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RESEARCH ARTICLE

Exploring the substrate spectrum of phylogenetically distinct bacterial polyesterases

Konstantinos Makryniotis[1] | **Efstratios Nikolaivits**[1] | **George Taxeidis**[1] | **Jasmina Nikodinovic-Runic**[2] | **Evangelos Topakas**[1]

[1]Industrial Biotechnology & Biocatalysis Group, Biotechnology Laboratory, School of Chemical Engineering, National Technical University of Athens, Athens, Greece

[2]Eco-Biotechnology & Drug Development Group, Laboratory for Microbial Molecular Genetics and Ecology, Institute of Molecular Genetics and Genetic Engineering, University of Belgrade, Belgrade, Serbia

Correspondence
Evangelos Topakas, Industrial Biotechnology & Biocatalysis Group, Biotechnology Laboratory, School of Chemical Engineering, National Technical University of Athens, Iroon Polytechniou 9, 15772 Athens, Greece.
Email: vtopakas@chemeng.ntua.gr

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Abstract

The rapid escalation of plastic waste accumulation presents a significant threat of the modern world, demanding an immediate solution. Over the last years, utilization of the enzymatic machinery of various microorganisms has emerged as an environmentally friendly asset in tackling this pressing global challenge. Thus, various hydrolases have been demonstrated to effectively degrade polyesters. Plastic waste streams often consist of a variety of different polyesters, as impurities, mainly due to wrong disposal practices, rendering recycling process challenging. The elucidation of the selective degradation of polyesters by hydrolases could offer a proper solution to this problem, enhancing the recyclability performance. Towards this, our study focused on the investigation of four bacterial polyesterases, including *Da*PUase, *Is*PETase, *Pf*PHOase, and *Se*1JFR, a novel PETase-like lipase. The enzymes, which were biochemically characterized and structurally analyzed, demonstrated degradation ability of synthetic plastics. While a consistent pattern of polyesters' degradation was observed across all enzymes, *Se*1JFR stood out in the degradation of PBS, PLA, and polyether PU. Additionally, it exhibited comparable results to *Is*PETase, a benchmark mesophilic PETase, in the degradation of PCL and semi-crystalline PET. Our results point out the wide substrate spectrum of bacterial hydrolases and underscore the significant potential of PETase-like enzymes in polyesters degradation.

KEYWORDS
1JFR, bacterial hydrolases, plastic pollution, polyester degradation, *Is*PETase

1 | INTRODUCTION

In the span of less than 30 years, the weight of plastic waste in the ocean is anticipated to surpass the fish residing therein.[1] The increasing number of ominous predictions concerning the fate of plastics in the environment accentuates the urgency of developing strategies to address the issue, emphasizing on the eco-friendly perspective, which lacks on the conventional management strategies that are already applied. Although plastics are well-known for their unique properties, including high durability and resistance to degradation,[2,3] enzymatic breakdown has gained prominence in recent years. Thus, the ability of enzymes to approach and "attack" the polymer bonds leading to breakdown in oligo- and monomers, is highly investigated.[2,4,5]

Konstantinos Makryniotis and Efstratios Nikolaivits contributed equally to this work.

Mainly present in water bottles, coatings and clothing, polyesters are the second most produced polymers worldwide,[6] indispensable in every aspect of our daily life. Thanks to their hydrolytically labile ester bonds, they are able to decompose in environmental conditions in variable periods, dependent on the polyester type.[7,8] In theory, all polyesters are deemed biodegradable, even though in practice, aliphatic polyesters, such as polycaprolactone (PCL) and polyhydroxy butyrate (PHB), exhibit considerably higher susceptibility to biodegradation compared to the aromatic ones, like polyethylene terephthalate (PET), as aromatic components in the polymer backbone impede enzymatic access and biodegradation process.[9,10]

Until today a variety of different enzymes with esterolytic activity are proven capable of degrading synthetic polyesters with varying yields and rates.[5,11] Various polyesterases from different families (cutinases, esterases, lipases, and proteases) can hydrolyze ester bonds of polylactic acid (PLA), polyhydroxyalkanoates (PHAs), poly (butylene succinate) (PBS), and PCL, leading, in some cases, to full degradation. Concerning, efficient PET and polyester polyurethane (PU) depolymerases, these are much more limited, with reduced performance.[12,13]

Industrial and urban plastic waste streams exhibit diverse compositions, containing mixtures of various polymers and other organic molecules.[14,15] The presence of impurities in plastic waste poses significant challenges during recycling, especially in the separation and classification of the different polymers, crucial step for the process's success. Among polyesters, PET stands out as a highly promising recyclable material, as it belongs to the minority of plastics that can be fully recyclable. However, PET waste as feedstock of the recycling system often contains impurities of other polymers, like PLA and PBS, while more biodegradable polyesters such as PHB and PCL are expected to insert these streams soon, primarily due to their recent extent use and the improper waste disposal practices. Even though most of these biodegradable polyesters can be recycled after use, the current strategies fail to achieve high-purity waste recovery. Consequently, the presence of polyester mixtures in the recycling process reinforces problems of chemical incompatibility and physical inhomogeneity, which significantly impact the recycling performance and the final structural and mechanical properties of the resulting materials.[16–18]

Considering the increasing adoption of biodegradable polyesters as eco-friendly alternatives to petroleum-based plastics which leads to higher disposal rates, it is anticipated that their existence in the recyclable waste streams of PET, polystyrene (PS), and polypropylene (PP), will significantly escalate.[18,19] Consequently, the selective enzymatic degradation of these biodegradable plastics could contribute to the purification of the recycling streams, improving process performance and enhancing the quality of the final recyclable plastic.

However, enzymatic degradation of polyester mixtures presents additional challenges due to the specificity of an enzyme towards different polyesters. These polymers can differ in both physical and chemical properties, such as crystallinity, glass transition temperature, molecular weight, and polarity.[20] So, even though ideally all polyesters can undergo enzymatic degradation through the same mechanism, that of ester bond hydrolysis, each hydrolase's characteristics, such as polarity, active site morphology, and thermostability, along with each polyester's traits can affect the depolymerization performance either enhancing or hindering it. Thus, it is crucial to thoroughly investigate the degradation capabilities of an enzyme across various polyesters to identify the optimal combination based on enzyme's selectivity.

In the present study, the selection of three polyesterases (*Da*PUase, *Is*PETase, *Pf*PHOase) and one putative polyesterase (*Se*1JFR), from different bacterial sources, belonging to different phylogenies, took place. The enzymes were recombinantly expressed, structurally analyzed and biochemically characterized, while their specificity towards individual non- and biodegradable synthetic plastics was investigated. The main aim of our research was to acknowledge the polymer substrate scope of these bacterial polyesterases, which could be a significant piece of knowledge for the development of synergistic enzyme cocktails tailored for the complete or selective degradation of polyester plastic waste mixtures.

2 | MATERIALS AND METHODS

2.1 | Tools for sequence and structural analysis of bacterial polyesterases

Protein Basic Local Alignment Search Tool (BLAST) was utilized to find homologues of the investigated target enzymes, while Clustal Omega program was used for multiple sequence alignment and the construction of the phylogenetic tree. Visualization of the tree took place through iTOL v6 tool.[21]

Prediction of target enzymes 3D structures was performed by ColabFold v1.5.2 (AlphaFold2 using MMseqs2) while UCSF Chimera v1.15[22] was used for visualization of structures as well as structural alignment and surface analysis.

