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Marine Bacteria as a Source of Polyester-Degrading Enzymes with Biocatalytic Potential

Thesis presented by

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for the degree of

Doctor of Philosophy

University College Cork

School of Microbiology

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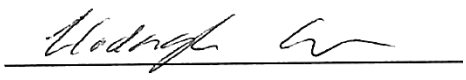
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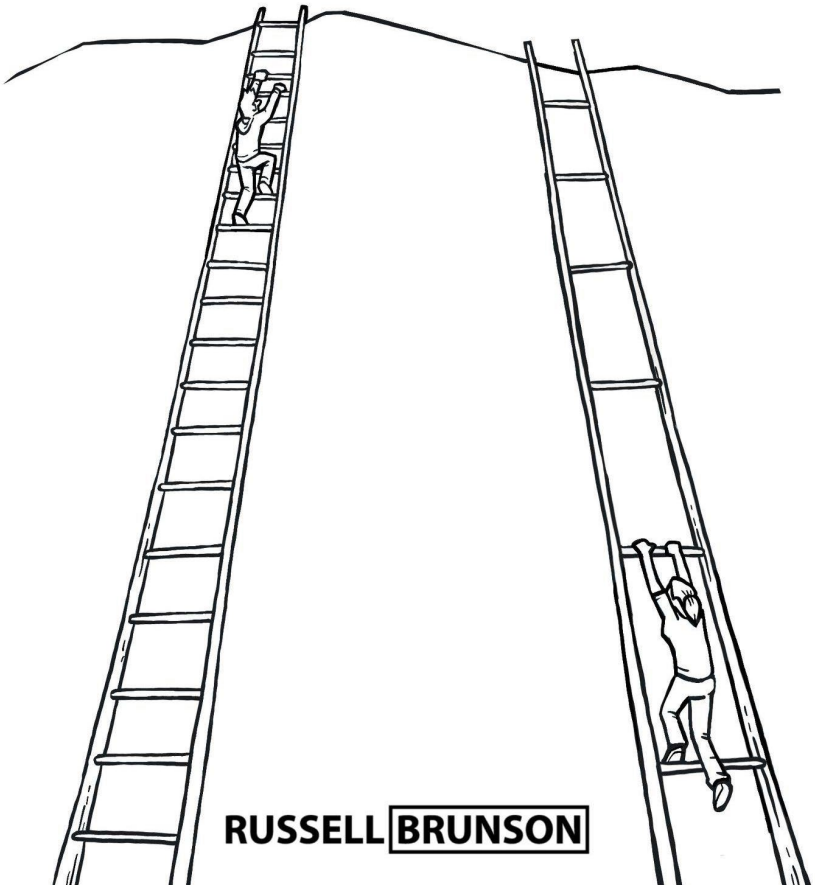
Declaration

"This is to certify that the work I am submitting is my own and has not been submitted for another degree, either at University College Cork or elsewhere. All external references and sources are clearly acknowledged and identified within the contents. I have read and understood the regulations of University College Cork concerning plagiarism and intellectual property."



Clodagh Carr

The Power of Small Steps



Acknowledgements

What's a journey without the people you meet along the way?

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Abstract

The issue of plastic waste continues to surge, as does our collective awareness of this global problem and interest in finding sustainable solutions for its control. Bacteria, which are among the earliest known forms of life, have evolved over millions of years to degrade organic matter found in the environment by producing enzymes that can catalyse the breakdown of various compounds for energy and nutrient gain. With biotechnological advances in recent decades, bacterial enzymes have emerged as a tool for the catalysis of chemical reactions, where they can aid in the development of safer, more efficient, and more environmentally friendly processes. The conventional recycling of plastic waste has typically consisted of a thermo-mechanical process, where waste is ground down, melted, and reformed into new, but lower-quality products that are less likely to be recycled multiple times. While alternative chemical methods can improve recyclability by facilitating recovery of the raw materials used to make plastic, enzyme-based treatments enable the same process to be conducted under milder, less energy-intensive reaction conditions without the use of hazardous reagents and solvents. Enzymes that are specialized to break apart ester bond-containing compounds in nature can be employed for the degradation of polyester-based plastics, allowing for the sustainable recycling of these materials after use. Polyester-degrading enzymes (termed polyesterases) have been studied with a particular focus on the recycling of polyethylene terephthalate (PET), a synthetic polyester which is mass-produced for use in food and beverage packaging or as a fiber in the textile industry. By an enzymatic hydrolysis mechanism, the PET polymer may be degraded into its short-chain oligomeric intermediates MHET and BHET and/or its constituent monomers ethylene glycol (EG) and terephthalic acid (TPA) which can subsequently be recycled into PET or upcycled into value-added products.

In chapter 1, a literature review was conducted on microbial PET hydrolase enzymes to assess existing knowledge in the field, identify key challenges, and determine important areas for future research. Thermophilic bacteria from the phylum Actinomycetota such as *Thermobifida fusca* emerged as a major source of PET-hydrolyzing enzymes as well as those with fungal and metagenomic origins, while *Ideonella sakaiensis*, a mesophilic bacterium isolated from a PET-contaminated site served as a model system featuring both PET and MHET hydrolases predicted to work in tandem. Relatively few PET-hydrolyzing enzymes were reported from marine environments, an aspect which we hoped to expand upon. In chapter 2, activity screening and genome mining of marine bacterial isolates led to the identification of a

polyesterase, BgP, from a deep-sea, marine sponge-derived *Brachybacterium* sp. isolate. BgP was explored as a structural homolog of cutinase-like enzymes, such as Cut190, LCC, and TfCut2, which had previously been reported for efficient hydrolysis of PET and its hydrolytic activity was confirmed on the PET model substrate polycaprolactone (PCL). In chapter 3, PET-hydrolyzing activity was confirmed for SM14est, a marine sponge-derived polyesterase from *Streptomyces* sp. SM14 with this enzyme exhibiting a preference for high-salt conditions and moderate temperatures (up to 45°C). In chapter 4, MarCE, a marine carboxylesterase was found encoded in the genome of a *Maribacter* sp. isolated from a sea sponge sample collected at Lough Hyne. MarCE was shown to hydrolyze polycaprolactone diol and putative binding of PET oligomers was demonstrated by molecular docking analysis. The work presented on BgP, SM14est, and MarCE makes a case for the continued exploration of marine-derived bacteria, in particular those found within the unique marine sponge ecosystem, as a source of potentially novel polyesterases with relevance for the biological degradation of synthetic polyesters among other biocatalytic applications.

Chapter 1

Microbial Polyethylene Terephthalate Hydrolases: Current and Future Perspectives

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1. Abstract

Plastic has rapidly transformed our world, with many aspects of human life now relying on a variety of plastic materials. Biological plastic degradation, which employs microorganisms and their degradative enzymes, has emerged as one way to address the unforeseen consequences the waste streams that have resulted from mass plastic production. The focus of this review is microbial hydrolase enzymes which have been found to act on polyethylene terephthalate (PET) plastic. The best characterized examples are discussed together with the use of genomic and protein engineering technologies to obtain PET hydrolase enzymes for different applications. In addition, the obstacles which are currently limiting the development of efficient PET bioprocessing are presented. By continuing to study the possible mechanisms and the structural elements of key enzymes involved in microbial PET hydrolysis, and by assessing the ability of PET hydrolase enzymes to work under practical conditions, this research will help inform large-scale waste management operations. Finally, the contribution of microbial PET hydrolases in creating a potential circular PET economy will be explored. This review combines the current knowledge on enzymatic PET processing with proposed strategies for optimization and use, to help clarify the next steps in addressing pollution by PET and other plastics.

2. Introduction

The use of plastics has become an integral part of modern society, with annual production exceeding 350 million metric tons (Danso et al., 2019; PlasticsEurope, 2019). Polyethylene terephthalate (PET), a crude-oil derived synthetic polymer, is currently one of the most extensively used plastics (Liu et al., 2019). In 2017, PET production capacity reached over 30 million tons per annum (PlasticsInsight, 2017). Approximately 485 billion PET bottles were produced in 2016, and an estimated 583.3 billion plastic bottles are predicted to be manufactured in 2021 (Garside, 2019). This polymer consists of repeating units of terephthalic acid (TPA) and ethylene glycol (EG) (Figure 1). Due to its light weight, durability and moldability, PET is convenient both in terms of its production and its utility; where it is used in containers, films, and fibers, in addition to bottles. However, the qualities which have made plastic an attractive resource, are equally as responsible for the damage that is typically caused once it becomes waste. Mismanagement of this PET-based material has resulted in its frequent disposal and accumulation in the environment where its robustness is problematic, as it is not particularly susceptible to biodegradation. The high stability of the polymer's backbone, together with its crystallinity and surface hydrophobicity are some of the underlying factors

which restrict the natural breakdown of this plastic (Kawai et al., 2019; Liu et al., 2019; Jaiswal et al., 2020).

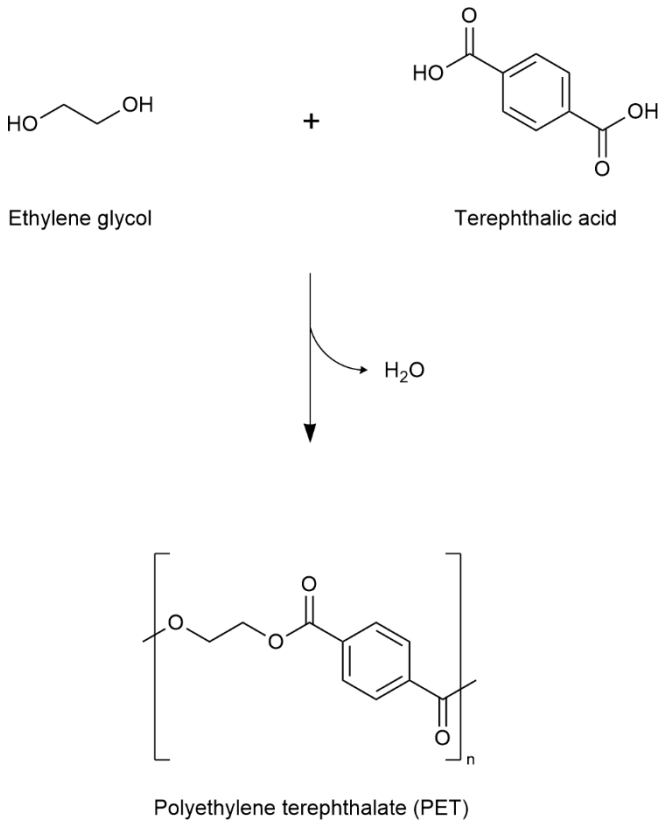


Figure 1. Structure of PET polymer. Polycondensation reaction of ethylene glycol (EG) and terephthalic acid (TPA) monomers gives polymeric polyethylene terephthalate (PET), with water as a byproduct.

As the primary use for plastics is packaging, PET is often used in short-term, single use applications. Despite the fact that recycling systems are in place, over half of the PET that is produced escapes collection, and instead ends up in landfills or is released into nature. Only a very limited amount of PET is recycled back into its original form, with the majority being downcycled into lower quality, non-recyclable plastics (Kawai et al., 2014). PET waste persists in both marine and terrestrial environments, which often results in harm or death of some of the inhabitants. Annually, plastic pollution is considered responsible for the death of around 1 million sea birds, as well as 100,000 marine mammals and turtles (Jaiswal et al., 2020). Furthermore, the partial breakdown of discarded plastics and treatment of synthetic fibers has resulted in the widespread shedding of microplastics and microfibers, respectively (Hiraga et al., 2019). In this form, plastics readily contaminate marine ecosystems that has resulted in them entering the animal and human food chain, which has been linked to various adverse health

effects, including immune disorders and congenital disabilities, as well as cancer (Jaiswal et al., 2020). It is now believed that soils may constitute an even larger sink for plastic, and since microplastics can readily leach into groundwater, or contaminate other water reserves, this problem is thought to affect organisms across the entire terrestrial ecosystem (Song et al., 2019). Recently, there has been a marked increase in awareness surrounding the potential detrimental environmental impact of plastics. Therefore, new solutions and technologies, which assist in managing plastic waste, are of major importance (Danso et al., 2019).

Synthetic polymers were once considered resistant to microbial degradation, however more recent studies have shown that certain microbes produce hydrolase enzymes that enable them to either break down or modify PET. Microbes have evolved a variety of hydrolytic enzymes that allow them to degrade and process polymers that are prevalent in nature, for example the waxy plant polyester cutin. In a somewhat similar way, some microorganisms are using enzymes to take advantage of PET as an energy source, despite the significant differences between synthetic and natural polymer structures (Danso et al., 2019; Kawai et al., 2019; Taniguchi et al., 2019).

Enzymes which display PET hydrolyzing activities include carboxylic ester hydrolase enzymes (EC 3.1.1) such as cutinases, lipases, and esterases (Guebitz and Cavaco-Paulo, 2008; Kawai et al., 2020). These hydrolases have been isolated from bacterial sources such as *Ideonella sakaiensis* and *Thermobifida fusca* as well as from fungi such as *Fusarium solani*, *Humicola insolens*, and *Aspergillus oryzae* (Wang et al., 2008; Korpecka et al., 2010; Zimmermann and Billig, 2010). They are typically serine hydrolases and are characterized by a catalytic triad in their active site that consists of serine, histidine, and aspartate amino acids and an α/β hydrolase fold (Remington et al., 1992; Wei et al., 2014a). Most of the PET hydrolases that have been functionally verified also contain a C-terminal disulfide bond which is responsible for promoting thermal and kinetic stability (Roth et al., 2014; Sulaiman et al., 2014; Then et al., 2016). In general, carboxylic ester hydrolases are defined by their activity on *p*-nitrophenol (*p*-NP) acyl esters, whereas lipases act on medium to long-chain acyl esters (>C_{10}), cutinases on short to medium-chain acyl esters (up to C_8-C_{10}), and esterases on short-chain acyl esters. Although PET hydrolytic activities are not correlated to *p*-NP acyl ester specificity, these substrates are useful for biochemical characterization of enzymes of interest (Kawai et al., 2020).

PET hydrolytic enzymes are generating a good deal of interest from a biotechnological perspective, particularly with respect to their potential applications in areas such as biorecycling, biocatalysis, waste treatment and sustainable polymer modifications. Cutinases (EC 3.1.1.74) and cutinase-like enzymes, which are capable of processing high molecular weight polyesters, have become an important focus of PET hydrolase studies (Taniguchi et al., 2019). Numerous PET hydrolyzing enzymes have been biochemically characterized to date and are shown (Table 1). Actinomycetes, especially *Thermobifida* species, are of interest for their PET hydrolytic cutinases and will be among the examples discussed. *Ideonella sakaiensis*, which was discovered in PET-contaminated sediment that was sampled near a Japanese recycling facility, combines a cutinase-like hydrolase enzyme termed "PETase" with mono-(2-hydroxyethyl) terephthalate degrading "MHETase" to enable the use of PET as a carbon and energy source (Yoshida et al., 2016; Taniguchi et al., 2019). Strictly speaking, cutinases are defined by hydrolysis of cutin, but this is often left undetermined as cutin is not commercially available. Cutin degradation is not crucial for synthetic polyesterase activity, and PET enzymes are often categorized as cutinases, based on showing near-identical structures in crystallographic studies (Kawai et al., 2020). Cutinases have been shown to hydrolyze cutin and various polyesters without the help of cofactors, under temperature conditions of 40-70°C and pH 7-9 (Furukawa et al., 2019).

Table 1. Native microbial PET hydrolytic enzymes that have been biochemically characterized and that are of known amino acid sequence.

Enzyme	Microbial Source	NCBI Accession Number or PBD Code	Rxn Temp (°C)	Substrate (Crystallinity)	Reported Degrad.	Ref.
BsEstB	*Bacillus subtilis* 4P3-11	ADH43200.1	40-45	3PET	TPA, MHET release	(Ribitsch et al., 2011)
Cut190	*Saccharomonospora viridis* AHK190	BAO42836.1	60-65	Amorphous PET film and package-grade PET	TPA, MHET release	(Kawai et al., 2014)
FsC	*Fusarium solani pisi*	1CEX	30-60	lcPET (7%) and bo-PET (35%)	5% lcPET weight loss	(Griswold et al., 2003; Silva et al., 2005; Eberl et al., 2009; Ronkvist et al., 2009; Yoshida et al., 2016)
HiC	*Humicola insolens*	4OYY	30-85	lcPET (7%) bo-PET (35%)	97 ± 3% lcPET weight loss	(Ronkvist et al., 2009; Ribitsch et al., 2012a)
***Is*PETase**	*Ideonella sakaiensis* 201-F6	GAP38373.1	20-45	lcPET (1.9%) and bottle-grade hcPET	TPA, MHET, EG release	(Yoshida et al., 2016)
LCC	Uncultured bacterium (from leaf-branch compost metagenome)	AEV21261.1	50-70	Amorphous PET film	≤25% weight loss	(Sulaiman et al., 2012; Sulaiman et al., 2014; Yoshida et al., 2016; Shirke et al., 2018)
PE-H	*Pseudomonas aestusnigri*	6SBN	30	Amorphous PET film	MHET release	(Bollinger et al., 2020)
PET12	Polyangium brachysporum	A0A0G3BI90	50	PET nanoparticle agar	Zone of clearance	(Danso et al., 2018)
PET2	Uncultured bacterium (marine metagenome)	C3RYL0	50	PET nanoparticle agar	Zone of clearance	(Danso et al., 2018)
PET5	*Oleispira antarctica* RB-8	R4YKL9	50	PET nanoparticle agar	Zone of clearance	(Danso et al., 2018)
PET6	*Vibrio gazogenes*	UPI0003945E1F	50	PET nanoparticle agar	Zone of clearance	(Danso et al., 2018)
Tcur0390	*Thermomonospora curvata* DSM 43183	CDN67546.1	50	PET nanoparticle suspension	Reduced turbidity	(Wei et al., 2014a)

Tcur1278	*Thermomonospora curvata* DSM 43183	CDN67545.1	50-60	PET nanoparticle suspension	Reduced turbidity	(Wei et al., 2014a)
TfCut1	*Thermobifida fusca* KW3	CBY05529.1	55-65	lcPET film	≤11% weight loss	(Then et al., 2015)
TfCut2	*Thermobifida fusca* KW3	CBY05530.1	55-65	lcPET film	≤12% weight loss	(Then et al., 2015)
TfH	*Thermobifida fusca* DSM43793	WP_011291330.1	55	Bottle-grade PET (10%)	≈ 50% weight loss	(Müller et al., 2005; Chen et al., 2008; Eberl et al., 2009; Silva et al., 2011)
Tha_Cut1	*Thermobifida alba* DSM43185	ADV92525.1	50	3PET	TPA, HEB, MHET release	(Ribitsch et al., 2012a)
Thc_Cut1	*Thermobifida cellulosilytica* DSM44535	ADV92526.1	50	3PET and PET film (37%)	MHET, TPA, HEB release	(Herrero Acero et al., 2011b)
Thc_Cut2	*Thermobifida cellulosilytica* DSM44535	ADV92527.1	50	3PET and PET film (37%)	MHET, TPA, HEB release	(Herrero Acero et al., 2011b)
Thf42_Cut1	*Thermobifida fusca* DSM44342	ADV92528.1	50	3PET and PET film (37%)	MHET, TPA, HEB release	(Herrero Acero et al., 2011b)
Thh_Est	*Thermobifida halotolerans* DSM44931	AFA45122.1	50	3PET	TPA, HEB, MHET release	(Ribitsch et al., 2012b)

Genomic and metagenomic strategies, which allow both genomic and gene expression analysis of unculturable and culturable microbes, have broadened the extent to which genetic information can be explored (Handelsman, 2004; Parages et al., 2016). The ever-increasing number of genomic and metagenomic datasets has led to the identification of genes of interest, including those encoding cutinases and PET hydrolase homologs (Hiraga et al., 2019). For example, functional screening of a leaf-branch compost metagenome enabled the isolation of LC-cutinase, a cutinase homolog with PET-degrading activity (Sulaiman et al., 2012). A metagenome mining approach that searches genome and metagenome databases has also recently been used to identify four novel PET hydrolase genes (Danso et al., 2018).

Genetic and protein engineering tools have commonly been used to increase the plastic degradation capacity of microorganisms and their enzymes, respectively (Wilkes and Aristilde, 2017; Jaiswal et al., 2020). For example, recombinant DNA techniques have enabled the expression of genes for pollutant degradation in a host that is better suited towards enzyme

production, thus allowing purification of large quantities for application or further study. Substrate range and activity can be further enhanced using technologies such as site-directed mutagenesis. At the protein level, enzyme engineering has been employed to change or modify the protein's amino acid sequence, which can lead to increased activity and tolerance to reaction conditions. The increased availability of enzyme structural information has greatly facilitated rational engineering approaches (Sharma et al., 2018a; Jaiswal et al., 2020). For example, by introducing mutations, LC-cutinase, actinobacterial TfCut2, and *Is*PETase variant enzymes have been generated, amongst others, and have displayed improved PET hydrolyzing activities, owing to factors such as optimized catalytic properties and the relief of product inhibition (Wei et al., 2016; Kawai et al., 2020; Tournier et al., 2020). In this review, microbial enzymes for PET hydrolysis will be examined, along with the current associated bottlenecks and considerations for progressing towards practical applications.

3. Microbial Sources of PET-Hydrolyzing Enzymes

3.1 *Ideonella sakaiensis* Enzymes

A team of Japanese researchers, based in the Kyoto Institute of Technology and Keio University, have proposed three strategies that could be employed in the bioremediation and bio-recycling of PET waste together with other potential applications including bioconversions, microplastic, and microbead degradation, as well as PET-surface treatment (Taniguchi et al., 2019).

The first strategy is based on a microbial consortium named "no. 46", which consists of bacteria, protozoa, and yeast-like cells, as revealed by light microscopy. This was isolated following extensive screening of environmental samples for PET-degrading microorganisms. The group screened wastewater, activated sludge, soil, and sediment from the site surrounding a PET bottle recycling plant (Taniguchi et al., 2019). The microbial consortium was found to both degrade PET and assimilate the degradation products into CO_2 and water (Yoshida et al., 2016). No. 46 was also shown to adhere to PET film and create significant changes in its morphology, which could be visualized as film decay and/or surface whitening. PET film degradation occurred under ambient temperature conditions at a rate of 0.13 mg/cm^2/day, with 75% of the carbon being catabolized (Yoshida et al., 2016). Furthermore, microbial consortium no. 46 was shown to retain PET degradation activity for at least 10 weeks and could be re-cultivated after freezing without losing activity. Of the estimated 20 types of bacteria within the consortium that have been identified, the following were investigated for their individual roles in the degradation

process: *Bacillus megaterium,* which creates a biofilm on the PET film surface; *Rhizopus* sp., which acts within the biofilm to cleave the ester linkages of the PET polymer to give BHET; *Pseudomonas* sp., which further degrades BHET (bis-(2-hydroxyethyl)-terephthalate) into the monomers TPA and EG; and *Pigmentiphaga* sp. and *Mycobacterium* sp., which assimilate TPA and EG, respectively (Taniguchi et al., 2019).

Ideonella sakaiensis 201-F6, a bacterial strain which was later isolated from the consortium, provides the basis of the next degradation system (Tanasupawat et al., 2016). This bacterium is PET-lytic and its growth on minimal medium containing PET film has been shown to be much greater than on control medium without PET (Taniguchi et al., 2019). When grown in liquid culture, detection of PET hydrolysates was negligible, demonstrating the ability of *Ideonella sakaiensis* to completely degrade and assimilate PET with CO_2 as a complete oxidation product. The rate of *I. sakaiensis* degradation was around twice that of the microbial consortium no. 46 from which it was isolated. These *I. sakaiensis* bacterial cells were shown to adhere onto PET film via appendages that may also facilitate enzyme delivery during the attack (Taniguchi et al., 2019).

The final approach is based on employing novel enzymes identified in *I. sakaiensis*. A single open reading frame sharing 51% amino acid sequence identity with TfH, a known *Thermobifida fusca* PET hydrolase, was identified in the *Ideonella sakaiensis* genome (Müller et al., 2005; Taniguchi et al., 2019). The corresponding recombinant protein released PET degradation products into aqueous medium and PET film exhibited crater-like pitting when exposed to the enzyme (Taniguchi et al., 2019). This *I. sakaiensis* cutinase-like enzyme, referred to as "PETase" (or *Is*PETase), was subsequently found to have the highest catalytic preference for PET, when compared with known PET hydrolytic enzymes such as TfH, *F. solani* fungal cutinase (FsC) (Silva et al., 2005) and LC-cutinase (LCC) (Sulaiman et al., 2012). These enzymes were assessed in terms of their activity against PET film and against highly crystallized, bottle-derived commercial PET (hc-PET), when incubated for 18 h at 30°C. When compared on this PET film, the activity of *Is*PETase was determined to be 120, 5.5 and 88 times as high as that of TfH, LCC and FsC, respectively (Yoshida et al., 2016). The efficiency and specificity of *Is*PETase with regards PET hydrolysis makes it a promising candidate for new biodegradation strategies (Taniguchi et al., 2019). However, it is important to consider that *Is*PETase operates well under moderate temperature conditions, whereas the other enzymes included in this study are optimally active at higher temperatures due to their thermophilicity.

The primary product of *Is*PETase hydrolysis is mono(2-hydroxyethyl) terephthalate (MHET), which is broken down into the monomers, terephthalate (TPA) and ethylene glycol (EG), by a second *I. sakaiensis* enzyme called MHETase. This MHETase is a member of the tannase enzyme family and has been shown to degrade MHET into TPA and EG, with a catalytic efficiency (k_{cat}/K_m) of 4200 ± 370 s^{-1} mM^{-1}. Interestingly, MHETase displayed little activity on other ester compounds. Both PETase and MHETase enzymes were assigned new Enzyme Commission (EC) numbers, 3.1.1.101, and 3.1.1.102, respectively, and their role in a PET metabolic model was subsequently proposed (Figure 2). *Is*PETase, which acts extracellularly, first converts PET into oligomers (mainly MHET). PET hydrolysates are then transported, through an outer membrane protein (e.g., porin) into the periplasmic space and further hydrolyzed by MHETase into PET monomers (Taniguchi et al., 2019). MHETase is predicted to be a lipoprotein, which is likely to be anchored in the outer membrane. A lid domain, which confers its specificity and activity towards MHET, has also been proposed (Yoshida et al., 2016). A TPA transporter coupled with a TPA-binding protein is likely to be responsible for taking TPA up into the cytoplasm (Hosaka et al., 2013). It is thought that TPA subsequently enters the central tricarboxylic acid (TCA) cycle via protocatechuic acid, while EG is also catabolized in the TCA cycle via glyoxylic acid (Mückschel et al., 2012).

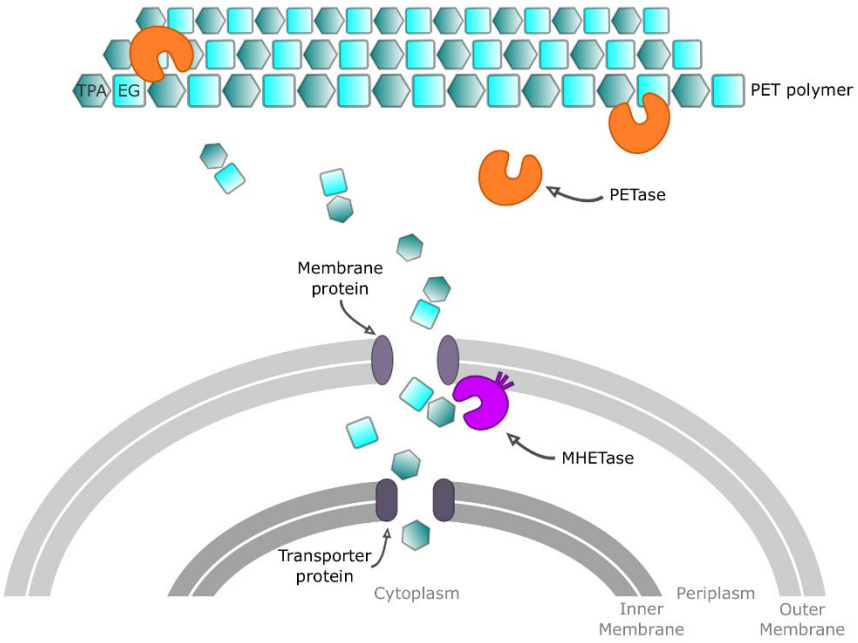


Figure 2. Proposed mechanism for PET processing in *Ideonella sakaiensis*. Extracellular PETase enzyme acts on PET polymer chains to give oligomeric mono-(2-hydroxyethyl) terephthalate (MHET), the monoester of terephthalic acid (TPA) and ethylene glycol (EG). Intermediates are transported through a membrane protein before reaching MHETase, which degrades MHET into TPA and EG. Finally, monomers are transported into the cytoplasm to be metabolized.

Structural analysis is important in providing useful information for subsequent protein engineering approaches, which may help in redesigning enzymes to make them more amenable for industrial applications (Taniguchi et al., 2019). Several groups have elucidated the crystal structure of *Is*PETase, revealing features shared by lipases (EC 3.1.1.3) and cutinases (EC 3.1.1.74), along with unique characteristics that differentiate the enzyme from cutinases previously recognized for PET hydrolysis (Han et al., 2017; Austin et al., 2018; Fecker et al., 2018; Joo et al., 2018; Liu et al., 2018). *Is*PETase has been expressed recombinantly from a codon-optimized gene in various *E. coli* host strains to facilitate generation of the crystal structure. The enzyme has a basic structure that resembles other PET hydrolyzing enzymes, featuring an α/β hydrolase fold and a nucleophile-His-acid catalytic triad. The α/β serine hydrolase family displays a catalytic mechanism based on a nucleophilic attack on the substrate's ester bond by serine, which is activated via the other triad residues (Taniguchi et al., 2019). This involves serine linking to the ester carbonyl group to form a covalent tetrahedral intermediate that is later stabilized by two hydrogen bonds between amide bonds of the residues in a nearby oxyanion hole (Jaeger et al., 1994; Chen et al., 2013). In the case of *Is*PETase, the α/β hydrolase fold consists of nine β-strands that form a twisted central β-sheet conformation, surrounded by six α-helices (Chen et al., 2018), while Ser160-His237-Asp206 comprise the catalytic triad residues (Joo et al., 2018; Liu et al., 2019).

Upon structural comparison with known PET hydrolytic enzymes, including TfCut2 and LCC, *Is*PETase exhibits a notably wider active site and has an elongated substrate binding cleft, which is composed of two subsites (I and II). Four MHET moieties bind at subsites I, IIa, IIb and IIc during PET degradation (Joo et al., 2018). Site I contains a cleavage site where ester bond breakage occurs. The enzyme also has an additional disulfide bond in its active site. The distinguishing features of *Is*PETase are detailed below (Taniguchi et al., 2019):

1. In subsite II, a pair of Trp159-Ser238 residues ensure there is adequate space for the substrate to bind. PET binding is obstructed by residues at the corresponding site in cutinases (e.g.,

His169-Phe249 pair in TfCut2) (Taniguchi et al., 2019). The width of the *Is*PETase substrate binding pocket has been reported to be crucial for effective hydrolysis of crystallized PET (Liu et al., 2018).

2. *Is*PETase has an extended connecting loop due to three additional residues, Ser245, Asn246 and Gln247. This also creates more space for PET to bind (Taniguchi et al., 2019).

3. *Is*PETase has two disulfide bonds, 1 and 2 (Taniguchi et al., 2019). Previously studied cutinases only possess disulfide bond 1, with disulfide bond 2 being unique to *Is*PETase. This extra disulfide bond connects the alpha and beta loops which contain the catalytic triad. It is also responsible for higher active site flexibility compared to PET-active cutinases (Taniguchi et al., 2019). This flexibility enables *Is*PETase to accommodate the rigidity of the PET substrate without impairing the enzyme's structural integrity. Removal of disulfide bond 2 results in reduced activity and weakening of the catalytic triad (Fecker et al., 2018).

Following a study based on structural and biochemical analyses of *Is*PETase, Joo and colleagues have proposed a detailed molecular mechanism for this enzyme (Joo et al., 2018). A four-MHET molecule called 2-HE(MHET)$_4$ was used to mimic PET in a covalent docking model to facilitate elucidation of the substrate binding mode. At the catalytic center, triad residue Ser160 functions as a covalent nucleophile which acts on the carbonyl carbon atom of the ester bond, as seen in other carboxylesterase enzymes. The nitrogen atoms of amino acids Tyr87 and Met160 form an oxyanion hole which stabilizes the tetrahedral intermediate of the reaction. This stimulates the substrate binding site to form a long, shallow cleft, which is primarily hydrophobic. The first MHET moiety is bound to subsite I, while the second, third, and fourth moieties are accommodated by subsite II. Binding is mediated and stabilized largely by hydrophobic interactions, although certain residues also lead to polar and π–π interactions. Various site-directed mutagenesis experiments were used to verify the key residues in catalysis and substrate binding. This group also presented a molecular mechanism for the degradation of PET, which involves two steps: nick generation and terminal digestion. Once the secreted *Is*PETase has bound to the PET surface via its hydrophobic substrate binding cleft, the scissile ester bond is positioned closer to the nucleophilic Ser160. This enables the cleavage of one ester bond, creating a nick in PET and resulting in two PET chains with different terminals (TPA-terminal and HE-terminal), that are then digested into MHET monomers. As there are some variations in the digestion process depending on the terminal type, and because the digestions occur in a combinatorial manner, MHET, TPA, BHET and EG accumulate, with

BHET undergoing further degradation into MHET and EG. Interestingly, although *Is*PETase does not hydrolyze MHET to TPA and EG, a significant amount of TPA accumulates during the degradation reaction, which is believed to be related to digestion of the TPA-terminal described in this mechanism (Joo et al., 2018).

