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Review

Enzyme discovery and engineering for sustainable plastic recycling

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The drastically increasing amount of plastic waste is causing an environmental crisis that requires innovative technologies for recycling post-consumer plastics to achieve waste valorization while meeting environmental quality goals. Biocatalytic depolymerization mediated by enzymes has emerged as an efficient and sustainable alternative for plastic treatment and recycling. A variety of plastic-degrading enzymes have been discovered from microbial sources. Meanwhile, protein engineering has been exploited to modify and optimize plastic-degrading enzymes. This review highlights the recent trends and up-to-date advances in mining novel plastic-degrading enzymes through state-of-the-art omics-based techniques and improving the enzyme catalytic efficiency and stability via various protein engineering strategies. Future research prospects and challenges are also discussed.

Highlights

Biocatalytic depolymerization mediated by enzymes has emerged as an efficient and sustainable alternative for plastic treatment and recycling, which aims to reduce adverse environmental effects and recover valuable components from plastic waste.

Metagenomic and proteomic approaches can be harnessed as powerful tools in mining enzymes capable of plastic depolymerization from a wide variety of environments and ecosystems.

Plastic-degrading enzymes can be optimized by protein engineering for improved performance, including enhancement of enzyme thermostability, reinforcement of the binding of substrate to enzyme active site, enhancement of interaction between substrate and enzyme surface, and refinement of catalytic capacity.

Biocatalysis as an emerging solution for the global plastic waste challenge

Plastic materials play a revolutionary role in the modern world, although the enormous manufacture and extensive use of plastic commodities inevitably generate an extraordinary amount of post-consumer plastic waste. Around 12 000 million metric tons of plastic waste are predicted to accumulate in landfills and the natural environment by 2050 [1]. Improper handling of plastic waste has caused a grand environmental challenge. The debris of plastic waste, especially **microplastics** (see Glossary), can impose hazardous effects on various organisms and eventually threaten human well-being [2–5]. In addition, the degradation resistance of plastics further escalates their adverse environmental impacts [6]. Therefore, it is urgent to develop innovative technologies for treatment and recycling of post-consumer plastics, to achieve both waste valorization and environmental protection.

Enzymatic biocatalysis has gained increasing attention as an eco-friendly alternative to conventional plastic treatment and recycling methods (Box 1) [7]. To date, various microbial plastic-degrading enzymes have been discovered, representing promising biocatalyst candidates for plastic depolymerization. Considering the ubiquity of plastics in different ecosystems and the tremendous metabolic and genetic diversity of microorganisms, microbial communities in various habitats have likely evolved capabilities in plastic decomposition and utilization. The plastic-degrading enzymes identified so far might only account for a small portion of the enzymes relevant to plastic depolymerization in the environment. Therefore, it is of ever-growing interest to explore diverse environments to discover new plastic-degrading enzymes with desirable properties and functionalities. However, naturally occurring plastic-degrading enzymes are not well suited for synthetic plastic degradation in industrial applications due to poor thermostability and low catalytic activity. Particularly, synthetic plastic materials usually possess distinct physical and chemical properties (e.g., high **crystallinity**) that render them more resistant to enzymatic attack than biogenic polymers. Therefore, protein engineering has been increasingly utilized to construct plastic-degrading enzymes with better catalytic efficiency and stability.

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Box 1. Approaches for plastic waste treatment and recycling

Currently, landfill is the main treatment approach and receives approximately 80% of the plastic waste [109]. However, traditional landfills can cause secondary pollution owing to the ineluctable release of plastic waste into natural environments. In addition, the large quantity of single use plastic commodities discarded into landfill would place extra burdens on the regional and global carbon budget for manufacture of new plastics from petroleum chemicals.

Recycling of plastic waste is developed as a more environmentally benign practice, which not only mitigates the pollution but also conserves natural resources. Current plastic recycling methods could be generally categorized into mechanical and chemical approaches [110]. Mechanical recycling has been commercialized for reclamation of plastic waste at large scale for years. This type of method converts plastics into raw materials for reuse through mechanical operations, including sorting, washing, grinding, and extrusion. However, mechanical recycling is usually a '**downcycling**' process because the quality of plastic materials will be dramatically deteriorated and produce lower valued end products. Chemical recycling is implemented to recover small component molecules from plastic waste via chemical reactions. This method is not widely employed because it typically performs under harsh reaction conditions and requires large energy input and costly chemical catalysts. Incineration is sometimes considered as a recycling approach because it recovers heat energy, but the concomitant release of greenhouse gases and toxic airborne compounds makes it less environmentally friendly.

By contrast, enzymatic conversion of plastic waste could operate under reaction conditions with lower temperature and pressure than the chemical recycling processes, thus significantly reducing the energy and reagent consumption. More importantly, it opens up a new avenue for 'upcycling' processes. Enzyme biocatalysis depolymerizes plastic substrates into oligomers and monomers which can be recovered as raw materials to manufacture new plastic products or synthesize other value-added chemicals in a circular economic manner. A vast majority of the enzymes reported so far degrade **hydrolyzable plastics**, such as polyesters, polyamides (PAs), and polyurethanes (PUs). These enzymes primarily belong to carboxylic ester hydrolases (EC 3.1.1) family, such as cutinases (EC 3.1.1.74), lipases (EC 3.1.1.3), and carboxylesterases (EC 3.1.1.1) [23,95,111]. As for **non-hydrolyzable plastics**, they are extremely resistant to biological cleavage, and thus only limited reports on the enzymes to degrade them are available [112].

Recent research efforts have made significant advances in discovering and engineering plastic-degrading enzymes, showing the great promise of enzyme biocatalysis for sustainable plastic treatment and recycling. This review highlights the up-to-date progress on the discovery of novel plastic-degrading enzymes by using state-of-the-art omics-based methods and the optimization of plastic-degrading enzymes via a variety of protein engineering strategies. This article is timely as it provides a holistic view of the current stage and emerging trends in obtaining innovative and effective biocatalysts for plastic degradation, which will inspire future research to address the critical challenges for plastic treatment and recycling. Future research prospects and challenges are also discussed.

