Chapter 8 Genetic improvement of polyester degrading enzymes

Capítulo 8 Mejoramiento genético de enzimas degradadoras de poliésteres

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Abstract

Synthetic polymers usage has increased according to modern society to have basic applications such as high technology in generating different plastic materials. Therefore, plastic debris accumulates in the environment while biodegradation occurs very slowly. Therefore, the application of hydrolases in the degradation of polyesters has been limited by the ranges of pH and temperature of the environment where these contaminants are found; for this reason, changes have been made in the sequence of some enzymes, resulting in modifications in the structure and change in its characteristics, using molecular techniques such as site-directed mutagenesis, error-prone PCR and random mutagenesis. Many enzymes with polyester degradation activity have been discovered, characterized and designed. However, the classification and integrated knowledge of these enzymes are of interest. For this reason, this paper summarizes the currently known improvement of polyester-degrading enzymes, focusing on their structural and activity modifications.

Mutagenesis, PCR, Enzyme, Mutation, Polyesters

Resumen

El uso de polímeros sintéticos como los poliésteres está en aumento acorde a la sociedad moderna, teniendo tanto aplicaciones básicas como de alta tecnología en la generación de diferentes materiales plásticos. Por lo tanto, los desechos plásticos se acumulan en el medio ambiente en grandes cantidades mientras que su degradación ocurre muy lentamente. La aplicación de hidrolasas en la biodegradación de polímeros sintéticos se ha visto limitada por los rangos de pH y temperatura del ambiente donde se encuentran estos contaminantes, por lo que se han realizado cambios en la secuencia de algunas enzimas, resultando en modificaciones en la estructura. y cambio en sus características, utilizando técnicas moleculares como la mutagénesis sitio-dirigida, la PCR propensa a errores y la mutagénesis aleatoria. Se han descubierto, caracterizado y diseñado enzimas con actividad de degradación de poliésteres. Sin embargo, la clasificación y el conocimiento integrado de estas enzimas son de interés. Por esta razón, en este artículo presentamos una revisión sobre el mejoramiento de enzimas degradadoras de poliésteres actualmente conocidas, centrándonos en sus modificaciones estructurales y de actividad.

Mutagénesis, PCR, Enzima, Mutación, Poliésteres

8.1 Introduction

According to modern society, synthetic polymers have increased to have had basic applications such as high technology. This increase is because of the low production costs of plastic from fossil feedstock, besides the material's high durability, being both relevant advantages and becoming a burden on the global ecosystem (Moharir and Kumar, 2019). Therefore, plastic debris accumulates in the environment to a large extent, while biodegradation occurs very slowly (Lebreton *et al*., 2018).

Among these contaminants are heteroatomic plastics such as polyamides, polyurethanes and polyesters, which contain groups of greater reactivity and therefore are more easily biologically degraded (Wei and Zimmermann, 2017). In recent years, enzymes have been used for the degradation of polyesters, such as carboxylic ester hydrolases (E.C.3.1.1) that have degraded PCL (polycaprolactone), PLA (polylactic acid) and PET (polyethylene terephthalate) (Nikolaivits *et al.* al., 2018). However, some polyesters have not been hydrolyzed efficiently, which is why the structure and functioning of enzymes have been investigated through enzymatic engineering to improve the hydrolysis activity of polyesters (Dubey *et al*., 2019).

The application of hydrolases in the degradation of polyesters has been limited by the ranges of pH and temperature of the environment where these contaminants are found; for this reason, changes have been made in the sequence of some enzymes, resulting in modifications in the structure and change in its characteristics, using molecular techniques such as site-directed mutagenesis, error-prone PCR and random mutagenesis (Lu *et al*., 2022).