2.2 | Polyesterases' expression and purification

Genes of a poly(ethylene terephthalate) hydrolase from *Ideonella sakaiensis* (*Is*PETase, UniProtKB ID: A0A0K8P6T7), a poly(3-hydroxyoctanoic acid) depolymerase from *Pseudomonas fluorescens* GK13 (*Pf*PHOase, UniProtKB ID: Q51718), a polyester polyurethane degrading esterase from *Delftia acidovorans* (*Comamonas acidovorans*) TB-35 (*Da*PUase, UniProtKB ID: Q9WX47) and a lipase-like enzyme from *Streptomyces sp.* WAC04770 (*Se*1JFR, UniProtKB ID: A0A3R9WEH4), were codon optimized for expression in *Escherichia coli*, excluding the native signal peptide, synthesized and cloned in the expression vector pET-22b(+), by GenScript Biotech B.V. (Netherlands). Transformation of chemically competent *E. coli* strains took place through heat-shock protocol and the transformants were grown in ampicillin supplemented Luria-Bertani (LB) plates for 16 h at 37°C. The transformants carrying *Is*PETase, *Pf*PHOase, *Da*PUase, and *Se*1JFR vectors were cultured in LB, Terrific Broth, 2 times yeast peptone

(2 × YT) and 1% w/v glucose supplemented LB nutrient mediums, respectively, at 37°C under agitation (180 rpm). Induction of the recombinant enzymes, except for *Se*1JFR, was initiated by the addition of 0.2 mM isopropyl β-D-1-thiogalactopyranosidase (IPTG), while *Se*1JFR expression was induced by 1.0 mM IPTG, for 16 h at 16°C.

Recombinant enzymes were purified as previously described by Dimarogona et al.[23], while homogeneity and purity of the isolated enzymes was established by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), using a 12.5% polyacrylamide gel. Protein quantification was performed by measuring the absorbance at 280 nm, using the molar extinction coefficients computed by the ProtParam tool from ExPASy.[24] The fractions containing the purified enzymes were dialyzed for 16 h at 4°C against a 25 mM Tris-HCl pH 7.4, 150 mM NaCl buffer (standard storage buffer).

2.3 | Biochemical characterization of recombinant polyesterases

Esterolytic activity of the recombinant polyesterases was assessed through enzymatic reactions with *p*-nitrophenyl fatty acid esters with varying chain lengths, including *p*-nitrophenyl acetate (*p*NP-C_2), *p*-nitrophenyl butyrate (*p*NP-C_4), *p*-nitrophenyl octanoate (*p*NP-C_8), and *p*-nitrophenyl decanoate (*p*NP-C_{10}), at 35°C for 10 min. Reactions consisted of 230 μL 0.1 M phosphate-citrate buffer pH 6.0 containing 1.1 mM of substrate, and 20 μL enzyme solution. The release of *p*-nitrophenol (*p*NP) was monitored at 410 nm using a SpectraMax-250 microplate reader (Molecular Devices, Sunnyvale, CA, USA) and connected with the SoftMaxPro software (version 1.1, Molecular Devices, Sunnyvale, CA, USA). Enzymatic activity was quantified in Units (U), representing the quantity of enzyme capable of releasing 1 μmol of *p*NP per minute.

The optimal temperature and pH of polyesterases were determined by assaying enzyme activity, using *p*NP-C_4 as substrate, following the standard *p*NP assay conditions, over a range of temperatures (7–70°C) and pH values (5.0–9.0). Employed buffer systems included 0.1 M citrate-phosphate (C-P, pH 5.0–6.0), 0.1 M sodium-phosphate (S-P, pH 6.0–8.0), and 0.1 M Tris-HCl (T-H, pH 8.0–9.0), while necessary calibration curves were constructed for the quantification of *p*NP in each buffer system.

The thermostability of polyesterases was investigated by assessing their remaining activity on *p*NP-C_4, after their incubation in 25 mM Tris-HCl, 150 mM NaCl pH 8.0 buffer, at temperatures ranging from 20 to 80°C for a duration of up to 72 h, under standard *p*NP-C_4 assay conditions. Likewise, to investigate the impact of pH on enzyme stability, the residual activity of these enzymes on *p*NP-C_4 was measured after incubation in various buffer systems, of pH 5.0 to 10.0, at 4°C for 24 h. The buffer systems used included 0.2 M citrate-phosphate (C-P, pH 3.0–6.0), 0.2 M sodium-phosphate (S-P, pH 6.0–8.0), 0.2 M Tris-HCl (T-H, pH 8.0–9.0), and 0.2 M glycine-NaOH (G-N, pH 9.0–10.0). The remaining activity was then compared to that of the standard storage buffer, as described above (paragraph 2.2).

2.4 | Target polymers: Origin, preparation, and characterization

Investigated polymers belong to the categories of polyesters, including aged PBS (initial grade NaturePlast PBE003, NaturePlast, France), PCL (CAPA 6500, Ravago Chemicals, Belgium), PET (PAPET clear, Lotte Chemical, UK), PHB (Biomer P226, Biomer, Germany) and PLA (4043D, NatureWorks, USA), as well as of aliphatic-aromatic polyether PU (LPR7560, Coim, Laripur). Preparation of target polymers in powder form as well as characterization of the investigated materials, took place following the methodology outlined by Nikolaivits et al.[25]

2.5 | Enzymatic degradation of investigated polymers

Enzymatic reactions with the target materials (PBS, PCL, PET, PHB, PLA, and PU) were conducted in 0.1 M sodium-phosphate pH 7.5 with 10 mg mL^{-1} of polymeric powder, at a final volume of 0.5 mL. The reactions were carried out in an Eppendorf Thermomixer Comfort (Eppendorf, Germany) at 30°C, under agitation (1350 rpm), initiated with the addition of 0.5 nmol of enzyme and supplemented with another 0.25 nmol of enzyme every 24 h, for 72 h. Biodegradation of investigated materials was evaluated through weight loss, alterations of polymer average molecular weights, determined by Gel permeation chromatography (GPC), and, in case of PET, quantification of the water-soluble hydrolysis products, namely terephthalic acid (TPA), mono-(2-hydroxyethyl) terephthalate (MHET), and bis-(2-hydroxyethyl) terephthalate (BHET), determined by high performance liquid chromatography (HPLC). Methodologies used for samples' preparation and analysis are described in the work by Makryniotis et al.[26]

3 | RESULTS AND DISCUSSION

3.1 | Selection of target polyesterases

In this paragraph, the criteria used for the selection of target polyesterases to be studied are discussed. Starting with *Is*PETase, a well-established bacterial PETase that is known to effectively degrade amorphous PET at low temperatures,[27] hence it was employed as a benchmark polyesterase in this study. Notably, *Is*PETase has demonstrated its capability to degrade PEF,[28] another semi-aromatic polymer, as well as the biodegradable PCL.[29] However, its activity on other aliphatic polyesters like PBS and PLA[28] seems marginal.

