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Discovery of Plastics-degrading Enzymes

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Abstract

Plastics are highly advanced materials that have a vast array of applications and are produced globally in an approximate amount of 350 to 400 million tons every year. Nevertheless, there are serious concerns about plastic waste and pollution as a result of the misuse and lack of control of their use in industries, including packaging, transportation, manufacturing, and agriculture. Approximately 1,000 years are required for plastic bags to decompose efficiently. Additionally, CO_2 and dioxins are released into the atmosphere by burning plastics, and they contribute to global warming. The Earth's environment is overwhelmed with waste, mostly from poor recycling practices and low circular usage, resulting in millions of tons of waste generated annually. To combat this, new technologies for recycling post-consumer plastics are desperately needed to decrease plastic waste and improve the environment, while also finding ways to utilise these materials. Due to the inadequate disposal methods currently available for plastic waste, there has been increased interest in the use of microorganisms and enzymes designed for the biodegradation of non-degradable synthetic polymers *via* biocatalytic depolymerisation indicating that plastics treatment and recycling can be more efficient and sustainable.

Keywords

Enzymatic degradation of plastics, enzymatic depolymerisation, circular bioeconomy, green bioprocesses, polymer biorecycling

1 Introduction

The Greek word *Plastikos,* which is the origin of the word plastic, means "mouldable". Plastic is defined as a polymer, which upon heating becomes mobile and thus can be cast into moulds. The manufacture and consumption of plastic commodities have profoundly altered the contemporary world. Nevertheless, it has created an extreme abundance of plastic waste. Plastic waste has become a major issue in recent years due to its non-biodegradable nature. If appropriate precautions are not taken, it is anticipated that 12 billion tonnes of plastic waste will be disposed of in landfills over the next 50 years. This could have devastating consequences for the environment and lead to long-term damage. Governments and organisations should take steps to reduce plastic waste, such as introducing bans or levies on single-use items. Additionally, the public should be educated on the importance of reducing their plastics consumption and disposing of waste correctly. This will help to ensure that plastic waste does not continue to accumulate in our landfills in the future. This is a major environmental concern, as current methods of handling plastic waste are proving insufficient. This build-up of plastic waste not only affects the environment but also affects the species that live in those areas, resulting in long-term implications that will be difficult to undo. The urgent need to solve this issue is critical, since the ongoing build-up of plastic waste will have a negative influence on our world and the lives of future generations.[1] In nature, petroleum-based plastics are practically non-degradable due to their high stability and durability.[2] However, both abiotic and biotic factors contribute to a very slow rate of decomposition.[3] Marine and wind waves, along with factors such as friction and collisions with solid materials like rocks, all contribute to the mechanical breakdown of plastics in the environment. This process, known as fragmentation, results in the plastic breaking down into smaller pieces. Micro- and nano-plastics formed by these effectors result in a much larger surface area for further degradation.[3,4] The presence of plastic waste poses a significant threat to the environment, particularly in the form of microplastics. These tiny particles can have a detrimental impact on a wide range of organisms, including marine life and wildlife, and can also pose a risk to human health.[5–7] To achieve waste value creation and environmental protection, innovative technologies are urgently needed for post-consumer plastics treatment and recycling. To date, plastics depolymerisation has been successfully achieved using various microbial enzymes that degrade plastics. It is likely that microorganisms living in a variety of habitats have evolved capabilities for decomposing and utilising plastics, given the widespread distribution of plastics in different ecosystems.[8] In terms of plastics depolymerisation, the identified plastics-degrading enzymes (PDEs) so far may only constitute a small fraction. Given the importance of finding new enzymes with specific properties and functions in a variety of settings, researchers are constantly seeking ways to improve natural plastics-degrading enzymes. However, current natural enzymes often lack the ability to withstand high temperatures, and have low catalytic activity, making them inadequate for use in industrial settings. Synthetic plastics are often more durable and resistant to decomposition compared to natural polymers because of their unique chemical and physical properties, which make them less susceptible to breakdown by enzymes. Thus, proteins engineered to achieve better stability and catalytic efficiency have become increasingly

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popular in the development of PDEs. The use of enzymes in biocatalysis has the potential to revolutionise the way plastics are treated and recycled, making the process more sustainable. In recent years, researchers have made significant strides in identifying and manipulating enzymes that can break down plastics, furthering the potential for these biocatalytic processes to be implemented on a larger scale.