Omics-based discovery of novel plastic-degrading enzymes

Mining plastic-degrading enzymes via metagenomics-based approaches

Metagenomics has demonstrated enormous potential to facilitate the discovery of new enzymes from various ecological habitats. The conventional culture-dependent method has been applied to discover most of the known plastic-degrading enzymes [8,9]. In the culture-dependent method, microorganisms expressing the desired enzyme are first enriched and isolated under proper cultivation conditions, followed by strain taxonomical classification, and identification of putative enzymes by molecular biological or computational approaches (Figure 1A) [10–12]. However, the culture-dependent method seriously limits the scope of finding new plastic-degrading enzymes because it is estimated that less than 1% of the total microorganisms on the planet have been cultured. By contrast, the culture-independent metagenomic approach has emerged as a powerful tool to explore the vast majority of microorganisms from diverse environmental sources. As summarized in Table 1, many genes encoding enzymes capable of depolymerizing different plastic materials have been retrieved from a wealth of environmental metagenome samples. Therefore, in this section we discuss the recent progress in deciphering novel plastic-degrading enzymes from the huge reservoir of natural biocatalysts through metagenomic techniques.

Glossary

Catalytic promiscuity: the capability of catalysts, such as enzymes, to utilize a particular substrate or catalyze a particular reaction other than those for which they originally evolved.
Crystallinity: the degree of structural order and regularity of the polymeric architecture of plastic materials. High crystallinity represents regular and orderly molecular orientation of the polymeric chains that form a tightly packed and recalcitrant structure.
Downcycling: the recycling process that converts the waste materials into their component units with lower quality and value.
Exoproteome: the entire protein repertoire that exists in the extracellular matrix of a given biological system formed via cellular secretion or other export pathways.
Glass transition temperature (T_g): the temperature at which the amorphous regions of the plastic structure change from the rigid and brittle glassy state into rubber-like soft state. The mobility of polymeric chains in the plastic structure would be remarkably increased when the temperature is raised from below T_g to above T_g.
Glycosylation: the post-translational modification process that enables the attachment of carbohydrate moieties to amino acid residues of a protein.
Hydrolyzable plastics: plastics containing hydrolyzable chemical bonds, such as ester and urethane bonds, in their backbone structures that are susceptible to abiotic or biotic hydrolysis.
Microplastics: small plastic particles with a size less than 5 mm that are usually formed through fragmentation of larger plastic pieces.
Non-hydrolyzable plastics: plastics with the backbone composed of inert C–C linkages without a reactive functional group for hydrolysis reaction, such as polyethylene (PE), polypropylene (PP), polystyrene (PS), and polyvinyl chloride (PVC).
Plastisphere: the ecosystem that is formed by microbial communities colonizing and inhabiting human-derived plastic debris in aquatic environments.
Stable-isotope probing (SIP): a technique that uses particular substrates enriched with a stable isotope (e.g., ^{13}C) that are consumed and assimilated by the active target microorganisms from

The overall workflow using metagenomics to discover plastic-degrading enzymes is illustrated in Figure 1B. Among these steps, selecting appropriate screening methods is pivotal to metagenomic mining. Generally, there are two commonly used methods to screen the metagenomic library, sequence-based screening and function-based screening [13,14]. Sequence-based screening takes advantage of sequence similarity comparison and functional gene annotation by searching bioinformatic databases [14]. For example, a poly(ethylene terephthalate) (PET) hydrolytic enzyme (PET2) was uncovered through *in silico* sequence-based screening from metagenome databases by using a search algorithm powered by a hidden Markov model [15]. More recently, a number of gene sequences similar to the ones encoding known enzymes with activity to degrade polyurethane (PU) plastics were retrieved from landfill-derived metagenomes [16]. *In silico* sequence-based metagenomic screening is relatively rapid and cost-effective for enzyme mining. However, its success is limited by the size and gene annotation quality of current databases of known plastic-degrading enzymes [14]. This method could also miss new families of plastic-degrading enzymes with low sequence similarity to previously characterized ones. In addition, sequence similarities do not guarantee plastic-degrading activity, so further characterization and validation of enzyme functionality is needed [17].

Alternatively, function-based screening uses activity assays to search for the desired phenotypes from metagenomic libraries (Figure 1B). This approach is particularly advantageous over sequence-based screening, in mining completely novel groups of enzymes for which the sequences are more divergent from existing homologous ones. For example, multiple enzymes phylogenetically belonging to entirely new esterase families were screened from environmental metagenomes by using function-based agar plate assays, which exhibited hydrolytic activity towards different polyesters, including poly(lactic acid) (PLA), poly(ε-caprolactone) (PCL), and poly(butylene succinate-co-adipate) (PBSA) [18] (Table 1). Traditional agar plate assays have limited capability in screening large-sized metagenomic libraries. Recent studies in developing high-throughput screening approaches might accelerate the discovery of new plastic-degrading microbes and enzymes [19,20]. When using a functional screening approach, it is important to select a proper host cell for constructing a heterologous gene expression library, with desirable expression level and library representativeness. *Escherichia coli* is most widely used due to its convenient cultivation and genetic manipulation [21]. Meanwhile, alternative expression systems might be employed to ensure functional enzyme expression. For instance, eukaryotic host cells, such as the yeast *Pichia pastoris*, could be used for functional expression of the plastic-degrading enzymes with disulfide bonds, as they are unsuitably expressed in common *E. coli* [22–24]. It is also critical to select a proper library type for successful functional screening. The type of library chosen is usually determined by two factors; library size and coverage. Due to the short length of the insert that a plasmid can harbor, plasmid-based libraries usually have a large size but relatively low coverage, which is unfavorable for functional screening. By contrast, longer DNA fragments can be inserted into phage or fosmid libraries. Moreover, the phage-based library is advantageous for heterologous expression of some toxic plastic-degrading enzymes in *E. coli* because the expression of target genes is concomitant with the lysis of *E. coli* cells and the enzyme activity can be screened for directly on the phage plaques.