*Se*1JFR was selected due to its high sequence homology to polyesterases/PETases. It is intriguing to note that *Se*1JFR originates from a bacterium belonging to a distinct phylum (Actinomycetota) when compared to *Is*PETase (Pseudomonadota). Furthermore, the availability of a known 3D structure for *Se*1JFR is an invaluable asset for investigations pertaining to structure-function relationships, obviating the need for constructing a structural model of the

protein. The crystal structure of the 1JFR enzyme, also known as SeL, has been explored in the context of its homology to mammalian platelet-activating factor acetylhydrolases,[30] and has been employed in a variety of in silico studies utilizing homology modeling, for the discovery of novel polyesterases.[31,32] Through this approach, SM14est, a PETase-like enzyme from *Streptomyces sp.* SM14, has been firstly identified, heterologously expressed and tested in PCL degradation, with promising results.[33] Furthermore, its biochemical characterization and PET degradation ability was reported recently.[34] Even though *Se*1JFR shares a 57% sequence homology with SM14est, its performance in polymer degradation remains untested.

*Pf*PHOase was isolated from the wild-type *P. fluorescens* strain and characterized,[35] while its gene was identified later.[36] This enzyme exhibits a notable preference for medium chain-length (mcl) PHAs, such as polyhydroxy octanoate (PHO) and its co-polymer with polyhydroxy decanoate, while displaying limited hydrolytic activity towards PHB, polyhydroxy valerate, and their co-polymers. The hydrolysis of PHO resulted in dimers as the main products.

*Da*PUase gene was identified by Nomura et al.[37] while the wild-type enzyme was characterized in a previous work,[38] showing it could degrade solid polyester PU, releasing diethylene glycol and adipic acid as the primary degradation products. This enzyme seems to degrade PU in a two-step fashion, commencing with hydrophobic adsorption to the polymer's surface, followed by the hydrolysis of the ester bond. In contrast, *Da*PUase seems to exhibit no activity on PHB and displayed only limited activity on low MW PLA (5000 g mol^{-1}).

3.2 | Sequence and structural analysis of selected polyesterases

In this section, sequence and structural analysis of the selected polyesterases is presented. To initiate this analysis, a sequence similarity search was performed by subjecting the sequences of these enzymes to the Uniprot database using BLASTp tool.

For *Is*PETase and *Se*1JFR the search yielded identical hits, albeit in different order, which included *Thermobifida* cutinases/PETases, *Amycolatopsis mediterranei* cutinase and *Moraxella* sp polyesterase. Even though *A. mediterranei* cutinase is not characterized, it is highly homologous (92% identity for 98% coverage) to the metagenomic PET40 enzyme (GenBank: WAU86704.1) deriving from an *Amycolatopsis* sp. *Is*PETase exhibited a homology of 45%–52% with these enzymes, whereas *Se*1JFR displayed a broader range of homology from 40%–75% (Table S1).

In contrast, *Pf*PHOase did not return any hits within the Uniprot database. Given this, the search was extended by subjecting the *Pf*PHOase sequence to the non-redundant protein sequence database. This revealed that *Pf*PHOase shares substantial homology (> 70%) with various proteins, including a dienelactone hydrolase from *Pseudomonas alcaligenes* (99.6%), a PHA depolymerase from *Prescottella equi* (96.9%), a PHA depolymerase from *Pseudomonas alcaligenes* (87.5%), an alpha/beta hydrolase-fold protein from *Pseudomonas* sp. (84.7%), a dipeptidyl aminopeptidase/acylaminoacyl-peptidase from *P. mendocina* (82.8%), an esterase from *Halopseudomonas* sp. (73%) and a PHA depolymerase from *P. luteola* (73%).

Regarding, *Da*PUase, top hits against Uniprot database were animal/human hydrolases with a homology of 32%–35%. Comparing this sequence against the non-redundant protein sequence database, homologies above 40% were found for proteins of the carboxylesterase family deriving from a wide array of bacterial genera.

Based on the insights gained from the homology search, the top hits were selected and employed for the construction of a phylogenetic tree (Figure S1). The resulting tree forms three distinct clades, one containing characterized PETases/polyesterases, another comprising *Da*PUase and uncharacterized bacterial carboxylesterases, and a third clade encompassing *Pf*PHOase and uncharacterized bacterial PHA depolymerases, dienelactone hydrolases, but also peptidases. This analysis sheds light on the evolutionary relationships and potential functional similarities between the selected polyesterases and their homologous enzymes, which is critical for understanding their roles and capabilities in polyester degradation.

The 3D structures of the studied enzymes were either downloaded from PDB (ID 6EQH for *Is*PETase and 1JFR for *Se*1JFR) or predicted with AlphaFold2 (for *Da*PUase and *Pf*PHOase). The two enzymes belonging to the PETase clade (*Is*PETase and *Se*1JFR) show a very similar structure with an overall rmsd of 1.065 Å (Figure S2). Both enzymes adopt a classical α/β-hydrolase fold, with a core twisted β-sheet consisting of nine β-strands and flanked on both sides by six α-helices. The catalytic triad consists of Ser120, His237 and Asp206 in *Is*PETase and Ser131, His209, and Asp177 in *Se*1JFR, with the nucleophilic serine, in each case, located in a sharp turn called the "nucleophile elbow," which is commonly observed in α/β-hydrolases.[39] The oxyanion hole is formed by Met161 and Tyr87 in *Is*PETase, while in *Se*1JFR by Met132 and Phe63.[28,30] *Is*PETase contains two disulfide bridges (Cys203-C239 and Cys273-Cys289), one of them also being present in *Se*1JFR (Cys242-Cys258).

According to DALI[40] server *Da*PUase is structurally related to liver carboxylesterases from *Homo sapiens* and *Sus scrofa* (rmsd 2.4–2.7 Å), along with a *Bacillus subtilis p*-nitrobenzyl esterase (PDB ID: 1QE3, rmsd 2.5 Å).[41] Out of the top structural homologues none is known to be implicated in polyester degradation. *Da*PUase (Figure 1), however, also belongs to the α/β hydrolase family, composed of a central 13-stranded β-sheet surrounded by 16 α-helices (above 3 residues long). Its putative catalytic triad is formed by Ser199, His432, and Glu323, with the glutamate replacing the usual aspartate residue as the active site carboxylate. The structure is stabilized by 3 disulfide bridges, namely Cys74-Cys92, Cys258-Cys265, and Cys389-Cys512 (Figure 1A). Nomura et al.,[38] when they first studied the enzyme, they had identified three regions with an increased number of hydrophobic residues. One of these regions is in fact homologues to the PHA binding domain of a PHA depolymerase (PhaZ1ple). The authors had previously shown that hydrophobic adsorption of the enzyme on the polymer was important for its degradation.[38] These three regions as shown in Figure 1A are Thr244-Asp262 (Ri), Glu272-Pro290 (Rii), and Leu333-Tyr366 (Riii) (Figure 1A). Surface hydrophobicity analysis by