1.1 Discovery of novel PDEs *via* metagenomics-based approaches

New enzymes from diverse ecological habitats can be discovered through metagenomics. Most of the known enzymes that degrade plastic have been discovered using culture-dependent methods, which involve enriching and isolating microorganisms that produce enzymes, sorting strains by taxonomy, and identifying putative enzymes using molecular biology techniques or computational methods.[9–13] It is estimated that only a tiny percentage of all microbes on our planet have been successfully grown in a laboratory setting. As a result, the method of discovering new enzymes that can break down plastic, which relies on cultivating microbes, is quite limited in scope. Culture-independent metagenomic analyses, on the other hand, have emerged as a powerful method for exploring microorganisms found in a range of different environments. A variety of environmental samples containing metagenomes have been found to contain genes encoding enzymes able to depolymerise plastic materials Table 1 (adapted from reference[8]).

A key component of metagenomic mining is selecting appropriate screening methods. Metagenomic library screening can be performed in two ways, sequence-based or function-based.[14,15] The first screening procedure uses bioinformatics databases to search for genes with similar sequences and functional annotations.[15] Sequence-based *in silico* screening of metagenome databases has led to the discovery of polyethylene terephthalate (PET) hydrolytic enzyme (PET2).[16] Also, several genes that encode enzymes associated with polyurethane (PU) degradation have recently been found in landfill-derived metagenomes.[17] As a method for mining enzymes from a metagenomic sequence, *in silico* sequence-based metagenomic screening offers excellent speed and cost efficiency. Nevertheless, its success has been limited by the small size of the existing plastics-degrading enzyme databases, as well as the quality of gene annotation that is currently available.[15] Because of this method, new families of enzymes that degrade plastics might also be missed that have little sequence similarity to enzymes already characterised. The sequence similarity between the two enzymes cannot guarantee plastics-degrading activity, so it is necessary to carry out further characterisations and validations of the enzymes to ensure their functionality.[18]

It is also possible to use function-based screening as an alternative method of searching metagenomic libraries for desired phenotypes by using activity assays. Compared with sequence-based screening, this approach is particularly advantageous for mining enzyme groups that are completely novel, whose sequences are very different from those of previously identified homologous enzymes, and particularly useful for mining new enzyme groups. For instance, researchers examined the genetic makeup of microorganisms in the environment for enzymes that can break down various types of polyesters, such as poly(ε-caprolactone) (PCL), poly(butylene succinate-co-adipate) (PBSA) and poly(lactic acid) (PLA) that were phylogenetically distributed across new esterase families.[19] When it comes to screening large metagenomic libraries, traditional agar plate assays are not as effective as they used to be. Recent studies have indicated that the process of discovering new plastics-degrading microbes and enzymes may be accelerated by using high throughput screening (HTS) methods. These methods involve the simultaneous testing of large numbers of samples, allowing for a more efficient and comprehensive exploration of potential candidates. By using HTS, scientists may be able to identify new plastics-degrading microorganisms and enzymes more quickly and effectively. This could potentially lead to significant advancements in the creation of innovative and efficient methods for dealing with plastic waste. Furthermore, it could also contribute to the overall sustainability and environmental health of our planet.[20,21]

When creating heterologous gene expression libraries, it is crucial to choose the right host cells to ensure the desired level of expression and representative nature of the library. Due to its ease of cultivation and genetic modifiability, *Escherichia coli* is one of the most frequently utilised host cells for protein expression. Its simplicity in growth conditions and ability to accept genetic modifications make it a popular choice among scientists and researchers in the field. Additionally, the extensive knowledge and understanding of *E. coli* biology and genetics have contributed to its widespread use as a host organism. This allows for efficient and effective manipulation of the library, resulting in a high level of control over the expression of the genes in the library.[22] In addition to utilising bacterial expression systems, alternative expression methods can also be employed to achieve the production of functional enzymes. It is possible to express plastics-degrading enzymes with disulphide bonds in eukaryotic cells, *e.g., Pichia pastoris,* as they have a more advanced cellular machinery that allows for the expression of enzymes with post-translational modifications like disulphide bonds, which are not typically found in *E. coli*.[23–25] Selecting an appropriate library type is essential for functional screening to be successful and yield the desired outcome. The selection of a library is generally based on two key considerations: the size of the library, and the coverage of the library. Plasmid-based libraries tend to be larger in size; however, they typically have a lower coverage, which can be detrimental for functional screening. When selecting a library for functional screening, it is crucial to consider not only the library's size, but also the diversity of sequences it contains. Merely a large-sized library may not be sufficient to identify the desired function if it is not diverse in its sequence coverage. To maximise the likelihood of successfully identifying the desired function, it is essential to choose a library that encompasses both a large size and a broad representation of different sequences. This ensures that a wide range of potential functions is included in the screening process.