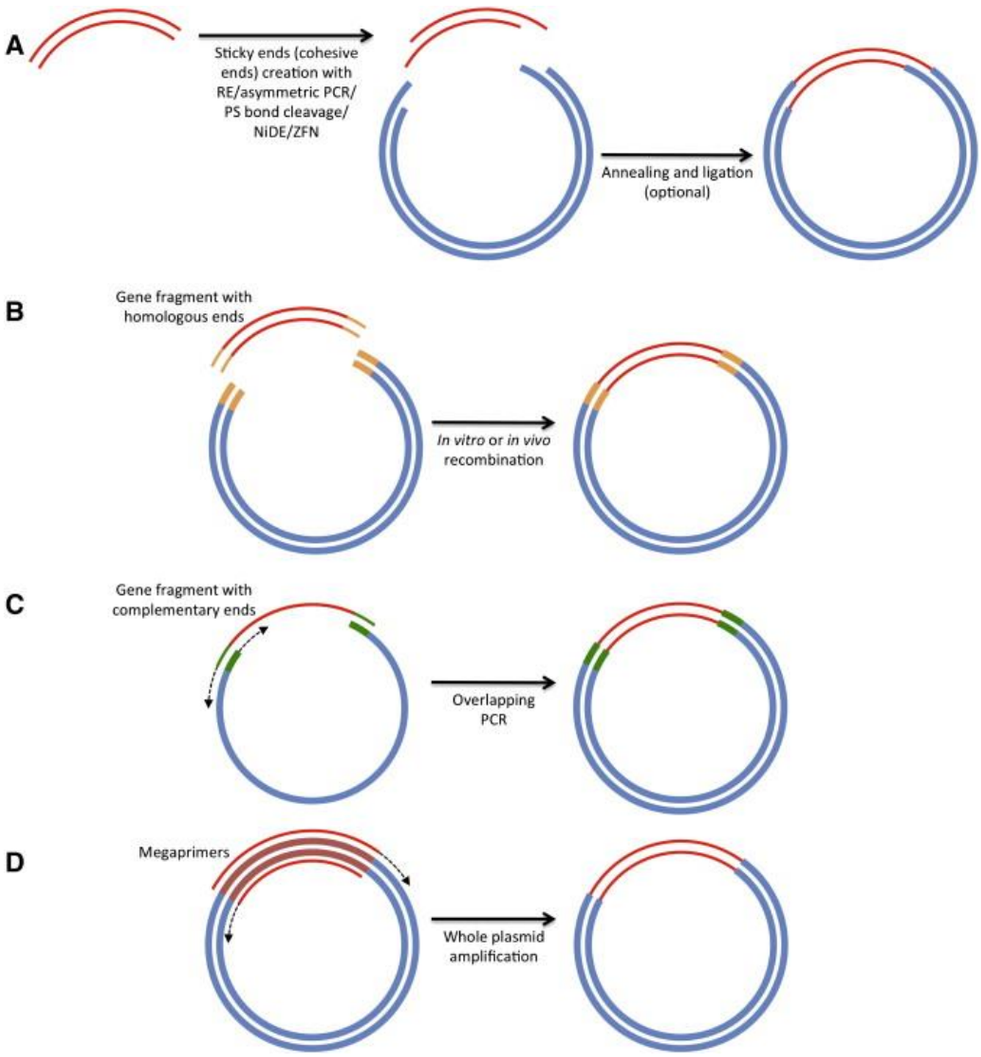
One of the most studied enzymes regarding the degradation of polyesters has been the esterase of *Ideonella sakaiensis*, which without making modifications, can degrade PET but only under certain conditions, for which they decided to make modifications in its sequence with the help of molecular techniques such as site-directed mutagenesis changing the amino acids (S160A, D206A, H237A) thus obtaining better results of enzyme degradation activity and stability (Liu *et al.*, 2019).

Many enzymes with polyester degradation activity have been discovered, characterized and designed. However, the classification and integrated knowledge of these enzymes are of interest. For this reason, we present a review of currently known improved polyester-degrading enzymes, focusing on their structural and activity modifications.

8.2 Site-directed mutagenesis

It is a technique that is one of the most used for the modification of enzymes, which allows the selective engineering of gene sequences and has been used to investigate the catalytic properties of proteins. One of the most critical steps in this technique is selecting and identifying the site of the mutations. Bioinformatics-based 3D modeling of the enzyme structure can be carried out using the PDB protein database (Dubey *et al.*, 2019; Liu *et al.*, 2008). In this technique, two independent PCR reactions are performed. The resulting first PCR products are used for an overlapping extension PCR reaction, with the products of the overlapping extension PCR reaction finally cloned into plasmids and expressed. Commonly in bacteria and yeasts, figure 8.1 (Tseng *et al*., 2008; Tee *et al*., 2013).

Figure 8.1 Principles of molecular cloning: (A) complementary overhangs, (B) homologous sequences, (C) overlapping PCR and (D) megaprimer