Besides the screening methods, metagenome sampling sources play an important role in determining the success of plastic-degrading enzyme discovery. Most of the natural environmental metagenomes investigated so far showed a low hit rate of genes related to plastic degradation (Table 1), representing a major challenge in metagenomic mining of novel plastic-degrading enzymes [13]. The analysis of different marine and terrestrial metagenomes worldwide revealed a broad distribution but an extremely low frequency for genes encoding PET hydrolytic enzymes, indicating the slow evolution of indigenous microorganisms to utilize anthropogenic PET plastics

which the isotope-labeled cellular components, such as DNA, can be selectively separated and recovered.
Targeted metagenomics: artificial manipulation of environmental samples to enrich the abundance of target genes of interest prior to metagenomic DNA extraction.
Upcycling: the recycling practice that transforms the discarded materials into compositional elements of higher quality and new value.

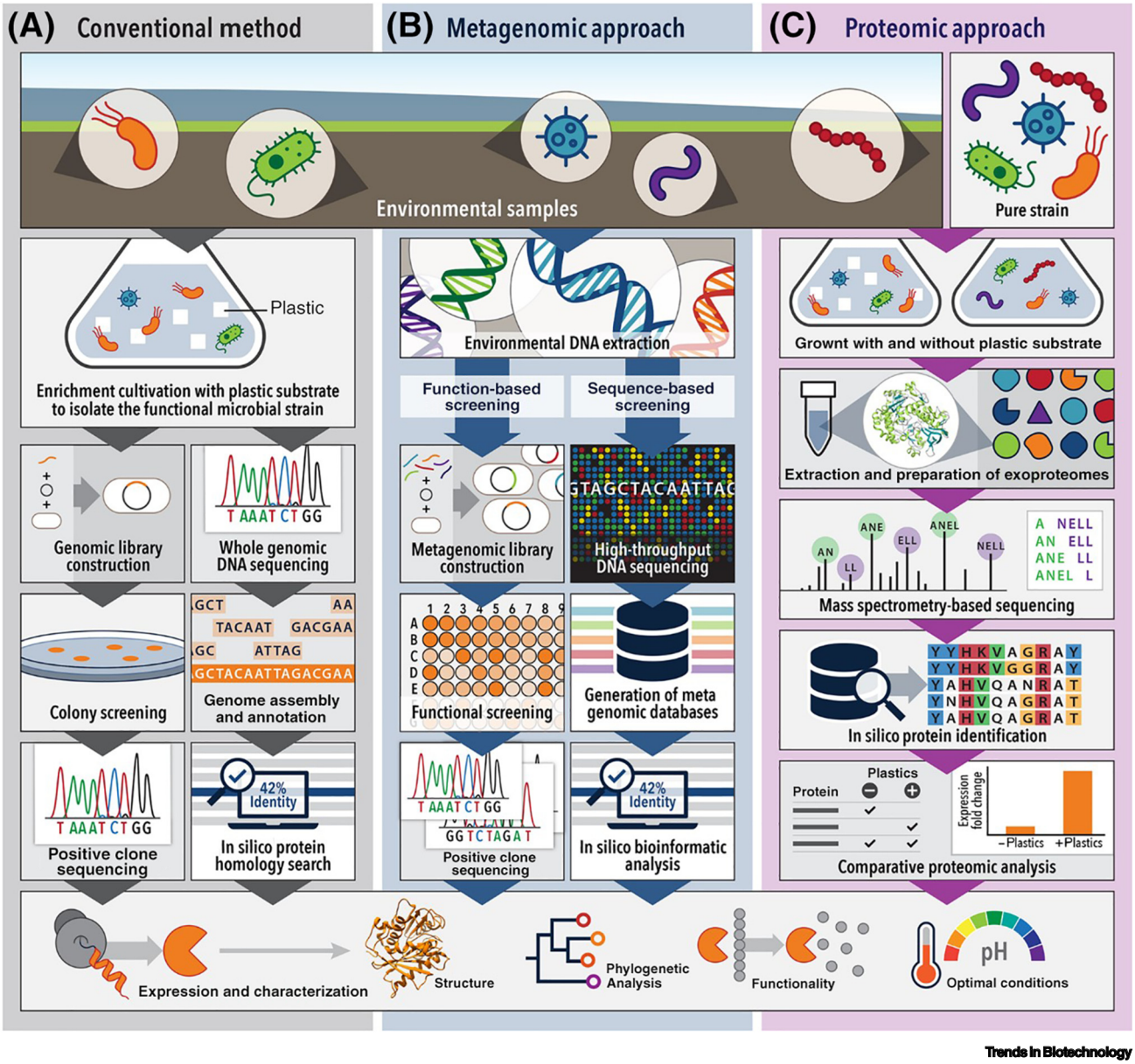

Figure 1. Schematic illustration of conventional and omics-based approaches for discovery of plastic-degrading enzymes.

[15]. By contrast, the likelihood for discovering plastic-degrading enzymes is greater from the environments abundant with biopolymeric substances. For example, a novel thermostable cutinase homologue, leaf and branch compost cutinase (LCC), capable of PCL and PET degradation, was identified from the metagenome of a leaf-branch compost with copious natural plant-derived polymers via function-based screening [25]. Likewise, esterases capable of hydrolyzing poly(diethylene glycol adipate) (poly DEGA) and synthetic copolyester poly(butylene adipate-co-terephthalate) (PBAT) were identified in metagenomic libraries constructed from soil compost and *Sphagnum* moss, respectively [17,26]. In addition, the **plastisphere** is a promising source for plastic-degrading enzyme discovery because the environment can select