Investigating the application of enzymes that break down plastics is a significant field of study, and metagenome samples are vital to this endeavour. By utilising metagenome

Table 1 – PDEs discovered by metagenomics[8]
Tablica 1 – PDE otkriveni metagenomikom[8]

Enzyme	Metagenome source	Library size	Heterologous host	Number of clones or sequences screened	Number of hit clones or sequences	Number of characterised enzymes	Target plastic substrate	Refs
Function-based screening								
Esterase	Seawater	1.43 Gbp	*E. coli*	$295 \cdot 10^3$	95	5	PHB, PLA, PCL, and PBSA*	36
	Marine environments, soils, and waste treatment facilities	7 Gbp	*E. coli*	$108 \cdot 10^4$	714	7	PLA and PCL*	37
	Sphagnum bog	NA	*E. coli*	$90 \cdot 10^3$	83	6	PBAT*	18
	Compost	0.1 Gbp	*E. coli*	$40 \cdot 10^3$	7	3	PLA*	31
	Compost	NA	*E. coli*	$13 \cdot 10^3$	10	1	PU*	27
Cutinase	Leaf-branch compost	0.7 Gbp	*E. coli*	$6 \cdot 10^3$	19	1	PCL and PET*	26
Sequence-based screening								
PHB depolymerase	Biofilms on marine plastics	245 Mbp	NA	$118 \cdot 10^3$	46	*	PHB*	38
PET hydrolase	Marine and terrestrial environments	16 Gbp	NA	*	349	4	PET*	16
PU esterase	Landfill	17.6 Mbp	NA	3072	6	*	PU*	17

NA: No available data. *PHB: polyhydroxybutyrate, PLA: poly(lactic acid), PCL: poly(ε-caprolactone), PBSA: poly(butylene succinate-co-adipate), PBAT: poly(butylene adipate-co-terephthalate), PU: polyurethane

samples and different screening techniques, researchers can uncover genes associated with plastics decomposition. Despite previous attempts, examination of the majority of metagenomes from the natural environment has not yet revealed a significant number of such genes. This suggests that more extensive research and advanced screening methods may be necessary to fully uncover the potential for plastics-degrading enzymes in natural environments (Table 1). Despite the widespread presence of genes responsible for the breakdown of PET plastics in both marine and terrestrial environments, the quantity of these genes is minimal, suggesting that microorganisms have not yet developed the capability to efficiently degrade PET plastics. It is likely that the absence of evolutionary changes observed in microorganisms capable of degrading PET plastics can be attributed to the relatively recent introduction of these synthetic polymers into the environment. Since PET plastics are not naturally occurring in nature, it stands to reason that microorganisms have not had sufficient time to adapt and evolve in response to this new type of organic substrate. Additionally, as a synthetic polymer, PET likely presents unique challenges for microorganisms that are not encountered in the degradation of naturally occurring organic materials. This lack of evolutionary adaptation may explain why current biodegradation rates of PET plastics are relatively slow. Furthermore, the slow degradation rate of PET plastics may also be due to the complex chemical structure of the polymer, which makes it difficult for microorganisms to break down. This lack of adaptation is likely due to the relatively recent introduction of PET as a man-made material, and the lack of selective pressure for microorganisms to develop the ability to degrade it. However, further research is needed to fully understand the reasons for the rarity of these genes in natural environments, and the implications for the persistence of PET in the environment.[16] In contrast to environments that are relatively scarce in biopolymeric compounds, those that are rich in such substances, present a higher likelihood of identifying plastics-degrading enzymes. A research was conducted to analyse the metagenome of compost created from a branch and leaves with a heavy amount of substances sourced from plants. The results of this study revealed the presence of a previously unknown thermostable cutinase homolog, specifically the leaf and branch compost cutinase. The discovery of this enzyme in a leaf-branch compost highlights the potential for such environments to harbour a diversity of plastics-degrading enzymes, and serves as a valuable resource for the ongoing development of biodegradable plastics.[26] In addition, metagenomic libraries constructed from soil compost and sphagnum moss were found to contain esterases capable of hydrolysing poly(diethylene glycol adipate) and poly(butylene adipate-co-terephthalate) (PBAT).[18,27] There is a developing curiosity in the area of plastics deterioration concerning the occurrence of plastispheres, which are communities of microorganisms that inhabit and settle on plastic waste produced by humans in watery environments. This is because these communities of microorganisms have the potential to serve as a valuable source of enzymes ca-

pable of breaking down plastic compounds. The process of metagenomic mining, which involves the identification and isolation of these enzymes through the study of the genetic makeup of plastisphere communities, is becoming increasingly important as a means of understanding and harnessing the plastics degradation capabilities of these microorganisms. The study of plastispheres and the application of metagenomic mining techniques therefore hold significant promise for the development of effective strategies for addressing the plastic pollution crisis.[28–30]