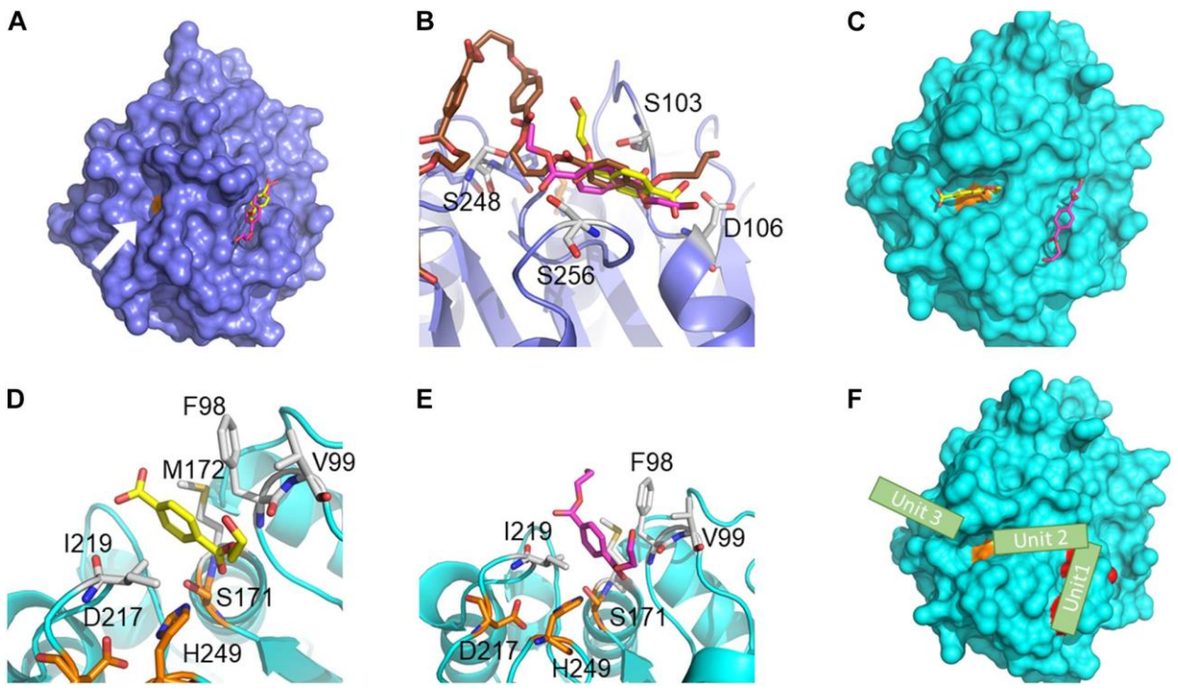
Source: (Tee et al., 2013)

The position of the bound ligand in the active site of RPA1511 and analysis of PLA hydrolysis products suggest that this enzyme can cleave PLA with both endo- and exoesterase. Using structure-based site-directed mutagenesis, we identified several residues of RPA1511 that were essential for PLA hydrolysis but not required for activity against a soluble monoester substrate. These residues represent a potential structural motif for PLA binding, and further characterization will help narrow the search for new PLA depolymerase based on sequence analysis (Hajighasemi *et al*., 2016).

Studies were also carried out on a *Clostridium botulinum* polyesterase in which the Cbotu_EstA zinc-binding domain was modified by site-directed mutagenesis, and a specific domain consisting of 71 amino acids at the N-terminus of the enzyme was also removed. A combination of substitution of residues in the zinc-binding domain synergistically increased enzyme activity in PET seven-fold when combined with truncation of 71 amino acids at the N-terminus of the enzyme alone. Compared to the native enzyme, the combination of truncation and substitutions in the zinc-binding domain leads to a 50-fold improvement in activity (Biundo *et al.*, 2018).

This technique was used with a carboxylic ester hydrolase from a marine bacterium *Pseudomonas aestusnigri* (VGXO14T), modified by mutagenesis to improve the potential for PET degradation, obtaining a variant (Y250S) in which changes were made to stabilize the MHET at the catalytic site with the help of hydrophobic interactions. After making these changes, the production of MHET was measured, resulting in 5.4 mg/L in 48 h at 30 ºC, compared to the unmodified enzyme, which was 4.2 mg/L of MHET (Bollinger *et al.*, 2020).