Furthermore, Chen and co-workers summarized recent crystallographic studies, in addition to their own work, to help uncover the key catalytic features of *Is*PETase and information regarding its underlying mechanism of action (Chen et al., 2018). Residue Trp156, which is found near the catalytic center, is thought to have a central role in binding the PET substrate. Although Trp156 is highly conserved across PET hydrolyzing enzymes, "Trp156 wobbling", whereby the residue displays three different conformations, is exclusive to *Is*PETase. In this enzyme, "Trp156 wobbling" is coupled to Ser185, which corresponds to a bulky His residue in homologous structures, that would usually prevent Trp from adopting more than one conformation. A variant created by mutating Ser to His at position 185 resulted in decreased activity and indicated that the performance of *Is*PETase is at least partially influenced by the smaller Ser185 residue, as it allows more space for Trp156 to rotate (Han et al., 2017; Chen et al., 2018). Using substrate and product analogs of PET to study the substrate-binding mode, the group have also proposed a mechanism of action for *Is*PETase whereby the enzyme first forms a shallow, superficial cleft, within which "Trp156 wobbling" takes place. Next, primarily via hydrophobic interactions, PET substrate binds with its carbonyl group positioned at the catalytic center and its O atom facing the oxyanion hole, while the Trp156 indole ring interacts with the aromatic TPA moiety of PET. Then, successive formation of acyl-enzyme intermediate and cleavage of the ester bond in a nucleophilic attack by water (hydrolytic reaction). Finally, the product is rotated and released from the catalytic center following interaction of the benzoic acid group with Trp156 (Chen et al., 2018).

With respect to PET hydrolase enzymatic mechanisms, while much progress has been made, the exact details remain to be conclusively determined. In response to (Joo et al., 2018), another group have argued that the flexible oligomeric 2-HE(MHET)$_4$ substrate employed in this study is not representative of the highly rigid PET polymer chain, and it is unlikely that the *Is*PETase catalytic mechanism involves simultaneous binding of all four MHET moieties and the occurrence of conformational changes in PET that perfectly match the binding cleft (Wei et al., 2019). Instead, Wei and co-workers propose that hydrolysis of amorphous PET is facilitated by weak interactions between its aromatic phenylene units and the nearby hydrophobic amino acids of the enzyme. This hypothesis is based on phenylene ring motions, which are more

inclined to arise compared with the rigid EG units that directly flank the ester bond (Wei et al., 2019). Although cutinase catalytic mechanisms have also been explored by studying enzyme structures complexed with inhibitors and natural substrate analogs, the precise binding mode of PET and the underlying mechanism of cutinase PET hydrolysis requires further investigation, as the structure of PET is very different to cutinase substrates, such as cutin and triglyceride (Han et al., 2017). While *I. sakaiensis* and its enzymes are well-studied and have gained much interest as a PET degradation system, other relevant microorganisms and enzymes have been identified and will now be discussed.

3.2 Actinobacterial Enzymes

Thermobifida fusca, a thermophilic actinomycete, is one of the multiple Actinobacterial strains that have been recognized as producers of enzymes for PET hydrolysis (Müller et al., 2005; Wei and Zimmermann, 2017a). *Thermobifida fusca* DSM43793 has been shown to produce two similar hydrolases, BTA-1 (commonly referred to as TfH) and a BTA-2, while *T. fusca* KW3, a moderate thermophile, has been reported to produce TfCut1 and TfCut2 cutinases (EC 3.1.1.74). *T. fusca* YX has been shown to produce Tfu_0882 and Tfu_0883, which are lipases (EC 3.1.1.3) (Wei and Zimmerman, 2017a). In one study, Thf42_Cut1 cutinase from *Thermobifida fusca* DSM44342 was reported to hydrolyze PET alongside Thc_Cut1 and Thc_Cut2 cutinases from another *Thermobifida* species, namely *T. cellulosilytica* (Herrero Acero et al., 2011). Additionally, *Thermobifida alba* and *Thermobifida halotolerans* are of interest for their PET hydrolyzing enzymes (Ribitsch et al., 2012a; Ribitsch et al., 2012b). Other known actinobacterial PET hydrolytic enzymes that have been described include those from the genera *Saccharomonospora* and *Thermomonospora* (Table 1).

TfH (BTA-1), a cutinase enzyme which was isolated by Müller and co-workers, was used to degrade melt-pressed PET bottles over a three week period at 55°C, with its weight being reduced by approximately 50% (Müller et al., 2005). This offered a marked improvement over the chemical hydrolysis of PET, which typically requires much higher temperatures, and had the added benefit of reducing unwanted side products and generating monomers of higher purity for repolymerization. While TfH shares 65% sequence similarity with a lipase from *Streptomyces albus*, it differs from lipases, which can only break ester bonds at a hydrophobic surface, in that it has additional esterase activity that enables hydrolysis of dissolved esters (Mueller, 2006).

Thermobifida fusca hydrolases BTA-2, Tfu_0882, TfCut1, TfCut2 and TfH have been assessed for their degradation capabilities on amorphous (non-crystalline) PET film (Wei and Zimmerman, 2017a). When incubated between 55 and 65°C for 48 h, film weight losses of up to 4%, 5%, 11%, 12% and 14% were observed for these five enzymes, respectively. (Then et al., 2015; Wei and Zimmermann, 2017a). TfCut2 variants have been shown to achieve 25% weight loss of the same film using temperature conditions of 65-80° for 48 h (Then et al., 2015) and 45% after 50 h incubation at 65°C (Wei et al., 2016). Both TfCut1 and TfCut2, along with their variants, have already been successfully applied in textile finishing processes in PET fiber surface modification (Zimmermann and Billig, 2010; Wei et al., 2014b).

In a study on TfCut2 cutinase, the enzyme was crystallized in both free and inactivated forms before structural and functional characterization (Roth et al., 2014). Combined with molecular models, the structural components responsible for substrate and product specificity were analyzed. TfCut2 displayed a standard α/β hydrolase fold and a S130-H208-D176 catalytic triad was found in a crevice on the enzyme surface and the highly resolved structure provided an appropriate foundation for enzyme engineering. The thermostability of TfCut2 was also investigated by comparison with a less stable *Thermobifida alba* AHK119 homolog. While the melting temperature (T_m) of TfCut2 appeared to be 70°C when measured by temperature-dependent CD spectroscopy, loss of activity was measured experimentally at 61°C (Roth et al., 2014). Despite this, TfCut2 displayed improved thermal stability when compared with other characterized bacterial cutinases (EC 3.1.1.74). It is believed that denaturation may be prevented in TfCut2 by the presence of a disulfide bridge between Cys241 and Cys259 which may stabilize a highly flexible region nearby that surrounds residues 245-247. Residues at the boundary of the active site also exhibit high levels of flexibility in relation to their overall structure, possibly helping to enable induced fit for catalysis. Flexibility in some areas can be susceptible to local unfolding which might explain the lower T_m seen in the activity assay. This analysis also suggested that the optimized amino acid sequence pattern and hydrogen bond network of this enzyme contributes to thermostability (Roth et al., 2014; Taniguchi et al., 2019).

Thc_Cut1, Thc_Cut2 and Thf42_Cut1, three cutinases which are closely related, were compared based on their hydrolytic activities towards bis(benzoyloxyethyl)-terephthalate (3PET), a short-chain PET model substrate, in addition to PET film with 37% crystallinity (Herrero Acero et al., 2011). The enzymes were incubated at 50°C with the 3PET assay proceeding for 72 h and the PET film treatment requiring 120 h, shaking at 130rpm. HPLC

(high-performance liquid chromatography) was used to quantify the resulting hydrolysates. Of the three enzymes tested, Thc_Cut1 was found to release MHET and TPA from both PET and 3PET to the greatest extent. For both Thc_Cut1 and Thf42_Cut1, TPA was found to be the major product of hydrolysis, indicating a more rapid processing of MHET, which was in higher abundance following treatment using Thc_Cut2. Although Thc_Cut1 and 2 had 93% homology, varying enzyme surface properties appeared to be responsible for the significant activity differences observed (Herrero Acero et al., 2011).

Another cutinase enzyme from *Thermobifida alba* called Tha_Cut1 was assessed for hydrolysis of 3PET and PET films (Ribitsch et al., 2012a), based on the previously mentioned methods used for Thc_Cut1 and 2 (Herrero Acero et al., 2011). For the 3PET assay, the well-studied fungal cutinase HiC was compared with Tha_Cut1 and although HiC released twice as many overall hydrolysates, it was determined than the two enzymes had distinct substrate specificities, with Tha_Cut1 releasing 35 times the amount of a hydrolysis product called 2-hydroxyethyl benzoate (HEB). This comparison provided further insights into the mechanism of Tha_Cut1, revealing the main products of 3PET hydrolysis to be MHET and HEB, indicating efficient hydrolysis of the ester bond between them. Tha_Cut1 displayed a high similarity to Thc_Cut1 from *T. cellulosilytica*, with a difference of just four amino acids that are located in a key region involved in substrate-enzyme interaction (Ribitsch et al., 2012a). In addition, two cutinase-like polyesterase enzymes Est1 and Est119 from tandem genes in *Thermobifida alba* AHK119 have been shown to have broad substrate specificity for various polyesters. Purified Est119 has been biochemically characterized, revealing highest kinetic activities (V_{max} and k_{cat}) towards short-chain *p*-nitrophenyl butyrate (*p*-NPB). However, the enzyme K_m values showed a higher affinity for longer chain (C_6 and C_8) acyl esters, indicating that Est119 is a cutinase-like enzyme rather than a true lipase or esterase (Thumarat et al., 2012). For both Est119 and Est1, optimum temperature and pH were recorded as 50°C and pH 6.0, respectively (Thumarat et al., 2015). Despite the enzymes displaying 98% similarity with each other, Est1 displayed higher activity and thermostability (Kawai et al., 2013). More recently, an Est1 (A68V/T253P) variant was shown to have the highest thermostability when compared with wild-type and mutant Est1 and Est119 enzymes. Est1 (A68V/T253P) was assayed with 3PET and PET film, shaking for 3 h at 50°C, and was noted for its PET surface hydrophilization and polyester degradation abilities (Thumarat et al., 2015).

Cut190 is a cutinase enzyme from *Saccharomonospora viridis* AHK190. Following mutational analysis, one variant Cut190 (S226P/R228S) that was generated displayed particularly high

activity and thermostability (Kawai et al., 2014). This variant remained stable up to 65°C and over a pH range of between 5-9 when incubated for 24 h, while also retaining 40% of its activity when incubated at 70°C for 1 h. Various aliphatic and aliphatic-co-aromatic polyester films were also shown to be efficiently degraded by Cut190 (S226P/R228S) and the enzyme even hydrolyzed PET film above 60°C. Both the wild-type and mutant Cut190 are dependent on Ca^{2+} ions to assist in their activity and thermostability (Kawai et al., 2014).

Tcur0390 and Tcur1278 are two hydrolase enzymes from *Thermomonospora curvata* DSM43183, the genes for which were identified following genome scanning analysis of the strain and were found to display 61% sequence identity with TfCut2 from *T. fusca* (Wei et al., 2014a). Enzyme activity was subsequently investigated using polycaprolactone (PCL) and polyethylene terephthalate (PET) nanoparticle assays. While Tcur0390 displayed higher hydrolytic activity than Tcur1278 at temperatures up to 50°C, only Tcur1278 was capable of hydrolyzing PET nanoparticles when temperatures were elevated to 55°C and 65°C. Relatively low thermal stability was observed for both Tcur1278 and Tcur0390 at their optimal temperatures (60°C and 55°C, respectively), with an irreversible activity loss of over 65% following incubation for just 10 min. The optimal pH for both enzymes is pH 8.5 (Wei et al., 2014a).

In a study that employed an *in silico*-based screening approach to interrogate 52 genomes from the *Streptomyces* genus, a potential PETase-like gene was identified in *Streptomyces* sp. SM14 (Almeida et al., 2019). Heterologous expression of the gene in *E. coli* resulted in the extracellular production of an enzyme,SM14est, that was shown to have polyesterase activity on polycaprolactone, the model substrate for plastic degradation (Almeida et al., 2019). SM14 is a marine sponge-derived isolate which may have been exposed to plastics and/or microplastics, resulting from its association with the filter-feeding sponge *Haliclona simulans*. Subsequent amino acid sequence analysis and comparison of SM14est with the well-described *Is*PETase revealed that the serine hydrolase motif Gly-x1-Ser-x2-Gly and the Ser-Asp-His catalytic triad are conserved in both enzymes. However, SM14 displays key differences in its catalytic sub-site II residues as well as an absence of the two disulfide bonds and the extended loop featured in *Is*PETase, which may contribute to a reduced efficiency in PET binding and hydrolysis (Joo et al., 2018; Almeida et al., 2019).

Bacterial enzymes of interest for PET hydrolysis are not limited to those derived from actinomycetes, with other examples including a *Bacillus subtilis* esterase (Ribitsch et al., 2011),

a cutinase from *Pseudomonas mendocina* (Yoon et al., 2002), and a *Burkholderia* spp. lipase (Lee and Chung, 2009). In 2019, the draft genome sequences of five bacterial isolates, from *Bacillus* and *Pseudomonas* species, were deposited in Genbank (León-Zayas et al., 2019). As a consortium, these isolates synergistically degraded 100mg of granular PET to give a weight loss of 3.15 mg (~3% reduction rate), following 6-weeks at 30°C (León-Zayas et al., 2019). Most recently, Bollinger and co-workers identified a polyester degrading carboxylesterase (EC 3.1.1.1) within the genome of the mesophilic marine bacterium *Pseudomonas aestusnigri* (Bollinger et al., 2020). The enzyme, named PE-H, was revealed as a PET hydrolytic enzyme (type IIa), based on amino acid sequence homology. The group also solved the enzyme's crystal structure, which featured the canonical α/β hydrolase fold and showed high homology to known polyesterases. PE-H was shown to hydrolyze amorphous PET film at 30°C, with intermediate MHET released as a product. Although the wild-type enzyme failed to hydrolyze a commercial PET bottle-based film, a variant of PE-H (Y250S), which displayed some hydrolytic activity towards PET in this form, was obtained using rational mutagenesis (Bollinger et al., 2020).

3.3 Fungal Enzymes

Cutinases (EC 3.1.1.74) from fungi have also shown activity towards PET as a substrate, with members of the *Fusarium* and *Humicola* genera in particular, representing the most significant sources of these enzymes (Danso et al., 2019).

The activities of *Fusarium solani pisi* cutinase (FsC) and *Humicola insolens* cutinase (HiC) have previously been evaluated, on both low-crystallinity and biaxially oriented PET films having 7% and 35% crystallinity, respectively (Ronkvist et al., 2009). Using a pH-stat based assay, the cutinases were shown to be 10-fold more active on low crystallinity PET over the biaxially oriented film. HiC was shown to be capable of completely degrading low crystallinity PET film with 97% (± 3%) weight loss being observed within 96 h. Further analysis of degradation products indicated that the water-soluble degradation products consisted exclusively of TPA and EG. Despite its preference for amorphous PET, the HiC enzyme was sufficiently active on crystalline PET regions to enable its full degradation. In comparison, a weight loss of just 5% was achieved by the FsC enzyme on low-crystallinity PET film. While FsC performed optimally at 50°C, HiC displayed higher thermostability, maintaining maximum initial activity between 70 and 80°C (Ronkvist et al., 2009).

More recently, HiC has been shown to act synergistically with *Candida antarctica* lipase B (CALB) to effectively hydrolyze PET to TPA (Carniel et al., 2017). Initial screening of 10

lipases using the PET intermediate bis-(2-hydroxyethyl)-terephthalate (BHET), revealed that CALB could complete its conversion to TPA, while HiC was limited by the last reaction step. When evaluated for PET hydrolysis, HiC displayed significant accumulation of the intermediate MHET, despite showing better potential for depolymerization of PET. By combining the CALB enzyme with HiC, PET degradation was shown to be more complete, resulting in a 7.7-fold increase in TPA yield from PET (Carniel et al., 2017)

Aspergillus oryzae, Candida antarctica and *Penicillium citrinum* are among other fungal enzymes that have been investigated for activity on PET and are considered most suitable for PET fiber and fabric treatments (Zimmermann and Billig, 2010; Kawai et al., 2019).

3.4 Metagenome-Derived Enzymes

Novel genes from the unculturable fraction of different environmental ecosystems can be accessed using metagenomic approaches. During a metagenomic study of leaf-branch compost, a gene with 57.4% sequence identity to Tfu_0883 cutinase from *Thermobifida fusca* was identified (Sulaiman et al., 2012). This gene was found to encode LC-cutinase (LCC), a novel cutinase homolog, following a functional based screening of a metagenomic library from the compost using tributyrin agar. LCC was subsequently heterologously expressed in *E. coli* and shown to hydrolyze various fatty acid monoesters optimally at 50°C, with a preference for short-chain substrates (up to C_4). This reaction was used to show that the optimal enzymatic activity for LC-cutinase is 50°C and pH 8.5. The enzyme's specific activity for *p*-NPB is comparable to that of the *T. fusca* cutinase, although slightly lower. LCC was also capable of degrading PCL and PET, with the enzyme displaying specific PET-degrading activity of 12 mg/h/mg of enzyme at 50°C and pH 8.0 (Sulaiman et al., 2012).

A dual enzyme system, which combined LC-cutinase with an immobilized *T. fusca* KW3 carboxylesterase (EC 3.1.1.1) called TfCa, was subsequently investigated for its ability to hydrolyze PET films (Barth et al., 2016). Typically, enzymatic PET degradation is restricted once the MHET intermediate is formed. The presence of immobilized TfCa was however shown to enable LC-cutinase hydrolysis of inhibitory MHET. HPLC analysis of degradation products indicated that the LCC-TfCa dual enzyme system gave a 2.4-fold higher yield of hydrolysates compared with one that combined TfCa with TfCut2 from *T. fusca*. In terms of total amounts of products released with the LCC-TfCa combination, an increase of 104% was observed versus PET enzymatic hydrolysis where TfCa was not added (Barth et al., 2016).

A recent study by the Streit group, involving a search for PET hydrolase genes across existing marine and terrestrial metagenomic datasets uncovered over 800 putative PET hydrolases (Danso et al., 2018; Danso et al., 2019). 13 potential PET homologs (PET1-PET13) were selected based on their sequence similarity to previously identified PET hydrolase enzymes. Of these, four enzymes, PET2, PET5, PET6 and PET12, were functionally verified as novel PET hydrolase candidates, with active clones giving clearance on PET nanoparticle plates. One of these enzymes, PET2, which was obtained from marine metagenomics data, was shown to have a temperature optimum of 70°C and retained its thermostability up to 90°C (Danso et al., 2018).

Screening of over 200 purified, uncharacterized hydrolytic enzymes from environmental metagenomes and sequenced microbial genomes was carried out in a recent study to find proteins with strong degradative activity against synthetic polyesters (Hajighasemi et al., 2018). The selected hydrolase genes were heterologously expressed in an *E. coli* host and purified by a combination of affinity chromatography and either ion exchange or size exclusion chromatography. Among the enzymes identified were MGS0156 and GEN0105, two metagenomic esterases capable of hydrolyzing polylactic acid (PLA), polycaprolactone and a PET model substrate, 3PET. Following determination of its crystal structure, MGS0156 was found to feature a modified α/β hydrolase fold, a highly hydrophobic active site, and a lid domain. Structure-based mutational studies revealed the key amino acid residues required for hydrolytic activity, including catalytic triad residues Ser232, His73 and Asp350, adjacent residues His231, Lys233 and Asp372. The side chains of Cys173 and Cys287 formed a disulfide bond which stabilizes an important protein lid domain. The polyesterase activity of a MGS0156 mutant (L169A) was twice as high as that of the wild-type (Hajighasemi et al., 2018).

Metagenomic tools have a powerful ability to tap into the microbial biodiversity of different environments. Thus, it is worth further developing search algorithms that explore metagenomic datasets for valuable activities as well as establishing consistent functional assays that can be run in parallel to detect PET hydrolase enzymes (Danso et al., 2019).

4. Challenges and Limitations

While the area of enzymatic PET hydrolysis is expanding rapidly, several major challenges remain which will need to be addressed to further advance the field.

4.1 Low Prevalence of PET Hydrolysis Activities

So far, relatively few microbial genera have been described which possess the ability to degrade PET, with those that do often only causing partial break down into oligomers (Wei and Zimmermann, 2017b). Current reports indicate that the PET degradation trait features in only a limited number of bacterial phyla, with most to date being found in members of the Actinobacteria (Herrero Acero et al., 2011).

In the aforementioned study targeting novel genes related to PET hydrolysis in marine and terrestrial metagenomics datasets, Danso and co-workers employed a search algorithm that detected 504 possible candidate genes from various databases. In addition, they performed a global search of over 16 Gb of metagenomic sequence data from the Integrated Microbial Genome (IMG) database, which resulted in the identification of 349 putative PET hydrolases. Both searches relied on a hidden Markov model and were followed by functional testing of potential PET hydrolase sequences (Figure 3). PET hydrolase frequencies ranged from 0.004 to 0.92 hits/Mb and 0.0001 to 1.513 hits/Mb for marine and terrestrial metagenome datasets, respectively. An average of 157 PET hydrolase homologs were present in terrestrial metagenomes, compared with an average of 42 homologs in marine metagenomes. A metagenomic sample from a crude oil reservoir offered the highest rate of sequence hits (1.5 hits/Mb). While the phylum Actinobacteria was the main provider of terrestrial-derived enzymes, many PET hydrolases predicted from the marine samples were linked to Bacteroidetes (Danso et al., 2018).

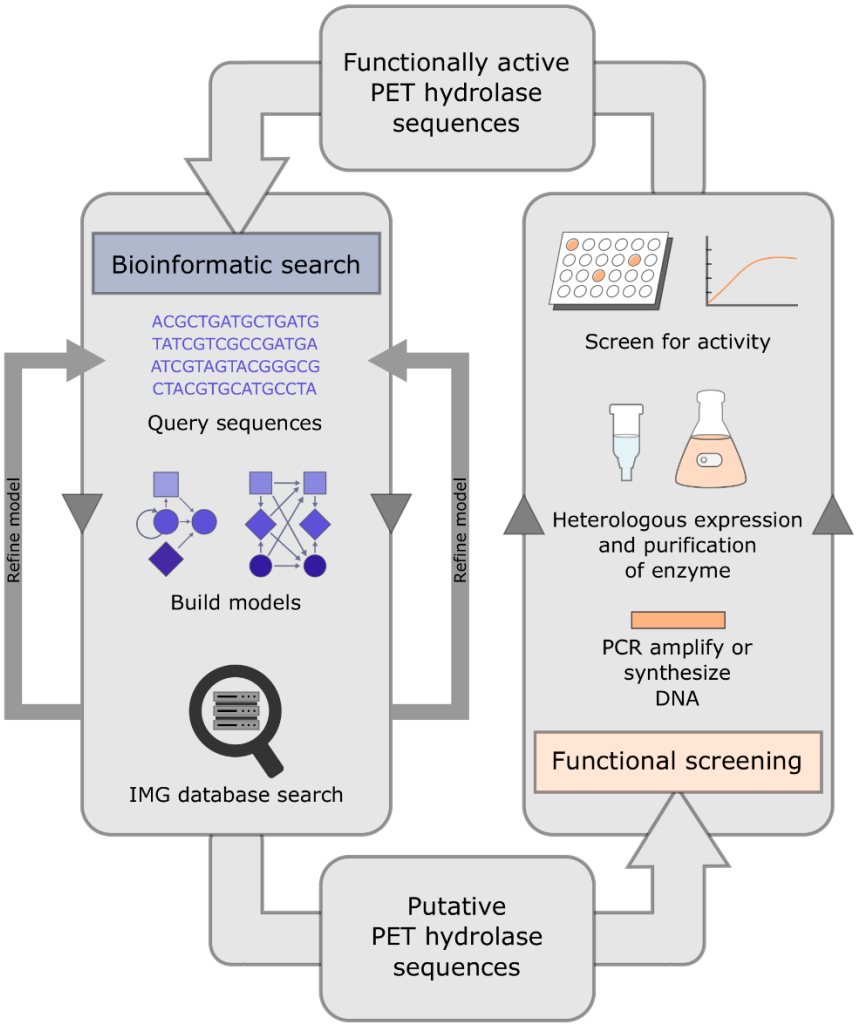


Figure 3. System for the identification and functional testing of candidate PET hydrolase enzymes. Potential PET hydrolase sequences may be uncovered with an initial bioinformatic search of existing databases, by employing hidden Markov models. Candidate genes are then cloned into a heterologous host to verify activity against substrates, e.g., PCL and PET-nanoparticles.

Bacteroidetes species, although not previously associated with PET hydrolysis, are known to be equipped with multiple hydrolases and binding molecules for degradation of other polymers such as xylan, cellulose, and pectin (Dodd et al., 2011; Thomas et al., 2011). The second most abundant phylum associated with both datasets was the Proteobacteria, comprising 20% and 30% of hits in the terrestrial and marine data, respectively. Beta-, Delta-, and Gammaproteobacteria were the main hosts harboring PET hydrolase genes within this phylum.

Since over 100 metagenomes were included in the analysis, an overall picture of the occurrence of PET hydrolase enzymes was generated. However, this data may not be an accurate representation of the expression of these genes in the natural environment. The low gene frequencies observed suggests that this trait may have only evolved quite recently and may not have had the opportunity to spread within different microbial populations. Overall, the data suggests that PET hydrolases are rare enzymes (Danso et al., 2018). Notwithstanding this, it is also likely that by exploiting global metagenomic data sets to gain access to dark matter proteins and non-culturable microbes, that there is potential to increase the diversity of microbes and enzymes that act on synthetic polymers (Danso et al., 2019).

4.2 Impact of PET Properties on Enzymatic Activity

The success of PET hydrolysis relies on a balanced combination of suitable enzyme structure and polymer chain flexibility (Zumstein et al., 2017). There are a few factors, relating to the nature of PET, which influence the extent to which it is hydrolyzed. PET crystallinity and orientation are among the key features which may affect the ability of an enzyme to break apart its building blocks (Webb et al., 2013; Kawai et al., 2019)

As with most polymers, PET is typically comprised of a complex structure with crystalline regions that feature tightly packed chains in parallel, and amorphous regions where the chains are disordered (Demirel et al., 2011; Robertson, 2016). Depending on the intended use of the final product, PET can have different degrees of crystallinity. Generally, PET employed to make bottles and textiles is associated with having a high crystallinity of between 30 and 40%. Conversely, PET which is used to create packaging has lower crystallinities, for example, PET-GF, a commercially available low-crystallinity PET film, is estimated to have 6-7% crystallinity (Kawai et al., 2014). Numerous reports indicate that PET hydrolase enzymes preferentially degrade the regions of PET that are amorphous in nature (Figure 4) (Welzel et al., 2002; Brueckner et al., 2008a; Ronkvist et al., 2009; Donelli et al., 2010; Gamerith et al., 2017). As the crystallinity of PET increases, the flexibility and movement of the polymer chains becomes more restricted, thus reducing the susceptibility of these chains to enzymatic attack. In spite of this, it has been suggested that hydrolysis of amorphous regions may impact on crystalline regions, such that both can ultimately be digested (Ronkvist et al., 2009; Kawai et al., 2019). Furthermore, the repeating units of bulky aromatic terephthalate, which are present in the PET backbone, restrict polymer chain mobility and therefore, degradability (Wei and Zimmermann, 2017b).

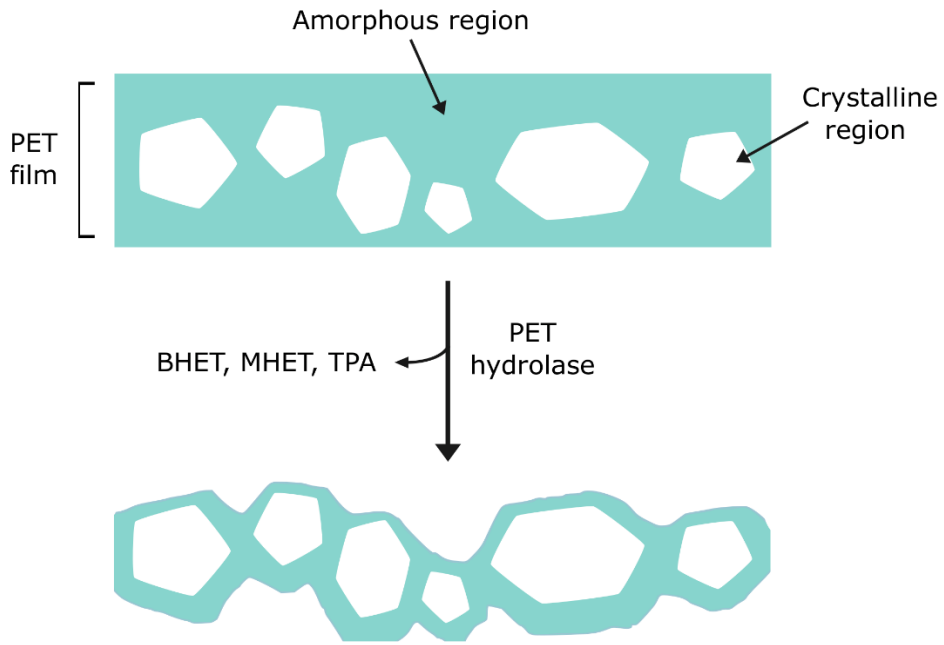


Figure 4. Enzyme preference for amorphous regions of PET over crystalline regions. PET hydrolase enzymes have been shown to favor lower crystallinity, amorphous areas of PET (shown in blue).

To achieve the desired form for bottles and textiles, the degree of orientation is increased by stretching to order the PET polymer chains (Kawai et al., 2019). PET can also be biaxially oriented (*bo*-PET) whereby the plastic undergoes additional stretching in two directions (Breil, 2013). In both cases, orientation results in strengthening of the hydrogen bonds between the polymers and further induces crystallization (Wei and Zimmermann, 2017a). In addition, crystallinity and orientation during production will influence surface topology, as seen in the manufacture of plastic bottles by injection moulding - different parts of the bottle will have different surface properties with varying orientations of polymer chains. While these methods enhance the physical properties of PET for application, it is very restrictive in terms of enzymatic degradation and there are currently no PET hydrolases known to directly digest PET in any of the forms just mentioned (Kawai et al., 2019).

The temperature range within which a polymeric substrate changes from a glass-like and rigid state to being soft but not yet melted, is referred to as the glass transition temperature (T_g) (Becker and Locascio, 2002). The application of heat above T_g is typically used to induce

crystallization, normally accompanied by molecular orientation. High crystallinity (and therefore increased resistance to degradation) is associated with higher glass transition temperatures, with amorphous PET having a T_g of 67°C and crystalline PET having a T_g of 81°C (Groeninckx et al., 1974; Demirel et al., 2011). However, when PET is in aqueous conditions, T_g values are lowered to 60-65°C as a result of the diffusion of water molecules between the polymer chains (Kikkawa et al., 2004; Kawai et al., 2014). This weakens the associated hydrogen bonds and thereby increases randomness, flexibility, and mobility of the chains. Enzymatic reactions are routinely performed in a solution, meaning that the T_g of PET decreases such that enzymes have increased accessibility to the polymer chains. Water absorbency of PET can vary between 0.1 and 1% depending on the form but will also increase under higher temperatures. Therefore, it is recommended to set high PET hydrolysis reaction temperatures, preferably above T_g, as this has been reported to give higher rates of degradation (Kawai et al., 2014; Oda et al., 2018; Kawai et al., 2019). On the other hand, it is then vital to employ a thermostable PET hydrolase. Thermostability is generally advantageous as catalysis is promoted at raised temperatures and biocatalysis can be maintained over a long period at ambient temperatures (Kawai et al., 2019).

Inhibition of the cutinase enzyme TfCut2 by the intermediate products of PET degradation has been investigated in a recent study aiming to identify the bottlenecks in reaching complete PET hydrolysis (Barth et al., 2015). MHET, BHET, EG, and TPA are recognized as the main hydrolysis products of PET films and fibers (Vertommen et al., 2005; Brueckner et al., 2008b; Eberl et al., 2009). The experiment involved TfCut2 digestion of a PET nanoparticle substrate in the presence of these four intermediates, with their inhibitory effect analyzed using reversed-phase HPLC and a model for heterogenous enzymatic polymer hydrolysis. MHET and BHET were shown to competitively inhibit hydrolysis by occupying the substrate binding site of the TfCut2 enzyme whereas TPA and EG had no impact on the ability of the enzyme to degrade the PET nanoparticles (Barth et al., 2015). One way of overcoming this challenge has been to use other enzymes in combination with PET hydrolases to improve substrate binding and catalytic characteristics (Wei et al., 2016; Carniel et al., 2017; Austin et al., 2018).

When studying and comparing PET hydrolyzing enzymes it is important to account for the variability in properties of different PET substrates. While commercial PET-GF film is regularly used and is a suitable standard for comparing the abilities of PET hydrolases, it has not been employed across all studies. It is also necessary to consider the chosen method for detecting and reporting activity i.e., weight loss, hydrolysis, etc. In addition, many enzymes,

including HiC, TfH, FsC, and Cut190 have been used in the textiles industry to achieve more desirable PET fiber characteristics and this requires only surface modification and avoids degrading the inner PET building block. While PET hydrolases may be used for surface treatments, they are distinct from PET surface-modifying enzymes. PET hydrolases should significantly degrade the building blocks of PET (by 10% at least) and result in visible change when observed by scanning electron microscope (SEM) (Kawai et al., 2019).

More recently, this group further discussed the need for clarification when categorizing an enzyme as a PET hydrolase (Kawai et al., 2020). They have recommended grouping PET hydrolyzing enzymes into, (1) PET hydrolases and (2) PET surface-modifying enzymes. They suggest that the only enzymes which can currently be considered as PET hydrolases are the following cutinases; HiC, LCC and variants of TfCut2 (Wei et al., 2016) and Cut190 (Oda et al., 2018). This is based on criteria for efficient PET degradation, namely thermostability (>65°C, or ideally >75°C**)** and the capacity for the open active site to bind multiple MHET monomers. It follows that mesophilic *Is*PETase, despite its structural similarities to these cutinases and observed hydrolyzing activities at 30°C, should not be regarded as a PET hydrolase, particularly with respect to application (Kawai et al., 2020). It was also recently demonstrated that while amorphous PET supports growth of *I. sakaiensis*, high crystallinity PET films and bottles do not (unless hcPET is melted and cooled rapidly to give amorphous plastic) (Wallace et al., 2020). TfH may still be grouped with PET hydrolases as it was the first enzyme reported for efficient PET hydrolysis and it is thermophilic.