Table 1. Plastic-degrading enzymes discovered by the metagenomic approach

Enzyme	Metagenome source	Library type	Library size	Heterologous host	Number of clones or sequences screened	Number of hit clones or sequences	Hit rate[a]	Number of characterized enzymes[b,c]	Target plastic substrate	Refs
Function-based screening										
Esterase	Seawater	λ ZAP phage	1430 Mbp	*Escherichia coli*	295 100	95	1:3106	5 (ranging from 314 aa to 505 aa)	PHB, PLA, PCL, and PBSA	[113]
Esterase	Marine environments, soils, and waste treatment facilities	λ ZAP phage and fosmid	7000 Mbp	*E. coli*	1 080 628	714	1:1513	7 (ranging from 308 aa to 501 aa)	PLA and PCL	[114]
Esterase	*Sphagnum* bog	Fosmid	N/A[d]	*E. coli*	90 000	83	1:1084	6 (ranging from 295 aa to 408 aa)	PBAT	[17]
Esterase	Compost	Plasmid	100 Mbp	*E. coli*	40 000	7	1:5714	3 (ranging from 283 aa to 431 aa)	PLA	[30]
Esterase	Compost	Fosmid	N/A	*E. coli*	13 000	10	1:1300	1 (570 aa)	PU	[26]
Cutinase	Leaf-branch compost	Fosmid	735 Mbp	*E. coli*	6000	19	1:316	1 (293 aa)	PCL and PET	[25]
Sequence-based screening										
PHB depolymerase	Biofilms on marine plastics	N/A	245 Mbp	N/A	118 520	46	1:2577	N/A	PHB	[115]
PET hydrolase	Marine and terrestrial environments	N/A	16 Gbp	N/A	N/A	349	0.0001–1.513 hits/Mbp	4 (ranging from 298 aa to 310 aa)	PET	[15]
Polyurethane esterase	Landfill	N/A	17.6 Mbp	N/A	3072	6	1:617	N/A	PU	[16]

[a]The hit rate means the ratio of the number of hit clones or sequences to the number of clones or sequences screened.
[b]This column represents the number of enzymes which were selected from the hit clones or sequences obtained by screening the corresponding metagenomic libraries and were further experimentally characterized regarding their ability in plastic degradation.
[c]The length of the enzyme is provided in parenthesis, and aa denotes amino acid.
[d]N/A indicates not applicable or not available for the data.

for microorganisms capable of using plastic compounds for survival and growth [27–29]. The plastisphere is currently underexplored but of growing interest for metagenomic mining of plastic-degrading enzymes.

Techniques such as **targeted metagenomics** and **stable-isotope probing (SIP)** might be helpful to increase the hit rate in metagenomic mining of new plastic-degrading enzymes. Targeted metagenomics can stimulate the presence of desired functions before DNA extraction, by *in situ* manipulation of the microbial habitat. For example, pre-incubation of target synthetic plastics in the native environment activated the prevalence of plastic-degrading microbial species and raised the likelihood of enzyme discovery [30]. Additionally, the SIP technique can be integrated with targeted metagenomics to further increase the hit rate [31,32]. Recently, ^{13}C-labeled plastic materials have been developed and used in biodegradation studies [33,34]. Using such compounds in targeted metagenomic studies would help pinpoint the functional microorganisms and enzymes participating in plastic degradation processes.

Mining plastic-degrading enzymes via the proteomics-based approach

The proteomics-based approach directly detects and quantifies protein expression and has proven its huge potential in mining new enzymes from a broad repertoire of microbial sources for biotechnological applications [35,36]. Figure 1C shows the commonly used workflow of the proteomic approach for mining plastic-degrading enzymes. First, the pure or environmental microbial consortia are grown with and without the plastic substrate, as the presence of plastics could differentially induce the functional microorganisms to express enzymes with plastic hydrolytic activity [19]. Proteins produced by the microbial cultures are extracted and digested into small peptides, which are subjected to sequencing, followed by protein identification via bioinformatic analysis. Typically, **exoproteome** is the principal target when screening for potential plastic-degrading enzymes because insoluble synthetic plastics are unable to enter the microbial cell and enzymes engaged in depolymerization are usually secreted extracellularly [23]. The effectiveness of the proteomic technique has been already demonstrated in identifying various enzymes involved in plant biopolymer degradation, inspiring its implementation in the discovery of novel plastic-degrading enzymes [37].

Comparative proteomics is most frequently used in mining plastic-degrading enzymes based on the presumption that incubation with plastics would stimulate the expression of enzymes involved in plastic depolymerization. For example, by comparatively analyzing the exoproteome of the bacterium *Pseudomonas pseudoalcaligenes* and fungus *Knufia chersonesos*, several novel putative polyesterases involved in PBAT degradation were identified, demonstrating the effectiveness of the method in mining plastic-degrading enzymes especially for microorganisms with unavailable annotated genomic data [38,39]. In another study, a polyhydroxybutyrate (PHB) depolymerase ALC24_4107 produced by *Alcanivorax* sp. 24 with activity in hydrolyzing a variety of natural and synthetic polyesters, was discovered via the comparative exoproteomic approach [40]. Proteomics-guided discovery of plastic-degrading enzymes is still in its infancy, and all of the currently reported studies were conducted with pure microbial cultures. Direct identification of plastic-degrading enzymes through metaproteomics from complex environmental samples is still challenging, due to difficulty in high-quality protein extraction and limited availability of databases for downstream bioinformatic analysis [41].

Protein engineering of plastic-degrading enzymes

Leveraging protein engineering techniques to improve the catalytic performance of plastic-degrading enzymes is a recently emerging topic. Protein engineering has two categories of approaches in general; rational design and directed evolution. Rational design modifies the protein of interest based on the knowledge of protein structure and mechanistic characteristics, computational simulation, and modeling. Almost all current reports on engineering plastic-degrading enzymes utilize rational design because of available structural and mechanistic information for many of these enzymes. The lack of efficient high-throughput screening techniques is a main barrier for directed evolution of plastic-degrading enzymes. The only attempt reported so far, employing direct evolution to engineer PHB depolymerase from *Ralstonia pickettii* T1, failed to acquire any variant with improved activity [42]. Therefore, this section will focus on discussing the rational design strategies for improving plastic-degrading enzymes. The strategies are illustrated in Figure 2 and examples are summarized in Table 2.