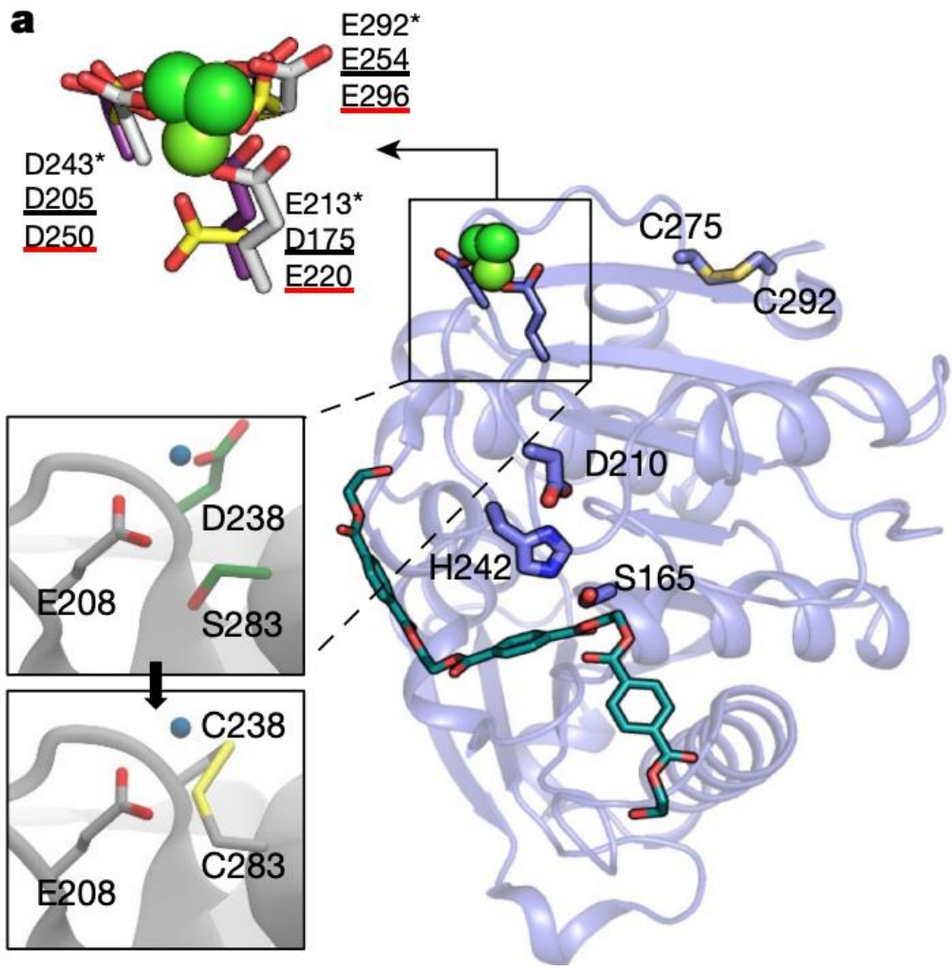
Figure 8.2 Predicted ligand binding modes in wild-type PE-H and variant Y250S. The predicted binding poses of BHET (magenta), MHET (yellow), and 2-HE(MHET)4 (brown) in WT PE-H (navy) and the variant Y250S (cyan). In (A, C, F), S171 is shown in orange, and in (B, D, E), the catalytic triad (S171, D217, and H249) is shown in orange and interacting residues are shown in white. (A) In wild-type PE-H, BHET and MHET bind to a groove adjacent to the catalytic site (white arrow). (B) BHET and MHET bind to the hydrophobic groove and are stabilized by hydrogen bonding interactions with S103, D106, S248, and S256. 2-HE(MHET)4 binds similarly to BHET and MHET in the groove adjacent to the catalytic site. (C) In the variant Y250S, MHET binds to the catalytic site, while BHET occupies the hydrophobic groove. (D) MHET binds to the catalytic site and is stabilized by hydrophobic interactions to F98, V99, M172, and I219 such that S171 can attack the carbonyl carbon for ester hydrolysis. (E) A second binding pose of BHET binds similarly to MHET. (F) Proposed mechanism of PET polymer interaction. Residues G254, Y258, and N259, which, when substituted, decrease esterase activity, are shown in red. One polymer unit (stylized green rectangle) binds to the groove adjacent to the catalytic site, a second unit bridges the distance to the catalytic site, and a third unit cleaves from the polymer chain at the catalytic unit



(Bollinger et al., 2020)

Another enzyme modified by this method was a PET hydrolase from I*deonella sakaiensis* (6THS), which managed to degrade 90% in 10 h, achieving productivity of 16.7 grams of terephthalate per liter per hour (200 grams per kilogram of PET suspension). , with an enzyme concentration of 3 milligrams per gram of PET), the changes made to this enzyme were in the amino acids D238 and S283, where these amino acids carry out the formation of disulfide bonds (Fig. 8.2), which allows the thermal stabilization of 94.4 ºC, 9.8 ºC higher than the wild-type enzyme, with a decrease in the activity of only 28% (Tournier *et al*., 2020).

Figure 8.3 Improvement of LCC thermostability by addition of a disulfide bridge. (a), The leading figure shows the locations of putative sites that coordinate divalent metal ions in the crystal structures of identified PET hydrolases. On wild-type LCC (ribbon), catalytic residues (S165, D210 and H242) and the C-terminal disulfide bond (C275–C292) are shown as blue rods. Divalent metal ions are shown as green spheres. In the upper inset, residues that bind metal ions in *Thermobifida alba* Est119 (PDB ID 3WYN) are shown as purple sticks, with the residues indicated with an asterisk; metal-binding residues in *Thermobifida cellulosilytica* Thc Cut1 (PDB ID 5LUI) are shown as yellow sticks, with the residues underlined in black; and the metal-binding sites in *Saccharomonospora viridis* Cut190 variants (PDB ID 4WFJ and 5ZNO) are shown as grey sticks, with residue names underlined in red. The left panels show a putative calcium-binding site formed by E208, D238 and S283 in LCC (with calcium shown as a blue sphere) and the disulfide bond (yellow) introduced here

