Review Article

Advancing PET-Degrading Enzymes through Directed Evolution to Combat Plastic Pollution

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ABSTRACT

Plastic pollution poses a significant environmental challenge due to its persistence and widespread distribution. Among various types of plastic, polyethylene terephthalate (PET) is especially problematic due to its resistance to natural degradation. PET-degrading enzymes, particularly PETases and cutinases, have emerged as promising solutions for enzymatic plastic recycling. However, their native catalytic efficiency and thermostability are limited. Directed evolution has enabled the development of improved enzyme variants through techniques such as error-prone PCR, DNA shuffling, and saturation mutagenesis. This review highlights recent advances in engineering Cutinase and PETases, focusing on enhancing catalytic efficiency and thermostability for PET plastic degradation by directed evolution. Key engineered variants, including HotPETase and optimized leaf-branch compost cutinase (LCC) mutants, demonstrate significant progress toward sustainable plastic recycling through enzymatic means. These advancements pave the way for scalable industrial applications, offering an environmentally friendly alternative to traditional plastic waste management methods.

Keywords: Directed Evolution; Plastic Pollution; Polyethylene Terephthalate; Error-prone PCR; DNA Shuffling; Saturation Mutagenesis; PETases; Cutinases

INTRODUCTION

Plastic pollution is a critical global issue nowadays with over 300 million tons of plastic discarded each year, resulting in substantial environmental buildup (1). A large portion of this waste exists as plastic particles. Research has shown that plastic particles have been detected in diverse environments, from the depths of the oceans to the summit of Mount Everest and in rainwater (2-5). Recently, many studies have shown that microplastics (plastic particles smaller than 5 mm) can be found throughout the human body including in the blood, saliva, liver, kidneys, and placenta, posing significant risk to human health (6).

Plastics primarily consist of synthetic polymers including polyethylene (PE), polypropylene (PP), polystyrene (PS), polycarbonate (PC), polyvinyl chloride (PVC), and poly(ethylene terephthalate) (PET). PET developed by DuPont in the mid-1940s, has become a major component of plastic waste due to its widespread use in textile fibers, beverage bottles, and food containers

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and its excellent mechanical properties and stability, while advantageous for these applications, also result in resistance to degradation. PET monomers are linked by ester bonds, which can be hydrolyzed by hydrolytic enzymes found in nature. Müller *et al*. reported the first discovered PET hydrolase, Cutinase isolated from the actinomycete Thermobifida fusca (7). Additionally, Sulaiman *et al* isolated the leaf-branch compost cutinase (LCC) from a leaf-branch compost thermophilic microbial habitat, which displays significant PET-degrading activity (8). Later in 2016, one of the first PET hydrolases, named PETases, was isolated from Ideonella Sakaiensis by Yoshida *et al* from a recycling center in Japan (9). Cutinase / PETases directly converts insoluble PET to a mixture of terephthalic acid (TPA) and ethylene glycol (EG), along with smaller quantities of incomplete hydrolysis products such as bis-(2-hydroxyethyl) terephthalate (BHET) and mono-(2-hydroxyethyl) terephthalate (MHET) (9, 10) (Figure 1).

Although Cutinase / PETases can convert PET to soluble monomers, their catalytic efficiency and thermostability are largely needed enhancement. Directed evolution has been utilized to engineer these enzymes for functional improvement. In this article, recent developments in directed evolution of PETases/Cutinase for higher catalytic efficiency and thermostability are thoroughly reviewed.

DIRECTED EVOLUTION: CONCEPTS AND TECHNIQUES

Directed evolution is a laboratory technique that harnesses the principles of evolution to engineer proteins or nucleic acids toward specific, user-defined objectives. By introducing random mutations and applying selective pressures, researchers can create a diverse library of variants. These variants are then screened or selected based on desired traits, such as increased activity, stability, or specificity. Directed evolution is a powerful tool in enzyme engineering, enabling the creation of optimized enzymes for diverse industrial and medical applications. Two key steps in this process are generating a large library of variants and efficiently screening or selecting those with desired traits. Error-Prone PCR, DNA shuffling and saturation mutagenesis are the most commonly used approaches for constructing libraries of variants (Figure 2).