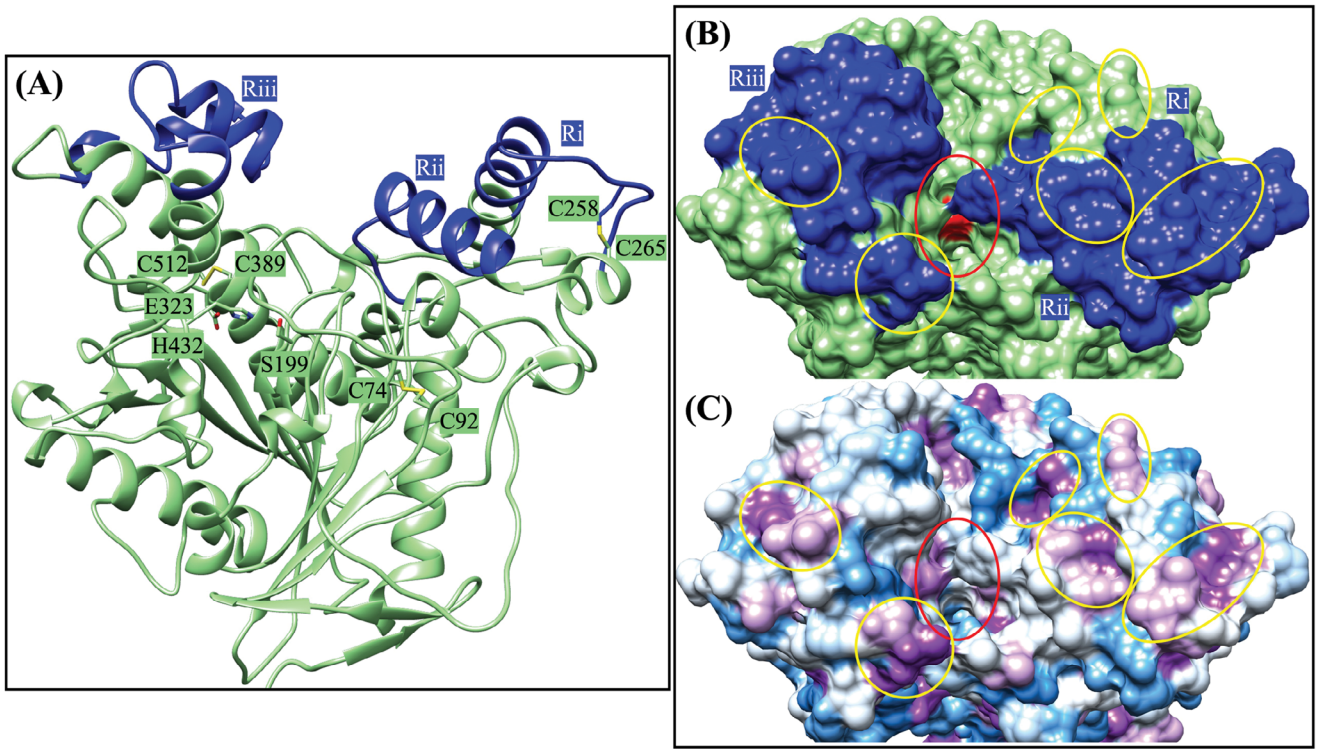

FIGURE 1 (A) Overall structure model of *Da*PUase as predicted by AlphaFold2. Stick representation of key residues forming the catalytic triad (Ser199, His432, and Glu323) and the three disulfide bridges (Cys74- Cys92, Cys258-Cys265, and Cys389-Cys512). Highlighted in blue the three hydrophobic regions as identified by Nomura et al., (B) Surface representation of *Da*PUase with highlighted (blue) predicted hydrophobic regions and catalytic residues (red) and (C) Surface representation colored by hydrophobicity as calculated by Chimera software (blue hydrophilic residues and purple hydrophobic residues). In (B) and (C) the red ellipse suggests the catalytic cleft, while the yellow ellipses surround hydrophobic regions as calculated by Chimera software.

UCSF Chimera shown that only parts of these regions are hydrophobic (Figure 1B and 1C).

*Pf*PHOase is another enzyme belonging to the α/β hydrolase family. It is composed of 8 central β-sheets flanked on each side by α-helices. In the central β-sheet, the last two β-strands are anti-parallel, while the center 8 β-strands are parallel. There are also 3 anti-parallel β-sheets at the N-terminus of the protein (Cys4-Cys19 and Ser67-Ser70), not belonging to its core. One of the two predicted disulfide bridges of the enzyme connects two of these three β-sheets (Cys4-Cys19), while the second stabilizes a long loop at the surface of the enzyme connecting two central β-sheets (Cys180-Cys185). The putative catalytic triad of *Pf*PHOase is Ser150, His238 and Asp206 (Figure S3A).

According to DALI server, *Pf*PHOase's structural homologues are mostly peptidases with an rmsd of 2.7–3.0 Å, along with a metagenome-derived esterase (PDB ID: 3WYD, rmsd 2.0 Å) and a PHB depolymerase (*Lt*PHBase – PDB ID: 8DAJ, rmsd 2.4 Å), with which it shares only 17% sequence identity. Comparison of *Pf*PHOase with *Lt*PHBase shows that the latter contains two more β-strands in the central β-sheet (D229-V258), while it doesn't share any of the disulfide bridges with *Pf*PHOase nor the 3 anti-parallel β-strand formation.[42] These, among other differences between the two structures are highlighted in Figure S3B.

Surface representation of the two structures shows that the catalytic cleft of *Lt*PHBase seems more accessible compared to *Pf*PHOase (Figure 2A). Additionally, the hydrophobicity of the residues surrounding the catalytic cleft is much more increased in *Pf*PHOase as analyzed by UCSF Chimera (Figure 2B). These differences can clearly affect the substrate binding properties of the enzyme and lead to diversified activities.

Given the hydrophobic nature of plastics, enzymatic approach and absorption to the polymer structure, which is the initial crucial phase of the degradation process, takes place through hydrophobic interactions.[43] Consequently, there has been a significant focus on introducing hydrophobic properties in polyesterases, such as cutinases and esterases, using surface engineering techniques. In various studies researchers have explored specific approaches, including amino acid substitutions, incorporation of binding modules or even curtailment of entire domains.[44] These efforts have shown promising results, by improving turnover rates and efficiency of polyesters degradation, as polyethylene terephthalate (PET)[31,45] and polyester poly(1,4-butylene adipate) (PBA).[46]

3.3 | Biochemical characterization of selected polyesterases

All polyesterases were recombinantly expressed, purified to homogeneity and concentrated in solutions, while their molecular weights