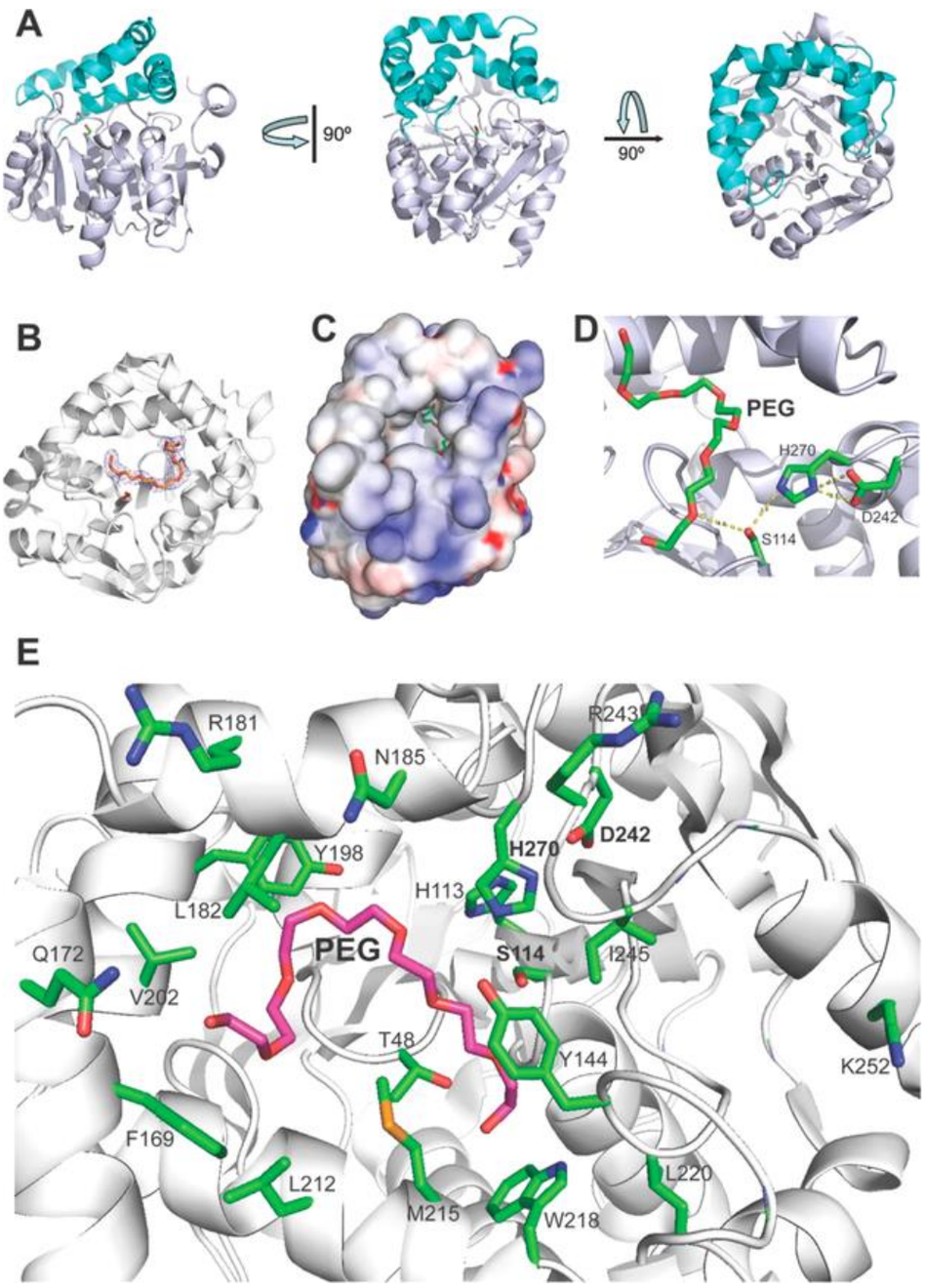


Source: (Tournier et al., 2020)

In 2019 Hajighasemi and collaborators conducted a study on 90 microbial hydrolases, selecting 2 enzymes (ABO2449 and RPA1511) from *Alcanivorax borkumensis* and *Rhodopseudomonas*, respectively. These were selected for the study due to the polyesters PLA (polylatic acid) and PCL (polycaprolactone) degradation power. These enzymes underwent site-directed mutagenesis changes where they identified several other residues necessary for the hydrolysis of both substrates, including His113, Leu182 and Tyr198 in RPA1511 and Leu32 and Leu249 in ABO2449. In the structure of RPA1511, the His113 side chain is located close to the catalytic Ser114. In contrast, the Leu182 and Tyr198 side chains are part of the alcohol-binding pocket, suggesting that these residues contribute to the binding of alcohol substrates. monoester and polyester.