New PDEs might be discovered by metagenomic mining using targeted metagenomics and stable-isotope probing (SIP). When the microbial habitat is altered *in situ*, it is possible to stimulate the emergence of specific functions prior to DNA extraction.[8] Plastics-degrading microbial species are promoted and enzymes are more likely to be discovered when target synthetic plastics are pre-incubated in their native environment.[31] A further increase in hit rate can be achieved by integrating the SIP technique with targeted metagenomics.[32,33] Biodegradation studies have been conducted recently with plastic materials that are ^{13}C-labeled, and the results are promising.[34,35]

1.2 Protein engineering of PDEs

It is a recently emerging topic to improve PDEs' catalytic performance utilising protein engineering techniques. Directed evolution (DE) and a rational design (RD) approach are the two general approaches to protein engineering. Rational design is a strategy that utilises the knowledge of the structural and mechanistic characteristics of enzymes to manipulate and improve their properties. This approach is particularly useful for enzymes involved in plastics degradation, as a significant amount of information on their structure and function is available. Consequently, many recent studies on the engineering of plastics-degrading enzymes have employed rational design techniques. The application of rational design not only allows for the optimisation of enzyme activity but also enables the exploration of new enzyme functions and the identification of potential biocatalysts for industrial applications. Furthermore, rational design can be used to overcome limitations of natural enzymes, such as low stability or poor substrate specificity. Overall, rational design is a powerful tool that can significantly advance the development of enzymes for plastics degradation and other industrial applications. A major issue that needs to be overcome by scientists involved in the field of directed evolution to generate plastics-degrading enzymes is the lack of efficient high-throughput screening approaches. While directed evolution has been employed in the past for the engineering of polyhydroxybutyrate (PHB) depolymerase, the results have been limited and no significant improvements in activity have been achieved.[39] This highlights the need for the development of more advanced high-throughput screening techniques that can increase the efficiency and effectiveness of directed evolution for the production of enzymes that can effectively degrade plastics. The examples of engineered PDEs by rational design strategies are summarised in Table 2 (adapted from reference[8]).

Semi-rational protein engineering offers a promising alternative to traditional methods such as rational design and directed evolution for generating novel proteins with desired properties. Rational design involves precise modifications to the amino acid sequence based on a thorough understanding of protein structure and function. Directed evolution, on the other hand, depends on the selection of favourable traits through random mutations. Nevertheless, both approaches have limitations when it comes to creating proteins with intricate or multiple desired characteristics. In contrast, semi-rational protein engineering combines the strengths of both techniques utilising computational tools to guide targeted mutations in regions where the desired effects are most likely to occur.[40] It also employs directed evolution to explore uncharted sequence space. As the field of protein engineering has advanced significantly with the increasing development of computer technology, user-friendly online tools, machine- and deep learning aided methodologies have been incorporated into protein engineering methodologies.[41–43] These hybrid strategies have proven successful in creating proteins with enhanced stability, activity, specificity, and other desirable attributes.[44–46]

1.2.1 Thermostability enhancement

Enzymes that are capable of breaking down plastics with high thermostability are highly desirable in the field of plastic waste management. As the temperature of the reaction reaches or surpasses the glass transition temperature of plastics (which is 65–70 °C for PET), the polymeric chains become more pliable and agile. This enhancement in flexibility and mobility of the polymers is a consequence of a diminishment in internal interactions between the polymer chains, which are accountable for the glassy state of plastics. Consequently, the enzymes having the capacity to depolymerise plastics at or beyond their glass transition temperature may be able to significantly boost the proficiency and effectiveness of plastic waste management programs.[71] PDEs in nature, however, are not very thermostable, which presents a major challenge for practical application. Scientists have taken advantage of the exclusive structural characteristics of thermophilic proteins to enhance the heat resistance of enzymes that degrade plastics. Enhancing the performance of those enzymes can be accomplished by the incorporation of disulphide bonds or ionic interactions.[72–75] The presence of disulphide bonds and salt bridges is essential to the proper folding and stability of proteins. These interactions allow proteins to achieve a thermostable conformation, meaning they can remain stable at higher temperatures. Thus, the formation of these covalent and ionic bonds is an important factor for proteins to be able to maintain their structure and function over time. Through rational design, the protein stability of *Thermobifida fusca* esterase was increased due to the introduction of a disulphide bond via D204C and E253C mutations. This modification has been observed to result in a substantial rise in the melting and plastics hydrolysis temperatures of the TfCut2 esterase. Such protein engineering provides new insights and opportunities for further research into improving enzyme stability for various industrial applications.[76] Additionally, the salt bridge between the N246D