It is also worth noting that solubilizers and other additives, which are often mixed into plastics to change their properties, may interfere with the activity of degradative enzymes and may also account for some weight loss during enzymatic digestion due to microbial break down of these chemical agents (Danso et al., 2019). While this does not usually apply to PET manufacturing, plasticizers may be used for some PET products (Hahladakis et al., 2018). A simple and robust PET powder-based suspension assay was recently used to quantify product release from PET, but product detection was shown to depend on choice of analytical technique. Therefore, it is advisable to use a combined approach. In this study, a plate reader method (for rapid screening, measurement of initial rates, and comparative biochemical studies) was well complemented by reversed-phase HPLC analysis (for more in-depth information on the aromatic monomers produced), which offered greater insight and avoided errors (Bååth et al., 2020).

Although these factors can create difficulty in making exact conclusions regarding the ability of an enzyme to hydrolyze PET, this is a relatively new field of research which should see greater harmony across different studies as it progresses into the future. In fact, the recommendations provided by Kawai and colleagues (Kawai et al., 2020), for distinguishing PET hydrolases from other enzymes may be worthy of further consideration in this area, particularly for comparative purposes when assessing the potential of new PET-active enzymes and when considering PET hydrolases for practical use.

4.3 Enzyme Properties, Catalytic Efficiency, and Thermal Stability

So far, the consensus in the area of PET hydrolysis has been that known PET hydrolase enzymes have low turnover rates, particularly towards highly crystallized forms of PET (Wei and Zimmermann, 2017a; Liu et al., 2018; Danso et al., 2019; Taniguchi et al., 2019). Even *Is*PETase, which has shown specificity for degradation of hc-PET, is considered to have a relatively low catalytic activity (Taniguchi et al., 2019). Furthermore, given that reaction temperatures above the T_g of PET have been advised for more rapid hydrolysis, candidate enzymes should ideally display high enzymatic efficiency while also retaining activity over 75°C (Yoshida et al., 2016; Taniguchi et al., 2019). For instance, *Is*PETase is heat labile but thermostable cutinases such as LCC, TfH, and HiC may fall short of *Is*PETase in terms of specificity for PET substrate, demonstrating the need for a combination of both traits (Wei and Zimmermann, 2017a; Liu et al., 2018; Taniguchi et al., 2019).

Enzyme adsorption, whereby the protein adheres to the polymer surface, is a key process in the hydrolysis of PET and other polymers. This binding results from contact between hydrophobic PET fibers and any hydrophobic amino acid residues located at the protein surface (Norde, 1996; Kim and Yoon, 2002; Stuart, 2003; Palonen et al., 2004; O'Neill et al., 2007) It was mentioned already that *T. cellulosilytica* cutinase, Thc_Cut2, displayed lower hydrolytic efficiency compared with Thc_Cut1 that could be attributed to the individual hydrophobic surface properties of these enzymes. Subsequently, site-directed mutagenesis was used to demonstrate that amino acids on the enzyme surface are important for PET hydrolysis, by exchanging selected Thc_Cut2 residues with those only present on the surface of Thc_Cut1 (Herrero Acero et al., 2013). Another group improved Thc_Cut1 PET hydrolysis through fusion of two hydrophobic binding domains to enhance the enzyme's adsorption abilities (Ribitsch et al., 2013). Also, microbial biofilm production can play an important role in plastic biodegradation due to the comparably high hydrophobicity of the associated cell surfaces,

which in turn facilitates their attachment to synthetic polymers. Given that cell surfaces are usually hydrophilic in nature, attachment is often enhanced by adding hydrophilic functional groups to the hydrophobic polymers. This increases access of the released hydrolytic enzymes to the plastic surface (Orr et al., 2004; Tribedi and Sil, 2014; Nauendorf et al., 2016; Wilkes and Aristilde, 2017). Once attachment is initiated, subsequent degradation steps, such as biodeterioration, biofragmentation, assimilation, and mineralization can proceed more easily (Jaiswal et al., 2020).

The area of enzymatic PET hydrolysis may be progressed either by continuing to mine for new PET hydrolases with improved efficiencies, or through the application of protein engineering strategies to enhance the characteristics of enzymes that have already been identified (Liu et al., 2018). To date, rational design has proven useful in modifying cutinase enzymes to improve their activities. For example, site-directed mutagenesis, has been used to deliver more space or hydrophobicity in the Tfu_0883 active site (Silva et al., 2011) and helped to achieve 2.7 and 3-fold increases in activity for TfCut2 and Thc_Cut2, respectively (Herrero Acero et al., 2013; Wei et al., 2016).

In a study that focused on six key residues around the *Is*PETase substrate-binding pocket, Ma and co-workers created and tested for highly efficient mutants using rational protein engineering (Ma et al., 2018). The selected mutations aimed at optimizing the residues around the active site by replacing them with smaller amino acids to create space for the bulky aromatic groups of PET, or by substituting with more hydrophobic amino acids to give a greater affinity towards hydrophobic PET. Of the mutants that displayed improved activities compared to wild-type *Is*PETase, one variant (I179F) achieved a 2.5-fold increase in degradation of biaxially oriented PET film (35% crystallinity). This was determined based on weight loss of film after 48 h at 30°C (Ma et al., 2018; Kawai et al., 2019).

Following structural analysis, (Liu et al., 2018) designed a series of *Is*PETase mutants using site directed mutagenesis. Targets included key residues in the substrate-binding pockets and those which help stabilize active site rigidity. With mutations that led to increased aromaticity on the binding pocket edge or increased space at the binding center, higher activities were observed over the wild-type enzyme towards PET bottles. Activity was detected in terms of hydrolysis following 48 h incubation at 30°C and included a 3.1-fold increase by one mutant (Y87A) (Liu et al., 2018; Taniguchi et al., 2019).

In another study, a glycosylation strategy was employed to inhibit LC-cutinase native aggregation (Shirke et al., 2018). While LCC displays high thermostability (T_m = 86°C), initial thermal deactivation testing revealed a high tendency for aggregation by electrostatic interactions, limiting this enzyme in terms of stability and therefore, practical application. Glycosylated LCC (LCC-G), which was produced post-translationally by engineering three known N-glycosylation sites and expression in *Pichia pastoris*, showed improvements in PET degradation under optimum temperature and concentration (Shirke et al., 2018).

Recently, Son and colleagues carried out a study to address the requirement for *Is*PETase variants with a highly increased capacity for PET hydrolysis and improved stabilities that enable the secreted enzyme to maintain its function in a harsh extracellular environment, over a long period at moderate temperatures (Son et al., 2019). To achieve this, the group designed a plan for rational protein engineering based on the existing crystal structure of *Is*PETase. Following screening, an *Is*PETase (S121E/D186H/R280A) mutant displayed particularly enhanced characteristics (Son et al., 2019). This variant was designed such that subsite IIc was extended to provide a non-protruding, hydrophobic cleft, and a flexible β6-β7 connecting loop was stabilized (Joo et al., 2018). Upon comparison of the mutant with wild-type *Is*PETase, degradative activity towards PET film was shown to be 14-fold higher at 40°C. In addition, the melting temperature of this variant increased by 8.81°C and high thermostability was further verified by a heat-inactivation experiment (Son et al., 2019). The same group have since introduced mutations N246D and S242T, generating a quadruple *Is*PETase variant which displayed a 58-fold increase in hydrolyzing activity over its wild-type at 37°C, and maintained activity under these conditions for 20 days (Son et al., 2020).

Previous work demonstrated that the addition of the cation Ca^{2+} (or Mg^{2+}) had a positive effect on activating and stabilizing enzymes for PET hydrolysis (Kawai et al., 2014; Sulaiman et al., 2014; Miyakawa et al., 2015; Then et al., 2015). More recently, (Oda et al., 2018) investigated the role of three Ca^{2+} ions bound to separate sites (1-3) on Cut190 (S226P/R228S) and found that the ions had different roles in thermal and structural stabilization as well as enzyme activation, generating an overall change in protein conformation that help increase activity. Furthermore, by introducing a disulfide bond in site 2 for thermostability, the melting temperature was increased by at least 20-30°C when mutants were Ca^{2+}-bound, with the bond mimicking the effect of Ca^{2+} (Oda et al., 2018).

Another group have demonstrated that pre-incubation of PET film with anionic surfactants attracted cationic *Is*PETase by helping to orient the enzyme active site with the hydrophobic PET surface (Furukawa et al., 2018). This accelerated enzymatic activity by 120-fold, with a 22% decrease in film thickness following incubation at 30°C for 36 h (Furukawa et al., 2018). In a follow-on study, they found that the activity of mutant TfCut2 (G62A/F209A) could be increased by the presence of a cationic surfactant. In this case, the enzyme has a negatively charged surface and is therefore brought closer to the PET film via electrostatic interactions upon addition of cationic surfactant (Furukawa et al., 2019). Moreover, when investigating the impact of different conditions on *Is*PETase, that may have relevance for industrial application, Liu and co-workers reported that organic solvents and detergents reduced the enzyme's activity, while salts and glycerol led to enhanced enzymatic activity. Of the salts used, Na_2SO_4 had the most positive effect, which may be due to its higher effective cation concentration (Liu et al., 2019).

From an industrial perspective, wild-type microbial enzymes are usually modified to withstand the harsh and variable reaction conditions, and to satisfy the catalytic needs of a given process (Shukla, 2019). A synthetic biology approach combines a range of molecular and computational tools to generate enzymes that meet industrial expectations. Microbial metabolism can be manipulated through engineering of regulatory and genetic elements (e.g., promoter, terminator, and binding sequences), to optimize gene expression and protein production. Nanotechnology is another synthetic biology tool, that has been used for enzyme immobilization onto nanoparticle beads. This can lead to improvements in enzyme activity, stability, and thus reusability, as demonstrated by an immobilized lipase from a *Fusarium incarnatum* strain, which retained 75% its degradation activity after 5 cycles when applied to treat waste cooking oil (Joshi et al., 2019). *In silico* studies and computer simulations bypass lab-scale investigations and are used, for example, to investigate enzyme-substrate interactions and substrate-binding efficiency (Shukla, 2019).

5. Future Perspectives

5.1 Bioremediation

Bioremediation refers to the use of living organisms or their enzymes to detoxify or restore contaminated sites, often by directing the natural capabilities of microbes towards environmental pollutants (Baba et al., 2016). Microorganisms have proven valuable in the remediation of environmental pollutants and various waste substances such as heavy metals

(Kang et al., 2016; Igiri et al., 2018), polychlorinated biphenyls (PCBs) (Sharma et al., 2018b; Elangovan et al., 2019) and even petroleum (Varjani, 2017; Koolivand et al., 2019). With respect to the contamination of marine and terrestrial environments by plastics, microplastics and their residues, it is vital to establish an efficient and carefully thought-out bioremediation strategy.

Thermobifida sp., *Fusarium* sp., *Saccharomonospora* sp., *Bacillus* sp., *and Pseudomonas* sp., are among the different microbial species that have to date been reported to degrade PET (Kumari and Chaudhary, 2020). Members of the *Pseudomonas* genus*,* which are noted for their extraordinary metabolic versatility, stand out as a natural degraders of synthetic substrates including different types of plastic and plastic associated monomers (Wierckx et al., 2015). In addition to hydrocarbons and other hydrophobic polymers, environmental *Pseudomonas* isolates have been shown to degrade polyethylene, polyethylene glycol, polypropylene, polyurethane, polystyrene, polyethylene succinate, polyvinyl alcohol, and polyvinyl chloride, to varying extents (Wilkes and Aristilde, 2017; Sharma, 2018). One microbial consortium, isolated from cow dung, consisting of thermophilic *Pseudomonas* sp., *Bacillus* sp., *Paenibacillus* sp., and *Stenotrophomonas* sp., was demonstrated to reduce the weight of high-density polyethylene (HDPE) and low density polyethylene (LDPE) by 55% and 77%, respectively, following 120 days incubation (Skariyachan et. al 2017). Several *Pseudomonas* spp., including *P. aeruginosa*, *P. fluorescens*, *P. protegens,* and *P. chlororaphis*, have been found to be polyurethane degraders, despite the high durability of this material (Wilkes and Aristilde, 2017).

Microbes for plastic degradation, especially those which have been enhanced through genetic engineering, should not be released into nature without assessing the risk of introducing foreign microorganisms, that could potentially become invasive. Moreover, the microbes cannot be expected to simply “seek out” and degrade plastic in the environment as there are likely to be other more easily accessible energy sources that they can consume (Jenkins et al., 2019). In the case of marine environments, where plastic waste most commonly accumulates, microorganisms for bioremediation may not be well adapted to operating under saltwater conditions. Therefore, the relevant enzymes may need to be produced in a better suited microbial cell factory (Moog et al., 2019). Another solution to facilitate bioremediation of ocean plastic is to use membrane-based systems featuring immobilized microbes or enzymes for plastic degradation. This would provide a “concentrated” source of plastic for the degradative microbes while also ensuring their containment (Jenkins et al., 2019).

Microorganisms are generally found living within complex communities that interact intensively and can adapt their overall metabolism to efficiently exploit the available resources (Jenkins et al., 2019). The use of a defined, but flexible mixed microbial consortia (MCC) can help extend this concept to biotechnology processes and offers the advantages of inexpensive culture preparation and maintenance, reduced susceptibility to contamination, improved conversion efficiencies, and an alternative to improvement by genetic modification. Biocontainment measures which enable the termination of the MMC or certain strains, such as passcode or dead-man kill switches, are required for their use in natural environments (Jenkins et al., 2019). In this respect the aforementioned microbial consortium no. 46 from *Ideonella sakaiensis*, may prove useful in bioremediation applications, either directly in the environment or in designated facilities for the treatment of PET waste and microplastics from marine and terrestrial sources (Hiraga et al., 2019). However, to fulfil the criteria for efficient bioremediation, natural microbial consortia require synthetic modifications to improve consortium interactions such as cell-cell signaling and metabolite exchange (Jaiswal et al., 2020).

Extensive research is still required for the bioremediation of PET-based environmental contamination and even with progress in this area, challenges will remain due to other types of synthetic polymers, present as waste in the environment. Although the potential of current candidates cannot be readily extended to large-scale bioremediation applications, the exploitation of their enzymes or metabolic pathways could be achieved through synthetic biology approaches and systemic biology, which utilizes multi-omics tools and biodegradation network databases, thus facilitating bioremediation of a complex substance like plastic (Jaiswal et al., 2020; Kumari and Chaudhary, 2020).

5.2 Biological Recycling

Unlike glass or aluminium, where material can be repeatedly recovered in its original form, it is difficult to close the loop on the recycling of plastics. However, PET can to some extent be recycled using mechanical, chemical, or biological routes. In mechanical recycling, collected and sorted PET waste can be shredded and ground into powders before melting and reprocessing to other forms (Koshti et al., 2018). The resulting fibers, films and sheets cannot however be further recycled and are often incinerated, subsequently contributing to increased CO_2 emissions. Chemical recycling aims to degrade PET into its basic monomers which can then be repolymerized. This method is unfavorable from an economic standpoint as the

recovered monomers are more expensive than those from crude oil and despite its drawbacks, mechanical recycling is much more cost effective (Awaja and Pavel, 2005; Hiraga et al., 2019). In addition, chemical methods require the maintenance of high temperature and pressure as well as employing toxic reagents and several preceding unit operations (Wei and Zimmermann, 2017a; Koshti et al., 2018). For both chemical and mechanical recycling, the temperatures used are usually over 100°C and often exceed 200°C. Unfortunately, there are also several contaminants, that significantly interfere with the effectiveness of these PET recycling methods, whether they arise from other waste collected, the recycling process itself or misuse by consumers (Awaja and Pavel, 2005; Webb et al., 2013). Overall, PET recycling remains an expensive and inefficient process, regardless of the advantages over waste management via landfill and incineration, which are associated with major environmental drawbacks such as pollution and atmospheric release of harmful compounds (Zhang et al., 2004; Webb et al., 2013).

While chemical and mechanical processing of PET are currently in widespread use, biological recycling is emerging as a more sustainable solution as it can be performed under mild pH and comparatively low temperature conditions, without the use of hazardous chemicals (Awaja and Pavel, 2005; Webb et al., 2013; Wei and Zimmermann, 2017b). Biological recycling (or bio-recycling) is based on microbial catalysis of polymer bond cleavage reactions, which results in the recovery of monomers that can be subjected to further processing (Koshti et al., 2018). Bio-recycling holds great potential for improved efficiency and cost effectiveness over the aforementioned mechanical and chemical methods as well as helping to eliminate the occurrence of secondary pollutants that come with landfill and incineration (Webb et al., 2013). The possibility exists that a biological recycling process could be achieved using PET hydrolysis and further metabolism of its monomers by *Ideonella sakaiensis* and other PET-degrading organisms and their enzymatic systems (Hiraga et al., 2019). However, bio-recycling is limited by the organism used, inherent polymer properties and the choice of pre-treatment. The future success of this process will rely on optimization and/or modification of these factors (Koshti et al., 2018). Given the time and variables associated with cultivating and maintaining microorganisms as well as extracting their enzymes and allowing the degradation reaction to proceed, biological recycling is relatively slow when compared with chemical or mechanical methods. Therefore, bio-recycling must currently be carried out in combination with at least one of these other approaches (Koshti et al., 2018; Farzi et al., 2019).

In one study, Farzi and co-workers assessed the biodegradation of PET by *Streptomyces* species in conjunction with a mechanical strategy. PET waste, in the form of bottles, was ground down to a powder form prior to the bacterial treatment, which comprised of 18 days incubation in a culture medium at 28°C. Powdered PET was separated by particle size (500, 420, 300, and 212 μm) with 50 mg samples of each size undergoing treatment. Through extraction of residual PET, final biodegradation percentages for the four groups were determined to be 49.2, 57.4, 62.4, and 68.8%, in order of decreasing particle size. Metabolites were analyzed via GC-MS to detect the hydrolysis products and to confirm the biodegradation process had taken place. In addition, a comparison between powdered and film sample degradation was undertaken using a PET film made from bottles, that was then subjected to the same biodegradation treatment, again for 18 days. Subsequent SEM analysis was used to visualize the surface of this film. While it was shown that degradation of the film surface did occur, results were not significant, particularly in contrast to the powdered samples. It was evident from this lab-scale assessment that a mechanical powdering treatment can be very effective, giving high degradation efficiency (Farzi et al., 2019)

In another study, which combined bio-recycling with alkaline hydrolysis, an alkali-resistant whole cell biocatalyst was employed (Gong et al., 2018). Alkaline hydrolysis is one of several methods used to achieve PET chemical recycling (Carta et al., 2003; Koshti et al., 2018). A *Comamonas testosteroni* F4 strain that had previously been isolated from the wastewater of a factory producing PET (Zhang et al., 2004), was shown to degrade PET fibers and was subsequently subjected to evolutionary engineering to first give *Comamonas testosteroni* F5, a strain which could utilize PET as a sole carbon source under alkaline conditions (Gong et al., 2012). Further modifications were completed to obtain the final alkali-tolerant strain, *Comamonas testosteroni* F6. With whole-cell bacterial degradation of PET, the products do not accumulate in the culture as they are used during growth of the strain, thereby circumventing any feedback inhibition. Micro-sized PET particles (<10μm) prepared by drying and grinding PET were used as the substrate, with no additional carbon sources. These particles were subjected to a 48-h fermentation at 37°C under three degradation conditions using: (1) the original F4 strain in a neutral medium; (2) the engineered F6 strain in an alkaline medium; (3) an alkaline medium without any bacterial culture. Under alkaline catalysis, the decomposition products were relatively simple, consisting mostly of TPA. Conversely, MHET, BHET, TPA, and methyl acrylate (MA) were the products obtained following degradation under biocatalytic conditions. The highest quantity of PET degradation products was observed under alkaline

conditions with *Comamonas testosteroni* F6, which was greater than the sum of both products under neutral conditions and alkaline hydrolysis (Gong et al., 2018).

Efficient biodegradation of highly crystallized PET was achieved by Chen and colleagues using a cell surface display-based strategy (Chen et al., 2020). An *Is*PETase-displaying yeast whole-cell biocatalyst was developed to overcome the low enzymatic activity of native *Is*PETase towards hc-PET, which limits its potential use in bio-recycling. Engineered *Pichia pastoris* yeast cells were constructed to functionally display *Is*PETase from a codon-optimized gene sequence. Endogenous glycosylphosphatidylinositol (GPI) proteins in the yeast cell wall were selected as anchor proteins, to which the enzyme could be connected via a flexible linker sequence (Chen et al., 2020). This strategy aimed to increase the probability of fully exposing the unique active-site cleft and substrate binding sites, which are crucial for *Is*PETase activity (Austin et al., 2018; Wu et al., 2018). A dramatic improvement in degradation efficiency was observed using this whole-cell biocatalyst system, with an approximated 36-fold increase in turnover rate compared with that of purified *Is*PETase. The whole-cell biocatalyst was shown to be robust and reusable, with the turnover rate remaining stable up to its seventh repeated use and under certain solvent/chemical conditions. In addition, the pH and thermostability of the enzyme increased following cell surface display. This approach presents a promising route for efficient bio-recycling and coupled with this, there is potential for activity to be further enhanced through similar cell-surface display of engineered *Is*PETase variants (Chen et al., 2020).

5.3 The Role of PET Hydrolase Enzymes in a Circular Bioeconomy

Plastics are currently one of the major challenges in establishing a circular economy. The aim of a circular economy is to promote sustainability and efficiency by creating loops which feed resources back into the economy to make the same or new products. Plastic production is relatively sustainable when compared with the resource-intensive processes associated with other materials such as glass and metal. That said, there is a huge need to re-evaluate plastic design, production, use and management in order to improve its ability to re-enter the system and to ensure that maximum value is being recovered (Hahladakis et al., 2020). Through a combination of biodegradation and biosynthesis, there is potential for a circular, bio-based PET economy that could play a significant role in reducing the associated detrimental environmental effects. In general, the low production cost of plastic has played a major role in accelerating its mismanagement as reuse does not offer an economic advantage. While current strategies remain

the most profitable option, a PET bioeconomy could match public expectations for alternative PET management, which is already leading to progress in terms of policies and regulations. In this way, the financial gap between strategies could be partially bridged, enabling an opportunity for further development of sustainable PET processing and therefore improving the environmental impact and rates of recycling in the longer term (Salvador et al., 2019).

In this respect, a strategy for movement towards a circularized PET economy which employs PET hydrolase enzymes to establish PET as a biotechnological feedstock has been proposed (Salvador et al., 2019). Due to the "bowtie" metabolic structure associated with microbes, there is great potential for biodegradation products to be transformed into a range of useful molecules by channeling the monomers into central metabolism and linking to relevant biosynthetic pathways. This could be achieved by using metabolic engineering and synthetic biology to incorporate PET hydrolase enzymes into a microbial biosynthesis chassis, thereby helping to create revenue from PET waste while also reducing its release into the environment (Salvador et al., 2019; Taniguchi et al., 2019; Blank et al., 2020). Recently, the photosynthetic microalga *Phaeodactylum tricornutum* was employed as a chassis to produce and secrete an engineered *Is*PETase which displayed degradative activity towards different PET substrates (Moog et al., 2019). These cell factories could be valuable in future applications, including the design of photobioreactors capable of PET biodegradation and synthesis of new PET from the resulting monomers. To date *Escherichia coli* and *Bacillus subtilis* are among other microbial systems that have been successfully utilized for the creation of synthetic *Is*PETase cell factories (Huang et al., 2018; Moog et al., 2019; Seo et al., 2019).

Prior to central metabolism, TPA is converted into protocatechuic acid (PCA) that can be further degraded via several different pathways/routes (Frazee et al., 1993; Maruyama et al., 2004; Kasai et al., 2009). This is important to consider when developing PET-based bioprocesses because different pathways will generate a distinct range of metabolites, with varied applications. For example, PCA itself has been used to synthesize adipic acid, which is an industrially relevant metabolite (Johnson et al., 2016; Salvador et al., 2019) and EG, which can be degraded into acetate or glyoxylate (GLA), has an even more diverse metabolism than TPA (Child and Willetts, 1978; Kataoka et al., 2001; Trifunović et al., 2016). Significantly, EG has been transformed into the bioplastic polyhydroxyalkanoate (PHA) by an engineered *Pseudomonas putida* KT2440 strain, presenting an opportunity to use PET monomers as a feedstock for the production of a biodegradable alternative (Franden et al., 2018; Blank et al., 2020).

Bio-PET, which refers to a PET polymer that is at least partially derived from biological sources, can be produced through the microbial synthesis of TPA and EG. By cutting down the dependence on fossil fuel-derived "virgin" PET, this method could make a significant contribution to a sustainable and circular PET economy (Salvador et al., 2019). While biosynthesis of aromatic compounds by microbes has not developed to the same extent as their degradation, it has been proposed to produce terephthalic acid by harnessing the shikimate pathway to provide *p*-toluate which could be transformed into TPA (Osterhout et al., 2014). It may also be possible to achieve sustainable TPA biosynthesis by using aromatics from renewable sources like lignin. However, many complexities are associated with biological TPA production and therefore, it is only EG that is currently produced biologically from renewable feedstocks to give bio-PET (Salvador et al., 2019). This has been achieved by engineering artificial pathways into microorganisms, enabling the use of renewable plant feedstocks, such as xylose (Cam et al., 2016) and corn stalk (Pang et al., 2011).

Most recently, the aforementioned LC-cutinase enzyme was engineered with the aim of maximizing catalysis of PET depolymerization and was subsequently shown to give 90% conversion within just 10 h. New PET was successfully synthesized from the TPA monomers obtained (Tournier et al., 2020). LCC was demonstrated to outperform four other PET hydrolytic enzymes, *Is*PETase, *Thermobifida fusca* hydrolases TfH (BTA-1) and BTA-2, and *Fusarium solani* cutinase when compared in terms of specific depolymerization rate of pre-treated amorphous PET film at 65°C. Then, following computational identification of amino acid residues for site-saturation mutagenesis, a range of variants were generated with the aim of introducing enhanced thermostability and specific activity towards bottle-grade PET. Four quadruple variants, ICCG, ICCM, WCCG and WCCM, were ultimately selected for further evaluation (Tournier et al., 2020).

These engineered enzymes were assessed under bioreactor conditions for degradation of post-consumer colored-flake PET (PcW-PET) waste. This is the residue that remains once clear PET waste for mechanical recycling has been removed (Tournier et al., 2020). PcW-PET was pre-treated using extrusion and micronization processes that are employed widely in plastics industries (Awaja and Pavel, 2005; Barboza Neto et al., 2014). The reaction temperature was set at 72°C to maximize kinetic turnover. WCCG and ICCG achieved the best conversion levels, with 85% and 82% conversion within 15 h and 20 h respectively. Wild-type LCC reached only 53% conversion in 20 h, owing to its lower thermostability that rapidly reduces the reaction kinetics after just 2 h. Following subsequent comparison at 3 milligrams of enzyme

per gram of PET, WCCG and ICCG demonstrated 90% depolymerization after 10.5 h and 9.3 h, respectively. This was further increased to a 150 L pilot-scale process using high content of PcW-PET (200 g/kg), which utilized 2 mg_{enzyme}/g_{PET} in a trade-off between enzyme cost and productivity (Tournier et al., 2020).

Initial process development investigated the recycling of terephthalic acid (TPA) only as this is the main component of PET by weight, with 1 ton of PET waste leading to 863 kg of TPA. Terephthalate monomers were purified to a level exceeding 99.8% through the use of industrially applied methods such as discoloration by activated carbon and crystallization (Meyer, 1966; Mohammad-Khah and Ansari, 2009). TPA monomers were used in the synthesis of "virgin" PET, which required three consecutive steps: esterification, polycondensation and solid-state polymerization. The resulting PET was used to blow new bottles, which exhibited improved lightness and similar mechanical characteristics to commercially available PET bottles, thus creating a closed-loop recycling process (Tournier et al., 2020).

Biological strategies, even in conjunction with established and emerging initiatives to curtail plastic pollution, both voluntary and regulatory, will not meet the current requirements. Instead, a combination of several different multi-tiered approaches will be essential in eliminating this issue (da Costa et al., 2020). The matter has been further complicated by the COVID-19 pandemic, whereby plastic-based personal protective equipment, such as masks and gloves, along with single-use plastics due to hygiene concerns and food packaging demands has resulted in delays in enforcing plastic reduction policies (Patrício Silva et al., 2020).

Overall, the prospects for establishing a PET circular bioeconomy are still promising, with several strategies underway that use PET hydrolytic enzymes (or whole cells) to give TPA and EG for synthesis of fresh PET or for conversion into value-added products. Biodegradation-based processes could be complemented by sustainable synthesis of TPA and EG monomers for bio-PET. Some of the potential routes for PET in the circular economy are summarized (Figure 5).

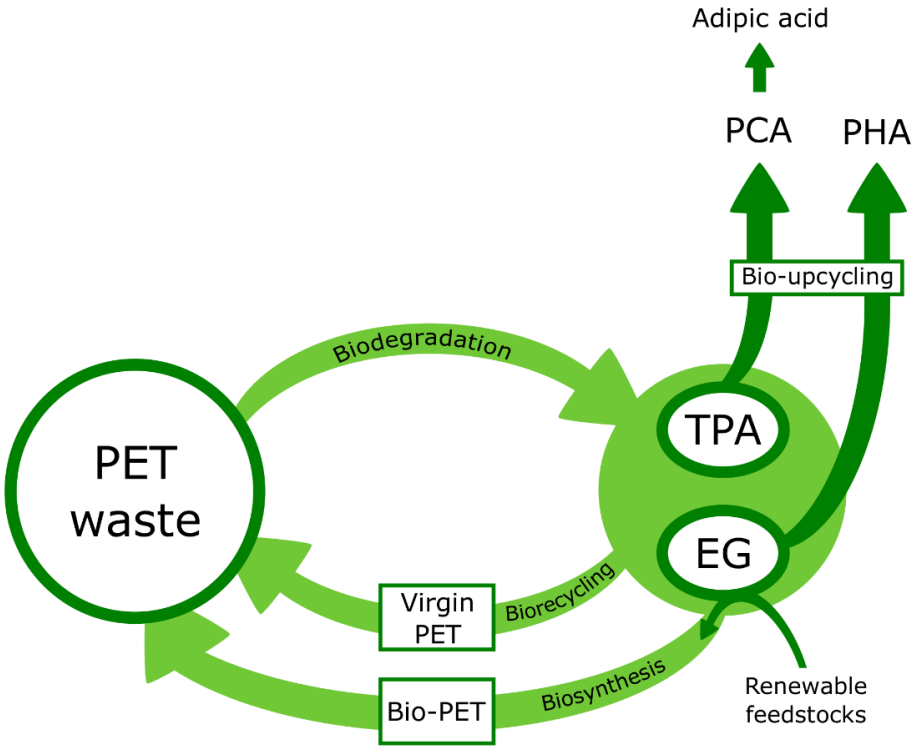


Figure 5. Prospective overview of PET in the circular economy. Monomers resulting from biodegradation of PET may be recovered for manufacture of virgin PET (biorecycling) or combined with microbially synthesized monomers for a bio-based alternative (biosynthesis). Adipic acid, an important chemical precursor used primarily in nylon production, and polyhydroxyalkanoates (PHAs), which are both bio-based and biodegradable, are among the value-added products that may be obtained through transformation of TPA and EG monomers (bio-upcycling).

6. Final Remarks

While the efforts to harness the power of microbial PET hydrolase enzymes are ongoing, research in this area has been encouraging and new knowledge has quickly accumulated. Many different groups have been working to identify and characterize PET hydrolyzing microorganisms and their enzymes to explore degradative activities, connect structure with function and uncover the mechanisms behind microbial processing of synthetic polymers. This work, together with the use of powerful enzyme technologies to create improved variants, has already improved the feasibility of practical PET degradation and will continue to underpin future applications as well as process optimization. Some of the most promising cases

highlighted in this review have included pilot-scale PET hydrolysis using engineered LC-cutinase together with *Ideonella sakaiensis* enzymes, various *Is*PETase mutants and *IsPETase* cell factories or display systems that may enable break down of high crystallinity PET under mild reaction conditions. Although enzymatic systems that resemble the *Ideonella sakeinsis* PETase-MHETase PET processing pair have not yet been observed in any other bacterial genome sequences, the chances of finding potential PET metabolic enzymes in the future is likely to increase with the aforementioned ongoing rapid expansion of genomic and metagenomic sequence data.

With the aim of addressing different types of PET waste, especially high crystallinity forms, there is a need for further investigation into strategies such as synergistic combined enzyme systems, use of ionic surfactants, circumvention of PET intermediate inhibition and the development of biosynthetic cell factories. It would also be useful to establish standard protocols and PET substrates to allow for more meaningful comparisons when assessing PET hydrolase candidates and determining optimal process conditions. The development of microbes and enzymes for biological recycling will help progress their implementation in other areas, for instance in bioremediation strategies. Despite the improvements that can be accomplished using enzyme engineering, “mining” for new PET hydrolases is still vital to gain a better understanding of plastic degradation in nature and to identify inherent activities that can be enhanced to enable treatment of PET waste in situ. Culture based screening strategies coupled with metagenomic based approaches targeting various PET or hydrocarbon contaminated environmental samples is likely to uncover even better evolved microbial activities for PET metabolism.

In recent years there has been a significant shift in awareness and concern surrounding plastic-related environmental issues. For the most part, plastic is not inherently “bad”, with alternative materials often having a higher overall impact when factors such as production requirements and bulk shipping are considered. Plastic packaging also provides the current best solution in niche sectors such as for example in helping prevent food spoilage in the food industry. Today’s plastic problem is rooted in a loss of control and the inability of waste collection, disposal, and recycling infrastructure to match the pace of the plastics industry. Therefore, a multi-faceted approach is essential in addressing the complexities of this issue. In the context of contributing microbial based solutions, there must be a balance between process optimization and environmental consequences, such that counterproductivity is avoided. A collaborative effort is needed to reimagine the life cycle of PET, where its value is maximized, and it is treated as

a feedstock rather than as a waste product after use. The development of systems which employ microbes for PET biodegradation and biosynthesis hold the potential to fulfil these requirements, at least partially. This shift from a linear to circularized system of PET production and processing could help meet public demands by reducing the need to “tap into” fossil fuel reserves and incentivizing better handling of PET waste.

This review serves as an important reminder to other researchers in this field, and to the wider scientific community, of the numerous PET hydrolase studies that have been carried out to date, with an emphasis on the most recent examples and advances. By highlighting the major challenges in studying and implementing enzymes for PET degradation, a clearer consensus can be developed to guide future work. Plastic pollution has become a global concern, and the interest in sustainable change is shared by the general public, environmental organizations, and by governments and industrial leaders. Therefore, it is hoped that the different perspectives presented in this review will play a role in helping to encourage a more multi-disciplinary approach to address the ongoing problem of plastic waste streams.