RPA1511-V202A showed significantly higher activity against PLA and lower activity towards α-naphthyl propionate than the wild-type protein, suggesting that the Val202 side chain might interfere with PLA binding. The crystal structure of RPA1511 revealed the presence of an active site-bound polyethylene glycol molecule near the Ser114 catalyst, likely mimicking the bound PLA substrate (Figure 8.4) (Clarke, D. J., y Dobson, A. D., 2020). In the case of the ABO2449 enzyme, it was able to completely degrade the solid and emulsified PLA substrates in 2 days.

Figure 8.4 Crystal structure of RPA1511: overall fold and catalytic triad. (A) The overall structure of the RPA1511 protomer is shown in three views related by a 90° rotation. The protein core domain is shown in gray, whereas the lid domain is colored in cyan. The position of the active site is indicated by the side chain of the catalytic Ser114. (B) RPA1511 protomer with bound PEG 3350 (dodecaethylene glycol, shown as sticks). 2Fo-Fc map contoured at 1.0 σ displayed (shown as a blue mesh) around the dodecaethylene glycol molecule (colored in orange). (C) The surface presentation of the RPA1511 protomer reveals the entrance into the active site with bound PEG 3350 (shown as green sticks). Electrostatic potential mapped onto the solvent-accessible protein surface with red color representing potential below 5 kT, blue above 5 kT, and white as neutral. (D) Close-up view of the dodecaethylene glycol molecule (PEG 3350) bound close to the catalytic triad Ser114, His270, and Asp242 (shown as sticks along the protein ribbon colored in gray). (E) Close-up view of the RPA1511 active site with bound PEG 3350

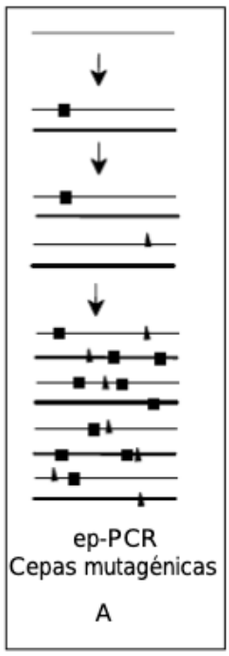


Source: (Clarke, D. J., y Dobson, A. D., 2020)

8.3 Error-prone PCR

This method introduces random mutations in a DNA segment >100 bp that are too long to be synthesized chemically (Wilson and Keefe *et al*., 2000). It is a method based on the polymerase chain reaction (PCR), in which the gene of interest is amplified under conditions that the enzyme adds wrong nucleotides randomly throughout the entire gene sequence and in the copies generated during replication cycles. The conditions that can change the fidelity of DNA polymerase $MgCl_2$, the presence of Mn_{2+}, a high concentration or asymmetric concentrations of deoxyribonucleotide triphosphates (dNTPs) and high concentrations of primers figure 8.4 (Cadwell & Joyce, 1992; Jaeger *et al*., 2001).

Figure 8.5 Asexual methods to generate point mutations. A) ep-PCR and mutagenic strains introduce errors at random positions along the gene sequence. Several random sequences are modified, so a group of mutagenic primers is used, hybridizing in the template's homologous regions