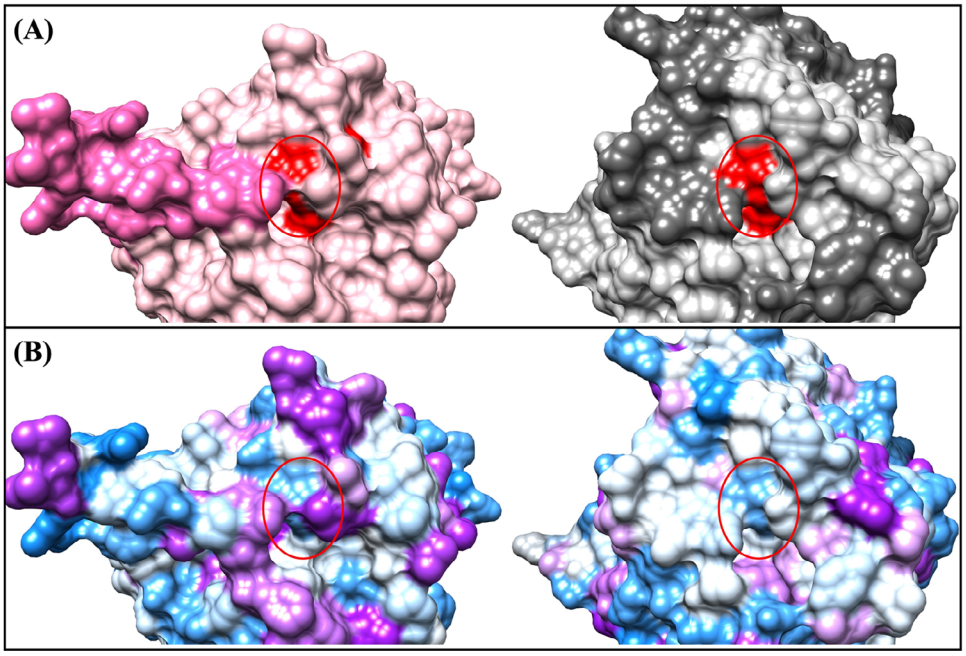

FIGURE 2 (A) Surface representation of of *Pf*PHOase (pink), and *Lt*PHBase (light grey - 8DAJ), highlighting the unique regions of each enzyme that are not superimposed (hot pink for *Pf*PHOase and dark grey for *Lt*PHBase), along with the catalytic residues (red), suggesting the catalytic cleft (red ellipse). (B) Surface representation of both enzymes colored by hydrophobicity as calculated by Chimera software (blue hydrophilic residues and purple hydrophobic residues).

(MW) were determined at 58 kDa (*Da*PUase), 30 kDa (*Se*1JFR), 29 kDa (*Pf*PHOase), and 28 kDa (*Is*PETase), via their appearance as single bands on 12.5% SDS-PAGE gel (Figure S4).

While literature has already explored the biochemical characterization of three out of the four selected enzymes, expressed natively (*Da*PUase and *Pf*PHOase) or heterologously (*Is*PETase), primarily emphasizing on plastic degradation optimal catalytic conditions, our study provides a comprehensive biochemical characterization of these polyesterases and the first one of *Se*1JFR, with a specific focus on their esterolytic activity. These characterization models could constitute a useful starting point for comparison of already existing or novel polymer degrading enzymes, of the lipase, cutinase, for example, and esterase family.

Regarding the optimum temperature, as illustrated in Figure 3A, *Is*PETase and *Se*1JFR exhibit their maximum activity at 35°C, while maintaining over 90% of this activity from 30°C to 40°C. In contrast, both *Da*PUase and *Pf*PHOase display significant esterolytic activity within a temperature range of 30°C to 55°C, reaching their peak at 45°C. At temperatures of 50°C and higher, the activity of both *Is*PETase and *Se*1JFR radically decreases, while *Da*PUase and *Pf*PHOase demonstrate a more thermophilic profile by remaining significantly active at 55°C while becoming completely inactivated at 60°C. Most of the obtained optimum temperature values align with those reported in the literature,[27,35,38] while SM14est, homologue of *Se*1JFR presents a higher optimal temperature of 45°C. Concerning the effect of pH on activity (Figure 3B), the optimum was determined to be pH 6.0 for *Da*PUase and *Pf*PHOase and pH 7.0 S-P for *Is*PETase and *Se*1JFR. At pH 9.0, *Da*PUase and *Pf*PHOase activity dropped abruptly while similar motif was observed for *Is*PETase in pH 5.0. *Se*1JFR activity was significantly affected in both acidic and alkaline pH conditions, with the enzyme retaining activity primarily around neutral pH.

Polyesterases' stability was investigated across a temperature and pH range. Among the tested temperatures spanning from 20 to 80°C, Figure S5A illustrates each enzyme's stability at the maximum temperature, at which it retained part of its activity after a 3-day incubation period. According to these results, *Da*PUase and *Pf*PHOase demonstrated greater thermostability compared to *Is*PETase and *Se*1JFR, retaining more than 70% of their activity at 40°C after 72 h. Over the same duration, at 30°C, *Is*PETase and *Se*1JFR retained about 55% and 65% of their activity, respectively. At 50°C, *Da*PUase, *Is*PETase, and *Pf*PHOase lost their activity after 24 h (Figure S6A-F), while at 60°C and higher temperatures, the enzymes were deactivated within minutes. Finally, *Se*1JFR exhibited extremely weak thermostability, instantly deactivating at 40°C (Figure S6G,H). Concerning stability at the investigated values of pH ranging from 5 to 10 (Figure S5B), it was observed that, for all polyesterases except *Da*PUase, the ionic strength of the buffer (200 mM) is more crucial for enzyme stability than the pH itself. In almost all cases, these enzymes exhibited higher activity compared to the standard storage buffer (150 mM), with neutral or slightly alkaline pH values optimizing storage stability. Conversely, *Da*PUase demonstrated the greatest stability under standard storage conditions of 150 mM salt concentration and pH 7.4.

Esterolytic activity of the polyesterases in relation to the substrate's size was evaluated through reactions with *p*-nitrophenyl water-soluble fatty acid esters of varying chain lengths, including *p*NP-C_2, *p*NP-C_4,

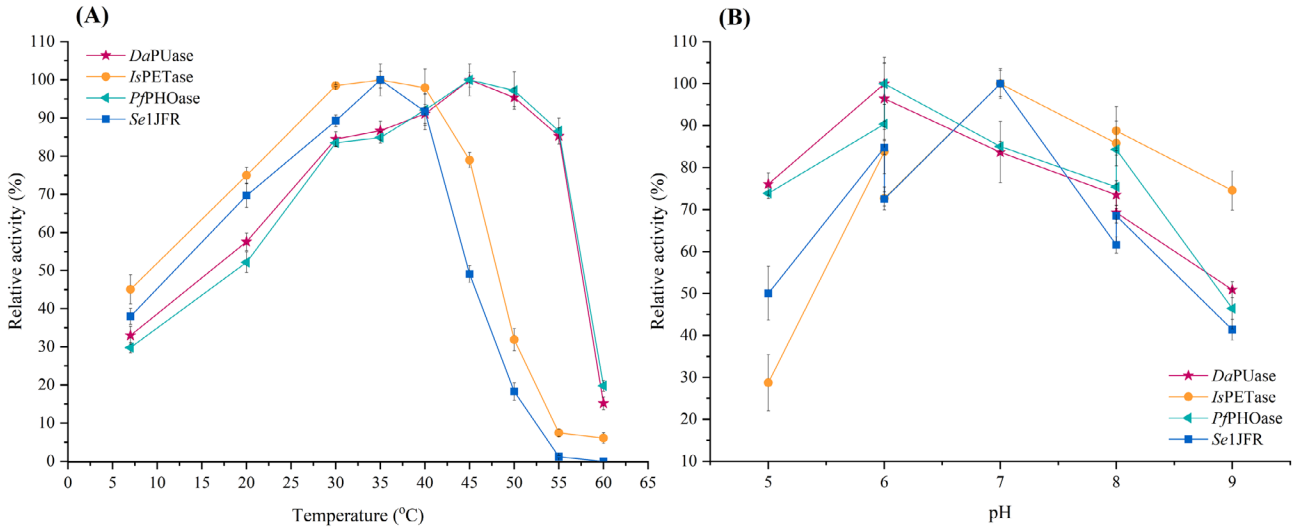

FIGURE 3 (A) Effect of temperature on the activity of *Da*PUase (★), *Is*PETase (●), *Pf*PHOase (◄), and *Se*1JFR (■). Relative activity was defined after assaying the enzymes with *p*NP-C4 in phosphate-citrate buffer (pH 6.0), at 35°C. (B) Effect of pH on the activity of *Da*PUase (★), *Is*PETase (●), *Pf*PHOase (◄), and *Se*1JFR (■). Relative activity was defined after assaying the enzymes with *p*NP-C4 in a variety of buffer systems, at 35°C. Buffer systems used were citrate-phosphate (pH 5.0–6.0), sodium-phosphate (pH 6.0–8.0) and Tris-HCl (pH 8.0–9.0).