Error-Prone PCR

Error-Prone PCR (EpPCR) is a widely used method for introducing random mutations during DNA amplification. It was first reported in 1989 and has since become one of the most widely used techniques for *in vitro* random mutagenesis (11). The method relies on low-fidelity DNA

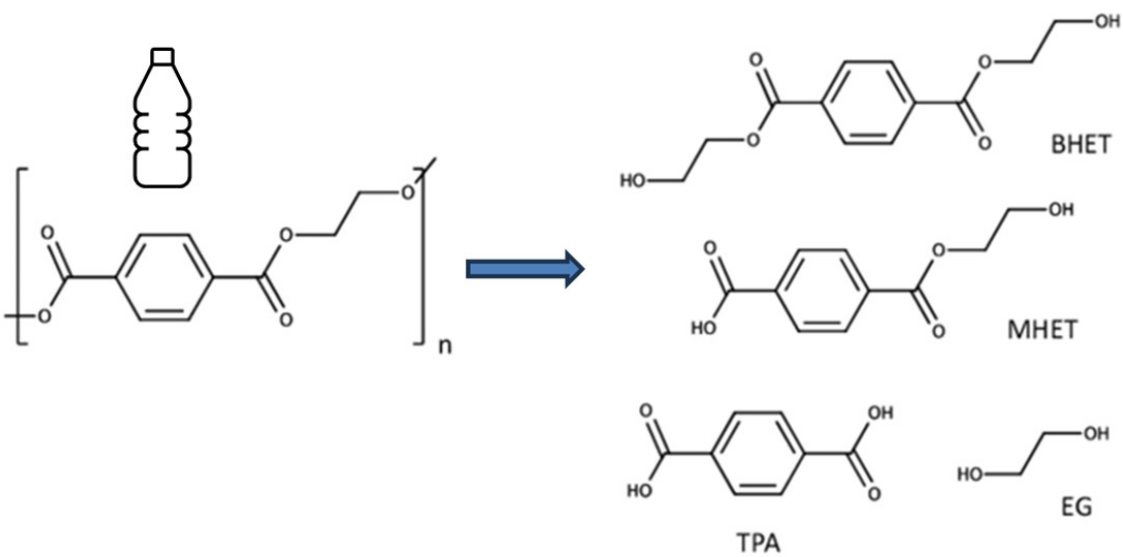

Figure 1. The enzymatic hydrolysis of PET by PETases/Cutinase produces a mixture of terephthalic acid (TPA) and ethylene glycol (EG), along with smaller quantities of incomplete hydrolysis products such as bis-(2-hydroxyethyl) terephthalate (BHET) and mono-(2-hydroxyethyl) terephthalate (MHET).

polymerases such as Taq and Mutazyme to introduce point mutations during PCR amplification under non-standard conditions (12-15). These conditions include specific additives, PCR cycle conditions and unbalanced dNTP ratios, all of which reduce the polymerase's fidelity and result in higher mutation rates (14, 16, 17).

Specific additives, such as manganese (Mn^{2+}) ions, are often used in error-prone PCR to increase the error rate of the polymerase (16). Mn^{2+} has been shown to reduce the proofreading ability of the polymerase, allowing for more frequent incorporation of incorrect nucleotides during DNA synthesis. Other additives, such as dimethyl sulfoxide (DMSO), is used in PCR to disrupt hydrogen bonds for secondary structure formation in the DNA template to reduce the accuracy of DNA replication (18). Moreover, PCR cycle conditions can also affect mutuation rate, which include the number of amplification cycles and the duration of the denaturation, annealing, and extension steps (19, 20). Longer cycles or altered temperatures can affect the fidelity of the polymerase, indirectly increasing the mutation frequency (20). Unbalanced dNTP ratios refers to an unequal mixture of the four deoxyribonucleotide triphosphates (dNTPs)—dATP, dTTP, dCTP, and dGTP—used during DNA synthesis (21). In a typical PCR reaction, the dNTPs are present in roughly equal amounts to ensure accurate DNA replication. However, in EpPCR, researchers intentionally use unbalanced dNTP ratios to increase the likelihood of introducing mutations (21). By adjusting the concentration of one or more dNTPs relative to the others, researchers can cause the DNA polymerase to incorporate incorrect nucleotides more frequently, leading to an increased mutation rate during DNA amplification (21).