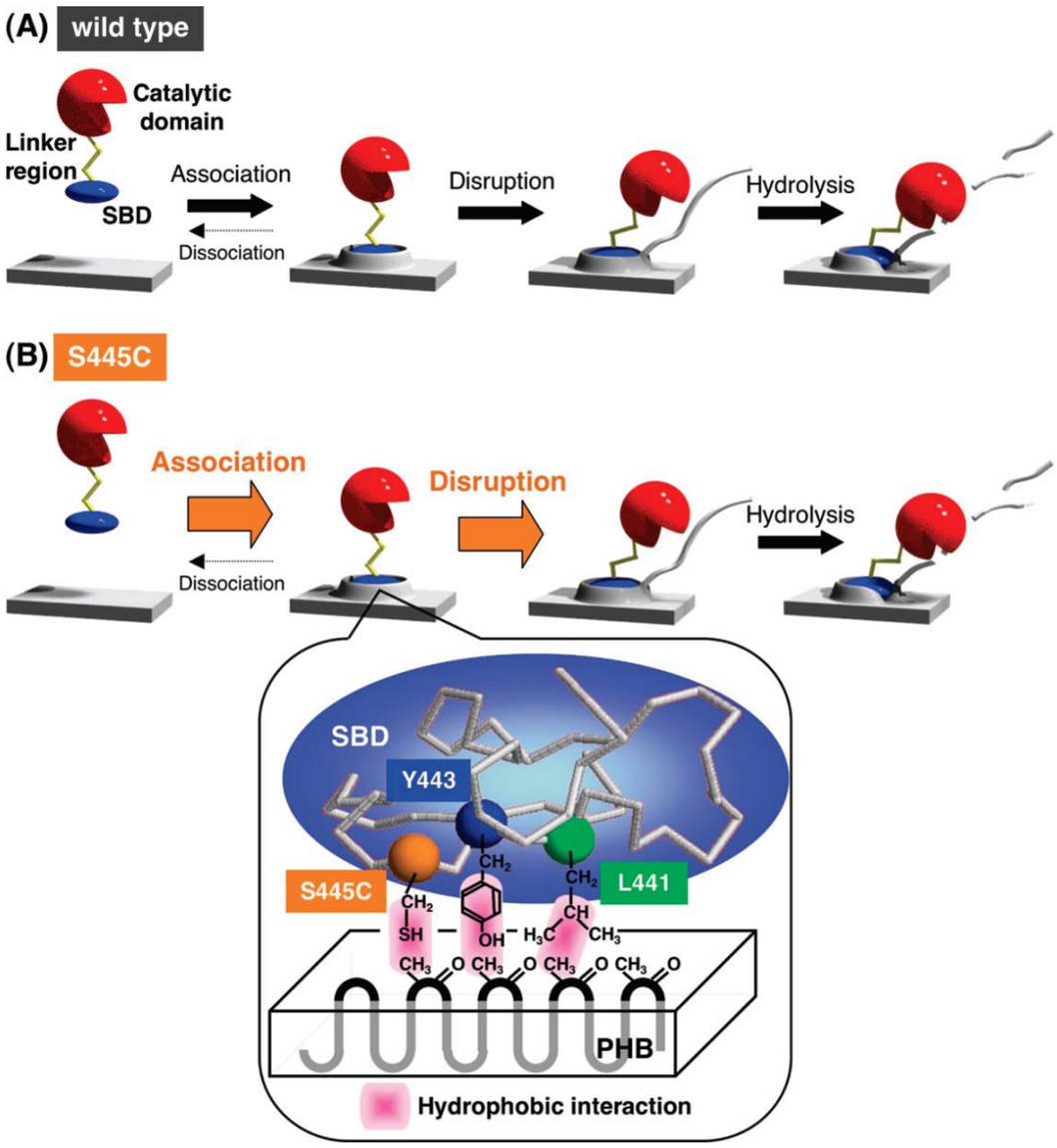


Source: (Cadwell y Joyce, 1992)

In 2006, the depolymerase enzyme obtained from *Ralstonia pickettii* responsible for degrading poly[(R)-3-hydroxybutyrate] (PHB) was mutated. It was modified to improve PHB degradation, changing amino acids found in its domain of Substrate binding (SBD), changing the Ser, Tyr, Val, Ala and Leu residues in the SBD for amino acids with high frequency in other enzymes, as can be seen in figure 8.4. The PhaZRiT1 variant was obtained from these mutations. It degrades PHB through hydrogen bonding between the hydroxyl groups of Ser on the enzyme and the carbonyl groups on the PHB polymer, but also hydrophobic interaction between the enzyme residues and the methyl groups on the PHB polymer. With these changes, the kinetic analysis of PHB degradation resulted in a decrease in the affinity of SBD towards dPHB (denatured PHB), causing a decrease in the degradation rate of dPHB without loss of its hydrolytic activity for the polymer chain (Hiraishi *et al.*,2006).

Similarly, in 2010 Hiraishi and coworkers investigated the effects of mutations in Leu441, Tyr443 and Ser445 on PHB degradation, as shown in Figure 8.5. They predicted that the modified residues would form a beta-sheet structure and be oriented in the same direction to interact directly with the PHB surface possibly. Mutant enzymes L441H, Y443H and S445C, were prepared. The hydrolytic activities for water-soluble substrates were identical to those of the wild-type enzyme, indicating that these mutations do not influence their structures and ability to break the bond ester.

Figure 8.6 Proposed mechanism of PHB degradation by PhaZRpiT1 (A) wild-type and (B) S445C enzymes based on the present kinetic studies. Inset shows a plausible model of the interaction between amino acid residues at positions 441, 443, and 445 in the SBD of the S445C enzyme and the PHB polymer chain. A predicted tertiary structure of SBD depicts only the R carbon, and the R carbons at positions 441, 443, and 445 in SBD of S445C are indicated in green, blue, and orange spheres, respectively



Source: (Hiraishi et al., 2010)

The PHB-degrading activity of the mutants differed from the wild-type enzyme, enzyme L441H and Y443H had lower PHB-degrading activity than their wild-type counterpart, while S445C had higher activity. Kinetic analysis of PHB degradation by the mutants suggested that hydrophobic residues at these positions are essential for the adsorption of enzymes on the PHB surface, and substitutions such as Y443H and S445C may more effectively alter the PHB surface to enhance the hydrolysis of PHB polymer chains than the wild-type enzyme (Hiraishi *et al*., 2010).