Table 2 – Examples of protein engineering of PDEs for improved biocatalytic performance[8]
Tablica 2 – Primjeri proteinskog inženjerstva na PDE radi poboljšanih biokatalitičkih svojstava[8]

Enzyme	Source	Plastic substrate	Method	Results	Refs
Enhancing enzyme thermostability					
Cutinase	*Thermobifida alba* AHK119 (PDB ID: 3VIS)	PBSA, PBS, PCL, PLA, and PET	Introducing proline residues	Increase in T_m value from 74 to 79 °C compared with the A68V variant	47,48
	Saccharomonospora viridis AHK190 (PDB ID: 4WFI)	PET	Introducing proline residues	Increase in T_m value by 3.7 °C compared with the wild-type enzyme	11,49
	Leaf-branch compost metagenome		Constructing disulphide bond	9.8 °C higher for T_m value than that of the wild-type enzyme	50
			Introducing glycan moiety	Resistant to thermal-induced aggregation at the temperature of 10 °C higher than the nonglycosylated enzyme	51
PETase	*Ideonella sakaiensis* (PDB ID: 6ILW)	PET	Forming hydrogen bond	Increase in T_m value by 7.21°C and improved enzyme activity at elevated temperature relative to wild-type PETase	52,53
Reinforcing the binding of substrate to enzyme active site					
PETase	*I. sakaiensis* (PDB ID: 6ILW)	PET	Increasing the hydrophobicity of active site	2.1- and 2.5-times increased improvement in catalytic efficiency compared with the wild-type enzyme	53,54
		PET and PEF	Narrowing the opening size of active site cleft	Enhanced capability in PET and PEF degradation	53,55
PE-H	*Pseudomonas aestusnigri* (PDB ID: 6SBN)	PET	Enlarging the opening size of active site cleft	Improved PET degradation activity as well as the capability of hydrolysing crystalline PET from commercial bottle	56
Cutinase	*Thermobifida fusca*	PET	Increasing both the opening size and hydrophobicity of active site	Higher hydrolysis efficiency than the wild-type enzyme	57
	Fusarium solani pisi (PDB ID: 1CEX)	PET and PA	Enlarging the opening size of active site cleft	Fivefold increase in enzyme activity compared with the wild-type enzyme	58,59
Improving the interaction between substrate and enzyme surface					
Polyamidase	*Nocardia farcinica*	PU	Tethered to an auxiliary binding module	Up to fourfold higher hydrolytic activity than the native enzyme	60
PHB depolymerase	*Ralstonia pickettii* T1	PHB	Modifying surface hydrophobicity	Improved degradation activity compared with the wild-type PHB depolymerase	61
Esterase	*Clostridium botulinum* (PDB ID: 5AH1)	PET	Modifying surface hydrophobicity	Enhanced hydrolysis efficiency relative to the wild-type enzyme	62,63
Cutinase	*T. cellulosilytica* (PDB ID: 5LUJ)	PET	Surface electrostatic potential adjustment	Increased hydrolytic activity compared with the wild-type enzyme	64,65
			Tethered to an auxiliary binding module	Over 16-fold increase of hydrolysis efficiency compared with wild-type enzyme	65,66
Refining other enzyme functionalities					
Lipase	*Thermomyces lanuginosus*	PCL	Constructing a bifunctional chimeric enzyme fusion	13.3 times higher hydrolysis efficiency than the native enzyme	67
PETase	*I. sakaiensis* (PDB ID: 6ILW)	PET	Constructing a bifunctional chimeric enzyme fusion	More than threefold increase in catalytic activity compared with the wild-type enzyme	53,68
Cutinase	*T. fusca* KW3	PET	Tailoring the substrate binding pocket	5.5 times lower binding ability to the inhibitory degradation product and 2.7 times higher degradation efficiency than the wild-type enzyme	69
	T. cellulosilytica (PDB ID: 5LUJ)	PA	Tuning the active site with more polar residues	Enhanced promiscuous amidase activity and up to 15-fold increase in hydrolysing insoluble model substrate of polyamide	65,70

and Arg280 residues of PETase from *Ideonella sakaiensis* lead to an increase in its thermostability.[73] It is noteworthy that the utilisation of both disulphide bonds and salt bridges in enzyme design may synergistically contribute to the enhancement of thermostability, ultimately resulting in enhanced enzyme performance at elevated temperatures. The presence of disulphide bonds, which are covalent interactions between cysteine residues, serve to increase the stability of the protein structure by forming a network of intramolecular interactions. Similarly, salt bridges, which are electrostatic interactions between positively and negatively charged amino acid residues also contribute to the overall stability of the protein structure by forming a network of intermolecular interactions. Therefore, the simultaneous incorporation of both disulphide bonds and salt bridges into enzyme design may result in a more robust protein structure, capable of maintaining its activity under harsh conditions.[76]

