase Activity by Protein Engineering, Engineering 4 (6) (2018) 888–893.
[23] Hyeoncheol Francis Son, et al., Rational protein engineering of thermo-stable PETase from Ideonella sakaiensis for highly efficient PET degradation, ACS Catal. 9 (4) (2019) 3519–3526.
[24] Lixia Shi, et al., Enhanced extracellular production of IsPETase in Escherichia coli via engineering of the pelB signal peptide, J. Agricult. Food Chem. 69 (7) (2021) 2245–2252.
[25] H. Seo, et al., Production of extracellular PETase from Ideonella sakaiensis using sec-dependent signal peptides in E. coli, Biochem. Biophys. Res. Commun. 508 (1) (2019) 250–255.
[26] Pan Liu, et al., Potential one-step strategy for PET degradation and PHB biosynthesis through co-cultivation of two engineered microorganisms, Eng. Microbiol. 1 (2021) 100003.
[27] R. Wei, et al., A high-throughput assay for enzymatic polyester hydrolysis activity by fluorimetric detection, Biotechnol. J. 7 (12) (2012) 1517–1521.
[28] M.T. Zumstein, et al., High-Throughput Analysis of Enzymatic Hydrolysis of Biodegradable Polyesters by Monitoring Cohydrolysis of a Polyester-Embedded Fluorogenic Probe, Environ. Sci. Technol. 51 (8) (2017) 4358–4367.
[29] Sophia A.H. Heyde, et al., Surface display as a functional screening platform for detecting enzymes active on PET, Microb. Cell Factories 20 (1) (2021) 93-93.
[30] C.F Earhart, Use of an Lpp-OmpA fusion vehicle for bacterial surface display, Methods Enzymol. 326 (2000) 506–516.
[31] Dominik Danso, et al., New Insights into the Function and Global Distribution of Polyethylene Terephthalate (PET)-Degrading Bacteria and Enzymes in Marine and Terrestrial Metagenomes, Appl. Environ. Microbiol. 84 (8) (2018) e02773-17.
[32] Carol Papworth, John Bauer, Jeffrey Braman, Site-Directed Mutagenesis in One day with >80% Efficiency, Strategies 9 (1996) 3–4.

[33] J.T. Sockolosky, F.C. Szoka, Periplasmic production via the pET expression system of soluble, bioactive human growth hormone, Protein Expr. Purif. 87 (2) (2013) 129–135.
[34] G. Miksch, et al., Factors that influence the extracellular expression of streptavidin in Escherichia coli using a bacteriocin release protein, Appl. Microbiol. Biotechnol. 81 (2) (2008) 319–326.
[35] Ikuo Taniguchi, et al., Biodegradation of PET: current status and application aspects, ACS Catal. 9 (5) (2019) 4089–4105.
[36] W.P Stemmer, Rapid evolution of a protein in vitro by DNA shuffling, Nature 370 (6488) (1994) 389–391.
[37] Ren Wei, et al., in: Mechanism-Based Design of Efficient PET Hydrolases, ACS Catalysis, 2022, pp. 3382–3396.
[38] X. Meng, et al., Protein engineering of stable IsPETase for PET plastic degradation by Premuse, Int. J. Biol. Macromol. 180 (2021) 667–676.
[39] M. Ostermeier, et al., Combinatorial protein engineering by incremental truncation, Proc. Natl. Acad. Sci. U. S. A. 96 (7) (1999) 3562–3567.