Enhancing enzyme thermostability

Thermostability of plastic-degrading enzymes is highly desired in industrial plastic depolymerization, especially for plastics with high **glass transition temperature (T_g)** (e.g., ~65–70°C for PET). When the reaction temperature gets close to or above the T_g of plastics, the polymeric chains would have considerably increased flexibility and mobility, facilitating their binding to the

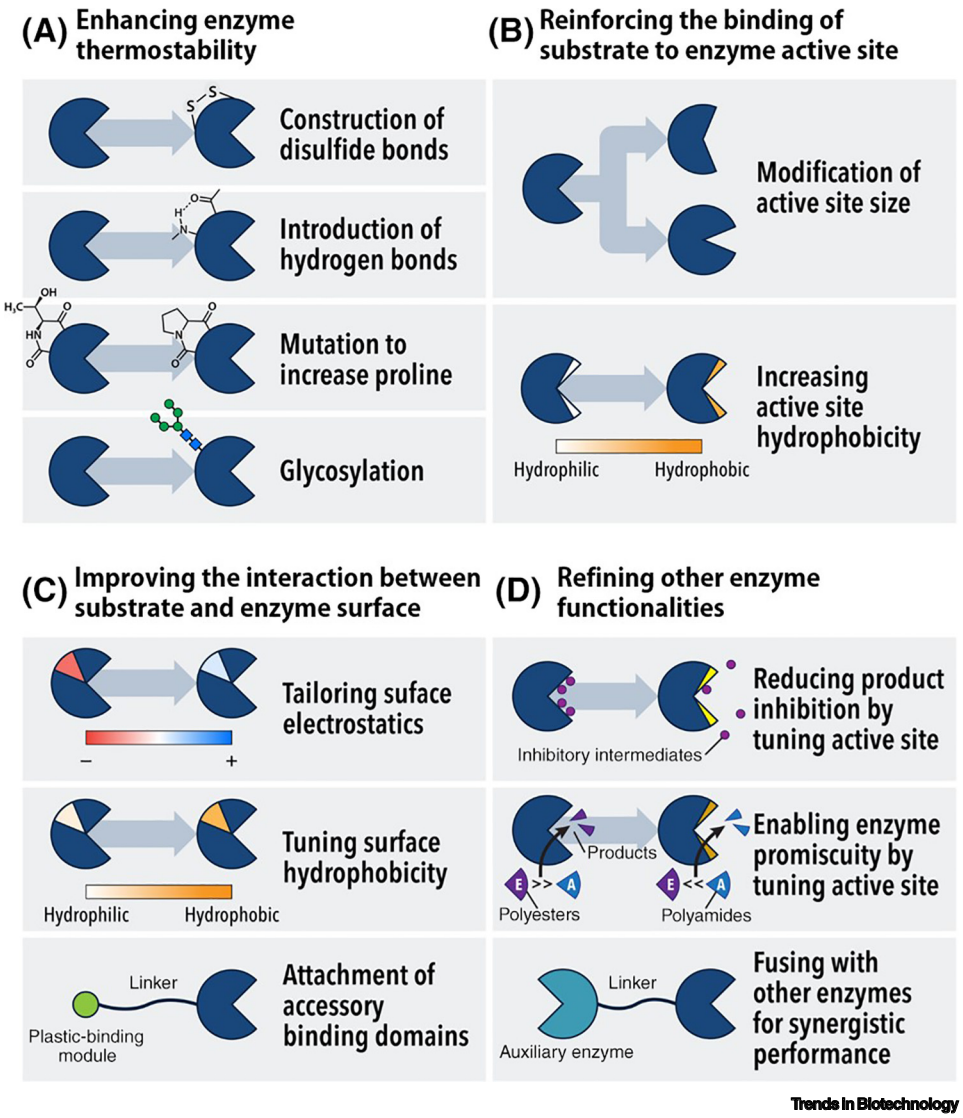

Figure 2. Typical protein engineering strategies for modifying plastic-degrading enzymes.

enzyme surface and accessibility to the active site for improved degradation efficiency [43]. However, the low thermostability of naturally occurring plastic-degrading enzymes is one major bottleneck for practical applications. Inspired by the unique structural features of thermophilic proteins, effective strategies have been designed to improve the thermostability of plastic-degrading enzymes, as detailed later.

Introduction of disulfide bonds or salt bridges can be beneficial to the enhancement of the thermostability of plastic-degrading enzymes (Figure 2A) [44–47]. Disulfide bonds and salt bridges are crucial for protein folding with the correct local or global conformation that could confer thermal resistance. Typically, the residues at a metal binding site responsible for protein thermostability might be replaced to introduce a disulfide bond. For example, the D204C and E253C mutations at the calcium binding site of an esterase TfCut2 from *Thermobifida fusca*

Table 2. Examples of protein engineering of plastic-degrading enzymes for improved biocatalytic performance