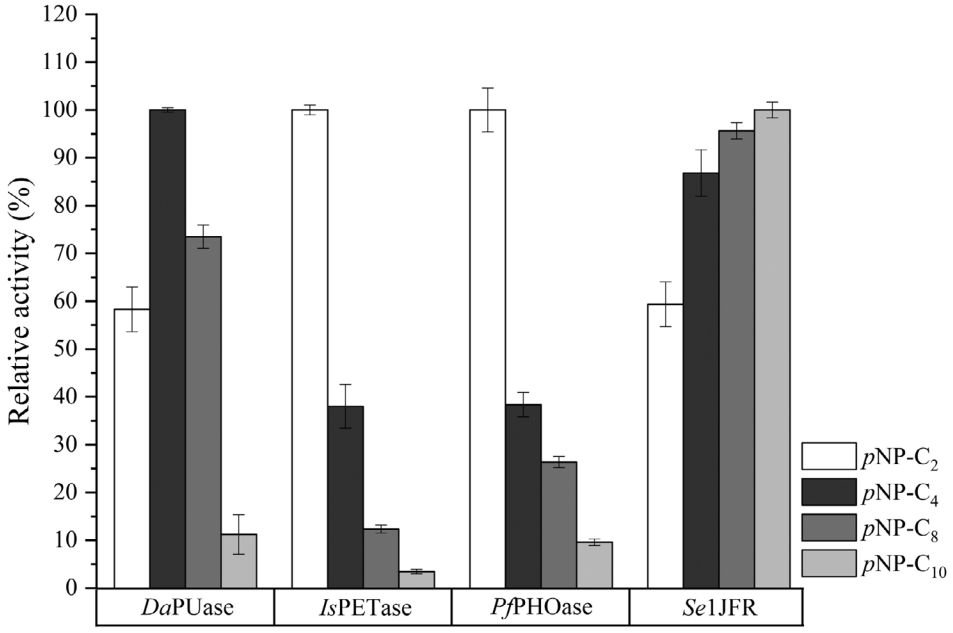

FIGURE 4 Effect of *p*NP fatty acid esters' chain length on the activity of *Da*PUase, *Is*PETase, *Pf*PHOase, and *Se*1JFR. Substrates used were *p*NP-C2 (white), *p*NP-C4 (black), *p*NP-C8 (dark grey), and *p*NP-C10 (light grey). Relative activity was defined after assaying the enzymes with the *p*NP substrates in phosphate-citrate buffer (pH 6.0), containing 1% v/v Triton-X and 0.125% w/v gum arabic, at 35°C.

*p*NP-C_8, and *p*NP-C_{10}. As depicted in Figure 4, the activity towards the different fatty acid esters varied for each polyesterase. *Is*PETase and *Pf*PHOase exhibited their highest enzymatic activity towards *p*NP-C_2, which significantly decreased for substrates of increasing chain length, reaching a 25- and 10-fold reduction on *p*NP-C_{10}, respectively. Conversely, *Se*1JFR displayed the lowest activity towards *p*NP-C_2, which escalated towards substrates of longer chain lengths, reaching the highest value, about 1.7-fold rise, for *p*NP-C_{10}. In contrast, *Da*PUase presented a distinct profile, as maximum activity was observed for *p*NP-C_4, while also displaying substantial activity for *p*NP-C_2 and *p*NP-C_8, and a notable 10-fold lower activity towards the substrate with the longest chain tested, *p*NP-C_{10}.