7. Advances and State of the Art

It has been projected that global plastics production will increase to 700 Mt in 2030, with demand increasing at an estimated rate of 5% each year (Tournier et al., 2023). Therefore, improvements are essential at all stages of the plastic supply chain, with bio-based technologies representing a key component in the future of sustainable polymer production and the management of postconsumer waste. Given their relative biodegradability compared to covalent (C-C) bond-containing polymers (e.g., PE, PVC, and PP), polyesters, polyurethanes, and polyamides have been important targets of in the area of enzymatic depolymerization, with various hydrolases known to act on C-O and C-N linkages (Orlando et al., 2023). PET remains the most common subject of such plastic hydrolysis studies, with continued worldwide efforts being made to uncover new PET hydrolases, engineer better-performing variants, elucidate their reaction mechanisms, and move towards feasible industrial PET processing at scale (Arnal et al., 2023).

An integrated *in vitro* and *in silico* approach, that employs rational design, directed evolution, and high-throughput screening has been necessary to optimize wild-type PET hydrolases for application, with further diversification of enzyme possible with advanced database mining and protein structural analyses and AI-guided design (Mican et al., 2024). LC-cutinase has represented the benchmark PET hydrolase in protein engineering and large-scale reactions, while *Is*PETase provided key structural insights, with numerous approaches used to generate improved variants of both enzymes, including by structure-guided mutagenesis and algorithm-based redesign (Cui et al., 2021; Erickson et al., 2022; Lu et al., 2022; Mican et al., 2024).

With respect to wild-type proteins, the PAZy database has been constructed for the collection of biochemically characterized plastics-active enzymes (Buchholz et al., 2022). While the discovery of new PET hydrolases from natural environments is often laborious in comparison to computational approaches, new assays have been developed for higher throughput detection of enzymatic activities, for example, using droplet microfluidics-, chemoselective probe-, and biosensor-based techniques (Bayer et al., 2022; Qiao et al., 2022; Dierkes et al., 2023; Hecko et al., 2023). Enrichment and metagenomics-based strategies have proven useful in identifying novel plastic-degrading microorganisms or enzymes, and will continue to be highly valuable in combination with omics technology, modern robotics, and AI-based analyses (Chow et al., 2023).

The versatility of PET hydrolase enzymes was recently exemplified by the upcycling of PET waste to calcium terephthalate, which may be employed in battery anode production, in a one-pot biocatalysis-based reaction (Xue et al., 2024). A cross-disciplinary effort has proven critical in the development and upscaling of enzymatic solutions to manage the issue of plastic waste (Herbert et al., 2022). Although the susceptibility of a given plastic to enzymatic recycling is highly dependent on the chemical backbone and degree of cross-linking exhibited by each type of polymer, the learnings that have emerged in the last decade from the study of PET hydrolases will help to inform the field of biological plastic degradation in general.

8. Author Contributions

CMC wrote the manuscript. ADWD and DJC supervised and corrected the manuscript. All authors read and approved the final manuscript.

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10. Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Chapter 2

Identification of BgP, a Cutinase-Like Polyesterase from a Deep-Sea Sponge-Derived Actinobacterium

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1. Abstract

Many marine bacteria produce extracellular enzymes that degrade complex molecules to facilitate their growth in environmental conditions that are often harsh and low in nutrients. Marine bacteria, including those inhabiting sea sponges, have previously been reported to be a promising source of polyesterase enzymes, which have received recent attention due to their potential ability to degrade polyethylene terephthalate (PET) plastic. During the screening of 51 marine bacterial isolates for hydrolytic activities targeting ester and polyester substrates, a *Brachybacterium ginsengisoli* B129SM11 isolate from the deep-sea sponge *Pheronema* sp. was identified as a polyesterase producer. Sequence analysis of genomic DNA from strain B129SM11, coupled with a genome "mining" strategy, allowed the identification of potential polyesterases, using a custom database of enzymes; that had previously been reported to hydrolyze PET and other synthetic polyesters. This resulted in the identification of a putative PET hydrolase gene, encoding a polyesterase-type enzyme which we named BgP; that shared high overall similarity with three well-characterized PET hydrolases – LCC, TfCut2, and Cut190, all of which are key enzymes currently under investigation for the biological recycling of PET. *In silico* protein analyses and homology protein modelling offered structural and functional insights into BgP, and a detailed comparison with Cut190 revealed highly conserved features with implications for both catalysis and substrate binding. Polyesterase activity was confirmed using an agar-based polycaprolactone (PCL) clearing assay, following heterologous expression of BgP in *Escherichia coli*. This is the first report of a polyesterase being identified from a deep-sea sponge bacterium such as *Brachybacterium ginsengisoli* and provides further insights into marine-derived polyesterases, an important family of enzymes for PET plastic hydrolysis. Microorganisms living in association with sponges are likely to have increased exposure to plastics and microplastics given the wide-scale contamination of marine ecosystems with these plastics, and thus they may represent a worthwhile source of enzymes for use in new plastic waste management systems. This study adds to the growing knowledge of microbial polyesterases and endorses further exploration of marine host-associated microorganisms as a potentially valuable source of this family of enzymes for PET plastic hydrolysis.

2. Introduction

Marine microbial communities play a central role in maintaining and supporting ocean ecosystems, largely by participating in biogeochemical processes, such as carbon cycling

(Arnosti et al., 2014). Moreover, by harnessing their ability to catalyze biological reactions, marine microorganisms can be used to reduce environmental pollutants, as well as the damaging effects of industrial activities (Carr et al., 2020; de Oliveira et al., 2020). Many marine enzymes offer novel biocatalytic properties, particularly when compared to those from terrestrial environments, due to the diversity of conditions in which they normally operate and the presence of unusual and often bulky substrates with distinct chemical substituents in marine ecosystems (Trincone, 2011; Parages et al., 2016; Trincone, 2017).

The plastics era, which evolved alongside fast-paced, modern lifestyles and transformative technological advancements, has contributed to a major shift towards a society of mass production and excess consumption. Over the past decade, 4.8-12.7 million metric tons (MMT) of plastic has been reported to enter the world's oceans on an annual basis, a figure which could accumulate to 250 MMT by 2025 (Yakimov et al., 2022). The "plastisphere", which refers to the microbial communities colonizing plastic debris, has attracted particular attention in the context of marine ecosystems (Amaral-Zettler et al., 2020; Li et al., 2021; Yakimov et al., 2022), with recent multi-omics analyses on the plastisphere highlighting this habitat as a promising source of plastic-degrading microorganisms (Suzuki et al., 2021; Wright et al., 2021; Zrimec et al., 2021).

Plastics are as versatile and easy to produce as they are problematic and difficult to discard. Unlike the broadly employed mechanical recycling methods that result in plastic "downcycling", chemical and biological recycling processes aim to facilitate virgin plastic production by recovering the original monomers (Carr et al., 2020). Thus, biological-based methods in plastic disposal or recycling strategies are gaining increased attention (Bahl et al., 2021). These methods, which may be either enzyme- or whole-cell catalyzed, are particularly attractive as they offer the possibility of comparatively mild reaction conditions, high specificity, and reduced energy requirements and greenhouse gas emissions, that are potentially more cost-effective when compared to chemical recycling (Carr et al., 2020; Singh et al., 2021).

Polyethylene terephthalate (PET) is a crude-oil derived thermoplastic synthetic polyester, consisting of terephthalic acid (TPA) and ethylene glycol (EG) monomers (Webb et al., 2013). PET, which is currently the most abundantly used polyester plastic in the world, has a predicted life span of up to 50 years (Pirillo et al., 2021). Its widespread use across packaging, textile, automotive, electrical and electronics sectors is primarily related to its light weight and high mechanical strength, together with its insulating properties, and capacity to act as both a gas

and moisture barrier (Webb et al., 2013; Liu et al., 2019a; Dissanayake and Jayakody, 2021). In particular, its durability and moldability are convenient for the production of PET containers, films, and fibres, together with bottles commonly used for carbonated soft drinks and water. However, the aforementioned qualities that make PET an attractive material for various industrial applications are also responsible for the problems it can cause if its disposal is mismanaged (Jaiswal et al., 2020). The backbone of the PET polymer is highly stable, which together with its surface hydrophobicity and crystallinity, restricts its natural breakdown (Liu et al., 2019a; Kawai et al., 2020).

Microbial enzymes with PET depolymerizing activity have however introduced the possibility of PET degradation or modification to allow the development of techno-economically feasible, microbial-based PET recycling processes (Tournier et al., 2020; Zimmermann, 2020). These PET-active enzymes commonly belong to carboxylesterase, lipase, and cutinase families (Danso et al., 2019), and to date have predominantly been identified in thermophilic Actinobacteria, particularly in the genus *Thermobifida* (Silva et al., 2011; Wei and Zimmermann, 2017). The best studied enzyme in this area is a PET hydrolase or "PETase" from *Ideonella sakaiensis* 201-F6 (*Is*PETase), on the basis that the strain was isolated from a PET-enriched environment and had potentially evolved specifically to process PET. This enzyme was subsequently biochemically and structurally characterized and adapted across a number of studies, in an effort to enhance its overall activity and stability (Han et al., 2017; Chen et al., 2018; Joo et al., 2018; Liu et al., 2019a; Urbanek et al., 2020).

Given the aforementioned studies highlighting the plastisphere as a promising source of plastic-degrading microorganisms, coupled with the fact that plastic constitutes up to 90% of the solid waste found in oceans (Oliveira et al., 2020); it is perhaps not surprising that microorganisms from marine ecosystems and holobionts, such as marine sponges and seaweeds, are likely to have been exposed to plastics or microplastics in marine environments and can be targeted as a potential source of PET-active enzymes. Our group has recently reported on a PETase-like enzyme with polycaprolactone (PCL)-degrading activity, which was identified in the marine sponge-derived *Streptomyces* sp. SM14 strain (Almeida et al., 2019). Additionally, a putative polyesterase named MorEst, from an Antarctic psychrotrophic bacterium from the genus *Moraxella* sp. has been reported to degrade a range of polyesters, including PCL (Nikolaivits et al., 2020). Also, a novel polyester hydrolase from the marine bacterium *Pseudomonas aestusnigri* with activity against PET film has been reported which, following mutagenesis, also displayed some activity against commercial PET bottles (Bollinger et al., 2020). Furthermore,

a study of marine metagenomic datasets has reported that genes encoding PET hydrolases are globally distributed in marine environments (Danso et al., 2018; Zrimec et al., 2021). Thus, marine microbiomes appear to be a potentially rewarding source of novel polyesterase and PETase enzymes and given the continued need to increase the diversity of enzymes and microorganisms acting on artificial polyesters, may prove a useful resource for these types of enzymes.

The deep sea, while one of the most vast biomes on our planet, still remains relatively unexplored. This environment, defined by seawater depths below 1,000 m and averaging at 3,800 m, is considered extreme; given its challenging physical conditions and unique composition (Jin et al., 2019). The microbial communities that can survive and thrive in the deep sea face a dynamic range of pressure, temperature, pH, and salinity, together with exposure to various chemicals and metals. The production of enzymes that tolerate these parameters contribute to the adaptive strategy of these microorganisms, and so the deep sea represents a promising source of stable and robust enzymes for industrial use (Jin et al., 2019). Furthermore, marine microbial communities and their hydrolytic enzymes may interact with synthetic polymer debris, within these ecosystems (Galloway et al., 2017). Plastic particles have been detected in deep-sea ecosystems, such as in Western Pacific Ocean sediment at depths between 4,601 and 5,732 m, where the most abundant microplastics were found to be poly(propylene-ethylene) copolymer (PP-PE) and PET (Zhang et al., 2020). It is worth noting that, although the ability to hydrolyze PET could potentially enhance the evolutionary fitness of microorganisms in the environment, this would require that PET is fully metabolized. In the case of the aforementioned *Ideonella sakaiensis*, the role of biofilms and microbial consortia, together with a dedicated PET metabolic pathway, have been explored to provide explanation for its ability to grow on PET as a sole carbon source (Taniguchi et al., 2019; Carr et al., 2020).

Following the screening of a number of marine sponge-derived bacterial isolates, with lipolytic activity that was initially observed on tributyrin agar, and with polyesterase activity subsequently being confirmed on polycaprolactone diol (PCD) and polycaprolactone (PCL) agar plates; a *Brachybacterium ginsengisoli* B129SM11 strain isolated from the deep-sea sponge *Pheronema* sp. which had been sampled at a depth of 2,129 m, was prioritized for further analyses. Genome mining of the B129SM11 strain revealed a putative PETase gene which, following computational protein analyses and homology modelling, was shown to encode a cutinase-like enzyme, named BgP. Polyesterase activity was subsequently confirmed

on ester and polyester substrates, following cloning and heterologous expression of BgP in *Escherichia coli*.

Cutinases and cutinase-like enzymes have become the focus of a number of polyester and PET hydrolysis studies, and are considered versatile biocatalysts with several uses, which are not limited to waste treatment and biorecycling, but also having other biotechnological applications, for example, in sustainable chemical synthesis, polymerization, and polymer modification (Chen et al., 2013; Nikolaivits et al., 2018; Molitor et al., 2020). This is the first report of a cutinase-like polyesterase being identified in a deep-sea sponge-derived *Brachybacterium* spp. isolate and this work provides further insight into enzymes for polyester degradation.

3. Materials and Methods

3.1 Sponge Sampling and Isolation of Bacterial Strains

The *R.V. Celtic Explorer* Irish research vessel and *Holland I* remotely operated vehicle (ROV) were employed for the collection of the marine sponge *Pheronema* sp., from the North Atlantic Ocean in the Irish Rockall Trough. The sponge sample was obtained from a depth of 2,129 m as part of a biodiscovery cruise in 2010. Following collection, sponge samples were rinsed with sterile artificial seawater (ASW) [Instant Ocean™, 3.33% (w/v)], macerated with a sterile razor blade, then placed in a tube with sterile glass beads and vortexed for 2 mins. Sterile ASW was added, and samples were vortexed again. Dilution series were performed to 10^{-5} with sterile ASW and microbial cultures were isolated by spread plating 100 µL of each dilution onto the following growth media; (i) starch-yeast-peptone seawater agar (SYP-SW): 1% (w/v) starch, 0.4% (w/v) yeast extract, 0.2% (w/v) peptone, 3.33% (w/v) artificial sea salts (Instant Ocean™), 1.5% (w/v) agar; (ii) modified marine agar (MMA): 0.005% (w/v) yeast extract, 0.05% (w/v) tryptone, 0.01% (w/v) β-glycerol phosphate disodium salt, pentahydrate, 3.33% (w/v) artificial sea salts (Instant Ocean™), 1.5% (w/v) agar and (iii) chitin agar: 4% (v/v) colloidal chitin, 1.5% (w/v) agar.

3.2 Enzyme Activity Screening

As part of a wider polyesterase screening study investigating 51 strains from a variety of marine sources, isolate B129SM11 from *Pheronema* sp. sponge was tested for enzyme activities against the following substrates: tributyrin (glyceryl tributyrate, Sigma Aldrich), polycaprolactone diol (PCD, Sigma Aldrich), and polycaprolactone (PCL, Sigma Aldrich),

using agar-based clearing assays previously detailed in (Molitor et al., 2020), with the following adaptations; marine agar (3.74% Marine Broth 2216, BD Difco™; 1.5% agar, Sigma) was supplemented with tributyrin (1.0%), PCD (3.0%), or PCL (0.1%). A Waring® laboratory blender was used to emulsify the tributyrin and PCD substrates with the media before autoclaving. PCL pellets were dissolved in acetone at 70 °C before adding dropwise, under fast stirring to autoclaved agar. Cultures were spot inoculated onto screening plates and incubated at 28 °C, then checked daily for zones of clearing for up to 7 days. Based on the observed activities, confirmed 16S rRNA gene identities, and considering its deep-sea origin, *Brachybacterium ginsengisoli* B129SM11 was subsequently selected as a suitable candidate for genome sequencing (Table 1).

Table 1. Confirmed hydrolytic activities for five isolates derived from deep-sea sponges, which were screened on ester (tributyrin) and polyester (PCD and PCL) substrates to identify potential polyesterase producers.

Isolate ID	Sponge Host	16S rRNA Gene	Depth (m)	Trib.	PCD	PCL
B129SM11	*Pheronema* sp.	*Brachybacterium ginsengisoli*	2,129	+	+	+
B226SK6	*Inflatella pellicula*	*Micrococcus* sp.	2,900	+	+	+
B226M5	*Inflatella pellicula*	*Agreia* sp.	2,900	+	+	-
B98C26	*Hexactinellida* sp.	*Jiella aquimaris*	1,480	+	+	-
B98SN116	*Hexactinellida* sp.	*Tsukamurella pseudospumae*	1,480	+	-	-

3.3 Genomic DNA Extraction

Genomic DNA (gDNA) was extracted from 5 mL cultures grown in Marine Broth 2216 for 24 h at 30°C, with shaking (125 rpm). Cells were pelleted by centrifugation (Eppendorf Centrifuge 5804R) at 4400 x *g* for 20 mins, then broth supernatants were discarded, and cell pellets were allowed to drain. The method used to obtain gDNA from isolate B129SM11 was based on a previously described phenol-chloroform-isoamyl alcohol extraction procedure (Jackson et al., 2018).

3.4 Genome Sequencing, Assembly, and Annotation

Next-generation sequencing was completed by Eurofins Genomics (Konstanz, Germany) using Illumina HiSeq technology (NovaSeq 6000 sequencing system), including library preparation and initial quality checks. FastQC (v 0.11.9) was used to evaluate the quality of the raw sequence reads. Reads were then assembled *de novo* using SPAdes Genome Assembler (v 3.15.0) (Bankevich et al., 2012), excluding contigs < 400 bp. QUAST (v 5.0.2) (Gurevich et al., 2013) was employed to assess overall quality of the final assembly, and completeness and contamination were determined using CheckM (v 1.1.3) (Parks et al., 2015). Initial annotation was completed using Prokka (v 1.14.6) (Seemann, 2014), while functional annotation of the predicted protein output was carried out with eggNOG mapper (v 2.0), against the eggNOG

database (v 5.0) (Huerta-Cepas et al., 2019), and with the BLASTKOALA (KEGG Orthology And Links Annotation) tool using the KEGG (Kyoto Encyclopedia of Genes and Genomes) database (Kanehisa et al., 2016). The Genome Database Taxonomy Toolkit (GTDB-Tk, v 1.5.0) was applied for phylogenomic analyses, operating based on an established set of single-copy conserved marker genes (Chaumeil et al., 2020).

3.5 Genome Mining

A reference dataset, containing the amino acid sequences of 26 PET hydrolases and homologous polyesterases, was used to produce a custom BLASTP database with the makeblastdb command-line tool (Table 2), following a previously described strategy for genome mining (Almeida et al., 2019). Potential homologs were identified in the genome of B129SM11 by performing a BLASTP search of the annotated Prokka output against the constructed database, employing an e-value threshold of 1e-30. The specific homology search results generated for BgP with three well-studied PET-hydrolyzing cutinases are presented in Table 3.

Table 2. Reference dataset of functionally verified polyesterases having activity on PET or PET model substrates, which was used to conduct BLASTP protein homology searches against the B129SM11 annotated genome and for further phylogenetic inferences.

Enzyme	Microbial Source	Uniprot Accession	Reference
TfH	*Thermobifida fusca* DSM43793	Q6A0I4	(Müller et al., 2005)
Tfu_0882	*Thermobifida fusca* YX	Q47RJ7	(Chen et al., 2008)
Tfu_0883	*Thermobifida fusca* YX	Q47RJ6	(Chen et al., 2008)
TfCut1	*Thermobifida fusca* KW3	E5BBQ2	(Herrero Acero et al., 2011)
TfCut2	*Thermobifida fusca* KW3	E5BBQ3	(Herrero Acero et al., 2011)
Est1	*Thermobifida alba* AHK119	D4Q9N1	(Thumarat et al., 2015)
Est119	*Thermobifida alba* AHK119	F7IX06	(Hu et al., 2010)
Thc_Cut1	*Thermobifida cellulosilytica* DSM44535	E9LVH8	(Herrero Acero et al., 2011)
Thc_Cut2	T*hermobifida cellulosilytica* DSM44535	E9LVH9	(Herrero Acero et al., 2011)
Thf42_Cut1	*Thermobifida fusca* DSM44342	E9LVI0	(Herrero Acero et al., 2011)
Tha_Cut1	*Thermobifida alba* DSM43185	E9LVH7	(Ribitsch et al., 2012a)
Thh_Est	*Thermobifida halotolerans* DSM44931	H6WX58	(Ribitsch et al., 2012b)
LCC	metagenome from leaf-branch compost	G9BY57	(Sulaiman et al., 2012)

Tcur1278	*Thermonospora curvata* DSM43183	D1A9G5	(Wei et al., 2014)
Tcur0390	*Thermonospora curvata* DSM43183	D1A2H1	(Wei et al., 2014)
Cut190	*Saccharomonospora viridis* AHK190	W0TJ64	(Kawai et al., 2014)
***Is*PETase**	*Ideonella sakaiensis* strain 201-F6	A0A0K8P6T7	(Yoshida et al., 2016)
BhrPETase	thermophilic bacterium strain HR29	A0A2H5Z9R5	(Xi et al., 2021)
SM14est	*Streptomyces* sp. SM14 (marine)	DAC80635.1 (Genbank)	(Almeida et al., 2019)
PE-H	*Pseudomonas aestusnigri* (marine)	A0A1H6AD45	(Bollinger et al., 2020)
BsEstB	*Bacillus subtilis* 4P3-11	D7R6G8	(Ribitsch et al., 2011)
PET12	*Polyangium brachysporum*	A0A0G3BI90	(Danso et al., 2018)
PET2	Uncultured bacterium (marine metagenome)	C3RYL0	(Danso et al., 2018)
PET5	*Oleispira antarctica* RB-8	R4YKL9	(Danso et al., 2018)
PET6	*Vibrio gazogenes*	A0A1Z2SIQ1	(Danso et al., 2018)
HiC	*Humicola insolens*	A0A075B5G4	(Ronkvist et al., 2009)

Table 3. Sequence similarities between BgP (290 aa) and bacterial polyesterases of interest for PET biorecycling, generated by a BLASTP search of annotated B. ginsengisoli B129SM11 proteins.

Protein	Microbial Source	Score (bits)	E-value	Identity (%)	Length (aa)
Cut190	*Saccharomonospora viridis* AHK190	312	9e-111	62	304
TfCut2	*Thermobifida fusca* KW3	288	9e-102	59	261
LCC	Leaf-branch compost metagenome	270	3e-94	56	293

3.6 Protein Analysis and Homology Modelling

Phylogenetic analysis of protein sequences was carried out using the MEGA-X program (Kumar et al., 2018) with the maximum likelihood statistical method and the WAG + G model, under 100 bootstrap replications and a 50% bootstrap cut-off value. T-COFFEE Expresso (Di Tommaso et al., 2011) was employed to generate amino acid sequence alignments and outputs were graphically represented and analyzed using ESPript 3.0 (Gouet et al., 1999). The Lipase Engineering Database (LED) BLAST resource facilitated the identification of the catalytic triad and oxyanion hole residues (http://www.led.uni-stuttgart.de/). The native protein signal peptide and corresponding cleavage site were predicted using the SignalP 5.0 server (Armenteros et al., 2019). Subcellular localization was inferred with PSORTb (Yu et al., 2010), Gpos-mPLoc (Shen and Chou, 2009), and FUEL-mLoc (Wan et al., 2017). The ExPASy-ProtParam tool was applied to predict physico-chemical properties such as molecular weight, theoretical isoelectric point (pI), amino acid composition, together with aliphatic and instability indexes (Gasteiger et al., 2005). InterProScan (https://www.ebi.ac.uk/interpro/search/sequence/), Pfam (http://pfam.xfam.org/), and SUPERFAMILY (https://supfam.mrc-lmb.cam.ac.uk/SUPERFAMILY/) databases were applied for protein family classification and to uncover functional protein domains.

The SWISS-MODEL homology-modelling server was run to predict a three-dimensional (3D) structure for the BgP protein (Waterhouse et al., 2018). A cutinase-like lipase, SeL, from *Streptomyces exfoliatus* (PDB Accession Code: 1JFR) (Wei et al., 1998), served as a template

for model construction. Model quality was initially assessed from SWISS-MODEL parameters, followed by validation using ERRAT (Colovos and Yeates, 1993), VERIFY3D (Eisenberg et al., 1997), and PROCHECK (Laskowski et al., 2006), which are tools within the Structure Analysis and Verification Server 6.0 (SAVES, https://servicesn.mbi.ucla.edu/SAVES/), in addition to the ProSA-web server (Wiederstein and Sippl, 2007). The active site pocket and potential binding residues were detected using Phyre2 (Colovos and Yeates, 1993) together with 3DLigandSite (Wass et al., 2010). Protein stability was predicted by SCooP (v 1.0), using a temperature-dependent dataset containing mesostable and thermostable proteins (Pucci et al., 2017). UCSF-Chimera was used to visualize the 3D model and enable structural comparison with the known polyesterase Cut190 (PDB code: 4WFI) ((Pettersen et al., 2004).

3.7 Cloning and Heterologous Expression

Snapgene (v 5.2.5.1) software (https://www.snapgene.com/) was employed to assist primer design and to simulate cloning. The GenScript Restriction Enzyme Map Analysis Tool (https://www.genscript.com/tools/restriction-enzyme-map-analysis) and the Molbiotools Restriction Analyzer (https://molbiotools.com/restrictionanalyzer.php) were used to assess the native gene sequence and aid in the selection of restriction enzymes. The Sigma-Aldrich OligoEvaluator™ (http://www.oligoevaluator.com/) and the Thermo Fisher Scientific Multiple Primer Analyzer (https://www.thermofisher.com/ie/en/home/brands/thermo-scientific/molecular-biology/molecular-biology-learning-center/molecular-biology-resource-library/thermo-scientific-web-tools/multiple-primer-analyzer.html) were run to predict possible secondary structures or primer-dimer pairings, and revealed the overall stability, melting temperature (T_m), and GC content. The New England BioLabs (NEB) T_m Calculator (https://tmcalculator.neb.com/) was used to evaluate specific annealing and melting temperatures for use of these primers with Q5® High-Fidelity Polymerase (NEB). Primer binding and PCR product length were predicted by uploading the *B. ginsengisoli* B129SM11 genome to Primer-BLAST (Ye et al., 2012).

The *bgp* gene was amplified from genomic DNA with a forward primer (5'-AAAAACATATGCACGCACAGACCCGCAGGATC-3'), containing an *Nde*I restriction site that replaces the gene sequence start codon, and a reverse primer (5'-AAAAAGCGGCCGCTTAATGGTGGTGGTGATGGTGGAACGGGCAGGTGGACCGGAC-3'), which incorporates a C-terminal His-Tag, a stop codon, and a *Not*I restriction site. The predicted BgP signal peptide was maintained in the construct design. The PCR product was

cloned into the pET20b(+) plasmid (Novagen ®), resulting in the pET20b(+):BgP vector construct, which was transformed into NEB® 5-alpha competent *E. coli* (New England Biolabs) for storage. The vector was subsequently conjugated into *E. coli* BL21-Codon Plus (DE3)-RIPL (Agilent Technologies) for heterologous protein expression. The insert was confirmed by a diagnostic restriction digest of Miniprep (Qiagen) purified plasmid, and by colony PCR, followed by Sanger sequencing of the amplified product (Eurofins LightRun).

Single colonies of the BL21 RIPL-(pET20b:BgP) recombinant clone were inoculated into 1 mL aliquots of Luria Bertani broth supplemented with 0.003% (w/v) chloramphenicol and 0.01% (w/v) ampicillin, and incubated at 37 °C overnight with shaking (225 rpm). After 14 h, 50 µL was subcultured in fresh 1 mL aliquots of LB (no antibiotic selection) and incubated for 2 h again at 37 °C with shaking. Activity was confirmed on LB agar plates containing 1% tributyrin and PCD, and 0.1 % PCL, following inoculation of 10 µL spots onto the plates, which were then incubated for 6 days and monitored carefully for substrate clearance.

4. Results and Discussion

4.1 Screening for Polyesterase Activities in Marine-Derived Bacterial Isolates

A recent report on the plastisphere of deep-sea samples in the Southwest Atlantic Ocean, involving the long-term colonization of plastic substrates by deep-sea microbes, has identified taxa in the core microbiome that may be related to plastic degradation. It also highlighted the fact that viable strains can be recovered from deep-sea conditions, which have the potential to be exploited for their plastic-degradation capacity (Agostini et al., 2021). A further indication of the potential of the marine environment as a source of novel polyesterase genes is reflected in a study of marine metagenomic datasets which reported that genes encoding PET hydrolases are globally distributed in marine environments (Danso et al., 2018). The capacity of marine microorganisms to degrade a range of polyesters is likely to be as a result of the high levels of exposure to plastics and microplastics that they have been and continue to be exposed to; with estimates indicating that over 250 thousand tons of plastic are currently floating in the oceans (Eriksen et al., 2014).

Previous reports have also highlighted the ability of marine-derived bacteria to degrade synthetic plastics, including *Bacillus* and *Rhodococcus* strains, together with a MorEst polyesterase from an Antarctic psychrotrophic bacterium which was able to degrade a range of polyesters, as well as a *Pseudomonas aestusnigri* isolate with a novel polyester hydrolase activity against PET film (Auta et al., 2018; Bollinger et al., 2020; Nikolaivits et al., 2020).

Screening of marine metagenomic data revealed an esterase, GEN0105, that was shown to hydrolyze bis(benzoyloxyethyl)-terephthalate (i.e., 3PET), along with polylactic acid (PLA), and PCL (Hajighasemi et al., 2018). Most recently, a bacterial consortium containing three marine bacterial species, namely *Exiguobacterium* sp., *Halomonas* sp., and *Ochrobactrum sp.* has been reported to degrade PET film with recombinant hydrolases and esterase genes from these strains demonstrating strong PET film degradation effects when heterologously expressed in *E. coli* (Gao and Sun, 2021). Thus, bacteria from marine environments represent a good source of polyesterases, which we should begin to sustainably exploit in the future.

Marine sponges (phylum Porifera) are generally sessile marine filter feeders, with some motile and carnivorous exceptions (Wilkinson, 1978; Lavrov and Kosevich, 2018; Vacelet, 2020). They harbor an abundant and diverse range of microbial symbionts, which can be responsible for up to 35% of the sponge biomass (Vacelet, 1975). Given that marine sponges, such as the deep-sea sponge *Pheronema* sp., filter large quantities of seawater (up to 24,000 L of water per day/Kg sponge) to obtain nutrients (Vacelet, 1975; Taylor et al., 2007), coupled with the fact that with typical densities of bacteria in seawater of up to 10^6 cells / mL, then there is the potential for sponges to ingest a total of 2.4 x 10^{13} bacterial cells on a daily basis (Hill, 2003). Thus, deep-sea sponges are likely to be a good source of such marine bacteria, with a number of bacteria from different genera having been isolated from them (Romanenko et al., 2008; Xin et al., 2011; Borchert et al., 2017; Williams et al., 2020).

There is clear evidence that deep-sea environments are exposed to microplastics, as evidenced by the report of the presence of a microplastic fibre very similar to PET in the shrimp *Eurythenes plasticus* recovered from depths of between 6010 and 6949 in the Mariana Trench in the Northwest Pacific Ocean (Weston et al., 2020). Thus, given the high levels of plastics and microplastics in the oceans, marine sponges are likely to be a good source of bacteria with potential to degrade polyesters. This is borne out by the fact that we recently reported on a PETase-like enzyme with polycaprolactone (PCL)-degrading activity which was identified in *Streptomyces* SM14 strain which was isolated from the sponge *Haliclona simulans* (Almeida et al., 2019).

A total of 51 bacterial strains that had previously been isolated from various marine sources, including shallow sea lough sponges (Jackson et al., 2012; Margassery et al., 2012), deep-sea sponges, and seaweeds (unpublished), were screened for polyesterase activities using tributyrin and PCD substrates, with either promising or interesting isolates subjected to further screening

on PCL (Molitor et al., 2020). Five deep-sea sponge isolates had their activities confirmed on the three substrates, with *Brachybacterium ginsengisoli* strain B129SM11 and *Micrococcus* sp. strain B226SK6 showing the best range of activities (Table 1). The isolate B226SK6 from *Inflatella pellicula* targeted tributyrin more specifically, with fainter activity towards the polyester substrates, whereas the isolate B129SM11 from *Pheronema* sp. sponge displayed superior activity across all three substrates. Based on these activities, the B129SM11 strain was chosen for genome sequencing and subsequent genome mining, in an effort to identify genes potentially involved in PCL degradation.

Tributyrin is a short-chain triglyceride that served as a suitable substrate for preliminary screening, given that polyesterases also display lipolytic activity. For more specific targeting of polyesterases, the aliphatic polyesters PCD and PCL were employed. Although these are aliphatic polyesters, with a simple composition compared to aliphatic-aromatic copolyesters like PET, many polyesterase enzymes have been reported to have activity towards both substrate types (Molitor et al., 2020).

The genus *Brachybacterium* is a high GC member of the *Dermabacteraceae* family, within the phylum Actinobacteria. *Brachybacterium* species have previously been identified from various environments including oil-contaminated coastal sand, lake sediment, more recently from deep-sea sediments in the Southern Ocean (Zhao et al., 2017; Ziganshina et al., 2018). *Brachybacterium* species have previously been reported in association with sponges (Kiran et al., 2014; Liu et al., 2019b), including deep-sea sponges from the Antarctic (Xin et al., 2011). While marine Actinobacteria have been reported to be capable of remediation of environmental pollutants including petroleum hydrocarbons and plastics (Rathore et al., 2021); members of the genus *Brachybacterium* have also been shown to possess the ability to degrade alkanes, phenols, and naphthalene (Velmurugan and Arunachalam, 2009; Wang et al., 2010; Wang et al., 2014). In addition, following the screening of Actinobacteria from a marine lake, a *Brachybacterium* sp. isolate was identified which displayed high lipolytic activity (Sadati et al., 2021). Thus, *Brachybacterium ginsengisoli* B129SM11 from the deep-sea sponge *Pheronema* sp. was expected to be an interesting candidate for further investigation in the context of polyester degradation.