Enzyme	Source	Plastic substrate	Method	Mutations/modifications	Results	Refs
Enhancing enzyme thermostability						
Cutinase	*Thermobifida alba* AHK119	PBSA, PBS, PCL, PLA, and PET	Introducing proline residues	A68V/T253P	Increase of T_m value from 74 to 79°C compared with the A68V variant	[53]
Cutinase	*Saccharomonospora viridis* AHK190	PET	Introducing proline residues	S226P	Increase of T_m value by 3.7°C with higher compared with the wild-type enzyme	[10]
PETase	*Ideonella sakaiensis*	PET	Forming hydrogen bond	S121E/D186H	Increase of T_m value by 7.21°C and improved enzyme activity at elevated temperature relative to wild-type PETase	[50]
Cutinase	Leaf-branch compost metagenome	PET	Constructing disulfide bond	D238C/S283C	9.8°C higher for T_m value than that of the wild-type enzyme	[58]
Cutinase	Leaf-branch compost metagenome	PET	Introducing glycan moiety	Glycosylation at N266 and N197[a]	Resistant to thermal-induced aggregation at the temperature of 10°C higher than the nonglycosylated enzyme	[54]
Reinforcing the binding of substrate to enzyme active site						
Cutinase	*Fusarium solani pisi*	PET and PA 6,6	Enlarging the opening size of active site cleft	L182A	Fivefold increase in enzyme activity compared with the wild-type enzyme	[60]
PET hydrolase	*Pseudomonas aestusnigri*	PET	Enlarging the opening size of active site cleft	Y250S	Improved PET degradation activity as well as the capability of hydrolyzing crystalline PET from commercial bottle	[61]
PETase	*I. sakaiensis*	PET and PEF	Narrowing the opening size of active site cleft	S238F/W159H	Enhanced capability in PET and PEF degradation	[65]
PETase	*I. sakaiensis*	PET	Increasing the hydrophobicity of active site	L88F and I179F	2.1 and 2.5 times increased improvement in catalytic efficiency compared with the wild-type enzyme	[66]
Cutinase	*Thermobifida fusca*	PET	Increasing both the opening size and hydrophobicity of active site	Q132A/T101A	Higher hydrolysis efficiency than the wild-type enzyme	[67]
Improving the interaction between substrate and enzyme surface						
Cutinase	*Thermobifida cellulosilytica*	PET	Tuning the surface electrostatic potential	R29N/A30V	Increased hydrolytic activity compared with the wild-type enzyme	[70]
PHB depolymerase	*Ralstonia pickettii* T1	PHB	Modulating the surface hydrophobicity	Y443F	Improved degradation activity compared with the wild-type PHB depolymerase	[74]
Esterase	*Clostridium botulinum*	PET	Modulating the surface hydrophobicity	Truncation of 17 residues at the N-terminus	Enhanced hydrolysis efficiency relative to the wild-type enzyme	[75]
Polyamidase	*Nocardia farcinica*	PU	Tethered to an auxiliary binding module	Fusion with a polymer binding domain from *Alcaligenes faecalis*	Up to fourfold higher hydrolytic activity than the native enzyme	[81]
Cutinase	*T. cellulosilytica*	PET	Tethered to an auxiliary binding module	Fusion with *Trichoderma* hydrophobins	Over 16-fold increase of hydrolysis efficiency compared with wild-type enzyme	[83]

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Table 2. (continued)

Enzyme	Source	Plastic substrate	Method	Mutations/modifications	Results	Refs
Refining other enzyme functionalities						
Cutinase	*T. fusca* KW3	PET	Tailoring the substrate binding pocket	G62A	5.5 times lower binding ability to the inhibitory degradation product and 2.7 times higher degradation efficiency than the wild-type enzyme	[91]
Cutinase	*T. cellulosilytica*	PA	Tuning the active site with more polar residues	I179A, I179N, and I179Q	Enhanced promiscuous amidase activity and up to 15-fold increase in hydrolyzing insoluble model substrate of polyamide	[96]
Lipase	*Thermomyces lanuginosus*	PCL	Constructing a bifunctional chimeric enzyme fusion	Tethered to a cutinase from *Thielavia terrestris*[a]	13.3 times higher hydrolysis efficiency than the native enzyme	[97]
PETase	*I. sakaiensis*	PET	Constructing a bifunctional chimeric enzyme fusion	Fused with MHETase	More than threefold increase in catalytic activity compared with the wild-type enzyme	[93]

[a]These engineered enzymes were expressed heterologously in yeast *Pichia pastoris*, and all the other engineered enzymes listed in this table were expressed heterologously in *Escherichia coli*.

formed a disulfide bond, considerably increasing the protein melting temperature and plastic hydrolysis activity [48]. Additionally, the formation of a salt bridge between the negatively-charged N246D residue and positively-charged Arg280 residue might contribute to the improved thermostability of the engineered PETase^{N246D} [45]. Moreover, disulfide bonds and salt bridge construction could work synergistically to further benefit the thermostability of the enzyme [48].

Engineering the formation of hydrogen bonds at the region responsible for a more stable enzyme structure is another method to gain enhanced thermostability [49]. The hydrogen bond can maintain protein higher-order structures, which can promote structural stability and improve resistance to high temperature. For example, the formation of a water-mediated hydrogen bond between S121E and N172 residues at the highly flexible β6–β7 connecting loop region of PETase could increase the regional rigidity and lead to substantially enhanced thermostability [50]. In another study, multiple mutations including T140D, W159H, I168R, and S188Q were implemented to introduce new hydrogen bonds in PETase, and the engineered PETase had a melting temperature 31°C higher than that of the wild-type enzyme [49].

Furthermore, introducing more proline residues is useful to increase the thermostability of plastic-degrading enzymes (Figure 2A) [51,52]. Formation of hydrogen bonds and hydrophobic interactions between proline and its adjacent residues can stabilize the protein tertiary structure against high temperature. Also, the cyclic structure of the proline side chain that reduces the conformational entropy opposing protein folding may contribute to higher structural rigidity. In one study, threonine was mutated into proline at the 235 position of a *Thermobifida alba* cutinase, and the enzyme melting temperature increased significantly and was accompanied by an increased hydrolytic activity to PET plastics [53].

For plastic-degrading enzymes expressed in eukaryotic microbial cells, introduction of **glycosylation** can potentially improve thermostability (Figure 2A). Glycosylation enhances enzyme thermostability through strengthening protein thermodynamic stabilization and preventing thermal protein aggregations. For example, the glycosylated LCC exhibited higher stability against thermal aggregation and higher PET hydrolysis activity at elevated temperature [54]. Notably, it is critical to determine an

appropriate glycosylation site because the glycan moiety would pose adverse effects if incorrectly placed [55]. For instance, the close proximity of the introduced glycan to the enzyme active site could hinder substrate accessibility, which causes enzyme activity loss for PCL hydrolysis despite achieving higher thermostability [56,57]. Therefore, it would be more beneficial to design the glycosylation positions at loop regions or hydrophobic patches relatively distant from the enzyme active site to minimize adverse steric effects.

It should be noted that engineering plastic-degrading enzymes with enhanced thermostability would occasionally impair catalytic efficiency, which might be due to interference with the active site [50]. To avoid such negative impact, profound understanding and analyses of enzyme structure–function relationships are needed. In fact, in most of the published studies, enzymes engineered with enhanced thermostability also had improved or unchanged plastic degradation efficiency [45,46,49,54,58]. For example, the engineered LCC variants exhibited both enhanced thermostability and increased PET degradation efficiency compared with wild-type LCC [58].