Another method to obtain enhanced thermostability is engineering hydrogen bonds within the enzyme structure.[44] It is known that hydrogen bonds promote structural stability, and can improve resistance to high temperatures by maintaining protein higher-order structures. PETase's highly flexible connecting loop region β6–β7 became more rigid and more thermostable through water-mediated hydrogen bonding between S121E and N172 residues.[52] Another study on PETase resulted in enzyme to have 31 °C higher melting temperature by introducing multiple mutations, T140D, W159H, I168R, and S188Q.[44]

The number of proline residues can also be increased on PDEs in order to increase their thermostability.[77,78] The establishment of hydrophobic interactions involving proline and its neighbouring residues can confer thermodynamic stability to the tertiary structure of proteins in the presence of elevated temperatures. Proline side chains also contribute to structural rigidity by reducing conformational entropy that opposes protein folding.[8] According to one study, the melting temperature of *Thermobifida alba* cutinase increased significantly when threonine was replaced by proline at position 235, along with an increase in PET hydrolysis activity.[47]

PDEs expressed in eukaryotes may become more thermostable *via* glycosylation, which is strengthening the thermodynamic stability of proteins and preventing thermal protein aggregates.[51] However, the glycosylation site must be chosen appropriately because incorrect placement of the glycan moiety could lead to detrimental effects.[79] An introduced glycan could interfere with substrate accessibility due to its proximity to the active site.[80,81] In order to minimise the negative effects of steric hindrance, it is crucial to strategically place glycosylation sites in loop regions or hydrophobic patches. This approach can effectively mitigate the potential impact of steric interactions, thus allowing for optimal protein function and stability. Additionally, it is important to note that the specific location and type of glycosylation can also influence the overall protein structure and activity, highlighting the importance of careful design and consideration in the glycosylation process.

Engineering thermostable enzymes for plastics degradation can occasionally interfere with the enzyme's active site, which might reduce catalytic efficiency.[52] The relationships between enzyme structure and function have to be deeply understood and analysed, in order to avoid such a negative impact. Many of the studies that have been published have shown that enzymes engineered to be more thermostable have the same or greater efficiency in plastics-degrading efficiency.[44,50,51,73,74]

1.2.2 Strengthened substrate binding

The active site region of an enzyme, where the substrate binds and undergoes chemical reactions, plays a crucial role in determining the efficiency of plastics degradation. As such, the active site has been identified as a key target for engineering and optimising PDEs. By understanding the specific interactions between the active site and substrate, scientists can design modifications to the enzyme that enhance its ability to degrade plastic. This approach of targeting the active site has the potential to significantly improve the efficiency of PDEs, making them more effective tools for addressing the global plastic pollution crisis. Additionally, it is worth noting that this area of research is rapidly advancing, and new insights and discoveries are being made to constantly improve the plastics-degradation enzymes.

This area, known as the enzyme's active site, is a key target for engineering plastics-degrading enzymes (PDEs) to enhance their performance. By understanding and manipulating the specific interactions between the active site and substrate, researchers can optimise the activity of PDEs and increase their effectiveness in breaking down plastic waste. This highlights the importance of studying the active site region in PDEs, and the potential for utilising this knowledge to improve plastics degradation processes. It is quite evident that the active site region of a PDE plays an imperative role in determining the efficiency of plastics degradation. This has made it a hot target for engineering PDEs for superior performance. As such, effective and efficient strategies are being developed to modify the active sites that lead to improved plastics degradation.[23,82] One of the most effective ways to enhance the ability of enzymes to interact with plastic substrates is by increasing the size of the active site. This is often accomplished by manipulating the structural elements of the enzyme, such as altering the conformation or orientation of amino acid residues that make up the active site. As a first step towards achieving this goal, cutinase from *Fusarium solani* was engineered.[58] L182A mutant of the enzyme had a wider active site than the wild-type enzyme, and was found to be more active towards PET and polyamide (PA) fibers.[58] The efficiency of PET and PBSA plastics degradation has been enhanced using similar strategies to engineer other PDEs.[56,83–85] There is no guarantee that larger substrate binding spaces will improve substrate binding due to the weak substrate affinity resulted from a too wide active site.[84] A narrower active site might be beneficial in some cases. Based on the results of one study, the double mutation S238F and W159H narrowed the active site of PETase and allowed for better degradation of PET and poly(ethylene furanoate) (PEF) by narrowing its active site.[55]