4.2 Genome Analyses and Mining of *Brachybacterium ginsengisoli* B129SM11

Genome mining, in combination with computational tools and dedicated databases, allows for the identification of new homologs of enzymes of biotechnological interest and offers an

efficient means to discover potential novel biocatalysts (Zaparucha et al., 2018). Recent examples of biocatalysts that have been uncovered using a genome mining based approach include a thermostable monoacylglycerol lipase from a deep-sea *Geobacillus* sp. (Tang et al., 2019) and a salt-tolerant, enantio-selective esterase from the actinobacterium *Dactylosporangium aurantiacum* that generated optically pure (R)-3-hydroxybutyrate (Wang et al., 2018).

Genomic DNA from *B. ginsengisoli* B129SM11 was sequenced using the Illumina HiSeq NGS system. The raw sequence data was evaluated in terms of overall quality prior to genome assembly and annotation. A high-quality draft genome was successfully assembled for strain B129SM11 in 22 contigs, with 100% completeness and very low contamination (0.58%). The assembled genome (3.98 Mb) displayed a high GC content of 71.55%. A total of 3,505 coding sequences (CDS) were annotated by Prokka (Supplementary Table S1). The B129SM11 genome properties are similar to those of other *Brachybacterium* spp. genomes deposited in the NCBI database in terms of genome size, GC content, and number of CDSs.

The genome of strain B129SM11 was mined for genes encoding potential PET polyesterases by first assembling a reference data set containing 26 PET hydrolyzing enzymes or homologs having functionally verified activities against some PET and/or synthetic polyester substrates (Table 2). This dataset was used to construct a database with the BLASTP suite tool to search against, including four thermophilic cutinases which have been recognized as the most promising candidates for PET biorecycling to date; HiC and LCC, together with TfCut2 and Cut190 mutants (Kawai et al., 2020) (Table 3). Cut190 and TfCut2 are actinobacterial enzymes, whereas LCC and HiC are derived from metagenomic and fungal sources, respectively.

BgP was identified by employing a BLASTP sequence similarity search of the Prokka-annotated protein output file generated for strain B129SM11, against the polyesterase database. BgP produced significant alignments with 24 of the 26 enzymes, including Cut190, TfCut2, and LCC. BgP was annotated as a “poly(ethylene) terephthalate hydrolase”, although it cannot be assumed based on this preliminary description that BgP is a true PET hydrolase. The protein shared high amino acid identity (>55%) with LCC, TfCut2, and Cut190 (Table 3). Of these three enzymes, BgP shared the highest sequence identity, that is 62%, with Cut190 from *Saccharomonospora viridis* AHK190 (UniProt Accession: W0TJ64) (Kawai et al., 2014). Given that HiC is a fungal cutinase, it was not expected to produce as significant an alignment

with BgP. The gene sequence for BgP was deposited in the GenBank database and can be found under the accession number ON000823.

4.3 Computational Protein Analyses of BgP Enzyme

Synthetic polyester plastics may be hydrolyzed by microbial lipases, carboxylesterases, and cutinases, which can therefore be collectively referred to as polyesterases (Gricajeva et al., 2021). Many polyesterases are PET hydrolyzing enzymes (PHEs), although cutinases are considered most effective in cleaving polyester bond linkages (Kawai et al., 2020). It has been suggested that PHEs should be categorized either as PET-surface modifying enzymes or PET hydrolases, based on their differing abilities to hydrolyze the inner building blocks of PET. The PET hydrolase group can be further divided into mesophilic and thermophilic members, with thermophilic PETases deemed more suitable for application in the biological recycling of PET, given that this process requires temperatures between 65 and 75° C where the polymer is flexible enough for enzyme access. On the other hand, mesophilic enzymes resembling *Is*PETase from *I. sakaiensis* 201-F6*,* which have been found in both marine and terrestrial environments, are deemed more appropriate for the decomposition of PET waste. However, this presents its own challenges, for instance when environmental temperatures are not compatible with the enzyme employed (Kawai et al., 2019; 2020; Kawai, 2021). There are four thermophilic PET hydrolases that have been considered appropriate for PET biorecycling; metagenomic LCC, and variants of Cut190 and TfCut2 from actinomycetes (Table 3), along with fungal HiC. More recently, two thermophilic polyesterases from metagenomic sources, namely PHL7 (Sonnendecker et al., 2021) and BhrPETase, (Xi et al., 2021) have emerged as promising candidates, with highly efficient activities towards amorphous PET films.

BgP was classified as an α/β hydrolase member using InterProScan, Pfam, and SUPERFAMILY. Most polyesterases fall into this α/β hydrolase superfamily, which is comprised of enzymes that are structurally similar, yet functionally diverse (Gricajeva et al., 2021). Based on the results generated from Gpos-mPLoc and FUEL-mLoc, the enzyme appears to be extracellular. SignalP predicted a Sec/SPI-type signal peptide (first 30 AA) for standard secretion, with a likelihood of 0.9571. A potential cleavage site (AFA-AD) was identified between position 31 and 32, based on the probability value of 0.9741.

In an effort to gain further insights into the potential functional and evolutionary relatedness of BgP to known polyesterases, a phylogenetic tree was constructed with protein sequences of BgP and PET-hydrolyzing enzymes or their homologs, using MEGA-X with CLUSTALW

sequence alignment (Figure 1) (Thompson et al., 1994). HiC is phylogenetically distinct due to its fungal origin and was therefore excluded from our phylogenetic reconstruction. All enzymes in the tree appear to originate from a common ancestor, with the exception of BsEstB, a PET hydrolyzing *p*-nitrobenzylesterase that is structurally different from most α/β hydrolases with a sequence length > 400 bp and where glutamate replaces the more commonly observed aspartate of the polyesterase catalytic triad. Proteins derived from thermophilic Actinobacteria are seen to form one clade, descending from the same node, while proteins from Proteobacteria comprise another clade, with these two clades labelled 1 and 2, respectively (Figure 1). Our polyesterase, BgP, appears to be located closer to clade 1, although derived from a different node, where it groups with SM14est from the *Haliclona simulans* sponge. Given that BgP and SM14est are also actinobacterial enzymes, their separation from clade 1 may be due to the mesophilic nature of the strains from which they were isolated.

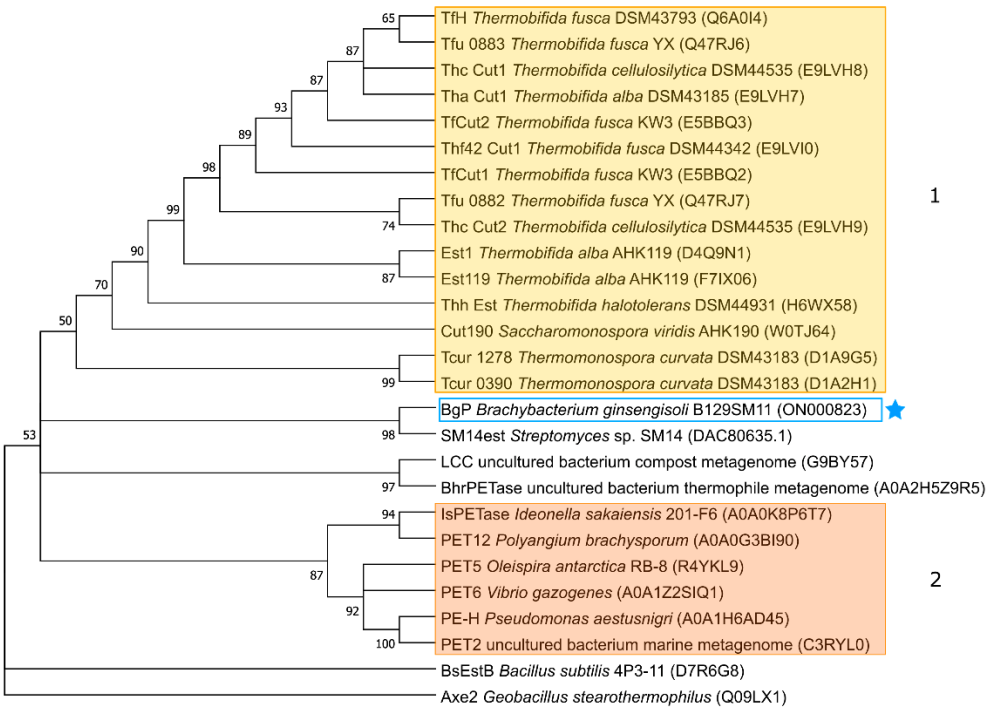


Figure 1. Maximum-likelihood (ML) phylogenetic tree of BgP among known PET-hydrolyzing enzymes and homologs from the reference dataset. Bootstrap consensus was inferred with 100 replicates, displaying only values above 50%, and the best-fit substitution model for this tree was WAG + G. Two main taxonomic clades are indicated; (1) proteins from thermophilic Actinobacteria (shaded in yellow) and (2) proteins from psychrophilic or mesophilic Proteobacteria (shaded in orange). BgP (starred) is tightly clustered with marine sponge-derived SM14est. LCC and BhrPETase from metagenomic thermophiles are also clustered together. A

Geobacillus stearothermophilus acetylxylan esterase, Axe2 (UniProt Accession Number: Q09LX1) served as an outgroup.

Multiple sequence alignments (MSA) were generated using T-COFFEE Expresso, which also incorporates structural information (Armougom et al., 2006). Most cutinases, (e.g., Cut190 and TfCut2) are designated as type I PETases, possessing one C-terminal disulfide bond, whereas type II PETases (e.g., *Is*PETase and PE-H) have an additional disulfide bond (Joo et al., 2018; Bollinger et al., 2020). Upon alignment with either the type I or type II PETases and visualization in ESPript, BgP was found to resemble the type I enzymes, with a single disulfide bond found near its terminal end (Supplementary Figure S2 and 3). Given that BgP appears closely related to SM14est, based on their phylogeny, an alignment was also produced for these enzymes (Supplementary Figure S4). This indicated that 53% of their amino acids are identical, and a further 29% of their residues are biochemically similar.

Cut190 was selected for amino acid sequence comparison with BgP (Figure 2), based on the BLASTP sequence identities and Phyre2 predictions, where it was ranked as one of the top structural templates. From the resulting MSA, it was inferred that 54% of the amino acids in BgP were identical to Cut190, and that 83% of the BgP residues share similar biochemical properties to those of Cut190. The pentapeptide motif GHSMG is conserved in both sequences, together with the serine hydrolase catalytic triad (Ser-Asp-His). Both enzymes possess C-terminal cysteine residues, which can potentially join covalently to form a disulfide bond. The MSA had perfect agreement across all alignment methods used, with the output having an average consistency score of 100 (from 0-100).

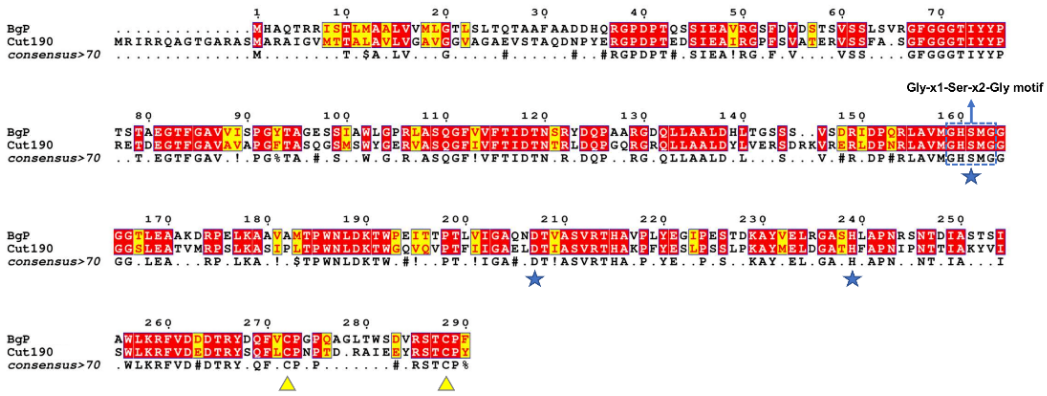


Figure 2. Amino acid sequence structural alignment of BgP and Cut190 generated with T-COFFEE Expresso and rendered using ESPript 3.0. Cut190 was indicated as a suitable structural homolog for BgP by the Phyre2 server. Amino acid residues shaded in red represent the ones strictly conserved between BgP and Cut190, while residues highlighted in yellow depict areas with an average level of homology. Catalytic triad residues are marked with a blue star, and disulfide bond cysteines are marked with a yellow triangle. The pentapeptide Gly-x1-Ser-x2-Gly serine hydrolase motif is outlined with a blue box.

Cut190 is a cutinase-like enzyme from *Saccharomonospora viridis* AHK190 that has been reported to hydrolyze PET (Kawai et al., 2014; Miyakawa et al., 2015). It is also a member of the lipase family and its thermostability and activity are enhanced by high concentrations of calcium ions, which are essential for the efficient enzymatic hydrolysis of amorphous PET (Miyakawa et al., 2015). Early mutagenesis studies of Cut190 found that a S226P/R228S substitution led to the highest activity and thermostability (Kawai et al., 2014). The resulting variant, named Cut190*, has been the target of multiple X-ray crystallography experiments and additional mutational analyses to elucidate the Ca^{2+}-binding mechanism and further improve the variant for PET hydrolysis applications (Kawai et al., 2014; Miyakawa et al., 2015; Kawabata et al., 2017; Oda et al., 2018). Cut190*SS, a variant generated by combined mutation (Q138A/D250C-E296C/Q13H/N202H), increased the thermostability of Cut190* from 63 °C to 70 °C, with a three-fold increase in PET film degradation. This suggests that there may be scope to undertake similar mutational modification of the BgP enzyme to generate variants with improved biochemical characteristics.

4.4 Protein Homology Modelling for BgP and Comparative Analysis with Cut190

Homology models that represent protein three-dimensional structure often offer insights into their conformation and functionality and enable the visualization of important features (Waterhouse et al., 2018). BgP was modelled using the SWISS-MODEL server, based on the top template, an enzyme named SeL, from *Streptomyces exfoliatus* (PDB code: 1JFR) (Figure 3). The signal peptide sequence of BgP was excluded in agreement with the SeL template, leaving 259 residues (32-290). The BgP model displayed a GMQE score of 0.87 (measured between 0 and 1, with higher numbers representing models with higher expected quality) and a QMEAN Z-score of -0.44 (with scores below -4.0 indicating low quality), indicating that the predicted model was reliable. Quality was also evaluated with tools from the SAVES suite, through which the BgP model was further validated. The ERRAT Overall Quality Factor was

calculated to be 94.7% and a VERIFY averaged 3D-1D score (≥ 0.2) of 100%, exceeding the threshold values in both cases. The overall PROCHECK G-score was predicted to be -0.12 (negative value desired) and the ProSA Z-score was -8.15 (within native conformation range), with both indicating good model quality. PROCHECK Ramachandran plot calculations revealed 92.6% of amino acid residues in the most favored regions, with an additional 7% in allowed regions, and 0% in disallowed regions.

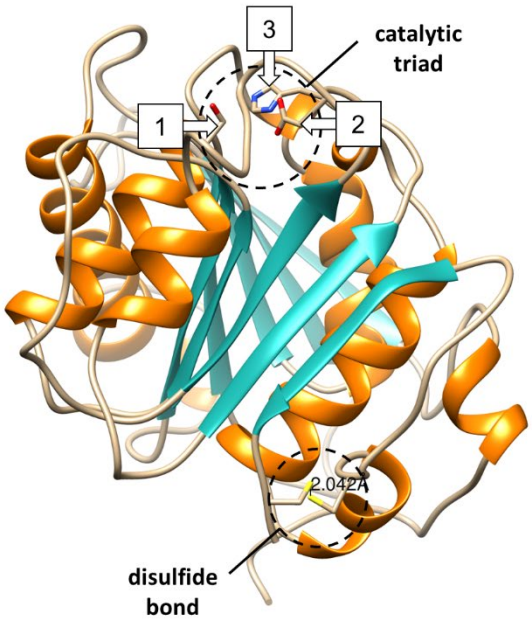


Figure 3. Three-dimensional structure of BgP based on the SeL (PDB code: 1JFR) template from Streptomyces exfoliatus, generated using UCSF-Chimera. Catalytic residues and disulfide bond cysteines are shown in stick form and circled. The calculated distance between the sulfur atoms of each cysteine is displayed in angstroms (Å). Secondary structures are shown as teal strands and orange helices, while coils are left uncolored.

Structural analysis of the BgP model was completed in UCSF-Chimera allowing the key features to be located. The BgP enzyme possesses six full α-helices which surround a central β-sheet consisting of nine strands. This flexible core is a signature fold within the α/β hydrolase superfamily and is considered responsible for the multifunctionality of its member enzymes (Rauwerdink and Kazlauskas, 2015; Gricajeva et al., 2021). Active site residues Ser130, Asp176, and His208 are found in close proximity to each other, forming the catalytic triad that is typical of serine hydrolases. The active site lies at the apex of the β-sheet, which is consistent

with polyesterases. All previously reported polyesterases display a Ser-Asp/Glu-His triad and follow the same catalytic mechanism where a nucleophilic serine initiates ester bond hydrolysis (Gricajeva et al., 2021). The Cys241 faces Cys257 at the C-terminal, bringing the residues into contact for disulfide linkage. The distance between these cysteine residues was measured to be 2.046 Å, which corresponds to the typical length of a disulfide bond (Chaney and Steinrauf, 1974). Unlike cutinases and PETases, the lipase and esterase enzymes involved in polyester hydrolysis do not possess a disulfide bond. Both lipases and esterases have a lid-domain formed by at least two α-helices. Although the lid facilitates adsorption onto hydrophobic polyesters, it also covers the active site in these enzymes, which is buried relatively deep within these enzymes. This lid structure is absent in most cutinases, which together with a more exposed active site near the enzyme surface, increases access to polyester substrates (Kawai et al., 2019). The *Streptomyces exfoliatus* template (1JFR) is an example of cutinase-like lipase that lacks a lid domain (Khan et al., 2017) and this alpha-helical structure also appears to be absent from the BgP enzyme.

The Chimera MatchMaker tool was used for structural comparison of BgP with Cut190 (Figure 4). The Ser-Asp-His catalytic triad is conserved between the two enzymes, positioned as Ser176, Asp222, and His254 in Cut190, and as Ser130, Asp176, and His208 in BgP. Disulfide bond residues were positioned as follows; Cys241 and Cys257 in Cut190, and Cys287 and Cys302 in BgP. The polyesterase active site is found within a pocket called the substrate binding groove (SBG) (Gricajeva et al., 2021). There is also a conserved methionine adjacent to each nucleophilic serine, at position 177 in Cut190 and at 131 in BgP (Table 4). Met177 has been reported as an oxyanion hole-forming amino acid in Cut190 (Kawabata et al., 2017), while results from the LED database also point to Met131 as an oxyanion residue for BgP. The oxyanion hole stabilizes the reaction intermediate during polyester hydrolysis and is regarded as an important structural determinant of catalytic efficiency (Gricajeva et al., 2021). In Cut190, the oxyanion hole is formed by Met177 together with another residue, Phe106 (Kawabata et al., 2017). There is no corresponding phenylalanine residue implicated at this position in BgP, but instead another hydrophobic residue, Tyr62, is present in its place (Table 4). Tyrosine participates in oxyanion hole formation in certain α/β hydrolases and replaces phenylalanine in other PHEs such as TfCut2 and *Is*PETase, where it is believed to play a similar role in intermediate stabilization (Bauer et al., 2020; Tan et al., 2021). The importance of Phe106 for the activity of Cut190 is reflected in the fact that during mutational analysis of Cut190, the replacement of Phe106 with tyrosine led to decreased turnover of the model substrate

poly(butylene succinate-co-adipate), or PBSA (Kawabata et al., 2017). Mutational and kinetic analyses will be required to investigate the potential role of Tyr62 in BgP during polyester hydrolysis, particularly if this residue has a similar impact on substrate turnover, as observed for Cut190.

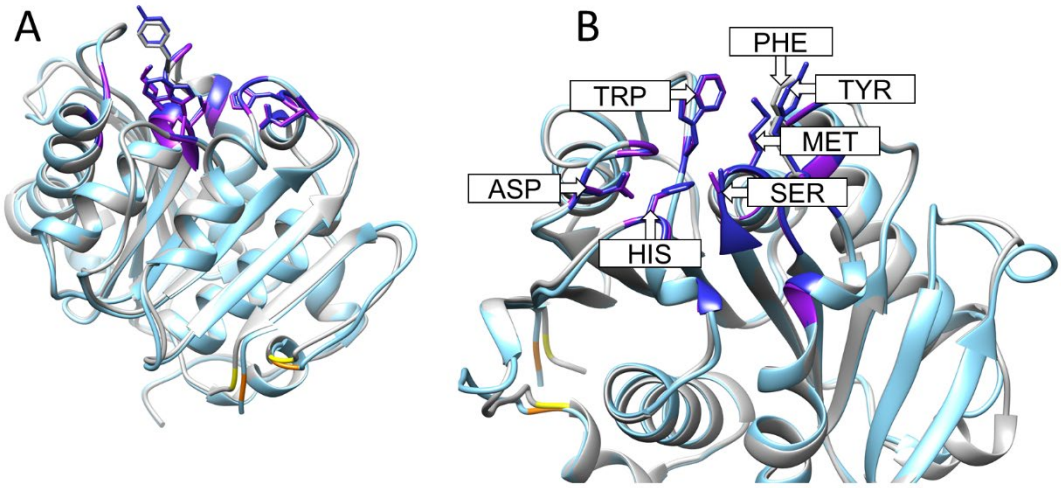


Figure 4. BgP structure (light blue) superimposed onto Cut190 (gray, PDB code: 4WFI) for comparative analysis. On the left view (A) the model is shown in full. On the right view (B), a close-up of the active site pocket is shown. Potential ligand binding residues are displayed for BgP in dark blue, while any corresponding Cut190 residues, that are also conserved in BgP, are colored in purple. Key amino acid residues are shown in stick format, and in view (B) they are labeled using their 3-letter code. With the exception of tyrosine in BgP, which is replaced by phenylalanine in Cut190, these highlighted residues are identical in both enzymes. Disulfide bond cysteines are highlighted in yellow for Cut190 and in orange for BgP.

Table 4. Comparative analysis of potential BgP binding residues, as predicted by 3DLigandSite, and the matching residue found at the same location in Cut190.

Predicted Binding Residue (BgP)	Corresponding Residue (Cut190)	Predicted Role (Cut190)[a]
Pro60	Pro104	Not fully investigated
Gly61	Gly105	Substrate interaction
Tyr62	Phe106	Substrate interaction
Thr63	Thr107	Not fully investigated
Ala64	Ala108	Not fully investigated
Ser68	Ser112	Only interacts in Ca^{2+}-bound form
Ile69	Met113	Not investigated
Gln94	Gln138	Substrate interaction
Arg98	Arg142	Not fully investigated
His129	His175	Only interacts in Ca^{2+}-bound form
Ser130	Ser176	Substrate interaction
Met131	Met177	Substrate interaction
Gly132	Gly178	Substrate interaction
Trp155	Trp201	Substrate interaction
Val178	Ile224	Substrate interaction
Ala179	Ala225	Not fully investigated
His208	His254	Only interacts in Ca^{2+}-bound form
Leu209	Phe255	Only interacts in Ca^{2+}-bound form
Asn212	Asn258	Only interacts in Ca^{2+}-bound form

[a] based on mutational analysis of Cut190 (Kawabata et al., 2017)

3DLigandSite, which employs structural results from the Phyre2 protein recognition server, was used to gain further insights into potential ligand binding residues in BgP. Excluding the catalytic triad residues, 12 of the 16 binding residues predicted by 3DLigandSite were found to be conserved in Cut190, including hydrophobic Pro60, Gly61, Ala64, Met131, Gly132, Trp155, and Ala179. In polyesterases, hydrophobic amino acid residues found in the substrate-binding groove form crucial interactions with the substrate. Trp155 lies directly opposite the oxyanion residue Tyr62. The corresponding residue in Cut190, Trp201, was previously highlighted as having a role in substrate-binding, with low activity observed upon mutation of this residue; with the suggestion that binding is influenced by the indole ring in tryptophan and that the loss in activity following mutation was caused by weakened enzyme-substrate interactions (Kawabata et al., 2017). Along with Trp201, active site Ser176, and oxyanion Met177, results from the same study also indicated Gly105, Gln138, and Ile224 as likely Cut190 interacting

residues using a partial PBSA structure called BABSBA. Gly105 and Gln138 are matched in BgP at positions 61 and 94, respectively. The Ile224 is replaced with a Val178 at the corresponding location in BgP. Although valine is smaller than isoleucine, both are hydrophobic amino acids, and share similar biochemical properties. In Cut190, mutation of Ile224 to Ala224, that is, the smallest hydrophobic residue, was shown to increase activity despite a decrease in substrate affinity (Kawabata et al., 2017).

Cut190 has been shown to undergo a conformational change upon addition of Ca^{2+} ions, which bind to the enzyme and result in its activation (Oda et al., 2018). Three Ca^{2+}-binding sites have been revealed by X-ray crystallography, involving the following amino acids; Ser76, Ala78, and Phe81 (site 1), Glu220, Asp250, and Glu296 (site 2), and Asp204 and Thr206 (site 3) (Oda et al., 2018). The serine and phenylalanine of site 1 are conserved in BgP (positions 31 and 37), together with the aspartate and threonine of site 3 (positions 158 and 160). Mutational analysis of Cut190 indicated that sites 1 and 3 are involved in activation, while sites 2 and 3 influence structural and thermal stability (Oda et al., 2018). It has also been reported that certain Cut190 residues only interact with model substrate when the enzyme is in the Ca^{2+}-bound state, namely Ser112, His175, Phe255, and Asn258 (Kawabata et al., 2017). In BgP, Ser68, His129, and Asn258 are conserved at the equivalent locations, with the exception of Phe255 which is replaced by Leu at BgP position 209. The presence of Ca^{2+} ions is an essential prerequisite for Cut190 in the context of PET polymer degradation, with this enzyme displaying active (Ca^{2+}-bound) and inactive (Ca^{2+}-free) states during PET hydrolysis (Kawai et al., 2014; Senga et al., 2021). The importance of metal ions (e.g., Ca^{2+} and Mg^{2+}) for increased stability and degradation of PET has also been highlighted for other polyesterases, including TfCut2, and LCC (Sulaiman et al., 2014; Then et al., 2015). Given the similarities between BgP and Cut190, we expect it would be worth investigating the influence of metal ions on BgP functionality, with the potential to further increase activity by engineering of the ion-binding sites.

The glass transition temperature (T_g) of PET is an important consideration for enzymatic hydrolysis, since its polymer chains display increased flexibility when reaction temperatures are set near or above T_g (Carr et al., 2020). For hydrolysis of amorphous PET under aqueous conditions, enzyme thermostability at 55 °C, or preferably above 60 °C, is recommended to facilitate efficient degradation, as exemplified by Cut190* (Kawai et al., 2014). During *in silico* protein analysis, BgP was classified as a stable protein based on the calculated instability index (32.44). The potential thermostability of BgP is indicated by its high aliphatic index (77.76), and its theoretical melting temperature (T_m) of 69.4 °C. However, further biochemical

characterization of BgP will be required to determine its temperature profile and evaluate its stability under various reaction conditions. In an effort to determine potential targets for the engineering of improved BgP variants, the activity enhancing mutations in the aforementioned Cut190* and Cut190*SS may be valid targets. Based on conserved residues at these positions, the following mutations may be appropriate; S226P, R228S, Q138A, and N202H. However, a random mutagenesis approach may be more rewarding, given that there is no guarantee that mutations at these positions would generate similar results in BgP and considering that there might be other targets in the enzyme that prove more worthwhile.

4.5 Cloning and Heterologous Expression of BgP

To determine whether the *bgp* gene does encode polyesterase activity, it was amplified from B129SM11 genomic DNA using primers that were designed to incorporate the native *Brachybacterium ginsengisoli* signal peptide, and a C-terminal His_6 tag. The gene was then cloned into the expression vector pET20b(+), generating the pET20b(+):BgP construct (Supplementary Figure S1). Following transformation into *E. coli* NEB® 5-alpha, the construct was conjugated into the BL21 (DE3)-RIPL expression host. Polyesterase activity was confirmed using tributyrin, PCD, and PCL plate clearing assays (Figure 5), with zones observed on all three types of agar, indicating the hydrolysis of each substrate. The negative control i.e., *E. coli* BL21 (DE3)-RIPL containing the pET-20b(+) plasmid without the insert did not display such activity towards any of the three substrates (Supplementary Figure S5).

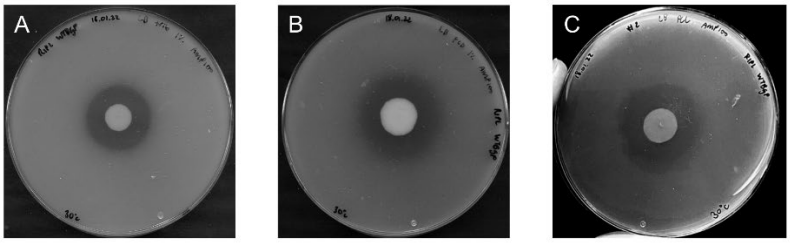


Figure 5. Heterologous expression by BL21 RIPL-(pET20b:BgP) clone plated on; (A) 1% tributyrin, (B) 1% PCD, and (C) 0.1% PCL, each prepared using LB agar and incubated for 6 days at 30°C.

In our previous study, SM14est was successfully exported by the *E. coli* heterologous host when its native *Streptomyces* sp. signal peptide was maintained in the expression construct (Almeida

et al., 2019). We encountered some difficulties with BgP expression, although it is not certain whether this relates to the use of the native signal sequence in the expression construct. While zones of clearing were observed, they developed slowly. In this case, clearing may not indicate extracellular secretion, but rather leaky expression from the T7 promoter or perhaps enzyme release following natural cell lysis. The addition of IPTG was not shown to improve expression, or at least improvements were not detectable on the plate assays used, despite various attempts to optimize both induction time and IPTG concentration (Supplementary Figure S5), and this will therefore require further investigation. The efficient expression and secretion of polyesterases from the heterologous host will not only be important in assisting with protein purification and biochemical characterization of BgP, but it may also facilitate the use of polyesterase producers in bioremediation-type applications. To our knowledge, this is the first report of a cutinase-like polyesterase enzyme being identified in a deep-sea sponge-derived *Brachybacterium* isolate, which we hope will further expand our current knowledge of enzymes for the degradation of synthetic polyesters.

5. Concluding Remarks

Polyester-degrading enzymes, which have to date been reported as either lipase, carboxylesterase, or cutinase family members, have become the subject of extensive research to achieve enzymatic hydrolysis of synthetic polyester plastics such as PET. Given the relatively recent development of synthetic plastic products and the exponential increase in plastic production and pollution that followed, the study of PET hydrolase enzymes has quickly gained traction. A number of PET-hydrolyzing polyesterases have to date been identified and characterized, which together with efforts to elucidate the mechanisms behind functionality and the engineering of improved variants, has advanced the field to the extent that the practical implementation of biological PET degradation systems is now being examined. However, there are many factors preventing the complete hydrolysis of PET by polyesterases, particularly due to the individual properties of different PET products and in particular due to the fact that most of our knowledge to date is limited to the enzyme's interactions with amorphous PET films and with model substrates. Furthermore, much of the research in this area is focused on PET biorecycling applications, with less known about polyesterases in the context of environmental degradation and potential for enzymatic remediation.

While most PET polyesterases that have been reported to date have primarily been from Actinobacteria, we report here on a novel polyesterase from a member of the genus

Brachybacterium which was isolated from the deep-sea sponge *Pheronema* sp. at a depth of 2,129 m. Activity testing and subsequent genome mining of *B. ginsengisoli* B129SM11, resulted in the identification of BgP, a cutinase-like polyesterase that successfully hydrolyzed tributyrin, polycaprolactone, and polycaprolactone diol substrates following heterologous expression in *E. coli*. Important functional and mechanistic insights were gained through comparisons with known PET hydrolases and by protein modelling of BgP. This work lays the foundation for future biochemical characterization and kinetic analysis, as well as mutational studies to determine structure-function relationships to potentially improve the enzyme for polyester hydrolysis and other biocatalytic applications. Furthermore, it would be interesting to explore the possible role of BgP in *B. ginsengisoli* in the context of its native deep-sea ecosystem, and specifically its function within the sponge microbiome. That said, we should consider that hydrolases are highly promiscuous, and enzymes such as BgP may not have specifically evolved to degrade PET, and its involvement in PET degradation would require additional enzymes and transporters as part of a specific PET catabolic pathway. Nonetheless, further exploration exploration of the role of BgP will broaden our understanding of polyesterases in marine environments and potentially facilitate the development of bioremediation-based applications in these ecosystems.

6. Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

7. Author Contributions

CC, BO, SJ, and AD conceived and designed the experiments. CC performed the experimental work. CC and BO analyzed the data. SJ and AD contributed reagents, materials, and analysis tools. CC, BO, and AD wrote the manuscript. AD and DC supervised. All authors read and approved the final manuscript.

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11. Supplementary Material

Table S1. Overall genome features of *Brachybacterium ginsengisoli* B129SM11.

Genome Property	**Value**
Size (bp)	3,987,864
Number of contigs	22
Largest contig (bp)	898,629
N50	651,468
L50	3
GC content (%)	71.55
Completeness (%)	100
Contamination (%)	0.58
Number of coding sequences	3,505
Number of hypothetical proteins	1,592
Number of rRNA genes	3
Number of tRNA genes	58
Number of tmRNA genes	1
Number of miscellaneous RNA genes	13



Figure S1. Vector map of the pET20b(+):BgP plasmid construct for expression of BgP in E. coli heterologous hosts. The insert and key features of the vector are graphically represented and labelled accordingly.