DNA Shuffling

DNA shuffling involves recombining fragments of genes from closely related sequences to create genetic diversity. DNA shuffling was first reported by Stemmer in 1994, and this technique allows for the exploration of combinations of beneficial mutations, increasing the chances of obtaining a superior variant (22). The principle of DNA shuffling involves fragmenting the DNA of related genes and then reassembling these fragments to create new combinations of sequences (23). By randomly recombining these genetic fragments, researchers can explore a vast sequence space that would be difficult or

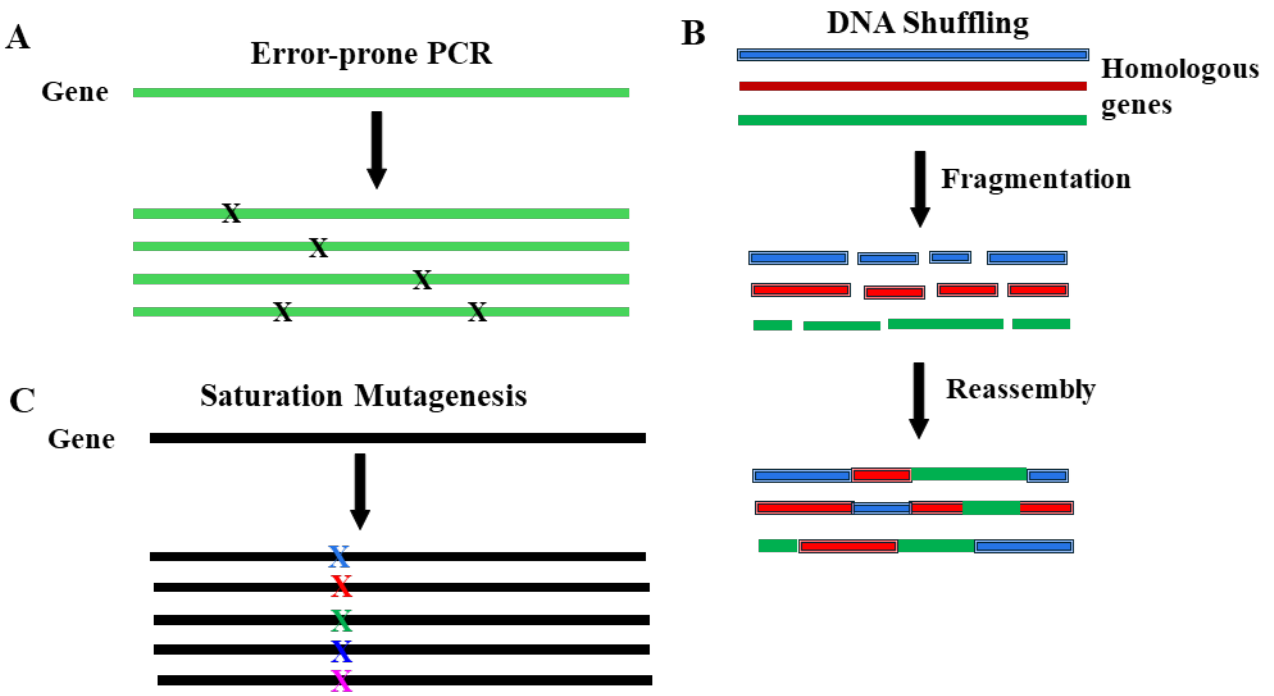

Figure 2. Three common approaches to generate mutated enzyme libraries are (A) error-prone PCR, (B) DNA shuffling and (C) Saturation Mutagenesis.

impossible to achieve through traditional mutagenesis. This technique allows for the exploration of combinations of beneficial mutations from different gene variants, thereby increasing the likelihood of obtaining a superior or optimally functional variant. This makes DNA shuffling particularly powerful in applications such as enzyme optimization (24).