Reinforcing the binding of substrate to the enzyme active site

The active site region is a hotspot for engineering plastic-degrading enzymes because the interaction between the enzyme active site and substrate is a critical factor dictating the efficiency of plastic depolymerization [22,59]. A common strategy is to create a wider opening of the active site to increase plastic substrate accessibility (Figure 2B). The first attempt to this end was made in engineering *Fusarium solani* cutinase [60]. A number of amino acid residues were mutated to generate an enlarged active site cleft, and the resultant L182A mutant with a wider opening of active site than the original enzyme showed increased hydrolytic activity towards PET and polyamide (PA) fibers [60]. Similar strategies have been successfully implemented in engineering various enzymes such as PETase, Cut190, MHETase, and a *Pseudomonas aestusnigri* hydrolase, to enhance their degradation activity towards PET and PBSA plastics [61–64].

However, larger substrate binding space does not always guarantee an improved catalytic performance because a too wide active site might cause weaker substrate affinity due to reduced binding ability [63]. In some cases, modifying the active site with narrower space might be favorable. One study reported that narrowing the active site of PETase by the double mutations S238F and W159H led to more efficient degradation of PET and its emerging replacement poly (ethylene furanoate) (PEF), because of the π-stacking interaction induced by S238F and the deeper sitting of the substrate in the active site cleft enabled by W159H [65].

Hydrophobicity of the substrate binding groove of the active site is also a potential engineering target (Figure 2B). Increasing hydrophobicity could be advantageous for plastic substrate binding due to higher affinity, resulting in enhanced degradation efficiency as demonstrated for PETase [66]. Furthermore, tuning the hydrophobicity and opening size of the active site simultaneously could synergistically improve enzyme catalytic performance. For example, the mutant Q132A/T101A derived from a *T. fusca* cutinase exhibited significantly higher PET hydrolysis efficiency, because the mutations created more space and increased hydrophobicity of the active site [67].

Improving the interaction between substrate and enzyme surface

Tailoring surface properties of plastic-degrading enzymes has been effectively exploited to improve biocatalytic efficiency through boosting enzyme–substrate interaction. The substrate binding process is dictated by electrostatic and hydrophobic interactions between the substrate molecules and amino acid residues on the enzyme surface [68,69]. Therefore, modification of surface electrostatic and/or hydrophobic properties are common strategies (Figure 2C). Specifically,

making the enzyme surface electrically neutral might reduce electrostatic repulsion between the enzyme and the plastic substrate, thus enhancing binding and degradation efficiency. For example, the mutation R29N of the cutinase Thc_Cut2 generated a more neutral enzyme surface and thus facilitated PET hydrolysis [70]. A similar result was also observed for the mutant R228S of Cut190 with electrical neutrality at the surface area proximate to the mutation [71]. By contrast, tuning the enzyme surface with increased hydrophobicity could also enhance the enzyme–substrate interaction [45,64,72]. As an example, replacement of serine and tyrosine with the more hydrophobic cysteine and phenylalanine in PHB depolymerase from *R. pickettii* T1, promoted adsorption of the enzyme onto the PHB surface and stimulated the efficiency of plastic hydrolysis [73,74]. In another study, after removing the N-terminal domain in a *Clostridium botulinum* esterase, a covered hydrophobic surface area became accessible for PET sorption, resulting in higher enzymatic hydrolysis [75]. However, introducing too many hydrophobic residues might impair catalytic activity due to enzyme aggregation or protein structure disruption caused by concomitant additional intermolecular hydrophobic interactions [72].

Another approach to enhance substrate–enzyme interaction is fusion of binding accessory to the enzyme surface (Figure 2C). This method is inspired by the fact that some enzymes involved in natural biopolymer degradation possess an auxiliary binding domain specialized in polymer substrate adhesion [76]. Therefore, plastic degrading enzymes intrinsically devoid of such function can be fused with heterologous binding modules to enhance their interaction with the plastic surface. Carbohydrate-binding modules (CBMs) originating from carbohydrate-active enzymes can promiscuously bind to various natural polymers and synthetic plastics [76,77]. Fusion of CBM from *Trichoderma reesei* to cutinase Thc_Cut1 achieved higher binding affinity to the PET surface and significantly increased hydrolysis efficiency [78]. Similar results were also reported when fusing the CBM from the *Cellulomonas fimi* cellulase CenA to a *T. fusca* cutinase with enhanced binding and catalytic efficiency towards PET fibers [79]. Besides CBMs, the polyhydroxyalkanoate binding module (PBM) from *Alcaligenes faecalis* was also used to create hybrid fusion with the Thc_Cut1 enzyme and a *Nocardia farcinica* polyamidase to hydrolyze different synthetic polymers [78,80,81]. Hydrophobins produced by fungal species are another class of polymer-binding biological macromolecule which promise to be engineered with plastic-degrading enzymes [82]. One study covalently tethered *Trichoderma* hydrophobins to Thc_Cut1 and drastically increased the PET hydrolysis rate by more than 16-fold compared with the native enzyme [83]. However, not all hydrophobins could improve plastic degradation and some of them exhibited limited or even inhibitory effects [84]. Such differences warrant reasonable selection of hydrophobins for plastic-degrading enzyme engineering. Additionally, some bioactive polypeptides originally recognized as antimicrobial peptides could strongly adhere to the surface of various synthetic polymers because of their amphipathic property [85,86]. For instance, a chimeric fusion was constructed by genetically linking Tachystatin A2 (TA2) peptide to a *Thermomonospora curvata* cutinase and achieved a 6.6 times higher efficiency in hydrolysis of polyester–PU nanoparticles than the wild-type cutinase [87]. Notably, directed evolution has been applied to these peptides to obtain potent variants with stronger binding affinity, which could be potentially used to engineer more efficient enzyme fusion for plastic degradation [88].