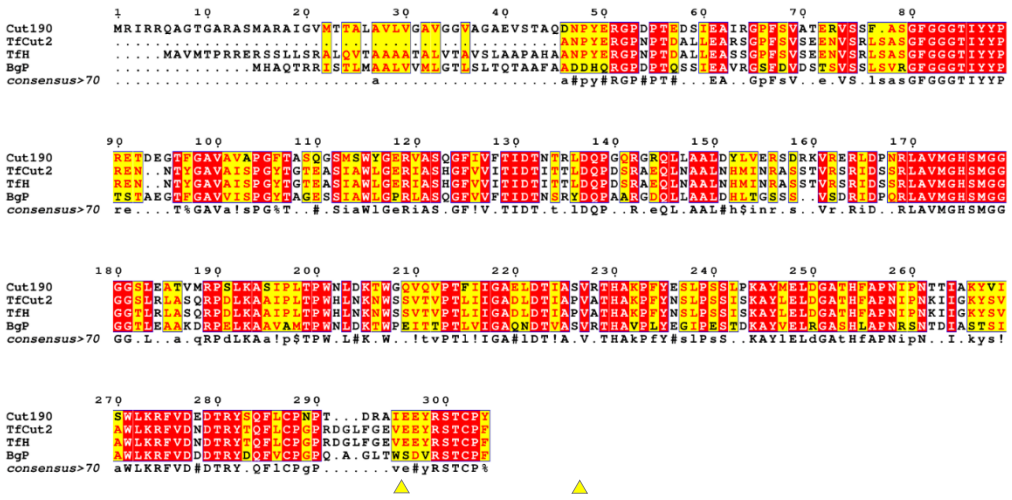


Figure S2. Amino acid sequence structural alignment of BgP and some "type I" PETases, generated with T-COFFEE Expresso and rendered using ESPript 3.0. Amino acid residues shaded in red depict strictly conserved residues, while residues highlighted in yellow represent areas with an average level of homology. Disulfide bond cysteines are marked with a yellow triangle.

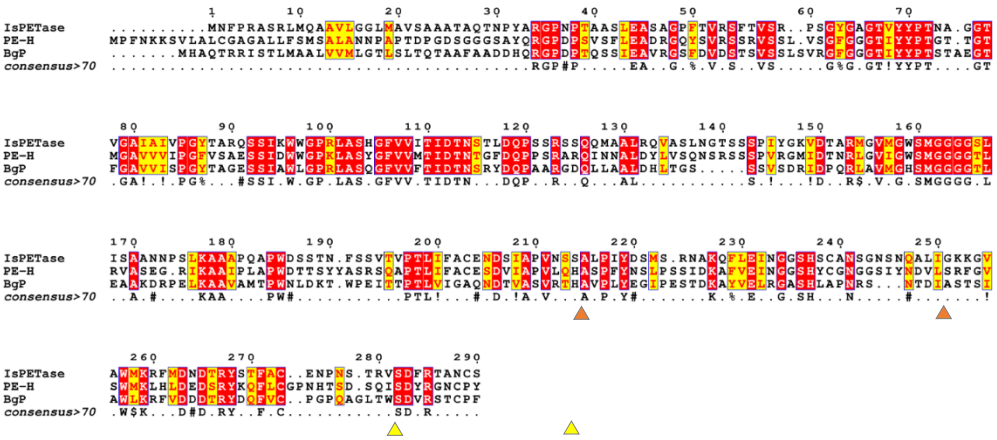


Figure S3. Amino acid sequence structural alignment of BgP and some "type II" PETases, generated with T-COFFEE Expresso and rendered using ESPript 3.0. Amino acid residues shaded in red depict strictly conserved residues, while residues highlighted in yellow represent areas with an average level of homology. The cysteine residues forming disulfide bond 1, which is common to both PETase types, are marked with a yellow triangle, while cysteines forming disulfide bond 2, which is specific to type II PETases, are marked with an orange triangle.

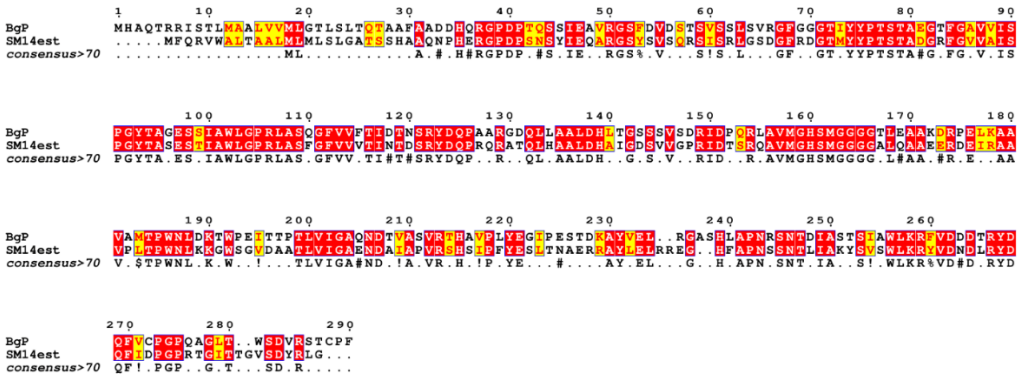


Figure S4. Amino acid sequence structural alignment of BgP, and SM14est from marine sponge-derived *Streptomyces* sp. SM14, generated with T-COFFEE Expresso and rendered using ESPript 3.0. Amino acid residues shaded in red depict strictly conserved residues, while residues highlighted in yellow represent areas with an average level of homology.

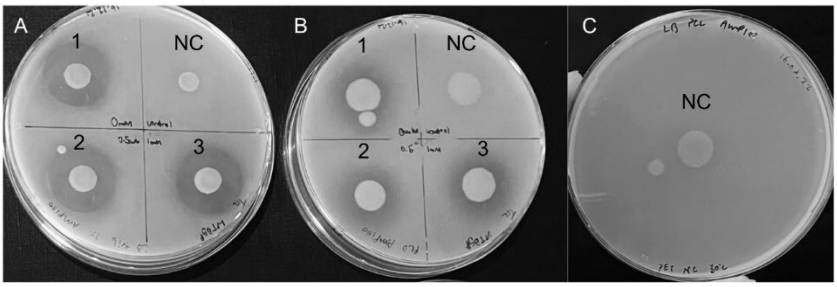


Figure S5. BL21-Codon Plus (DE3)-RIPL *E. coli* negative control (labelled NC), growing on; (A) tributyrin, (B) PCD, and (C) PCL. In the case of (A) and (B), the negative control is plated alongside the BgP production strain, which was being investigated under three different conditions, labelled 1, 2, and 3 (where 1 = no IPTG added, 2 = 0.5 mM IPTG added, 3 = 1 mM IPTG added).

Chapter 3

Purification and Biochemical Characterization of SM14est, a PET-Hydrolyzing Enzyme from the Marine Sponge-Derived *Streptomyces* sp. SM14

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1. Abstract

The successful enzymatic degradation of polyester substrates has fueled worldwide investigation into the treatment of plastic waste using bio-based processes. Within this realm, marine-associated microorganisms have emerged as a promising source of polyester-degrading enzymes. In this work, we describe the hydrolysis of the synthetic polymer PET by SM14est, a polyesterase which was previously identified from *Streptomyces* sp. SM14, an isolate of the marine sponge *Haliclona simulans*. The PET hydrolase activity of purified SM14est was assessed using a suspension-based assay and subsequent analysis of reaction products by UV-spectrophotometry and RP-HPLC. SM14est displayed a preference for high salt conditions, with activity significantly increasing at sodium chloride concentrations from 100 mM up to 1000 mM. The initial rate of PET hydrolysis by SM14est was determined to be 0.004 s^{-1} at 45 °C, which was increased by 5-fold to 0.02 s^{-1} upon addition of 500 mM sodium chloride. Sequence alignment and structural comparison with known PET hydrolases, including the marine halophile PET6, and the highly efficient, thermophilic PHL7, revealed conserved features of interest. Based on this work, SM14est emerges as a useful enzyme that is more similar to key players in the area of PET hydrolysis, like PHL7 and IsPETase, than it is to its marine counterparts. Salt-tolerant polyesterases such as SM14est are potentially valuable in the biological degradation of plastic particles that readily contaminate marine ecosystems and industrial wastewaters.

2. Introduction

The *Streptomyces* genus has proven to be a worthwhile source of secondary metabolites, ranging from antimicrobial to cytotoxic compounds, with human health needs being the primary driver of the discovery efforts for these bioactive products (Lacey and Rutledge, 2022). As with other actinomycetes, *Streptomyces* species are also important producers of industrially relevant enzymes (Kumar et al., 2020), although perhaps they have not yet been as well explored in this regard to date. The last decade has seen a notable shift away from terrestrial ecosystems towards the marine environment as a source of microorganisms harboring novel biological activities (Dharmaraj, 2010; Yang et al., 2020; Olaniyan and Adetunji, 2021). Nevertheless, given the vast and varied nature of the Earth's oceans, we are still in the early stages of the quest to describe the full potential of the marine microbiome (Abreu et al., 2022). In particular, marine invertebrates such as sponges have been shown to represent a hotspot of microbial biodiversity and activity, brought about by the mutualistic interactions that exist between microbial

symbionts and the host sponge, which have the potential to be sustainably exploited for biotechnological purposes (Amelia et al., 2022).

Marine sponges harbor diverse communities of bacteria, archaea, and unicellular eukaryotes, which inhabit sponge tissues at densities of over 10^9 cells/cm^3 and contribute to as much as 35% of the total sponge biomass (Hentschel et al., 2012). Sponge-associated microbes are known to be quite prolific producers of natural products, as well as extracellular enzymes, which are believed to contribute positively to the metabolism and overall health of their sea sponge host (Taylor et al., 2007; de Oliveira et al., 2020; Nnaji et al., 2022). Such enzymes are often hydrolase family members and are likely to be produced in an attempt to degrade the wide variety of substrates present in the large quantities of seawater being filtered by the sponge, providing both symbiotic partners with a source of energy and nutrients (Wang, 2006; Pita et al., 2018; Birolli et al., 2019). In the marine environment, heterotrophic microbes are responsible for the biogeochemical cycling of dissolved organic carbon, an abundant reservoir comprising thousands of distinct chemical structures. However, dissolved organic carbon compounds are often quite complex and recalcitrant to degradation, thus requiring adaptable enzymes for their utilization as substrates (Wagner et al., 2020). Marine microbial hydrolases must also withstand the changeable and challenging conditions associated with ocean waters, further supporting the potential applicability of these resilient enzymes across a range of new biotech products and services (Sana, 2015).

PET hydrolase enzymes have, for the past number of years, been the focus of extensive research and engineering efforts to support the biological recycling of polyester plastics, with increasing success since the identification of TfH, a PET-hydrolyzing enzyme from *Thermobifida fusca*, in 2005 (Wei et al., 2022). LC-cutinase (LCC) from a leaf compost-derived metagenome has represented a highly active benchmark PET hydrolase (Andler et al., 2022; Wei et al., 2022). The LCC variant, ICCG, which can achieve 90% degradation of pre-treated post-consumer PET within 10 hours, has even been implemented as a catalyst in pilot-scale PET bio-recycling (Tournier et al., 2020). Most recently, HotPETase and FAST-PETase, which are thermostable variants of IsPETase from the mesophilic bacterium *Ideonella sakaiensis*, have been engineered to match the activities of LCC and ICCG (Bell et al., 2022; Lu et al., 2022). During enzymatic hydrolysis, the polyethylene terephthalate (PET) polymer is primarily converted into the intermediate products MHET (mono(hydroxyethyl terephthalate)) and BHET (bis(hydroxyethyl terephthalate)), which may then be further degraded into the monomeric subunits terephthalic acid (TPA) and ethylene glycol (EG) (Figure 1). TPA and EG can also be

further abbreviated to T and E, respectively, which allows for short-hand notation of the PET breakdown products that reflects their composition and length, for example, where ET is MHET and ETE is BHET (Schubert et al., 2023).

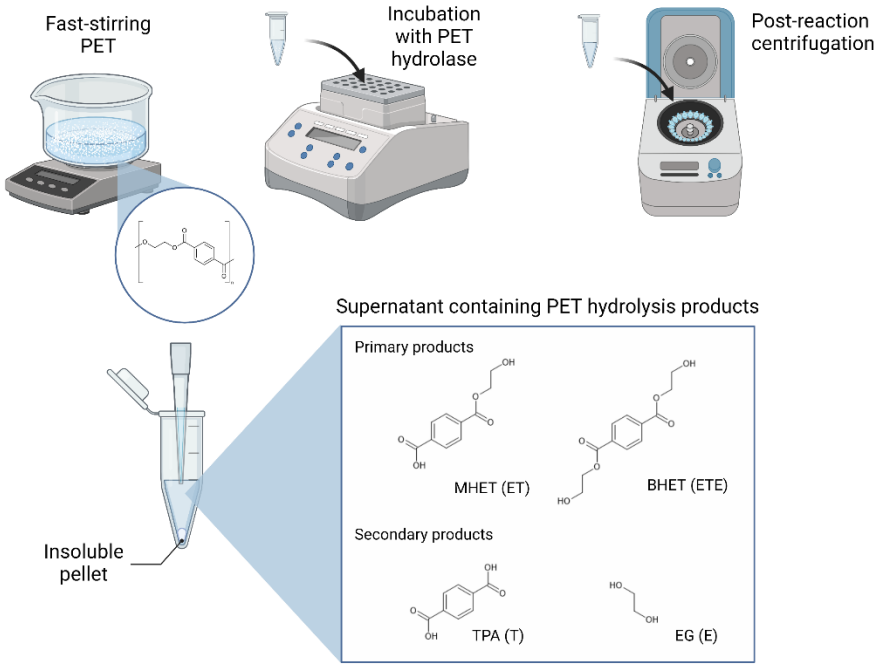


Figure 1. Experimental setup for the detection of the soluble products of PET hydrolysis. Polymeric PET substrate is suspended in the reaction buffer and homogeneity is maintained with fast stirring. The PET substrate and PET hydrolase of interest are incubated with continuous shaking under the selected temperature and time parameters. Upon successful PET hydrolysis, the soluble fraction will include the shortest oligomers, MHET and BHET, and the simplest monomeric units, TPA and EG, which are separated from insoluble PET material by centrifugation. The aromatic ring-containing hydrolysis products may be detected from the supernatant, for example, by UV-spectrophotometry alone or coupled to RP-HPLC for separation of individual products.

Within the realm of enzymatic plastic degradation, several groups have investigated marine-derived microbes for polyester hydrolases, particularly those capable of breaking the ester bonds that link the subunits of the synthetic polyester PET (Blázquez-Sánchez et al., 2022; Meyer Cifuentes et al., 2022; Weigert et al., 2022). PET waste is widely distributed in the marine environment, both in macro and microplastic forms, thereby posing a significant threat to marine life and ultimately to humans (Nabi et al., 2022). While it is interesting to consider the enzymatic degradation of PET by marine microorganisms, both from an ecological and

biotechnological perspective, it is not yet well understood whether this capacity has evolved specifically in response to plastic pollution in our oceans or if this is among the many unconventional side reactions that hydrolases can facilitate when deprived of simpler substrates that are more easily metabolized (Barzkar et al., 2021; Dittmar et al., 2021; Jiménez et al., 2022).

Our group had previously identified SM14est, a polyesterase from the marine-sponge derived *Streptomyces* sp. SM14, during the mining of 52 *Streptomyces* genome sequences for PET hydrolase-encoding genes. Polycaprolactone (PCL) was used to confirm polyesterase activity of the heterologously expressed enzyme in an agar-based plate clearing assay (Almeida et al., 2019b). Although petroleum-based PCL and PET are both employed to produce semi-crystalline thermoplastics, the biodegradable polycaprolactone only serves as a model substrate for the more complex PET, which is an aliphatic-aromatic co-polyester (Niaounakis, 2017; Molitor et al., 2020). Based on computational characterization and molecular docking with BHET, the ester of PET monomeric units EG and TPA, hydrolytic activity towards PET was predicted but not confirmed (Almeida et al., 2019b).

Here we present biochemical and kinetic data for SM14est, obtained using a suspension-based assay that allows for the detection of PET hydrolysis products. A *Bacillus subtilis* heterologous host was employed to produce and secrete recombinant SM14est, which was his-tagged and subsequently affinity purified. The purified SM14est was found to hydrolyze PET following incubation at 45 °C in sodium phosphate buffer (pH 8), with products measured by UV-spectrophotometry. This activity towards PET was enhanced with increasing concentrations of sodium chloride (100-1000 mM) and further progress curve analysis revealed a 5-fold increase in the initial rate of PET hydrolysis when sodium chloride (500 mM) was included in the reaction.

3. Materials and Methods

3.1 Enzyme Expression and Purification

SM14est (Genbank: DAC80635.1) was codon optimized for heterologous expression in *Bacillus subtilis* and produced as previously described (Jensen et al., 2010), with modification during construct design such that the native SM14est signal peptide was replaced by AmyL (FJ556804.1) from *B. licheniformis* and a hexa histidine-tag was incorporated at the C-terminus of the protein. The recombinant SM14est protein (29 kDa) was purified by Ni-affinity chromatography on a HisTrap FF 5 mL column (Cytiva) using the ÄKTA™ Pure system. A

peristaltic pump (MiniPuls 3, Gilson®) was used for the initial column wash and loading steps. The purified protein was desalted using Spectra/Por® dialysis membrane (MWCO 12-14 kDa). The molar extinction coefficient of 39880 M^{-1} cm^{-1} was predicted by ExPASy-ProtParam and was used to calculate the concentration of purified enzyme from the absorbance measured at 280 nm. The SM14est sample was visualized following SDS-PAGE of 2.5 µg of purified protein on a Tris-Glycine 4-20% gel (Bio-Rad Laboratories). Thermal denaturation experiments were conducted in triplicate with 10 µM of enzyme and analyzed by nano differential scanning fluorimetry (nanoDFS) using a temperature slope of 2 °C min^{-1} (Prometheus NT.48, Nano Temper).

3.2 Suspension-Based PET Activity Assay

A suspension-based assay (Bååth et al., 2020) was used to investigate the hydrolytic activity of SM14est towards semi-crystalline PET powder reported to have > 40% crystallinity with a particle size of < 300 µM (Goodfellow Co, product number ES306031/1). Depending on the required assay setup, this microPET powder was suspended either in the selected buffer or Milli-Q water and pipetted while under vigorous stirring. The PET substrate suspension was transferred to low-binding microplates (Greiner Bio-One™ 655901) or microtubes (Sarstedt 72.706.600), followed by additional buffer, and finally the purified enzyme.

The reactions were performed in triplicate, with samples incubated in a thermomixer (Eppendorf) at 1100 rpm. Based on preliminary results, assays were typically carried out at 45 °C using 0.5 µM enzyme, 10 g L^{-1} PET, and 50 mM sodium phosphate buffer pH 8 in a final reaction volume of 250 µL. Samples were centrifuged to quench the reaction, then supernatants (100 µL) were transferred into a UV-transparent microplate (Corning) to measure soluble aromatic products of PET hydrolysis, such as TPA, MHET, and BHET, at absorbance 240 nm in a plate reader (Molecular Devices SpectraMax Paradigm). As previously described, products were quantified from the absorbance readings by comparing against standard curves for BHET and converting to BHET equivalents (µM), providing a platform for screening and initial characterization of potential PET hydrolase enzymes (Bååth et al., 2020). It should be noted that it is not possible to differentiate between the individual soluble products using this method, however, BHET has been previously employed as a standard to reflect the pooled absorbance of soluble products (Thomsen et al., 2022).

3.3 Progress Curve Analyses

Supernatant time-point samples were taken at t = 1, 3, 5, and 7 h incubation for measurement at 240 nm (as previously described). An additional 100 µL was retained from the remaining supernatant, treated with hydrochloric acid (2 µL, 5M) to quench the reaction, and stored at -20 °C until further quantification using reversed-phase high-performance liquid chromatography (RP-HPLC). (Thermo Scientific™, Vanquisher: Capital HPLC). Formic acid (7.5 mM) and acetonitrile (5% v/v) were used during the injection of samples (20 µL) at a ratio of 1:5 for 7.5 min, prior to elution using acetonitrile for 12.5 min, with a flow rate of 1 mL min^{-1} maintained at 40 °C. The analytes were detected by UV-spectrophotometry at 240 nm and Chromeleon chromatography studio software (version 7.3.1) was used to perform peak analysis. The mono-aromatic products of PET hydrolysis were quantified against standards of TPA, MHET, BHET, while longer di- and tri-aromatic compounds were quantified against standards of TETE and TETET. All reactions were performed in duplicate and substrate blanks were included for the quantification of autohydrolysis.

3.4 Computational Protein Analyses

Protein sequences were aligned using T-COFFEE (Di Tommaso et al., 2011) in Expresso (structural alignment) mode, and outputs were visualized with ESPript 3.0 (Gouet et al., 1999). Alpha-Fold, which combines a deep learning-based algorithm with well-curated experimental knowledge from pre-existing structure and sequence databases, was used to model the SM14est protein as it is considered to be highly accurate with respect to atomic structure (Jumper et al., 2021). The AlphaFold-predicted structure for SM14est was verified as a good quality model based on the per-residue confidence scores (pLDDT) and the Predicted Aligned Error (PAE) plots (Supplementary Figure 1). The SM14est model was visualized in UCSF-Chimera and the MatchMaker tool was used to facilitate structural comparison with other PET hydrolases (Pettersen et al., 2004).

4. Results

4.1 Purification of SM14est and T_m Analysis

The expression and purification of SM14est was evaluated by SDS-PAGE analysis, which displayed a single band at around 29 kDa that corresponded to the predicted molecular weight of the recombinant SM14est protein (Figure 2A). Nano differential scanning fluorimetry was employed to study protein denaturation and the thermal transition midpoint (T_m) of SM14est

was subsequently determined to be 55 °C in sodium phosphate buffer, pH 8 (Figure 2B). Here, the intrinsic fluorescence of aromatic amino acid residues, primarily tryptophan, was used to track protein unfolding by measuring and recording changes in the fluorescence intensity ratio (350:330 nm), yielding a value for T_m when plotted as a function of temperature (Wen et al., 2020). The inflection point and isolated peak observed following the nanoDFS scan further validated the purity of the SM14est protein sample, given that a mixed sample would have generated a linear F350/330 signal and multiple first derivative peaks.

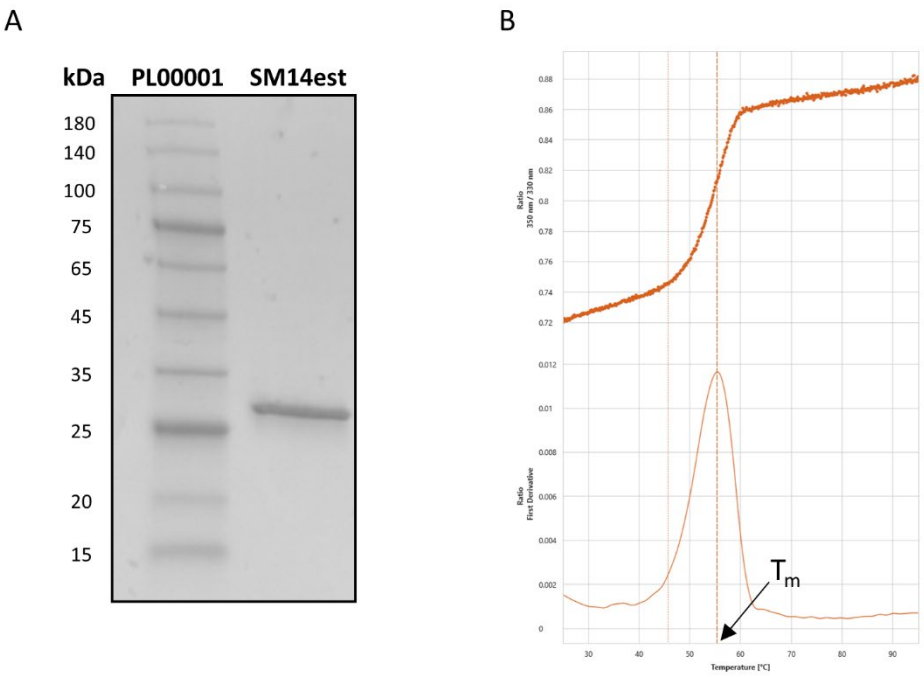


Figure 2. Post-purification analyses of SM14est by (A) SDS-PAGE, with pre-stained protein marker in lane 1 and SM14est in lane 2 and (B) NanoDFS, with the F350/330 ratio and its first derivative shown for Tm determination.

4.2 Investigating the Effect of Temperature, Salt, and pH on SM14est PET-Hydrolyzing Activity

The PET-hydrolyzing activity of the recombinant SM14est was assessed in the temperature range 10-45 °C (Figure 3A), as well as in the presence of increasing concentrations of NaCl (Figure 3B), and in the alkaline pH range 8.5 to 10.5 (Figure 3C). SM14est was shown to hydrolyze PET, to varying extents, at a number of different temperatures; 10, 28, 37, and 45 °C (Figure 3A). SM14est activity increased with the addition of NaCl, using concentrations up to 900 mM, at which point the effect appeared to plateau (Figure 3B). A marked decrease in the

PET-hydrolyzing activity of SM14est was observed at pH 9.5 when compared to that measured at pH 8.5 and the enzyme performed poorly when tested at pH 10.5 (Figure 3C).

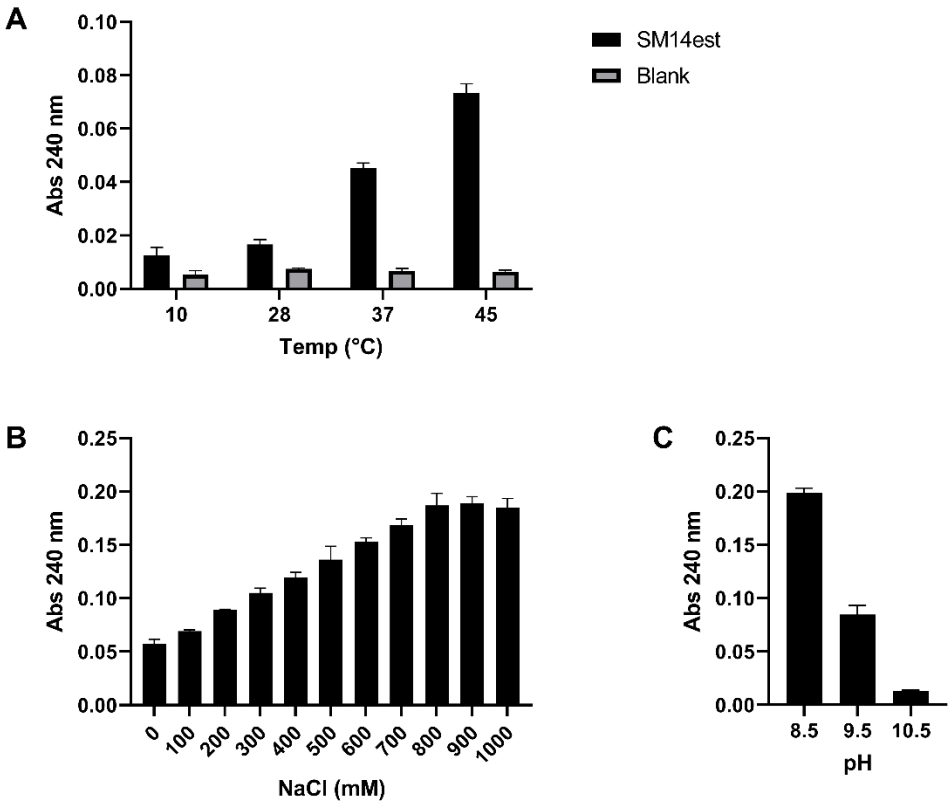


Figure 3. Effect of (A) temperature, (B) sodium chloride concentration, and (C) alkaline pH on the PET-hydrolyzing activity of SM14est. Reaction products were detected by absorbance at 240 nm following incubation of SM14est (0.5 μM) with PET (10 g L−1) in 50 mM sodium phosphate buffer, pH 8 except for (C) where 50 mM glycine buffer (with 500 mM NaCl added) was used. Incubations were set up as follows; (A) 1 h, 10–45°C and (B, C) 2 h, 45°C. Substrate blanks with no enzyme added served as a control. The data was evaluated by paired *t*-test for (A) and one-way ANOVA for (B) and (C), with $P < 0.05$ in each case.

4.3 Characterization of SM14est Activity Towards PET

Given that SM14est polyesterase activity increased at higher sodium chloride concentrations, the recombinant enzyme (0.5 μM) was subsequently assayed with PET (10 g L^{-1}) as substrate over a 7 h period at pH 8 and 45 °C, both with and without the addition of sodium chloride (500 mM) (Figure 4). In absence of NaCl, reasonable levels of PET degradation were observed, reaching 56 μM product in the t_7 sample. However, higher levels of activity were generated in the presence of NaCl, with 270 μM of product having accumulated in the equivalent t_7 sample.

The progress curves produced provide an insight into the initial rates of PET hydrolysis by SM14est, which were calculated to be 0.02 s^{-1} for the reaction with NaCl and in 0.004 s^{-1} in its absence (R^2 = 0.9976 and 0.8959, respectively). This represents a 5-fold difference in the initial rate of product formation, and thus PET-hydrolyzing activity, when sodium chloride is included in the reaction versus without the addition of salt.

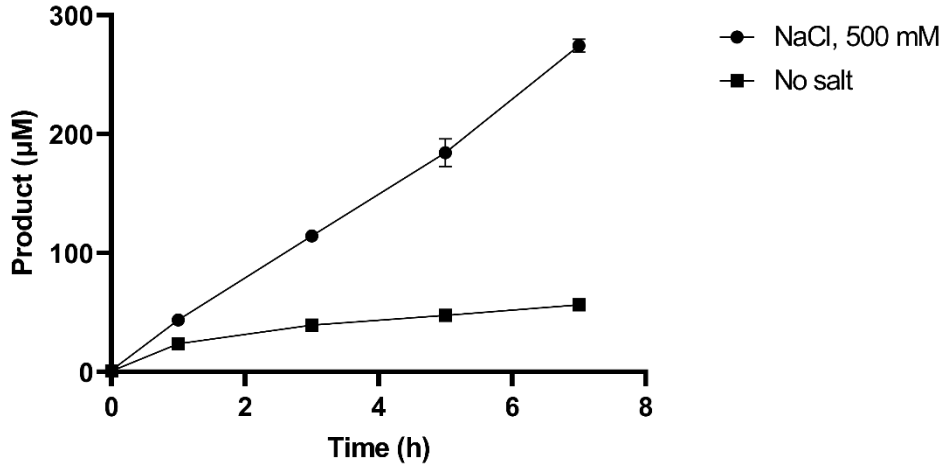


Figure 4. Product release over 7 h incubation of SM14est (0.5 µM) and PET (10 g L−1) in 50 mM sodium phosphate buffer, pH 8 with sodium chloride (500 mM) and without added salt at 45°C, quantified using a BHET standard curve.

RP-HPLC-based analysis (Figure 5) was then performed to identify the reaction products generated during the 7-hour time-course assay in Figure 4. This provides a breakdown of individual hydrolysis products, namely TPA, MHET, and BHET (shown here as T, ET, and ETE, respectively); as well as soluble di-aromatic oligomers which may include TET, TETE, ETETE. Tri-aromatic oligomers were not detected in any of the samples. It is clear from this analysis that SM14est displayed increased activity in the presence of sodium chloride, reflected in the generation of much higher quantities of PET degradation products. MHET was detected as the primary product of hydrolysis in both the presence (Figure 5A) and absence (Figure 5B) of sodium chloride; with minor secondary conversion of MHET to TPA, being observed with time.

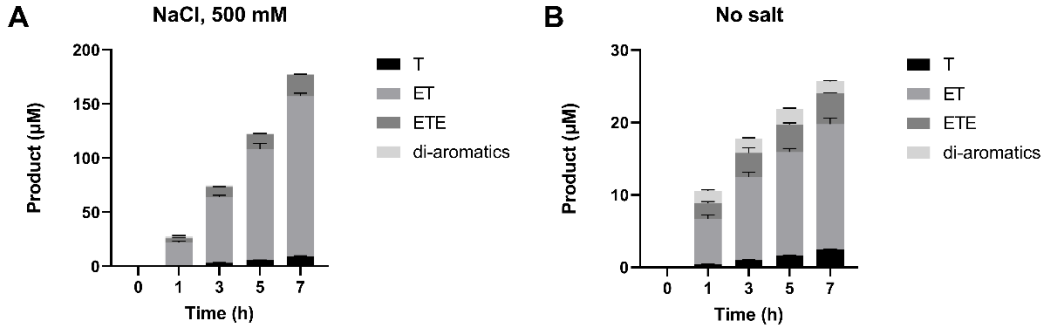


Figure 5. Individual products released during the 7 h time-course assay (A) with 500 mM NaCl added and (B) without additional salt, as detected by RP-HPLC. Mono-aromatic TPA, MHET, and BHET are represented by T, ET, and ETE, respectively (where T, terephthalic acid and E, ethylene glycol).

5. Discussion

To date, known PET-hydrolyzing enzymes are derived from esterases, lipases, and cutinases, with thermostable proteins being prioritized since PET is most easily hydrolyzed when reaction conditions are set in accordance with the glass transition temperature (T_g) of the plastic polymer (Lu et al., 2022). While thermotolerant enzymes have yielded promising results for the development of improved, bio-based recycling processes, it remains crucial to study enzymes that can efficiently degrade PET, among other plastics, under a variety of conditions to address the widespread issue of residual plastic that finds its way into natural ecosystems (Carr et al., 2022).

In the suspension-based assay employed for the characterization of SM14est, the aromatic ring structure present in MHET, BHET, and TPA enables UV-detection of these products at 240 nm, thus providing insight into the enzyme's PET hydrolase activity (Bååth et al., 2020). The substrate used here is of similar crystallinity to that of post-consumer PET waste (30-40 %), but exists in a powder form which is more readily depolymerized due to the particle size and available surface area (Gamerith et al., 2017). Pre-treatment by grinding and micronization are currently required for efficient enzymatic recycling of PET and, importantly, a large proportion of PET already exists as microparticles in the environment (Kawai, 2021).

While PET-hydrolyzing activity was observed at each temperature tested, SM14est performed best at 45 °C (Figure 3A). Semi-crystalline PET is composed of both amorphous and crystalline regions and its T_g is 65-70 °C in water (Kawai, 2021). The amorphous chains of PET have

increased mobility around the T_g, where they exist in a softened, rubber-like state (Thomsen et al., 2022). As reaction temperatures increase towards the T_g, PET becomes more malleable and enzymes can better access its constituent chains, increasing the efficiency of hydrolysis (Figure 3A). SM14est has a T_m around 55 °C, facilitating the high levels of activity observed at 45 °C. By comparison, wildtype IsPETase, the mesophilic model PET hydrolase, has a lower T_m of 49 °C, and displays optimal activity between 30 °C and 40 °C (depending on the substrate) and loses activity after 1 h at temperatures above 40 °C (Son et al., 2019; Kawai, 2021).