Saturation Mutagenesis

Saturation mutagenesis is a powerful technique that involves substituting an amino acid at a specific position in a protein with each of the other 19 possible amino acids, excluding the original (25). This generates a diverse set of mutants, each with a unique substitution at the targeted site. By evaluating how these changes affect key protein properties—such as stability, catalytic activity, and substrate binding—researchers can uncover the roles of individual residues. This allows for the identification of amino acids critical for enzyme catalysis, substrate recognition, and other functional aspects of the protein. By mapping how specific substitutions influence protein function, saturation mutagenesis aids the design of proteins with enhanced stability, activity, or specificity.

DIRECTED EVOLUTION OF PET-DEGRADING ENZYMES

Since the discovery of the PET hydrolase from the culture supernatant of Thermobifida fusca in 2005 (7), numerous thermostable PET hydrolases and their homologs from the cutinase group have been investigated for their potential to degrade PET. The cutinase group of enzymes, originally identified for their role in degrading cutin, the plant polyester, has been recognized for its ability to hydrolyze polyesters such as PET, making them promising candidates for biorecycling applications. Researchers have explored several thermostable cutinase homologs derived from bacterial species, such as Thermobifida fusca, Thermonospora curvata, and Ideonella sakaiensis, as well as fungal sources, including Fusarium solani, Humicola insolens, and Aspergillus oryzae (26-31). These enzymes exhibit distinct catalytic activities and substrate specificities, which make them valuable for applications in the degradation of recalcitrant plastic waste. Among all these enzymes, IsPETase identified from bacterial strain Ideonella sakaiensis 201-F6.9 and LCC isolated from a leaf-branch compost have been extensively engineered to improve their efficiency for large-scale industrial applications, particularly in breaking down the highly crystalline forms of PET that constitute most plastic waste (8). Directed evolution techniques have been widely applied to enhance the catalytic efficiency, stability, and substrate specificity of these enzymes, aiming to overcome limitations such as low degradation rates and poor accessibility to the polymer surface.

Direct Evolution of IsPETase

IsPETase is a mesophilic enzyme with an optimal reaction temperature lower than the glass transition temperature (Tg) of PET, limiting its efficiency in degrading the polymer. Brott *et al*. utilized error-prone PCR to generate a mutant library of IsPETase, which was screened with the commercially available polyester-polyurethane Impranil DLN W50 to identify more thermostable variants (32). This led to the discovery of four mutants with higher melting points. The melting point refers to the temperature at which the enzyme's structure begins to destabilize, resulting in the loss of its functional conformation and enzymatic activity. Among these variants, IsPETaseTMK95N/F201I stood out, exhibiting a melting point 5.0°C higher than that of the parent IsPETaseTM. Although this variant showed a slight reduction in activity at lower temperatures, its enhanced thermostability allowed it to maintain high activity at elevated temperatures up to 60°C. They further compared this and other variants with previously published IsPETase mutants, evaluating their thermostability and hydrolytic activity against PET nanoparticles and amorphous PET films (32). Their findings underscore the importance of thermostability as a critical factor in the development of effective PET hydrolases, especially for industrial applications requiring enzyme activity at higher temperatures for efficient plastic.

In parallel, Bell *et al.* developed an automated, high-throughput directed evolution platform for engineering IsPETase, using catalytic activity at elevated temperatures as the primary selection pressure (33). They performed sequential rounds of saturation mutagenesis to individually randomize between 24–30 residue positions per cycle. In total, 106 of the 264 residues present in IsPETase were targeted for mutation throughout evolution (33). Through this approach, they created a thermostable IsPETase variant, termed HotPETase, with a melting temperature (Tm) of 82.5°C. HotPETase operates efficiently at the glass transition temperature of PET, enabling it to depolymerize semicrystalline PET more rapidly than previously reported PETases. Furthermore, it can selectively deconstruct the PET component of laminated multimaterials, a task that other enzymes have struggled to accomplish (33). Structural analysis of HotPETase

revealed specific adaptations that significantly enhanced both its thermotolerance and catalytic performance.