Refining other enzyme functionalities

Efforts in optimizing plastic-degrading enzymes have also been made in other aspects including reducing product inhibition effects, enabling the enzyme **catalytic promiscuity**, and creating multifunctional biocatalysts (Figure 2D). First, plastic degradation intermediates or products can inhibit enzyme activity [39,89,90], and such inhibition can be mitigated by modifying the active site architecture. For example, mutation G62A in the substrate binding groove of TfCut2

resulted in 5.5 times decrease in the binding constant for the inhibitory hydrolysis product, mono (2-hydroxyethyl) terephthalate (MHET), thus leading to improved PET degradation [91]. Besides, fusion with an ancillary enzyme capable of decomposing the intermediate compounds was demonstrated to be effective in reducing product inhibition [92]. For instance, the chimera composed of PETase and MHETase showed increased PET hydrolytic activity, which could be attributed to the role of MHETase in degrading the intermediate MHET that inhibits PETase activity [93].

Enabling catalytic promiscuity is a meaningful approach to expand the capacity of plastic-degrading enzymes to use different plastic substrates, especially for plastics with few enzymes known to efficiently degrade them (such as PAs and PUs) [94,95]. Recently, a pioneering study reported manipulation of the Thc_Cut1 cutinase to develop its promiscuous amidase activity towards depolymerizing artificial PAs [96]. The residues possibly obstructing the interaction of water molecules with the transition state were identified for mutation, which generated a six- to 15-fold higher hydrolytic activity [96].

Additionally, fusion of a plastic-degrading enzyme with another auxiliary enzyme has been exploited to create bifunctional biocatalysts for improved depolymerization efficiency. For example, a lipase–cutinase fusion protein (Lip–Cut) with remarkably higher PCL hydrolysis activity was constructed, based on the rationale that lipase is primarily involved in the cleavage of the polymeric backbone of PCL into low molecular weight oligomers while cutinase subsequently hydrolyzes these low molecular weight oligomers into soluble monomers [97]. Similarly, synergistic fusion of PETase and MHETase can improve PET hydrolysis efficiency by threefold when compared with wild-type PETase [93].

Concluding remarks and future perspectives

Enzyme biocatalysis provides a green chemistry alternative for sustainable plastic waste management and recycling. The enzyme-mediated biocatalytic degradation could be potentially integrated into the plastic recycling process to cooperate with or replace current chemical recycling. After mechanical pretreatment, the plastic materials would be transferred into bioreactors containing plastic-degrading enzymes for biocatalytic depolymerization. The produced chemical molecules can be used either as building block monomers to synthesize new plastic products in a closed-loop recycling method or as feedstocks for conversion into high-value chemicals in an open-loop **upcycling** method [7]. The rapid advances in 'omics' techniques, synthetic biology, and protein engineering offer a wide array of powerful toolkits to discover, characterize, and modify plastic-degrading enzymes, opening up new possibilities to acquire novel biocatalysts with ideal properties for efficient and cost-effective plastic depolymerization. While recent research efforts have made significant progresses to this end, there are critical challenges remaining before applying enzyme biocatalysis for plastic recycling at industrial levels (see Outstanding questions).

First, high-throughput screening methods are needed to facilitate the identification of plastic-degrading enzymes of interest from metagenomic or mutant libraries since the current agar plate-based method has relatively low sensitivity and throughput [98]. Development of new screening methods to identify novel plastic-degrading enzymes more efficiently and accurately might benefit from the recent advances in high- and ultra-high throughput platform techniques such as cell-as-compartment, micro- and pico-droplet based, and microchamber-based screening methods [20,99]. Meanwhile, finding proper substrates compatible with advanced screening platforms is also a challenge. Several encouraging examples of chromogenic and fluorogenic substrates recently developed for high-throughput analysis of plastic-degrading enzymes might inspire future substrate design [100–102].

Outstanding questions

How can we develop innovative high-throughput screening methods for efficient discovery of plastic-degrading enzymes from metagenomics or mutant libraries?

How can we exploit computational techniques such as machine learning and artificial intelligence to assist the design of protein engineering strategies for optimizing plastic-degrading enzymes effectively and accurately?

How can we achieve scalable production and regeneration of plastic-degrading enzyme biocatalysts for efficient and economically feasible industrial plastic recycling applications?

Regarding engineering of plastic-degrading enzymes, the current rational design approaches are largely empirical with uncertainties because of limited understanding of the enzyme structure–function relationships. It is challenging to precisely predict the effect of potential mutations, and thus sometimes researchers have to arduously prepare a large library of mutants for screening. The growing knowledge of the enzyme structure–function relationship and continuous advancement of computer-assisted modeling and simulation could revolutionize plastic-degrading enzyme engineering. Notably, the emerging computational approaches, such as machine learning and artificial intelligence, hold great promise in deciphering protein features and functions and may guide protein engineering in a more predictive and precise way [103,104].

A successful enzyme biocatalytic system for real-world applications requires high efficiency, robustness, and reuse/regeneration of the biocatalysts. It is far from an economical option to directly use free enzymes in large-scale reactions due to the relatively short enzyme lifetimes and difficulty in enzyme recovery and reuse [105]. Engineering whole-cell biocatalysts constantly producing functional plastic-degrading enzymes could be a strategy to overcome the problem of short enzyme lifetimes [106,107]. Identifying and engineering microorganisms that naturally degrade plastics could be another potential solution for this issue. However, it is challenging to keep the microorganisms active under harsh industrial plastic degradation conditions (e.g., relatively high temperatures for efficient degradation of plastics with a high T_g). To this end, thermophilic microorganisms are needed, but genetic engineering toolboxes for such unconventional host microorganisms are still in development. Additionally, ecological risk assessment of using genetically engineered microorganisms is necessary for real-world applications. In this respect, using enzyme biocatalysts has attracted more attention. To improve enzyme stability and reusability, immobilization techniques such as crosslinking and cell surface display could be reasonable options as they can retain enzyme accessibility to solid plastic substrates [108]. Future research is needed to address the challenges of scalable preparation and regeneration of biocatalysts for practical plastic recycling.

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Declaration of interests

No interests are declared.

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