Based on the observed effect of increasing salt concentration, it appears the SM14est enzyme is halophilic and displays good salt tolerance (Figure 3C). The average salinity of seawater is commonly reported as being ~3.5% or 600 mM NaCl, and the general performance of the marine-derived SM14est seems to improve in accordance with this concentration range. The beneficial effect of sodium chloride on the activity of SM14est is further supported by the progress curves and product profiles generated in the presence and absence of salt (Figures 4 & 5). In a previous study the halophilic PET hydrolase, PET6, from *Vibrio gazogenes,* was reported to exhibit a preference for very high salt concentrations, with optimum product release occurring between 1 and 1.5 M sodium chloride, when incubated at 30 °C in PET-coated microtiter plates while using the well-studied IsPETase as a reference (Weigert et al., 2022). At a fixed concentration of sodium chloride (50 mM), PET6 exhibited distinct optimal activity between 45 °C and 50 °C, while IsPETase displayed optimal activity at 30 °C. In general, IsPETase displayed optimal activity at low salt concentrations, which rapidly decreases as ionic strength increases, while the opposite effect was observed for the PET6 enzyme. In the case of PET6, higher salt concentrations appeared to confer thermal stability, with a T_m increase from 49.8 to 57.7 °C, and hence superior activity (Weigert et al., 2022).

Given the observed increase in SM14est activity with the addition of sodium chloride, high salt conditions may elicit a stabilizing effect on SM14est, as seen with the halophilic PET6. Multiple sequence alignment of SM14est with PET6 demonstrates 75.89% similarity between both their protein sequences (Table 1), including conservation of the serine hydrolase catalytic triad (Ser163-Asp209-His241 in PET6; Ser131-Asp177-His209 in SM14est) and GXSXG motif (GWSMG in PET6; GHSMG in SM14est) (Supplementary Figure 2A). The shared α/β fold arrangement, which was visualized by superimposing the AlphaFold model for SM14est onto the already resolved PET6 crystal structure (chain A), is well-conserved between these two enzymes (Figure 6). Structural analysis of PET6 previously revealed binding sites for monovalent ions, where one sodium ion interacts with Arg61, Ala63, and Phe65 residues, a

second sodium ion is associated with Asp282, Ser284, and Val287, each occurring within a small loop via the amino acid backbone carbonyl groups (Weigert et al., 2022). In SM14est, Arg32, Ser35, and Phe38 correspond to the first ion site in PET6, while Gly248, Thr250, and Val253 are found in a loop around the second site (Figure 6). In PET6, a chloride ion sits in a shallow pocket comprised by Ala208, Asp209, Ala210, Val211, Ser240, His241, Phe242, which is similarly present in SM14est by Asn176, Asp177, Ala178, Ile179, Gly208, His209, and Phe210 (Figure 6)(Weigert et al., 2022). This comparison indicates structural similarity between the two enzymes, supporting their potential adaptation to saline environments, which, when combined with the observed promotion of activity by sodium and chloride ions, provides further insights into marine-derived polyester hydrolases.

Table 1. Sequence similarities generated for SM14est against six functionally verified PET hydrolases.

Enzyme	**Origin**	**Identity (%)**	**Similarity (%)**	**Reference**
PHL7	*Thermoanaerobacter* sp.	46.13	79.41	(Sonnendecker et al., 2022)
IsPETase	*Ideonella sakaiensis*	41.03	78.21	(Yoshida et al., 2016)
LCC	Leaf-compost metagenome	38.51	76.40	(Sulaiman et al., 2012)
PET6	*Vibrio gazogenes*	34.40	75.89	(Danso et al., 2018)
PE-H	*Pseudomonas aestusnigri*	33.63	73.66	(Bollinger et al., 2020)
Ple629	*Marinobacter* sp.	32.26	72.87	(Meyer Cifuentes et al., 2022)

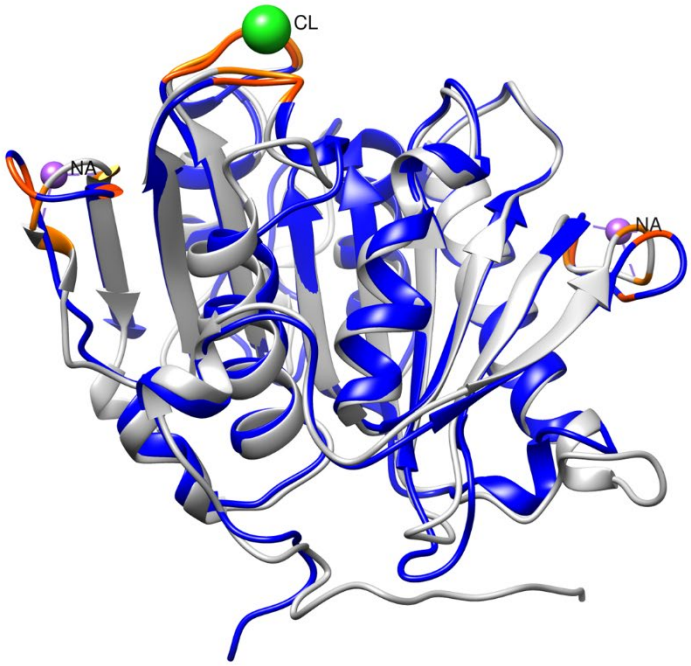


Figure 6. Superimposition of SM14est model (blue) onto PET6 structure (gray, PDB code: 7Z6B). Sodium and chloride ions (colored in purple and green, respectively) are shown at their PET6 binding sites, with relevant residues colored in orange. The corresponding residues in SM14est are colored in red-orange.

The Plastics-Active Enzymes Database (PAZy) has recently been established as a repository for enzymes with experimentally confirmed activities towards synthetic polymers, primarily those which act on PET (Buchholz et al., 2022). PET6, together with PE-H from *Pseudomonas aestusnigri*, and Ple629 from *Marinobacter* sp. are among the marine-derived PET hydrolases in the PAZy collection. Although SM14est shares over 70% amino acid sequence similarity with these three marine enzymes and between 32 and 34% sequence identity, it shares almost 80% similarity and just over 46% sequence identity with one of the top performing PET hydrolases, PHL7 (Table 1 & Supplementary Figure 2B). An engineered variant of PHL7 displays comparable activity to mutant LCC (ICCG), generating high levels of degradation products on a millimolar scale (Kawai et al., 2022). The identity and similarity percentages generated between SM14est and the benchmark enzymes LCC and IsPETase also ranked above those of the aforementioned marine examples, PET6, PE-H, and Ple629. This may indicate a potential for SM14est to represent an interesting “middle-ground” in the study of marine PET hydrolases, where its sea-sponge origin is complemented by its similarity to enzymes which have the highest known capacity for PET degradation. The product profiles obtained for

SM14est by RP-HPLC (Figure 5) are comparable to those generated for thermophilic PET-hydrolyzing cutinases in a previous study, further evidencing the PET degradation ability of SM14est (Bååth et al., 2020).

Structural analysis and molecular docking experiments have previously been conducted to identify important amino acid residues in PHL7, particularly those contributing to its degradation efficiency and those with binding affinity for PET (Sonnendecker et al., 2022). The residues in the active site are highly conserved between SM14est and PHL7 (chain B) (Figure 7). Within the substrate-binding cavity, Gly62, Thr64, Gln95, His130, Ser131, Met132, Trp156, Asp177, Ile179, Ala180, His209, Leu210, and Asn213 (catalytic triad residues underlined) are identical in SM14est and PHL7 (colored in magenta), with differences at just three positions; Tyr63, Thr69, and Tyr93 in SM14est correspond to Phe63, Ser69, and Leu93 in PHL7 (colored in orange) (Figure 7). In PHL7, Leu210 was proposed as a particularly important residue for substrate binding and was experimentally verified to contribute to higher activity of PHL7 over the near-identical enzyme PHL3, which differs only by four amino acids overall. When this leucine was substituted for phenylalanine, which is found at this position in other type I PET hydrolases, the activity of PHL7 decreased to that of native PHL3 (Sonnendecker et al., 2022). The occurrence of leucine at position 210 in SM14est may have similar implications for PET binding and catalysis. Based on the PAZy-defined “PET hydrolase core domain”, Tyr63 in SM14est is likely to be an aromatic oxyanion hole residue involved in stabilizing the PET hydrolysis reaction intermediate, which occurs as tyrosine in 51% of homologous core domains (e.g., Tyr95 in LCC) and as phenylalanine in 46% (e.g., Phe63 in PHL7) (Buchholz et al., 2022). In another study of PHL7, the replacement of phenylalanine with tyrosine at this position resulted in a variant with decreased hydrolytic activity but further investigation would be required to determine the role of Tyr63 in SM14est (Pfaff et al., 2022).

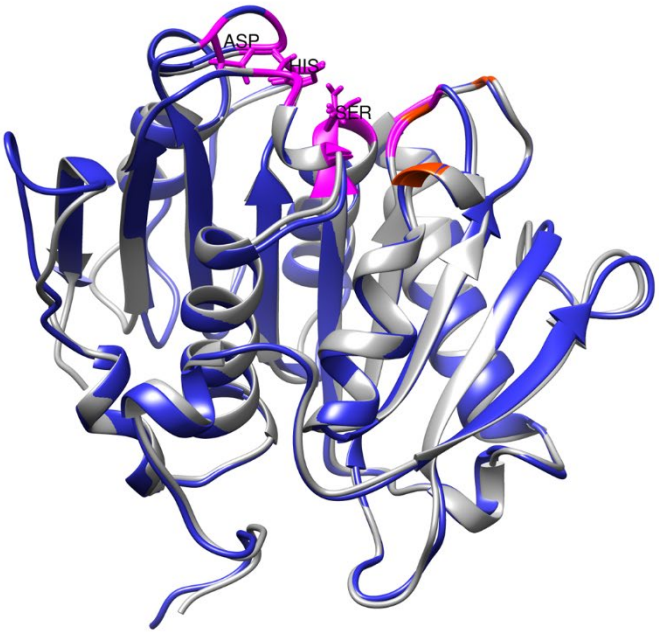


Figure 7. Superimposition of SM14est model (blue) onto PHL7 structure (gray, PDB code: 7NEI). Conserved active site residues are colored in magenta, distinguished from non-identical pocket residues, which are colored in orange. The catalytic triad residues (Ser131-Asp177-His-209) are labeled and shown in stick format.

Since the identification of IsPETase in 2016, there has been an ongoing debate regarding the relevance of mesophilic PET hydrolases in the context of plastic waste management (Wallace et al., 2020; Kawai, 2021; Wei et al., 2022). Previously, it was highlighted that mesophilic PET hydrolases may be unsuitable for enzymatic PET recycling given the temperature requirements for efficient hydrolysis and furthermore, that attempts to improve the thermostability of IsPETase were contradictory to the original claims that this enzyme could enable PET hydrolysis under mild conditions (Kawai, 2021). Based on a study of LCC (ICCG) mutant and IsPETase molecular reaction mechanics, it has been suggested that the superior activity of known thermostable PET hydrolases is primarily due to the greater accessibility of PET polymer chains at higher temperatures, as opposed to their specific interaction with or affinity for the substrate (Boneta et al., 2021).

During the construction of the PAZy database, a PET hydrolase core domain was determined by Hidden Markov Model-based profiling and used to identify 2930 homologs in the Lipase Engineering Database (LED), which was then assessed in terms of amino acid conservation

(Buchholz et al., 2022). Using the LED PETase tool within PAZy, SM14est was aligned with IsPETase to compare positions that have previously been proposed to have structural or functional involvement in PET hydrolysis (Supplementary Figure 3). The isoleucine residue corresponding to Ile179 in SM14est has been implicated as a key residue for IsPETase substrate interaction. The tryptophan residue of subsite I, found at position 156 in SM14est, is referred to as the "wobbling tryptophan" in IsPETase since it is hypothesized to adopt three different conformations which confers increased flexibility within the substrate binding site. This effect is likely restricted in SM14est due to nearby His185 and Phe189 residues, but in other cases these have been successfully substituted for Ser and Ile (the neighboring residues in IsPETase) to enhance tryptophan mobility and thus, PET-hydrolytic activity (Aboelnga and Kalyaanamoorthy, 2022; Brott et al., 2022). Nonetheless, this tryptophan is believed to form stacking interactions with the aromatic component of PET and it is conserved in 77% of PET hydrolase core domain homologs (Buchholz et al., 2022). While many of the subsite I and II residues are equivalent in both IsPETase and SM14est, SM14est does not display the same extension in the second alpha-helix, another structural hallmark linked to superior IsPETase activity at ambient temperature (Sagong et al., 2021).

Recently, quantum mechanics/molecular mechanics (QM/MM) calculations have been employed for a more in-depth examination of the PET hydrolysis reaction pathway and the key structural features required for efficient catalysis (Jerves et al., 2021; Aboelnga and Kalyaanamoorthy, 2022). The IsPETase molecular features investigated, which enable the accommodation of PET in an advantageous bent conformation and promote tightened hydrogen bond interactions between catalytic triad residues, could prove useful in the rational engineering of SM14est. In particular, emphasis is placed on the role of the unique disulfide bond present near the IsPETase active site. Interestingly, the typical type I PET hydrolase disulfide bond (DS1) and the additional disulfide bond of IsPETase (DS2) are both absent from SM14est, which does not possess any disulfide bridge. Since we have now demonstrated that SM14est can use PET as a substrate, the introduction of DS1, DS2, or both could offer further insight into the role of such bonds in PET hydrolysis. Furthermore, the overall structure of SM14est could be modified using a machine learning-based approach to generate improved variants. A self-supervised, structure-based convolutional neural network called MutCompute was recently implemented to identify stabilizing mutations that resulted in FAST-PETase (functional, active, stable, and tolerant PETase$^{S121E/D186H/R224Q/N233K/R280A}$), which emerged as an excellent candidate for in situ PET degradation when compared with wild-type IsPETase, previously

engineered variants ThermoPETase and DuraPETase, as well as LCC and mutant LCC (ICCM), across a pH range of 6.5-8.0 and ambient temperatures of 30-40 °C (Lu et al., 2022).

Halophilic enzymes are more likely to be resistant to proteolysis and denaturation by organic solvents and detergents, and often display a longer half-life that is particularly advantageous during the relatively slow process of plastic degradation (Atanasova et al., 2021). Plastic frequently contaminates marine and other salt-rich natural environments, together with high-salinity wastewaters, including those derived from agriculture, aquacultural, pharmaceutical, and chemical industries, that already pose a threat to surrounding ecosystems (Raju et al., 2018; Zhao et al., 2020; Atanasova et al., 2021). This highlights the need for bioremediation strategies that employ plastic-degrading bacteria and their enzymes; such approaches are under-represented in the field of PET hydrolases, where the primary goal to date has been to facilitate biological plastic recycling (Atanasova et al., 2021). Marine actinomycetes, including *Streptomyces* spp., that display halophilicity and halotolerance have previously been isolated from saline environments and shown to be both physiologically and metabolically distinct from their terrestrial counterparts (Almeida et al., 2019a). The work presented here on the PET-hydrolyzing activity of SM14est supports the need for the continued investigation of marine microorganisms and their enzymes, not only for the biological degradation of PET and other plastics, but also for the identification of industrially relevant biocatalysts.

6. Concluding Remarks

While rapid advancements have been made in the study of PET hydrolases, the problem of plastic waste is multi-faceted and requires a diverse suite of microbes and enzymes to enable both environmentally sound, closed-loop recycling as well as biological remediation of plastic-contaminated sites. Here, the hydrolytic activity of the marine polyesterase SM14est is characterized using PET as a substrate. SM14est can hydrolyze PET at a range of temperatures, most efficiently at 45 °C, but even as low as 10 °C, which may be indicative of an ability to act on PET in its native environment. Furthermore, SM14est displayed a strong preference for pH 8 and high salt concentrations, each aligning with the typical conditions found in ocean waters. Following structural protein analyses, SM14est was shown to share similarities as well as key differences with other well-characterized PET hydrolases, including those from marine sources. Although there is a possibility that, as a result of microbial evolution, the enzymatic machinery in marine bacteria could become adapted to degrade the increasing levels of plastics and microplastics to which they are being exposed, we suspect that it is the promiscuous nature of

hydrolase enzymes such as SM14est that primarily facilitates the acceptance of an unnatural substrate like PET. Nonetheless, given that marine-specific actinomycetes have been studied and shown to populate a variety of habitats, which remains a largely neglected resource as of yet, this work on the sea sponge-derived *Streptomyces* enzyme SM14est provides further incentive to explore different types of marine ecosystems as a source of novel enzymes with potentially interesting biotechnological applications.

7. Conflict of Interest

The authors declare that there are no conflicts of interest. KJ and BP are employed at Novozymes A/S, a major manufacturer of industrial enzymes.

8. Author Contributions

CC designed the experiments with the support of MK, BP, KJ, PW, and AD. Experimental work was performed by CC, BP, SS, and KC. CC, SS, and KC analyzed the data. KJ, PW, and AD contributed reagents, materials, and analysis tools. CC and AD wrote the manuscript. PW, DC, and AD supervised the study. All authors approved the final manuscript.

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11. Data Availability Statement

The raw data supporting the conclusions of this article will be made available by the corresponding authors upon request.

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13. Supplementary Material

A



B

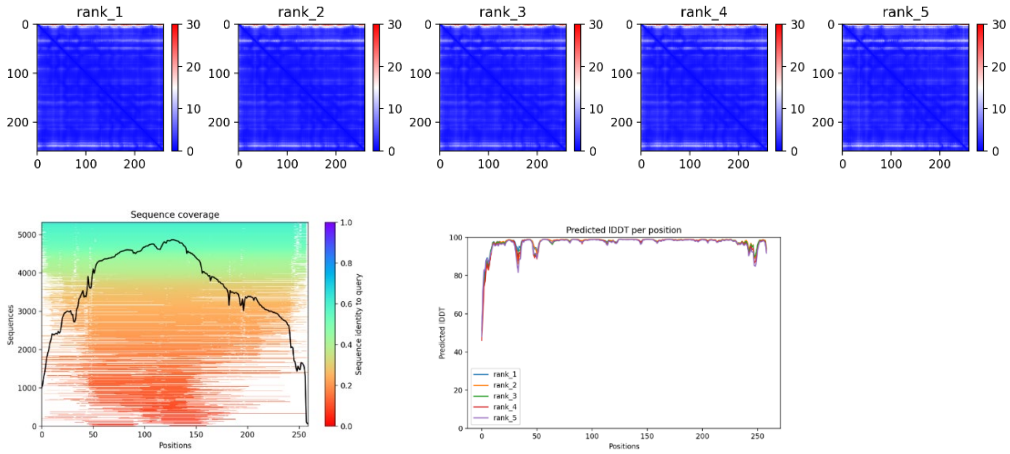
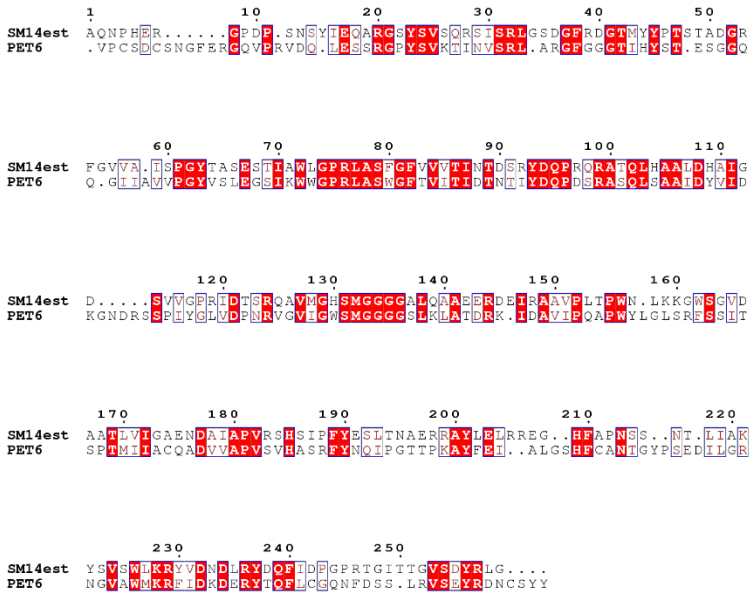


Figure S1. AlphaFold model of SM14est three-dimensional protein structure (A), where the central beta-sheet is colored in cyan and alpha-helices colored are in orange. The complementary quality data is shown (B), with Predicted Aligned Error (PAE) matrices, sequence coverage plot, and per-residue confidence measure (pLDDT) included for the top five ranked models.

A



B

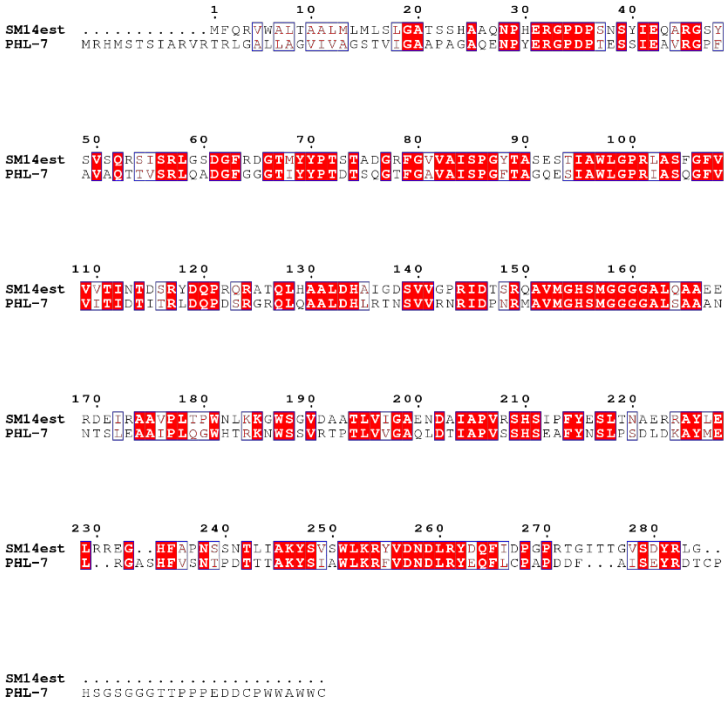
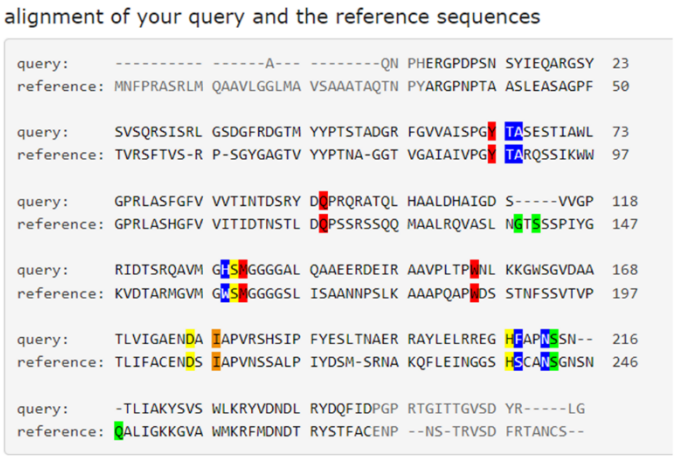


Figure S2. Amino acid sequence alignments of SM14est with (A) PET6, and (B) PHL7.



Residue (SM14est)	Residue (IsPETase)	Feature
Tyr63	Tyr87	Subsite I, aromatic clamp/oxyanion hole
Thr64	Thr88	Subsite II
Ala65	Ala89	Subsite II
Gln95	Gln119	Subsite I
-	Gly139	Extension in alpha-helix 2
-	Ser141	Extension in alpha-helix 2
His130	Trp159	Subsite II
Ser131	Ser160	Catalytic triad, nucleophilic serine
Met132	Met161	Subsite I, oxyanion hole
Trp156	Trp185	Subsite I, aromatic clamp
Asp177	Asp206	Catalytic triad
Ile179	Ile208	Substrate interaction
His209	His237	Catalytic triad
Phe210	Ser238	Subsite II
Asn213	Asn241	Subsite II
Ser214	Ser242	Extended loop
-	Gln247	Extended loop

Figure S3. PAZy-LED output, generated by aligning SM14est (query) sequence with IsPETase (reference) sequence.

Chapter 4

Identification, Expression, and Activity of MarCE, a Promising Marine Carboxylesterase for the Degradation of Synthetic Polyesters

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1. Abstract

Bacteria and other microorganisms produce enzymes called carboxylic ester hydrolases which facilitate the breakdown of ester-linked compounds in nature. Microbial CEHs such as carboxylesterases and lipases are of industrial interest, with use in the biodegradation of synthetic polyesters and for the sustainable catalysis of chemical reactions across various industries. Here, we report on a carboxylesterase-like enzyme named MarCE, which was found encoded in the genome of a *Maribacter* sp. isolate from the marine sponge *Stelligera stuposa*. MarCE shares >70% amino acid similarity with two carboxylesterases (TfCa and BsEstB) that have been previously shown to efficiently degrade short-chain oligomers of the most widely used polyester plastic, polyethylene terephthalate (PET). Protein modelling and comparative analyses revealed key structural and catalytic features, including a serine hydrolase Ser-Glu-His triad, a Gly-Gly oxyanion motif, and a pentapeptide GESAG sequence, while molecular docking showed putative binding of PET oligomers. MarCE was heterologously expressed in *E. coli*, purified from culture supernatant, and confirmed as active on polycaprolactone diol clearing assays. *Maribacter* species may represent a promising source of novel CEHs, that are uniquely adapted to the marine environment, whose discovery will help to expand and diversify the selection of enzymes available for biological recycling among other biocatalytic applications.

2. Introduction

The class of enzymes termed carboxylic ester hydrolases (CEHs, EC 3.1.1.x) represents a wide range of proteins that are ubiquitously distributed across living systems and that are known to act on ester and ester-linked substrates (Bornscheuer, 2002). Microbial CEHs tend to be highly diverse in terms of their biological roles, with potential involvement in various cellular interactions, metabolic processes, and signalling pathways (Oh et al., 2019). This multi-functionality can be leveraged for biocatalysis, biodegradation, and biotransformation purposes, where alternative or non-native substrates can be processed by these enzymes. In particular, the degradation of synthetic polyesters by microbial CEHs has been explored with the aim of developing systems for the biorecycling or bioremediation of polyester-based plastic materials (Urbanek et al., 2020). Polyester-degrading enzymes are collectively termed "polyesterases" and include CEHs homologous to carboxylesterases (CEs), lipases, and cutinases. Cutinase-like polyesterases have emerged as the most efficient degraders of recalcitrant polyesters like polyethylene terephthalate (PET), but complete hydrolysis of waste

plastic is unlikely to be achieved by a single enzyme. Therefore, dual enzyme systems, synergistic cocktails, multi-enzyme cascades, and immobilization strategies have been proposed to assist the process, together with physical pretreatment of the plastic to be degraded (Gricajeva et al., 2022).

Marine ecosystems have become increasingly valued as a source of microbial biocatalysts. With respect to marine CEHs, a number of enzymes have been identified and characterized, including examples from *Pseudoalteromonas*, *Vibrio*, *Bacillus, Erythrobacter*, *Pseudomonas*, and *Oleispira* species together with those from metagenomic sources (Lee et al., 2010; Rodrigues et al., 2017). In a study investigating marine metagenomic libraries prepared from crude oil-contaminated seawater enrichment cultures, five particularly active carboxylesterases that exhibited broad substrate profiles and biochemical diversity were identified (Tchigvintsev et al., 2015). These CEHs efficiently hydrolyzed a range of mono- and polymeric esters and were also shown to withstand low temperatures and the presence of certain salts, solvents, and detergents. More recently, a carboxylesterase named E93 was identified from a marine strain of the bacterium *Altererythrobacter indicus* and investigated in terms of specificity towards CPT11 and NPC ester prodrug substrates, together with *p*NP-esters; expanding the knowledge of hydrolysis of such compounds by microbial enzymes, which have to date been largely underreported in comparison to human carboxylesterases for prodrug activation (Li et al., 2023c). In addition, marine-derived bacterial CEHs such as Ple628, Ple629, PE-H, and SM14est have been shown to hydrolyze PET among other polyesters, as reported in the Plastics-Active Enzyme Database (PAZy) (Buchholz et al., 2022). In addition, based on a metagenomic study, genes encoding PET hydrolase homologs appear to be globally distributed across both terrestrial environments, primarily occurring within the phylum *Actinomycetota*, and marine-associated ecosystems, most commonly detected in the phylum *Bacteroidota* (Danso et al., 2018).

There is a keen interest in sequence-based prediction and computational design of highly efficient enzymes, whether for use as green catalysts in chemical manufacturing or for the degradation of synthetic pollutants. Even so, it remains important to harness natural ecosystems for enzyme discovery and to consider the environmental and/or microbial origins of a given enzyme, which can often provide clues regarding its biochemical properties and native ecological function (Ogawa and Shimizu, 1999; Reen et al., 2015). Marine microorganisms and their enzymes are still relatively underutilized from a biotechnological standpoint, particularly when compared to their terrestrial counterparts. Nonetheless, efforts to characterize marine

microbial activities have to date proven worthwhile, leading to increased diversity in the pool of available biocatalysts and the uncovering of enzymes with novel properties including high salt tolerance, temperature adaptivity, barophilicity, and unique substrate specificities (Rodrigues et al., 2017; Gavin et al., 2019). In particular, microbial enzymes from marine sponges are rarely reported on, but represent a promising biodiscovery resource, especially considering the important role they play in the degradation of organic matter that is extracted by the sponge from the large quantities of seawater that are pumped through its filter-feeding structures for energy and nutrient gain (Wang, 2006; de Oliveira et al., 2020).

Following screening and genome mining of bacterial isolates from marine samples for polyesterase activity, MarCE, a putative carboxylesterase from a marine lake sponge-derived *Maribacter* sp. was identified, cloned, and heterologously expressed. Computational protein analyses and molecular docking were carried out to gain functional and evolutionary insight into MarCE, which supports its biocatalytic potential. MarCE displays hydrolytic activity towards the synthetic polyester polycaprolactone diol (PCD), and based on docking of bis(2-hydroxyethyl) terephthalate (BHET), bis[2-(benzoyloxy)ethyl] terephthalate (3PET), and diethyl phthalate (DEP) exhibits potential to degrade oligomers of PET and other synthetic esters.

3. Materials and Methods

3.1 Culture Isolation and Polyesterase Screening

Pure bacterial cultures were previously obtained from four different Irish marine sponges, namely *Stelligera stuposa* and *Eurypon major* collected from marine waters at Lough Hyne, Co. Cork (Jackson et al., 2012; Margassery et al., 2012) as well as *Inflatella pellicula* and *Pheronema* sp. sampled from deep waters of the North East Atlantic Ocean (Kennedy et al., 2014; Carr et al., 2022), and from *Laminaria digitata* and *Ascophyllum nodosum* seaweeds found on the west coast of Ireland (unpublished). The isolates were screened to identify potential polyesterase producers and six strains were selected for next-generation genome sequencing based on the observed enzyme activities. Substrate clearing on tributyrin, polycaprolactone diol (PCD), and polycaprolactone (PCL) marine agar-based assays was monitored to assess hydrolytic activity, as previously outlined in (Carr et al., 2022).

3.2 Genome Sequencing, Assembly, and Analysis

Genomic DNA (gDNA) was extracted by phenol-chloroform-isoamyl alcohol extraction as previously described (Jackson et al., 2018) or in some cases by QIAamp® DNA Mini Kit (Qiagen) from 5 mL cultures grown by shaking for 24 h in Marine Broth 2216 at either 28 or 30°C. Next-generation Illumina HiSeq sequencing (NovaSeq 6000 system), library preparation, and initial quality control were performed by Eurofins Genomics (Konstanz, Germany). The Genome Taxonomy Database Toolkit (GTDB-Tk, v 1.5.0) was used to classify each isolate (Chaumeil et al., 2020). The sequence read quality was confirmed by FastQC (v 0.11.9) [http://www.bioinformatics.babraham.ac.uk/projects/fastqc] prior to genome assembly (of contigs >400 bp), which was completed using the SPAdes algorithm (v 3.15.0) (Bankevich et al., 2012). The resulting assemblies were evaluated by QUAST (v 5.0.2) (Gurevich et al., 2013) and CheckM (v 1.1.3) (Parks et al., 2015) before initial annotation was performed using Prokka (v 1.14.6) (Seemann, 2014). The predicted protein outputs were functionally annotated using eggNOG mapper (v 2.0) (Huerta-Cepas et al., 2019).

3.3 Genome Mining, Protein Analyses, and Protein Modelling

Each genome was mined for homologs of known polyester-degrading enzymes using a custom BLASTP database, as previously described by our group for the identification of the cutinase-like polyesterase BgP from a deep-sea sponge-derived Actinobacterium (Carr et al., 2022). The reference dataset used to construct the polyesterase database was made available as part of the

above-mentioned work on BgP. MarCE was identified in the genome of the *Maribacter* sp. isolate J2146c as a homolog of the carboxylesterase BsEstB (Table 1), which is known to degrade 3PET (Ribitsch et al., 2011).

The SignalP 5.0 server was used to predict the native signal peptide sequence, which was subsequently removed (Almagro Armenteros et al., 2019). Pfam [http://pfam.xfam.org/], InterProScan [https://www.ebi.ac.uk/interpro/search/sequence/], and SUPERFAMILY [https://supfam.mrc-lmb.cam.ac.uk/SUPERFAMILY/] tools were used for protein classification and functional prediction analysis. Protein structural alignments were generated by T-COFFEE Expresso (Di Tommaso et al., 2011) and graphically rendered using ESPript 3.0 (Gouet et al., 1999), while MEGA-X was employed for phylogenetic analysis (using MUSCLE alignment, maximum likelihood statistical method, WAG + G model, 100 bootstrap replications, 50% bootstrap cut-off value) (Kumar et al., 2018). Alpha-Fold2 (MMseqs2 mode, v1.3.0) was run via ColabFold to predict the structure of MarCE and the model reliability was assessed based on the sequence coverage, per-residue confidence (pLDDT), and predicted aligned error (PAE) outputs (Mirdita et al., 2022). The model was visualized in UCSF Chimera and the Chimera MatchMaker tool was used to facilitate structural comparison with homologs of interest (Pettersen et al., 2004).