Another potential engineering target is the hydrophobicity of the substrate binding pocket. PETase demonstrated that

increased hydrophobicity results in higher binding affinity, resulting in higher degradation efficiency.[54] It is feasible to increase the catalytic efficiency of enzymes by optimising both the hydrophobicity and the active site opening size in a complementary way. Mutations in the active site of the cutinase from *Thermobifida fusca* increased hydrophobicity and created more space, resulting in a notable improvement in the efficiency of PET hydrolysis.[57]

1.2.3 Enzyme surface-substrate interaction improvement

Enhancing enzyme-substrate interactions has been effective in improving PDEs efficiency. An enzyme's surface amino acid residues are interacted with substrate molecules electrostatically and hydrophobically to maintain substrate binding.[65,86] Consequently, one of the most typical strategies is to alter the electrostatic and/or hydrophobic characteristics of the outer layer. Electrostatic repulsion between enzymes and plastic substrates might be reduced if the enzyme surface is made electrically neutral.[8] Mutation R29N in Thc_Cut2 enabled PET hydrolysis by generating a more neutral enzyme surface.[64] Similarly, electrical neutrality was observed on the surface area of the R228S mutant of Cut190.[87] On the other hand, increasing the hydrophobicity of the enzyme surface may improve the interaction between the enzyme and substrate.[73,85,88] PHB depolymerase from *Ralstonia pickettii* was shown to be more effective at hydrolysing plastics when Tyr and Ser residues were replaced with more hydrophobic Cys and Phe amino acids.[61,89] Nevertheless, if too many hydrophobic residues are included, the incorporation of further hydrophobic residues within the same protein might alter its structure or reduce its catalytic capacity.[88]

1.3 Applications of PDEs

The use of enzyme biocatalysis presents a promising and environmentally friendly solution for managing and recycling plastic waste. Through the utilisation of biocatalysts, plastic can be broken down into its individual components, allowing for efficient and cost-effective recycling. Recent advancements in fields such as omics, synthetic biology, and protein engineering are providing new opportunities for the development of novel biocatalysts with optimal properties for plastics depolymerisation. These advancements in technology are helping to pave the way for more sustainable and eco-friendly plastic waste management strategies.

As there is a loss in quality in every cycle, current recycling methods can only recycle clear plastic in closed loops. This makes it difficult to convert 100 % recycled PET into new products. Enzyme-initiated biocatalysis is an innovative strategy that could be incorporated into the plastics recycling procedure as a supplement or substitute to the standard chemical or thermomechanical methods. Open-loop upcycling uses the generated molecule as feedstock for the production high-value chemicals, or closed-loop recycling uses them as building block monomers for new plastic products.[90] Bioreactors containing PDEs are able to depolymerise the plastic materials after mechanical pre-treatment.

An engineered variant of leaf-branch compost cutinase with enhanced thermostability (Table 2) was successfully utilised in an enzymatic recycling process of PET into various plastics or textiles at a pilot scale *via* innovative technology of Carbios.[91] A range of bottle-grade PET standards were propitiously reached by obtaining the terephthalic acid monomers, which comprised 86 % of PET waste, with 99.8 % purity. Depolymerised PET plastic waste and PET polyester textile waste were used to make batches of transparent PET bottles. Newly synthesised PET had similar molecular weight averages and viscosities as PET manufactured with petrochemical terephthalic acid. In terms of mechanical properties, similar standards with commercial PET bottles were reached when produced from enzymatically recycled PET. Additionally, a kilogram of recycled PET produced 0.6 kg of sodium sulphate during this process may lead to fulfilling a part of the demand for Na_2SO_4 in the glass, paper, and detergents industries. With this innovative technology, PET waste can be infinitely recycled, and manufactured products would be 100 % recycled and 100 % recyclable. Based on the cost of producing a cellulase[92] the cost of the enzyme needed for recycling one ton of PET is calculated at approximately 4 % of the ton price of virgin PET.[50]

1.4 Characterisation of PDEs

The enzymatic activity characterisation of plastics-degrading enzymes is a critical step in understanding their mechanisms of action and optimising their use in bioremediation applications. Several methods are currently used to determine the activity of these enzymes, including spectrophotometric,[11,26,99,50,74,93–98] turbidimetric,[100–102] fluorimetric,[103–107] titrimetric[50,108,109] methods, as well as high-performance liquid chromatography (HPLC).[13,26,50,51,85,110–113] Spectrophotometry and fluorimetry are commonly used to measure the degradation of coloured and fluorescent substrates, respectively, whereas HPLC is used to separate and quantify the reaction products. These methods provide valuable information on kinetics, substrate specificity, and optimal conditions for plastics degradation. However, there are still limitations in the characterisation of plastics-degrading enzymes, particularly in the assessment of their effectiveness in degrading complex plastic waste streams. Therefore, the development of novel and more comprehensive methods for enzymatic activity characterisation of plastics-degrading enzymes is crucial to improving our understanding of these enzymes and their potential applications in plastic waste management.