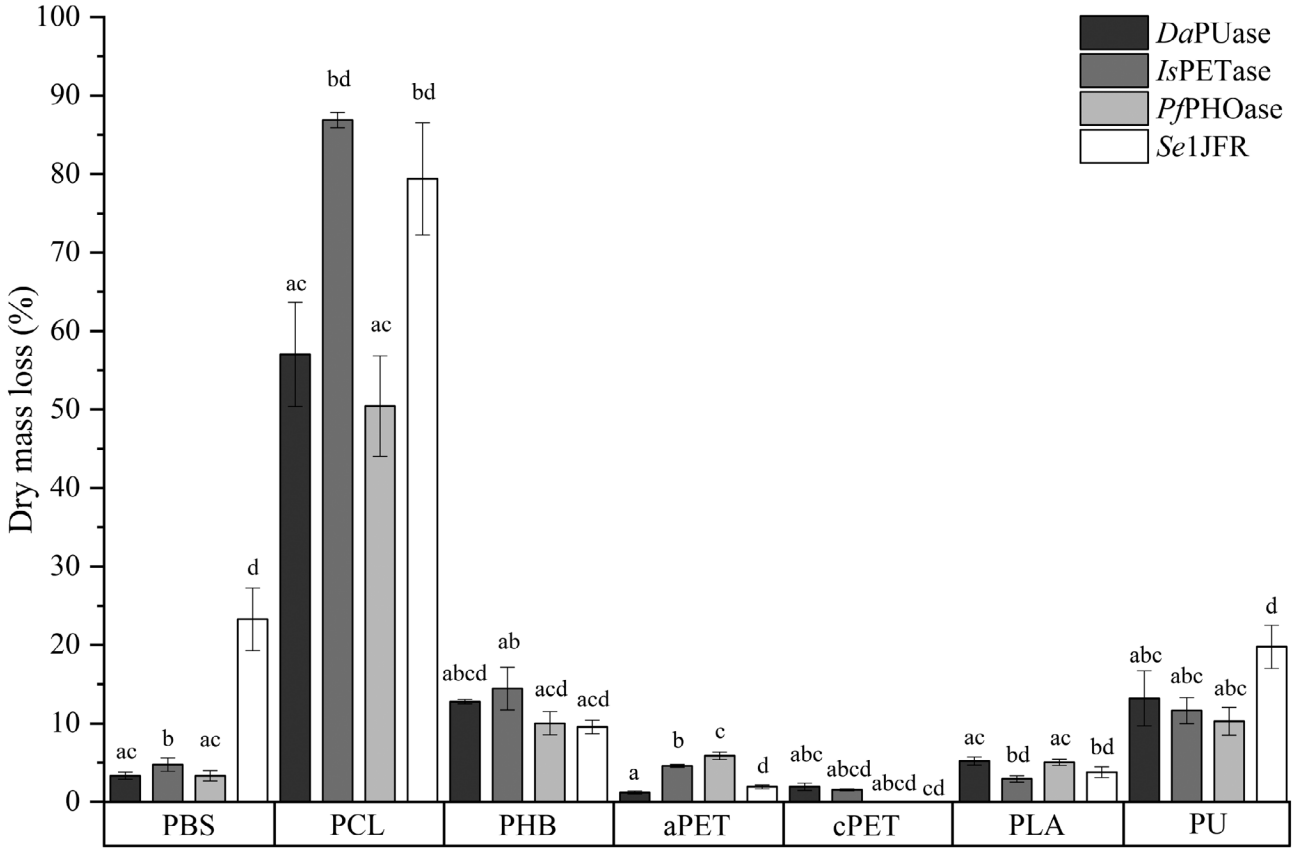

FIGURE 5 Dry mass loss (%) of different synthetic polymers after their treatment with *Da*PUase (black), *Is*PETase (dark grey), *Pf*PHOase (light grey), and *Se*1JFR (white). Reactions took place at 35°C, for *Is*PETase and *Se*1JFR, and at 45°C, for *Da*PUase and *Pf*PHOase, for 72 h (except from PCL, for 24 h). Control reactions in the absence of enzyme were performed under the same conditions and dry mass loss, whenever existed, was subtracted from the enzymatic reactions' results. Letters a, b, c, and d are signifying *Da*PUase, *Is*PETase, *Pf*PHOase, and *Se*1JFR degradation results caused in each polyester respectively and were used to represent statistically significant differences between corresponding values, according to IndependentSamples *t*-Test with a significance level of p-value < 0.05. Bars that share the same letter are not statistically different from each other based on the statistical test performed.

3.4 | Depolymerization capability of investigated polyesterases

Our main research focus was the investigation of the substrate specificity of bacterial polyesterases, which was achieved by conducting enzymatic reactions on various polyesters, most of which are not reported in existing literature of the investigated polyesterases, and assessing their depolymerization degree based on the percentage dry mass loss and changes in molecular weights ($\overline{M_n}$, $\overline{M_w}$), presented in Figure 5 and Table S2, respectively.

3.4.1 | Degradation of biodegradable synthetic polyesters

Concerning the biodegradable polyesters, mass loss results (Figure 5) indicate that PCL emerged as the most susceptible to enzymatic degradation by all investigated enzymes. *Is*PETase and *Se*1JFR nearly completely degraded the material, resulting in a mass loss exceeding 80% within just one day of incubation, using 0.5 nmol of enzyme. Likewise, *Da*PUase and *Pf*PHOase induced significant breakdown of the polymer, accounting for approximately 50% mass loss. Notably, *Da*PUase results were also accompanied by alterations in the molecular weight of the remaining material, particularly in $\overline{M_n}$, with a 5% decrease (Table S2). The fact that this reduction is comparatively minor in relation to the observed mass loss, highlights the possibility of a competitive inhibition of *Da*PUase by the products generated during PCL degradation. Enzymatic treatment of PHB powder led to a 10%–15% mass loss by all the investigated polyesterases, while no variations of the remaining material's molecular weight were detected, indicating a possible exo-activity (end scission) of all polyesterases. PBS degradation exhibited limited success, evidenced by a minimal mass reduction in the powder, except from *Se*1JFR action which led to a distinct 23% mass loss. Inefficiency of the investigated polyesterases in PBS degradation might seem surprising, given the documented success of various bacterial hydrolases, particularly lipases and cutinases, in achieving complete breakdown of this biodegradable polyester of similar crystallinity grades (57% to 65%),[47–49] However, it is noteworthy that the quantity of enzymes employed in these studies is approximately 10 to 50-fold higher than in our investigation. Even though the enzy-

matic degradation of PBS was not obvious from the alterations in material's weight, all polyesterases reduced powder's $\overline{M_n}$, from 3% to a maximum 9%, a statistically significant alteration, caused by *Is*PETase. Polyesterases' action on PLA was mainly implied by significant alterations on the polymer's molecular weight. *Se*1JFR and *Is*PETase led to a 26% and 20% $\overline{M_n}$ loss, both statistically significant reductions, while *Is*PETase also reduced polymer's $\overline{M_w}$, by 9%. Similarly, *Pf*PHOase degradation capability was detected by 16% reduction of PLA $\overline{M_w}$. Allegedly, these polyesterases, especially *Se*1JFR and *Is*PETase, possess the ability to cleave PLA chains in an endo-manner. However, they do not exhibit exo-activity, which would lead to the release of water-soluble products and, consequently, a distinct reduction in polymer mass. Thus, concerning mass loss, all polyesterases caused marginal mass reduction of the material, with the results of *Da*PUase and *Pf*PHOase particularly standing out as statistically higher.

Upon comparing the mass loss results induced by the investigated enzymes across the spectrum of biodegradable polyesters, it is evident that a discernible trend is emerging. More specifically, *Da*PUase and *Pf*PHOase consistently lead to statistically similar degradation outcomes across all biodegradable polyesters, forming a distinct subgroup separate from the other two enzymes. A singular difference between *Da*PUase and *Pf*PHOase, is the reduction in the average molecular weight ($\overline{M_w}$,) observed in PLA due to the latter, which points out the potential of the enzyme in PLA degradation. A similar grouping pattern is evident in the case of *Is*PETase and *Se*1JFR, where both enzymes' depolymerase action, results in comparable mass loss results in most biodegradable polyesters. Concerning the degradation of PCL and PLA, both enzymes induced statistically similar degradation levels, while results mainly differ for PBS degradation, where *Se*1JFR exhibited significantly superior degradation (approximately 25%) compared to the other polyesterases.

3.4.2 | Degradation of non-biodegradable, PET, and polyether based PU

Regarding the non-biodegradable polyester, PET, some interesting results came out. All polyesterases presented indications of degradation of the low crystallinity PET (x_c of 5%), by slight (*Da*PUase and *Se*1JFR) or distinct (*Is*PETase and *Pf*PHOase) mass reduction (Figure 5), accompanied by the release of water-soluble hydrolysis products (Table S3). *Is*PETase, proposed in literature as a benchmark mesophilic PET hydrolase, was able to release 33.5 $\mu g_{products}/mg_{PET}$, about 10-fold higher than the newly studied *Se*1JFR (3.07 $\mu g_{products}/mg_{PET}$). Following this, *Pf*PHOase and *Da*PUase depolymerase action resulted in a considerably lower release of hydrolysis products, at 0.6 and 0.1 $\mu g_{products}/mg_{PET}$, respectively. Degradation of semi-crystalline PET (x_c of 41%) was restricted, an expected outcome taking into consideration the limited performance of known PETases towards depolymerization of PET substrates with x_c of 20% or higher, as documented in literature.[50] Specifically, *Is*PETase degraded the material releasing 7.77 $\mu g_{products}/mg_{PET}$ of hydrolysis products, just 3-fold higher than *Se*1JFR (2.75 $\mu g_{products}/mg_{PET}$), while *Pf*PHOase and *Da*PUase led to the release of 0.11 and 0.04 $\mu g_{products}/mg_{PET}$, respectively. Upon initial inspection, it is evident that the mass loss results and the accompanied hydrolysis products release do not align. So, even though *Pf*PHOase and *Da*PUase action led to the highest dry mass decrease of aPET (5.88%) and cPET (1.94%), respectively, the concentration of the released hydrolysis products was relatively low compared to *Is*PETase and *Se*1JFR, in both PET samples.