Another enzyme modified by the error-prone PCR method was a PETase obtained from *Ideonella sakaiensis* (IsPETase), where a mutant library based on a thermostable triplet mutant (TM) of IsPETase was obtained. Four variants showed higher melting points; the most promising IsPETAse TMK95N/F201I had a melting point of 5 ºC higher than IsPETAseTM. This variant showed slightly lower activity in PET at lower incubation temperatures; its thermostability makes it a more active PET hydrolase at higher action temperatures of up to 60°C.

The degradation of the amorphous PET film was carried out with the TMK95N/F201I variant at an incubation temperature of 60 ºC for 72 h, where a higher concentration of products (2500 mM) was observed, which are the sum of MHET, TA, and BHET released (Brott *et al*., 2022).

8.4 Modified enzyme degraded polyesters

Enzymes that can degrade polyesters have amino acids and critical bonds that allow them to carry out this activity; some enzymes are modified using the techniques mentioned in the previous sections. Depending on the requirement or use of the enzymes, they can be modified so that these enzymes change their characteristics, such as thermostability, halotolerant or increase the affinity to the substrate. In Table 8.1, mutated polyester-degrading enzymes are shown; PETases can be observed first since these were studied for their significant activation on PET (Han *et al*., 2017).

Table 8.1 Modified enzymes for improvement of polyesters degradation

Enzyme	Mutation	Substrate	Reference
IsPETase	C203S, C2395,W185A, S214H, I208A, W159A, M161A, Y87A, T88A, W168H.	PET Film	Han *et al*., 2017
IsPETase	S160A, D206A, H237A, Y87A, M161A, W185A, I208A, W159A, S238A, N241A, R280A, C203S, C239S, S238F, W159H	PET Film	Joo *et al*., 2018
IsPETase	S238F/W159H, W185A.	PET Film	Austin *et al*., 2018
IsPETase	P181A, S121D/D186H, S121E, D186H, D186F, P181G, P181S, P181A/S121D/D186H.	PET Film	Son *et al*.,2019
IsPETase	S160A,D206A, H237A, Y87B, M161A, W185A, I208A, W159A, S238A, N241A, R280A, C203S, C239S, S238F, W159H.	BHET	Sagong *et al*., 2020
IsPETase	S160A, D206A, H237A, W159A, W159H, M161A, A209I, Q119A.	BHET	Liu *et al*., 2018
IsMHETase	R411K, F415S, F424D, F424E, F424H, F424I, F424L, F424N, F424T, F424V, R411K/F424N, R411K/F424V, F415H/F424N.	MHET	Sagong *et al*., 2020
	S225A, D492A, H528A, W397A, R411A, R411Q, F415A, F415H, S416A, S416G, S419G, F424Q, H488A		Palm *et al*., 2019
PaPETase	G254S, S256N, I257S, Y258N, N259Q, Y250S,	*p*-NPB	Bollinger *et al*., 2020
PaPETase	S256N, I257S, Y258N, N259Q, Y250S	BHET	Bollinger *et al*., 2020
PaPETase	G254S, S256N, I257S, Y258N, N259Q, Y250S	Amorphous PET	Bollinger *et al*., 2020
CutinasaSvCut190	S226P/R228S/S176	Succinate	Numoto *et al*.,2018
Cutinasa SxCut190	S226P/R228S	NPB	Kawai et al.,2014
CutinasaSvcut190	S226P/R228s/E184R	PBSA, PET	Oda *et al*., 2018
Cutinasa TcCut1	S131A	PBA	Perz *et al*., 2015
Cutinasa TcCut1	R29D/A30V	*p*-NPA	Herrero *et al*.,2013
Esterase TaEst1	A68V/t253P	3PET	Thumarat *et al*., 2015
Esterase TaEst1	A68V/T253P	3PET	Thumarat *et al*., 2015
Esterase TaEst1	A68V/M259K	3PET	Thumarat *et al*., 2015
Lipase TlLip	E109A	*p*-NPB	Skjold *et al*., 2017
Lipase TlLip	I108c/I277c	*p*-NPDecanoate	Skjold *et al*., 2017
Esterase TfCa	E184Q	*p*-NPB	Billig *et al.*, 2010
Esterase TfCa	E319D	*p*-NPB	Billig *et al.*, 2010
Esterase TfCa	E184H/A186M	*p*-NPB	Billig *et al*., 2010

8.5 Acknowledgment

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8.7 Conclusions

The enzymes have been improved to meet industrial requirements. These improvements have been made through various molecular techniques, such as site-directed mutagenesis and error-prone PCR. For the modifications, it is necessary to consider strategies and selective practices imitating the evolutionary processes of nature, thus creating enzymes with new characteristics of industrial interest.

For the use of enzymes in an industrial context, high temperatures close to the glass transition temperature of polyesters are desirable. For this reason, modified enzymes are required for the efficient hydrolysis of polyesters at high temperatures and in extreme conditions since most polymeric contaminants are found in extreme environments such as wastewater.

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