3 Conclusion

Despite the advancements made in recent research efforts, there remain significant obstacles that impede the ability to effectively implement industrial-scale enzyme biocatalysis in the production of plastics. These critical challenges must be addressed in order to fully realise the potential of this technology and its ability to revolutionise the plastics industry. Some of these challenges may include developing more efficient enzymes, improving the stability and durability of enzymes in industrial settings, and addressing the

economic feasibility of implementing enzyme biocatalysis on a large scale. Overcoming these challenges will require continued research and innovation, as well as collaboration among scientists, engineers, and industry leaders.

The current method of using agar plates is not very effective and does not allow for a large number of PDEs to be identified from metagenomic or mutant libraries. Utilising high-throughput screening techniques will make it easier to identify PDEs from these sources.[114] Using techniques such as cell-as-compartment methods and micro-droplet-based approaches may also improve the efficiency and accuracy of identifying novel PDEs.[20,115]

Currently, the lack of a comprehensive understanding of how enzyme structure affects its function hinders the ability to design PDEs through rational means. This leads to a reliance on creating a vast collection of mutated enzymes for experimentation, as it is difficult to predict the precise impact of mutations. However, with the ongoing development of computer-aided modelling and simulation, coupled with an increasing understanding of enzyme structure-function relationships, there is the potential for a significant change in the field of PDE engineering.

For real-world applications, biocatalytic systems must be highly efficient, robust, and biocatalysts must be reusable/regenerable. Free enzymes have short lifetimes and are difficult to recover and reuse, making them far from an economical option in large-scale reactions.[116] A strategy to overcome short enzyme lifetimes could be to engineer whole-cell biocatalysts to continuously produce plastics-degrading enzymes.[117,118] One potential solution would be to identify and engineer microorganisms that naturally degrade plastics. Nonetheless, microorganisms are difficult to keep active under harsh conditions during industrial plastics degradation.[8] Considering this fact immobilisation techniques could be a viable option for improving enzyme stability and reusability. As the circular economy becomes a reality, plastic and textile waste are becoming precious raw materials.

All of these efforts are aimed at providing scientific solutions to address one of the most pressing environmental challenges of our time.

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Popis kratica
List of abbreviations

HTS – high-throughput screening
– probir visoke propusnosti

PA – polyamide
– poliamid

PBAT – poly(butylene adipate-co-terephthalate)
– poli(butilen adipat-ko-tereftalat)

PBSA – poly(butylene succinate-co-adipate)
– poli(butilen sukcinat-ko-adipat)

PCL – poly(ε-caprolactone)
– poli(ε-kaprolakton)

PDE – plastic degrading enzyme
– enzimi koji razgrađuju plastiku

PEF – poly(ethylene furanoate)
– poli(etilen furanoat)

PET – polyethylene terephthalate
– polietilen tereftalat

PHB – polyhydroxybutyrate
– polihidroksibutirat

PLA – poly(lactic acid)
– poli(mliječna kiselina)

PU – polyurethane
– poliuretan

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SAŽETAK

Otkriće enzima za razgradnju plastike

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Plastika je daleko najnapredniji materijal kad je riječ o primjeni i svojstvima, a procjenjuje se da se svake godine globalno proizvede 350 do 400 milijuna tona. Ona je postala ozbiljan problem s obzirom na odlaganje plastičnog otpada i onečišćenje, zbog njezine nekontrolirane upotrebe u različite svrhe tijekom posljednjih desetljeća, kao što su pakiranje, transport, industrija i poljoprivreda. Za učinkovitu razgradnju plastičnih vrećica potrebno je otprilike 1000 godina. Osim toga, izgaranjem plastike u atmosferu se ispuštaju CO_2 i dioksini koji doprinose globalnom zatopljenju. Zemaljski kopneni ili morski okoliš akumulira milijune tona otpada svake godine zbog lošeg recikliranja i niske kružne upotrebe. Inovativne tehnologije za recikliranje otpadne plastike prijeko su potrebne za smanjenje plastičnog otpada i postizanje ciljeva kvalitete okoliša uz valorizaciju potrošne plastike. Zbog trenutačno neadekvatnih metoda zbrinjavanja plastičnog otpada povećan je fokus na upotrebu mikroorganizama i enzima dizajniranih za biorazgradnju nerazgradivih sintetičkih polimera putem biokatalitičke depolimerizacije, što ukazuje na to da obrada plastike i recikliranje mogu biti učinkovitiji i održivi.

Ključne riječi
Enzimatska razgradnja plastike, enzimatska depolimerizacija, kružna bioekonomija, zeleni bioprocesi, biorecikliranje polimera

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