It is possible that such phenomenon could be correlated with the mechanism of each enzyme. For instance, *Pf*PHOase and *Da*PUase degradation capability of PET, might also lead to the release of other water-soluble oligomers, which cannot be detected through the performed analysis method, but could contribute to distinct mass loss. In parallel, *Is*PETase and *Se*1JFR, which appear to be the most active in PET degradation, through the release of TPA and MHET, might mainly lead also to the release of high concentrations of insoluble oligomers of higher molecular weights, a fact justifying the slight mass decrease of the samples but their significant PETase activity. In such case the synergistic effect of these enzymes with an MHETase could lead to a higher degradation performance.[51,52]

With a primary focus on the released hydrolysis products, it is concluded that the investigated enzymes can be divided in two groups, exhibiting analogous results in non-biodegradable PET degradation (*Is*PETase and *Se*1JFR, *Da*PUase and *Pf*PHOase). This grouping mirrors the previously observed patterns in the degradation of biodegradable polymers. *Se*1JFR was previously characterized as a PETase-like enzyme given its high homology with *Is*PETase and other PET degrading esterases. Hereby, we observed that *Se*1JFR presents comparable results in low and semi-crystalline PET degradation with *Is*PETase, the only well-known mesophilic PETase in literature, rendering *Se*1JFR as an interesting and promising platform for PET degradation in mild temperatures. However, SM14est, which shares high sequence homology with *Se*1JFR, presents superior PET activity, leading to same degradation level of crystalline PET powder utilizing 10-fold less enzyme concentration than *Se*1JFR, in different reaction conditions, upon addition of 0.5 M sodium chloride at 45°C.[34]

Considering polyether PU, all the polyesterases exhibited evidence of material degradation, resulting in mass losses ranging from 10% to 20%. While the degree of degradation among the enzymes did not vary radically, *Se*1JFR stood out as the polyesterase with a statistically significant difference in mass reduction, measuring at 19%. The fact that polyether PU, which encompasses not only ether bonds but also urethane bonds, presented indications of degradation by all enzymes, points out the ability of polyesterases to cleave urethane bonds. This aligns with existing literature, where esterases have been demonstrated to break the C-O bond of urethane moieties, emphasizing their versatility.[53,54] Surprisingly, in total, enzymatic polyether PU degradation was more feasible than that of PLA, which is a biodegradable polyester. However, irrespective of PLA's polyester nature, it is established that proteases are the powerhouse of its degradation, surpassing the efficiency of esterases and cutinases.[5]

In general, all investigated enzymes demonstrated indications of polyesters' degradation. The novel enzyme, *Se*1JFR, exhibited the most

efficient overall results, particularly in the degradation of PBS, PCL, and polyether PU. *Da*PUase, previously proposed by literature for polyester PU degradation,[55] exhibited limited efficiency in polyether PU degradation. This outcome suggests that the enzyme may primarily break down ester bond in polyester polyurethanes, with reduced capability for urethane bond cleavage in polyether polyurethanes. Moreover, *Da*PUase presented major results in PCL degradation as well as indications of PET degradation.

Meanwhile, *Pf*PHOase showed restricted results in PHB degradation, a result which aligns with Schirmer et al.,[35] who reported that native *Pf*PHOase exhibited limited degradation of PHB, PHV, and their co-polymers. While, to our knowledge, *Pf*PHOase has not been further investigated in other synthetic plastics' degradation, our research highlighted the potential of the enzyme also in PCL and PLA degradation, with notable alterations in materials' mass and molecular weight, respectively. A similar profile has been reported for *Lt*PHBase, a PHB depolymerase which, as aforementioned, shares low sequence homology, but high structural similarity with *Pf*PHOase. Utilizing a turbidometric assay, *Lt*PHBase was proved capable of depolymerizing both PLA and PCL.[42]

Notably, *Is*PETase, recognized as a mesophilic PETase, displayed noteworthy outcomes not only in PET degradation but also in the degradation of PCL and PLA, highlighting its esterolytic activity across multiple polyester substrates. Whereas PCL depolymerization by *Is*PETase has been previously documented,[29] our study marks the first report of PLA degradation by it. In contrast to Austin et al.,[28] who were unable to observe PLA degradation by *Is*PETase through SEM analysis, our research documented the enzymatic degradation of the material through reduction of both molecular weights, using GPC. Overall, it seems that *Is*PETase and *Se*1JFR, exhibited the most promising results, a fact highlighting the potential of PETases in various synthetic polymers' degradation.

4 | CONCLUSIONS

This study delves into the structural and biochemical analysis of four bacterial polyesterases—specifically, *Da*PUase, *Is*PETase, *Pf*PHOase, and *Se*1JFR. The novel lipase-like *Se*1JFR presented substantial structural and sequence homology with *Is*PETase, a benchmark mesophilic PETase. Despite their distinct bacterial origin, both enzymes were classified in a distinct clade of characterized PETases/polyesterases. Conversely, *Da*PUase, a proposed from the literature polyester PU degrader, did not present any similarities with known polyesterases. Structural wise, the enzyme exhibited three hydrophobic regions around its catalytic site, one of which is homologue to the binding domain of a PHA depolymerase. *Pf*PHOase, a PHO depolymerase, displayed sequence similarities with couple bacterial PHA depolymerases. Both, *Da*PUase and *Pf*PHOase, created their own sub-branches in the phylogenetic tree. Biochemical characterization of the polyesterases demonstrated a mesophilic profile of *Is*PETase and *Se*1JFR and a slight thermophilic behavior of *Da*PUase and *Pf*PHOase. All hydrolases presented indications of various polyesters breakdown, proving the wide polymer degradation spectrum of bacterial hydrolases. While, no clear substrate specificity was elucidated, *Se*1JFR stood out in degradation performance of most investigated polyesters, particularly PCL, PBS, PLA, and polyether PU. Additionally, the enzyme exhibited comparable results to *Is*PETase in the degradation of semi-crystalline PET, positioning *Se*1JFR as an intriguing platform for PET degradation at moderate temperatures.

CREDIT AUTHORSHIP CONTRIBUTION STATEMENT

Konstantinos Makryniotis: Methodology, Validation, Formal analysis, Investigation, Writing – original draft, Writing – review & editing, Visualization. Efstratios Nikolaivits: Conceptualization, Methodology, Validation, Investigation, Writing – original draft, Writing – review & editing, Visualization, Supervision, Project administration. George Taxeidis: Methodology, Validation, Formal analysis, Investigation. Jasmina Nikodinovic-Runic: Methodology, Resources, Supervision, Funding acquisition. Evangelos Topakas: Conceptualization, Resources, Writing – review & editing, Supervision, Project administration, Funding acquisition.

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CONFLICT OF INTEREST STATEMENT

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

DATA AVAILABILITY STATEMENT

The authors declare that the data supporting the findings of this study are available within the paper and its Supplementary Information files. Should any raw data files be needed in another format they are available from the corresponding author upon reasonable request.

ETHICAL APPROVAL DECLARATION

This article does not contain studies with human participants performed by any of the authors.

ORCID

Evangelos Topakas https://orcid.org/0000-0003-0078-5904

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