Additionally, Wang *et al*. isolated and characterized a PET hydrolase, SbPETase, from Schlegelella brevitalea sp. nov., based on its sequence similarity to IsPETase. Given the differences in substrate binding sites between SbPETase and IsPETase, they initially generated several SbPETase mutants through site-directed mutagenesis to improve its catalytic efficiency. This effort led to the discovery of three mutants (SbPETaseW132H, SbPETaseR259A, and SbPETaseL61T) with enhanced catalytic performance. These mutants were then combined to create three double mutants and one triple mutant, with the triple mutant demonstrating the highest catalytic efficiency toward PET film, although its activity remained significantly lower than that of IsPETase. Subsequently, directed evolution via DNA shuffling of both SbPETase and IsPETase, combined with a high-throughput screening platform using a secretion system in E. coli BL21(DE3), resulted in the generation of the IsPETase mutant S139T, which exhibited enhanced catalytic activity.

Liu *et al* reported a two rounds of error-prone PCR followed by dual fluorescence-based high-throughput screening (HTS) assay of a library consisting of 2,850 IsPETase variants for improved catalytic activity (34). They identified six mutant IsPETases exhibiting 1.3 to 4.9 times improved activity compared to the wildt type IsPETases.

Directed Evoluton of LCC

The LCC enzyme, originally isolated from a leaf-branch compost thermophilic microbial habitat, exhibits significant PET-degrading activity under optimal conditions of pH 8.0 and a hydrolysis temperature of 50°C (8). Cribari *et al*. applied error-prone PCR to create an extensive library of enzyme mutants, enabling the exploration of diverse variants for enhanced polymer degradation (35). They developed a high-throughput yeast surface display platform capable of screening over 10^7 enzyme mutants. This platform allows individual yeast cells to present unique enzyme variants on their surface, with activity detected through fluorescence changes caused by the cleavage of a synthetic probe resembling the target polymer. Mutants with high activity are selected using fluorescence-activated cell sorting (FACS) and further characterized through DNA sequencing, providing a robust method for identifying optimized enzyme candidates (35). Through this platform, the authors identified mutations that significantly enhanced the degradation kinetics of solid polyethylene terephthalate (PET) films. Biochemical assays and molecular dynamics simulations of the most effective variants revealed that the H218Y mutation improves enzyme binding to PET, highlighting its critical role in boosting enzymatic performance (35). Meanwhile, Pirillo *et al* reported the generation of LLC variants using a semi-rational protein engineering approach, starting with a truncated form of LCC lacking the secretion signal (ΔLCC) (36). They isolated single and double mutants that exhibited enhanced activity on PET. The F243T ΔLCC single variant depolymerized approximately 67% of amorphous PET film, producing 21.9 mm of products after 27 hours at 72°C (36). The S101N/F243T ΔLCC double variant further improved PET degradation. Interestingly, the highest conversion yield for both variants was achieved at 55°C. Kinetic studies and molecular dynamics simulations suggested that the superior performance of the S101N/F243T variant was due to a slight reduction in its affinity for PET, linked to increased flexibility in the active site near position 243 (36).

The continued combination of directed evolution with computational modeling and enzyme engineering represents a promising avenue for the development of more efficient PET-degrading enzymes. As these efforts evolve, these enzymes could play a crucial role in the sustainable recycling of plastic waste, offering a more environmentally friendly alternative to traditional recycling methods.

CONCLUSION

With plastic pollution getting more severe, the development of enzymes for plastic degradation has become a critical research priority. Among these, PETase and LCC, enzymes capable of PET, hold considerable promise but face challenges in stability and catalytic efficiency. Directed evolution has emerged as a powerful strategy to overcome these limitations, enabling the creation of more robust enzymes with enhanced performance.

Through directed evolution, researchers have engineered PETase variants with significantly improved stability and catalytic efficiency, allowing for faster and more effective plastic degradation. Advanced PETases and engineered cutinases, such as HotPETase, exemplify the transformative potential of enzyme-based recycling technologies. These engineered enzymes possess increased thermostability and catalytic efficiency, enabling them to function at higher temperatures, a key requirement for industrial-scale applications. Engineered LCCs have shown exceptional performance, even

degrading crystalline PET, which is typically resistant to enzymatic breakdown. Their integration into industrial recycling processes could substantially reduce the environmental impact of plastic waste by enabling closed-loop recycling systems.

Future research should prioritize optimizing enzyme-substrate interactions, expanding substrate specificity, and enhancing stability under industrial conditions. Advances in computational modeling, protein design, and high-throughput screening will accelerate the development of next-generation enzymes tailored for large-scale applications.

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