3.4 Cloning, Heterologous Expression, and Purification of MarCE

The *marCE* gene was amplified by Q5® High-Fidelity DNA Polymerase (NEB) from gDNA and cloned into a pET20b(+) plasmid (Novagen®) using *XhoI* and *BamHI* restriction sites, designed in-frame with the vector signal sequence (pelB) and C-terminal His-Tag. The pET20b:MarCE construct (Supplementary Figure S3) was transformed into NEB® 5-alpha competent *E. coli* (New England Biolabs) and subsequently conjugated into the expression host *E. coli* BL21-Codon Plus (DE3)-RIPL (Agilent Technologies). Diagnostic restriction digest, colony PCR, and subsequent Sanger sequencing (Eurofins LightRun) were carried out to confirm the presence of the insert and 20% glycerol stocks were prepared to store the recombinant clone at -80°C.

Fresh cultures of the BL21 RIPL-(pET20b:MarCE) clone and its negative control (BL21 RIPL carrying an empty pET20b vector) were prepared by inoculating single colonies into 20 mL Luria Bertani (LB) broth (0.003% w/v chloramphenicol, 0.01% w/v ampicillin). After overnight incubation at 37°C with shaking (225 rpm), 10 mL was subcultured into fresh LB (1 L, ampicillin only), then further incubated for 4 h before inducing at 20°C with 0.5 mM IPTG

(Melford). Following 20 h induction, the culture broths were pelleted by centrifugation (17700 x *g*) for 10 mins, at 4°C (Beckman Coulter, Avanti JXN-26, JA-10 rotor), then supernatants were collected and kept on ice. The supernatants were sterile filtered (0.22 μM) and concentrated using Vivaspin® 20 centrifugal units (MWCO 10 kDa, Sartorius) prior to purification at 4°C using the Pierce™ High-Capacity Ni-IMAC Resin (Invitrogen). Purified MarCE was desalted using Cytiva™ Disposable PD10 columns to remove imidazole, which can catalyze *p*NP-ester hydrolysis (Smith et al., 2021), and concentrated as before.

The ExPASy ProtParam tool was used to predict the molecular weight and molar extinction coefficient of MarCE. The concentration of purified enzyme was measured by absorbance at 280 nm and 2.5 μg of sample was analyzed by SDS-PAGE, prepared with 10% resolving gel, 5% stacking gel, tris-glycine-SDS running buffer, and 2X β-mercaptoethanol sample buffer. Enzyme activities of the supernatant and purified preparations (10 μL) were confirmed on polycaprolactone diol (PCD_{Mn530}, 1%) agar, while a preliminary temperature profile was generated from the MarCE culture supernatant by incubating at 4, 20, 25, 28, 30, 37, and 50°C.

3.5 Molecular Docking

AutoDock Vina (v 1.1.2) was employed to dock several substrates into the putative binding site of MarCE, namely the terephthalate oligomers BHET and 3PET, and the ortho-phthalate ester DEP. The search box was set around the center of the catalytic triad, with an edge length of 15 Å (or 25 Å in the case of 3PET) and an exhaustiveness value of 8. The binding modes were visualized in UCSF ChimeraX and BIOVIA Discovery Studio.

4 Results

4.1 Identification and Classification of MarCE

Marine-derived bacteria from shallow water sea-lake sponges, deep-sea sponges, and coastal seaweeds were assessed for hydrolytic activities and six candidates were subsequently chosen for genome sequencing (Table 1 and Supplementary Table S1). Of these, the *Maribacter* sp. isolate (sample ID = J2146c), associated with a *Stelligera stuposa* sponge from the marine waters of Lough Hyne, was noted as a promising strain with activity being observed on all tested substrates (Supplementary Table S1). The closest relative of the J2146c strain was determined to be *Maribacter litoralis* using the GTDB-Toolkit and its genome statistics indicate that a high-quality draft genome was obtained (Table 1 and Supplementary Table S3), with similar properties to previously deposited *Maribacter* spp. genomes. Using an E-value cutoff of 1e-30, a putative enzyme in the J2146c genome produced a significant alignment with BsEstB, one of the reference enzymes in the custom polyesterase database constructed for genome mining. T-Coffee Expresso alignment revealed a mean amino acid identity of 30.47% between their sequences and a mean similarity of 72.90%. The other five candidate genomes were again mined using this approach and another hit was detected in the *Brachybacterium* sp. genome (sample ID = B129SM11) which has already been reported as the cutinase-like enzyme BgP (Carr et al., 2022).

Table 1: Genome classification and total number of coding sequences predicted for the six selected marine bacterial isolates of interest, together with the hits detected during the mining of their genomes for polyesterase homologs (E-value cutoff set to 1e-30).

Genome Information			**Genome Mining**					
Isolate ID	Classification (by GTDB-Tk)	#CDS	Prokka Annotation	Length (AA)	Top Hit	Score	E-Value	Assigned Name
J2009s	*Bacillus altitudinis*	3809	-	-	-	-	-	-
J938L	*Bacillus pumilus*	3653	-	-	-	-	-	-
B129SM11	*Brachybacterium ginsengisoli*	3505	Poly(ethylene terephthalate) hydrolase	290	Tcur1278	342	9e-122	BgP
			Hypothetical protein	493	BsEstB	160	1e-46	-
B226SK6	*Micrococcus luteus*	3460	-	-	-	-	-	-
J2146c	*Maribacter litoralis*	4149	Fumonisin B1 esterase	555	BsEstB	200	8e-61	MarCE
J2163c	*Cobetia amphilecti*	2257	-	-	-	-	-	-

The *Maribacter* sp. CEH was annotated by Prokka as a "Fumonisin B1 esterase", while eggNOG mapper assigned it as a carboxylesterase family protein. A general NCBI BLASTP search of its sequence was conducted against the non-redundant database together with narrowed searches against the Swissprot database (Supplementary Table S2). Each of the top matches falls under the carboxylic ester hydrolase (CEH) class of enzymes but their individual descriptions vary from carboxylesterase to acylcarnitine and pyrethroid hydrolase to para-nitrobenzyl esterase. Based on InterProScan, Pfam, and SUPERFAMILY outputs, the enzyme of interest appears to be an α/β hydrolase of the carboxylesterase (CE, EC 3.1.1.1) family. Considering the search results, the *Maribacter* sp. enzyme was designated as MarCE.

4.2 Phylogenetic and Structural Protein Analyses

Phylogenetic analysis was conducted to gain insight into the evolutionary relationships between MarCE and a selection of homologous carboxylesterases (Supplementary Table S4). The resulting consensus tree is comprised of two main clades (labelled 1 and 2, Figure 1). The first clade features carboxylesterases from CFB (Cytophaga, Flavobacterium, Bacteroides) group bacteria, which are associated with the phylum Bacteroidota and that are considered to be important contributors to the degradation of marine organic matter (Brettar et al., 2004). In clade 1a, MarCE is clustered with its closest *Maribacter* spp. homologs and those from two closely related CFB members, *Saonia flava* and *Fulvivirga sedimenti*. The clade 1b consists of sequences belonging to Flavobacteriaceae family members, while clade 1c proteins come from the Cytophagia class of bacteria. The reference carboxylesterases (i.e., those sourced from Swissprot or PDB databases for which experimental or structural data is available, or that have at least been previously studied in the literature) are found in clade 2, where carboxylesterases from the species *Thermobifida fusca* and the family *Bacillaceae* form 2a and 2b, respectively. The other clade 2 proteins can be assigned as Actinomycetota (*R. erythropolis* and *P. oxydans*), Alphaproteobacteria (*S. macrogoltabida* and *A. indicum*), or Bacillota (*H. hathewayi*) phyla members. A metagenomic enzyme from marine sediment is found between clade 1 and clade 2, while IsPETase serves as an outgroup.

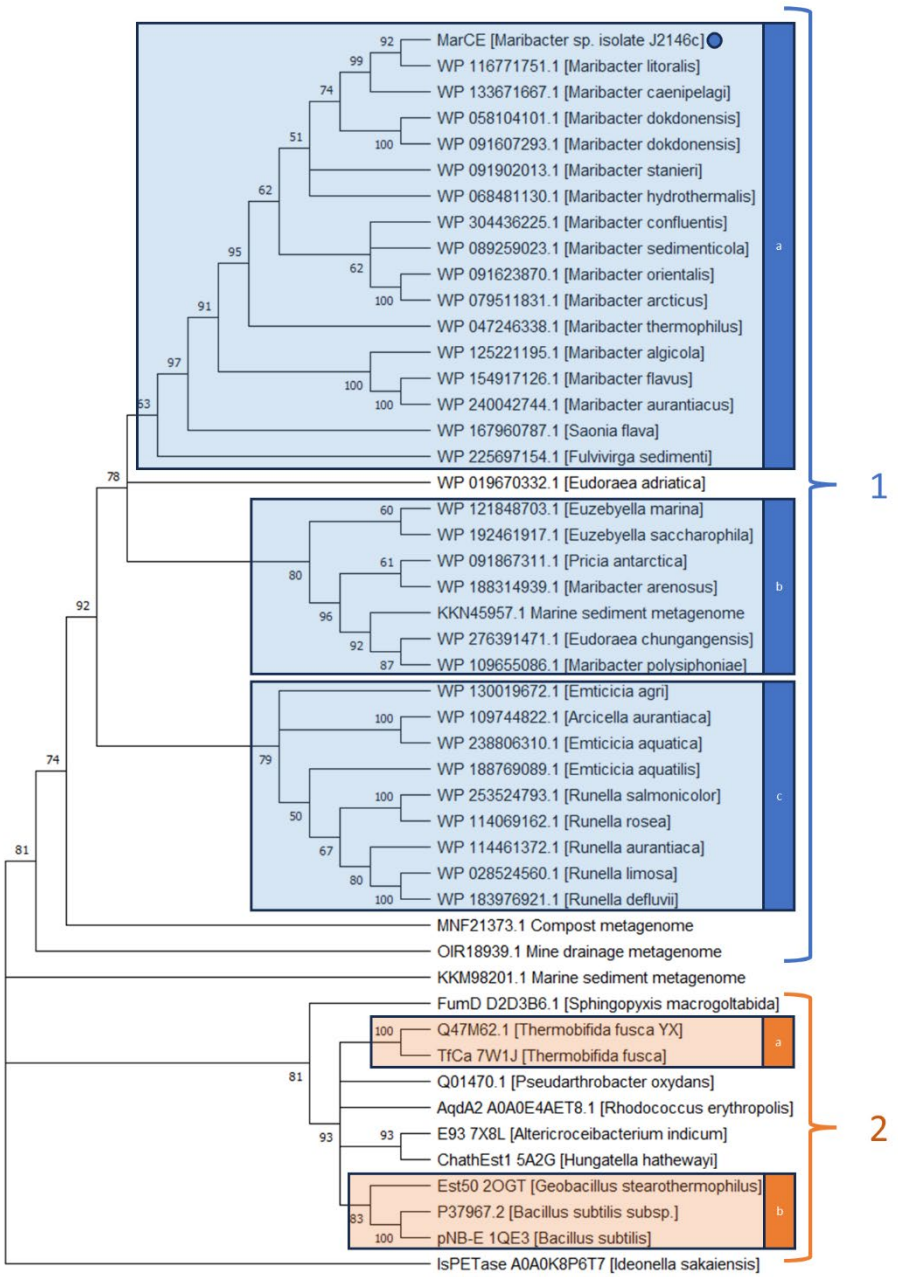


Figure 1: Phylogenetic consensus tree generated from the protein sequences of MarCE and 46 homologous carboxylesterase-like enzymes, rooted with IsPETase (NCBI accession: A0A0K8P6T7) as an outgroup. The maximum-likelihood method was employed to infer probabilities, with bootstrap values above 50% displayed on the tree for 100 replicates. The two main clades are numbered 1 and 2, within which smaller clades are highlighted in blue (1a, 1b, and 1c) and orange (2a, 2b), respectively. The MarCE sequence is marked by a blue circle.

The three-dimensional structure of MarCE predicted by AlphaFold was indicated as being of good overall quality, with most regions expected to be modelled to high accuracy (pLDDT

score > 90) and with lower confidence regions still expected to be modelled well (pLDDT > 70) for the best ranked output (Supplementary Figure S1). The PAE is relatively low, indicating that the position and orientation of each residue has been predicted with certainty, while the coverage is good overall with over 16000 sequences found to match the full query protein. The carboxylesterase TfCa from *Thermobifida fusca*, which has been biochemically characterized and recently investigated for PET oligomer degradation, was selected for comparison with MarCE (von Haugwitz et al., 2022). Following structure-based amino acid sequence alignment, MarCE was found to share a mean similarity of 70.76% with TfCa and a mean identity of 27.08% (inferred from ESPript output, Supplementary Figure S2). The predicted MarCE structure was superimposed onto the elucidated crystal structure for TfCa and the conserved serine, glutamine, and histidine residues forming the catalytic triad were located in MarCE at positions 198, 314, and 432, respectively (Figure 2). The carboxylesterase Gly-Gly oxyanion motif can also be observed in both enzyme sequences (G106 and G107 in TfCa, G111 and G112 in MarCE, Supplementary Figure S2).

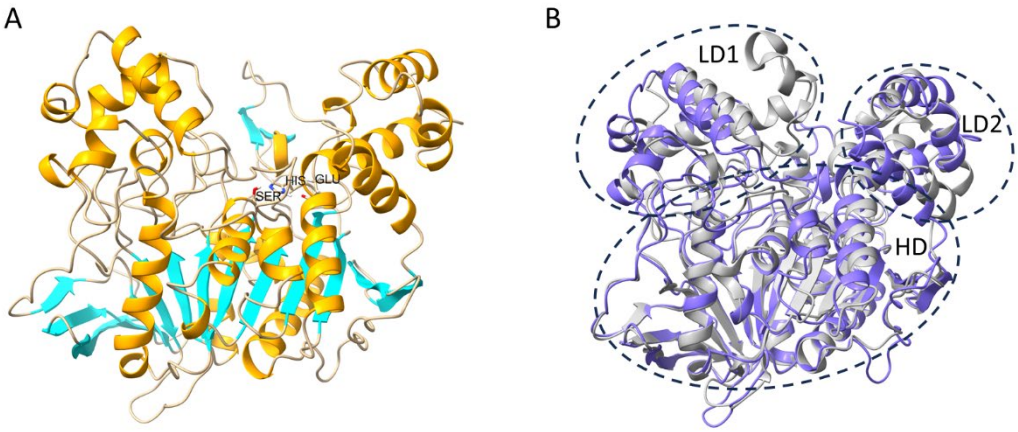


Figure 2: Structural analysis of MarCE, visualized using Chimera software. (A) The 3-D model predicted for MarCE, with alpha helices shown in orange, beta strands in cyan, and coils in beige. Catalytic triad residues are labelled and displayed as sticks. (B) Comparison of MarCE (in blue) with the homologous carboxylesterase TfCa (PDB code: 7W1J, in grey) facilitated by superimposition of their structures. The lid domains (LD1 and LD2) and the hydrolase domain (HD) are labelled to indicate their general position within each enzyme structure.

4.3 Molecular Docking and Activity Confirmation

Docking experiments generated five binding modes with affinity ≤ -5.0 kcal/mol for MarCE with BHET as its ligand. The most stable BHET docking pose (binding mode 1, RMSD value = 0.0) had a corresponding binding energy of -5.8 kcal/mol. For 3PET, nine binding modes with affinity ≤ -7.0 kcal/mol were generated, where -8.7 kcal/mol was calculated for the best pose. The top solution for DEP had a binding affinity of -5.2 kcal/mol. MarCE is shown in the surface representation and with each of these three ligands bound (Figure 3). Based on docking of BHET, the MarCE binding pocket residues were identified in Discovery Studio and the equivalent TfCa residues were located using their superimposed structures, providing an insight into the potential role of key amino acids based on the previous description of TfCa reported by the Bornscheuer group (Table 2).

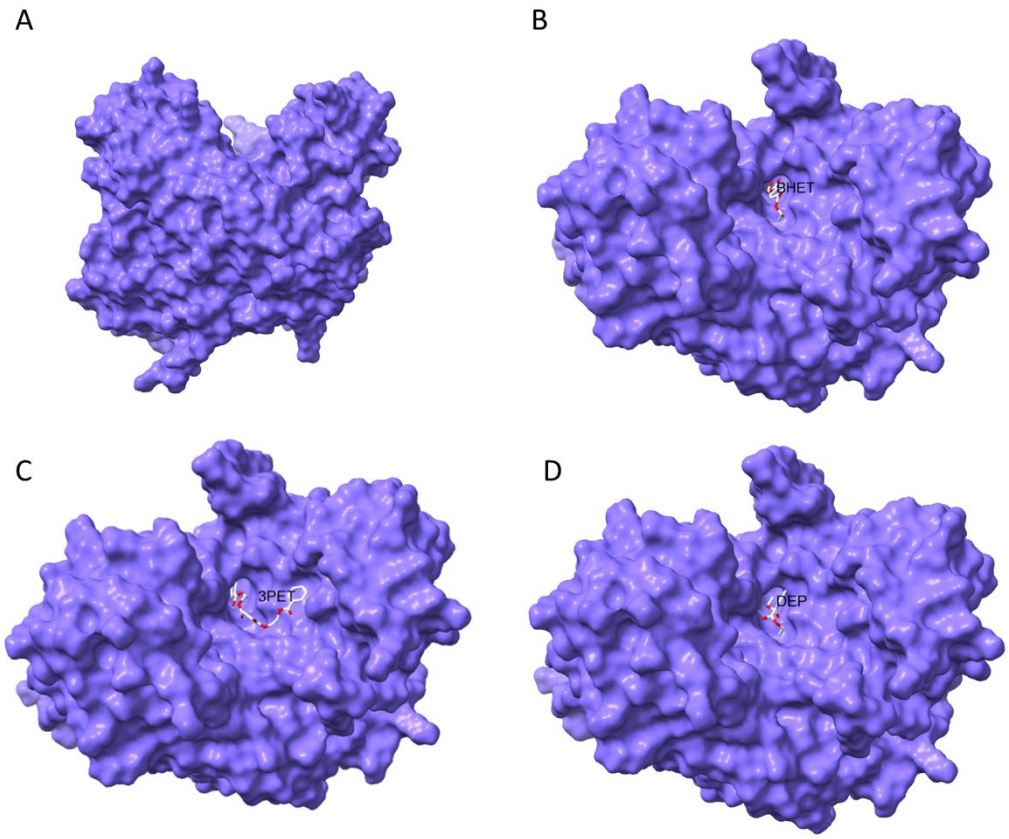


Figure 3: Molecular docking analysis facilitated by AutoDock Vina and visualized in ChimeraX. The MarCE model is shown in surface representation (A), with BHET, 3PET, and DEP ligands bound at active site pocket regions (labelled in B, C, and D, respectively).

Table 2: Comparative analysis of predicted binding pocket residues based on the molecular docking of BHET onto MarCE and each residue found at the corresponding position in TfCa carboxylesterase.

Putative Pocket Residue (MarCE)	**Corresponding Residue (TfCa)**	**Role/Importance (TfCa) (von Haugwitz et al., 2022)**
Phe73	Ile69	Mutagenesis target
Gly111	Gly106	Substrate binding
Gly112	Gly107	Substrate binding
Gly113	Ala108	Substrate binding
Ser198	Ser185	Catalytic triad
Ala199	Ala186	Not described
Ile202	Met189	Mutagenesis target
Thr231	-	-
Met232	-	-
Phe277	Leu282	Hydrophobic cleft
Phe369	-	-
Ile370	Ile372	Not described
Leu410	-	-
Ala411	-	-
His432	His415	Catalytic triad
Ala433	-	

Following heterologous expression from *E. coli* BL21-Codon Plus (DE3)-RIPL and subsequent purification, MarCE was visualized as a single band around its predicted molecular weight following SDS-PAGE (58 kDa, Figure 4). The supernatant and purified samples obtained for MarCE were confirmed to be active on PCD-based agar (Supplementary Figure S4). No such protein bands or activities were detected for the empty vector negative control samples. MarCE displayed activity from 4-37°C, with an optimum around 20-30°C and minimal activity detected following incubation at 50°C.

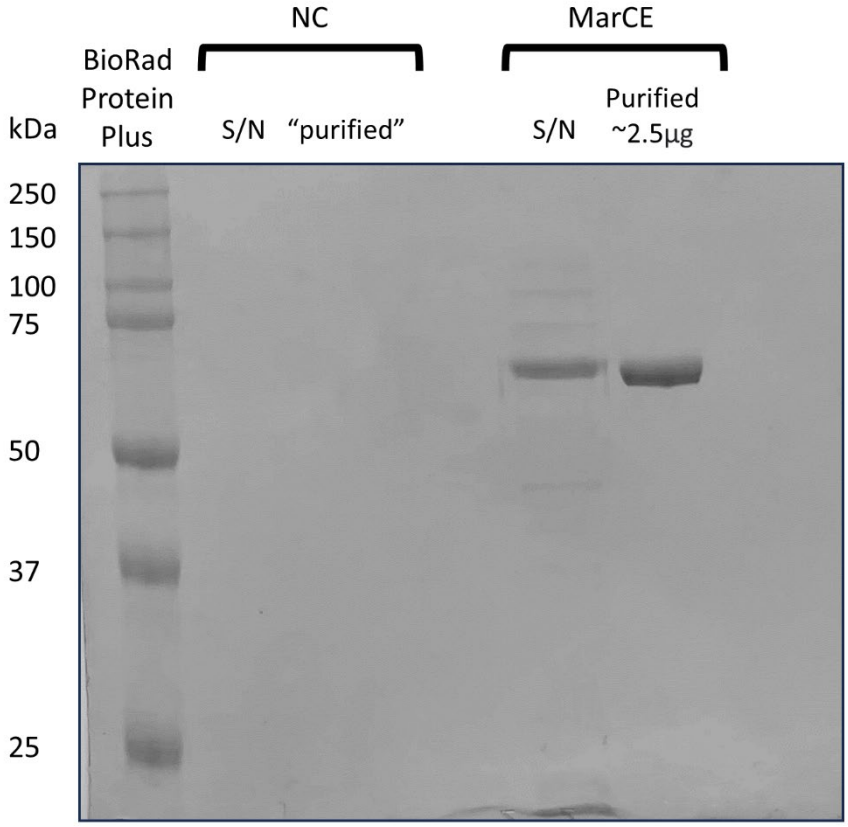


Figure 4: SDS-PAGE analysis. Lane 1: Bio-Rad marker, lane 2: culture supernatant from induced negative control (expression host carrying empty vector), lane 3: "purified" negative control preparation (followed protocol for purification from NC supernatant in parallel with MarCE), lane 4: culture supernatant from induced *E. coli* BL21 RIPL-(pET20b:MarCE), lane 5: purified MarCE (desalted, concentrated, ~2.5 µg loaded).

5. Discussion

Petroleum-based, non-biodegradable synthetic plastics have an environmental half-life that ranges from several to a few thousand years. Since PET plastic is employed in packaging and textiles for its strength and physicochemical resistances, PET-based materials are slow to degrade in nature, whether by microbial-based or abiotic processes (Li et al., 2023a). The conversion of waste plastics into high-purity monomers or value-added products has gained much traction as a way to sustainably manage and circularize the life cycle of synthetic materials, with biocatalytic approaches of particular interest to enable depolymerization under mild reaction conditions (Lee et al., 2023). The biological recycling and upcycling of PET is made viable by the capacity of enzymes termed polyesterases to break the ester bonds that link each polymer subunit (Wei et al., 2022). Polyester-hydrolyzing biocatalysts are innately promiscuous and are also potentially useful for the bioconversion of organic compounds to yield valuable pharmaceutical intermediates (Sanchez and Demain, 2011; Biundo et al., 2019).

Following screening of bacterial isolates from a variety marine of ecosystems, namely sea sponges and seaweeds, six isolates were chosen for genome sequencing based on their activities towards tributyrin, polycaprolactone diol (PCD), and polycaprolactone (PCL) model substrates using agar-based plate assays. Taxonomic classifications were assigned from genome sequences based on the Genome Taxonomy Database. While each isolate was derived from a marine source, we were particularly interested in samples from genera that have been specifically linked to marine environments, in this case *Maribacter* (ID = J2146c) and *Cobetia* (ID = J2163c) species. Each of the six genomes were assembled and their predicted protein sequences were mined for polyesterases, which in the case of J2146c revealed a putative carboxylic ester hydrolase, later named MarCE (Table 1).

The MarCE-producing *Maribacter* sp. strain was isolated from a *Stelligera stuposa* sponge found in Lough Hyne, which is a salt-water lake connected to the Celtic Sea of the Atlantic Ocean by a narrow tidal channel (Plowman et al., 2020). As Europe's first Marine Nature Reserve, Lough Hyne is regarded as a biodiversity hotspot, with a notable abundance of sponge assemblages across its individual habitats that display comparable species richness to tropical, polar, and temperate sites for which similar data is available. This fully marine lake is subjected to an unusual, but predictable tidally-generated flow regime that is restricted compared to that of the open Atlantic coast, which is thought to contribute to the observed sponge diversity and abundance patterns (Bell, 2007). *Stelligera stuposa* is a species of demosponge with robust

branching morphologies that facilitate enhanced feeding and adaptation, for example, to current flow (Bell et al., 2002). A previous pyrosequencing study from our group of microbial communities from the same *Stelligera stuposa* sponge revealed members from eight bacterial phyla, including five genera of Bacteroidota, with Flavobacteriaceae noted as a reservoir of β-lactamase genes and *Flavobacterium* species predicted to play a role in nitrite and nitrate reduction (Jackson et al., 2012).

The genus *Maribacter*, which belongs to the family Flavobacteriaceae of the phylum Bacteroidota, was first described in 2004 and currently consists of 30 validated species (https://lpsn.dsmz.de/genus/maribacter, accessed September 2023) (Nedashkovskaya et al., 2004). *Maribacter* spp. appear to be exclusively associated with marine environments, including a diverse range of isolates having been identified from seawater (Yoon et al., 2005), algae (Zhang et al., 2020), sponge (Jackson et al., 2015), deep-sea sediment (Fang et al., 2017), seaweed (Weerawongwiwat et al., 2013), Arctic (Cho et al., 2008), and Antarctic (Zhang et al., 2009) sources. To date, the majority of published work on the genus *Maribacter* has been related to the description of a new species, the deposit of genomic sequences, or their interactions with macroalgae, but with only a small number of *Maribacter* sp. enzymes being specifically characterized, primarily for their ability to degrade polysaccharides (Lee et al., 2016; Lu et al., 2022). To our knowledge there are no reports describing other types of enzymes from *Maribacter* species, for example those with activity towards ester and polyester substrates. Thus, MarCE from our *Maribacter* sp. strain was prioritized for further investigation.

The MarCE enzyme was initially annotated as a fumonisin B1 esterase by Prokka (prokaryotic annotation) but subsequent NCBI BLASTP search of bacterial proteins in the Swiss-Prot database indicated a sequence identity of 32.12% to the functionally characterized example of a fumonisin B1 esterase, while similar identities were observed for other types of carboxylic ester hydrolases (CEHs) and slightly higher identities were observed for non-microbial CEH homologs (Supplementary Table S2). A wider search against the NCBI's non-redundant database showed that the best matches are with other *Maribacter* sp. carboxylesterase-type enzymes, which share significantly higher sequence identities of around 97%. Such *Maribacter* proteins appear to be available as result of their deposition in genome databases but have not as of yet been further studied or biochemically characterized in terms of their activity. InterProScan, Pfam, and SUPERFAMILY enable classification by comparison with protein signature or structural domain collections. Using these computational tools, MarCE was broadly classified as a carboxylesterase family member belonging to the α/β hydrolase

superfamily, which encompasses enzymes whose core structure typically folds into a β-sheet (consisting of as many as 13 strands) surrounded by a several α-helices and whose catalytic triad is composed of a nucleophile (Ser, Asp, or Cys), an acid (Asp or Glu), and a histidine (His) (Jochens et al., 2011; Bauer et al., 2020). Carboxylesterases (CEs, assigned to EC 3.1.1.1) catalyze the cleavage of carboxyesters (RCOOR') into their carboxylic acid (RCOOH) and alcohol (R'OH) components and the reverse reaction, primarily targeting ester bonds and to some extent thioester, amide, and carbamate bonds (Aranda et al., 2014). Based on the Arpigny and Jaegar classification scheme for microbial carboxylesterases, MarCE belongs to the carboxylesterase family VII (Sood et al., 2016). The pentapeptide GESAG motif, which is the main feature used to identify family VII members, was located and is highlighted in the MarCE protein sequence around the nucleophilic Ser198 residue (Supplementary Figure S2). Microbial family VII carboxylesterases have gained industrial interest for their capacity to act on polyurethanes as well as sterically hindered esters of tertiary alcohols (Kang et al., 2011).

Phylogenetic comparison of MarCE with the selected protein homologs suggests an evolutionary divergence between *Maribacter* sp. carboxylesterase-like enzymes and their closest functionally characterized relatives, as indicated by their separation into two distinct clades (labelled 1 and 2, Figure 1), likely resulting from the adaptation of *Maribacter* species to their marine environment. MarCE is found in clade 1a with proteins from *Maribacter litoralis* (marine sediment-derived strain) and *Maribacter caenipelagi* as its closest relatives, with other clade 1a sequences forming a number of sister groups derived from the same node, featuring entries from *Maribacter dokdonensis* (strain from seawater), *Maribacter hydrothermalis*, *Maribacter thermophilus*, and *Maribacter aurantiacus* (from marine sediment). The *Maribacter algicola* and *Maribacter flavus* strains in clade 1a are potential symbionts, linked to marine algae and cyanobacterial pond samples, respectively. Two other genera appear in clade 1a, *Saonia flava*, another Flavobacteriaceae family member, and *Fulvivirga sedimenti* of the class Cytophagia. The clade 1b consists of Flavobacteriacea sequences exclusively belonging to marine-associated genera (*Pricia*, *Eudoraea*, *Euzebyella*, and *Maribacter* spp.) (Yu et al., 2012; Siamphan et al., 2015; Zhang et al., 2017) or in one case, coming from a marine-derived metagenomic sample. The proteins in clade 1c each belong to the Cytophagia class of bacteria, featuring genera that are not specific to the marine environment (*Arcicella*, *Emticicia*, and *Runella* spp.), which have instead been associated with terrestrial, freshwater, or wastewater samples (Nikitin et al., 2004; Ten et al., 2019; Yang et al., 2020).

The carboxylesterases clustered in clade 2 of the phylogenetic tree represent the reference sequences selected from Swissprot and PDB databases, which share lower levels of homology (<35% amino acid identity) with MarCE but that have been previously studied in the context of their function and/or structure (Figure 1). FumD from *Sphingopyxis macrogoltabida* has been characterized in detail and commercialized (FUMzyme®) for its ability to degrade fumonisin mycotoxins, which occur as a contaminant of maize in food and feed industries (Heinl et al., 2010; Alberts et al., 2019), while the phenmedipham hydrolase from *Pseudarthrobacter oxydans* is a carboxylesterase that has been investigated for the degradation of phenylcarbamate herbicides (Pohlenz et al., 1992) and AqdA2 from *Rhodococcus erythropolis* for its potential role in the disruption of *Pseudomonas aeruginosa* quorum signalling (Müller et al., 2015). TfCa from *Thermobifida fusca* KW3 and its close homolog from the *T. fusca* YX strain are found in clade 2a. During initial biochemical studies, TfCa was shown to hydrolyze cyclic PET trimers and PET nanoparticles (Billig et al., 2010; Oeser et al., 2010), but this carboxylesterase has since proven to be more useful as a secondary biocatalyst in combination with higher efficiency PET hydrolases such as TfCut2 and LC-cutinase (Barth et al., 2016). From clade 2b, a *p*-nitrobenzyl esterase from *Bacillus subtilis* subsp. *subtilis* (99.18% identical to the aforementioned BsEstB) has been functionally characterized as a para-nitrobenzyl esterase and the structure of its close homolog (pNB-E) has also been elucidated, while Est50 from *Geobacillus stearothermophilus* has been shown to activate the prodrug irinotecan (CPT11) by hydrolysis to enable its use in cancer therapy (Zock et al., 1994; Spiller et al., 1999; Liu et al., 2007). The marine-derived E93 and Chath_Est1 from *Clostridium hathewayi* are clustered together despite coming from different phyla (Pseudomonadota and Bacillota, respectively). E93 has also been investigated for anticancer prodrug activation, where it can hydrolyze CPT11 and its derivative NPC (Li et al., 2023), while Chath_Est1 has been found to degrade the synthetic polyester PBAT (poly(butylene adipate-co-butylene terephthalate) (Perz et al., 2016)..

Numerous microbial CEHs have been shown to efficiently depolymerize PET and other polyesters such as polycaprolactone, PBSA (poly(butylene succinate-co-adipate)), and polylactic acid (Hajighasemi et al., 2016; Li et al., 2023b). BsEstB and TfCa are two carboxylesterases known to act on PET oligomers. While MarCE shares slightly higher amino acid similarity values to BsEstB than it does with TfCa (72.90% vs. 70.76%), the latter has been studied in greater detail as a (tere)phthalate-ester hydrolase and for its complementary role in PET degradation by cutinase-like enzymes where it promotes the hydrolysis of intermediate reaction products such as MHET and BHET, and was therefore selected for comparative

analysis (von Haugwitz et al., 2022). The AlphaFold-generated MarCE model appears to have a similar overall structure to that which has been described for TfCa, consisting of one α/β hydrolase domain and two lid domains (Figure 2B). Similar to TfCa, the hydrolase portion of MarCE features a mixed β-sheet composed of 12 full strands, 7 of which run parallel in the center with inversion at either end where the structure runs in anti-parallel. In general, anti-parallel β-sheets are thought to contribute increased flexibility over the parallel conformation (Emberly et al., 2004). Two additional anti-parallel β-strands are found in MarCE, as part of a loop away from the α/β fold, that are not observed in TfCa. These isolated β-strands may have structural and thus, functional implications for MarCE. Further structural and spatial differences can be seen between the two enzymes, for example, in the helical lid domains and in other variable loop regions. The loops carrying the catalytic triad (S198-E314-H432) and oxyanion residues (Gly111-Gly112) are more highly conserved, as is generally the case for α/β hydrolase superfamily members (Figure 2 and Supplementary Figure S2)(Mindrebo et al., 2016).

While cutinase family members are currently known as the most efficient degraders of PET, the associated reaction does not typically result in the full hydrolysis of the polyester to its constituent monomers (i.e., terephthalic acid (TPA) and ethylene glycol (EG)). Instead, there tends to be an accumulation of MHET and BHET intermediates which are not as easily depolymerized by such enzymes (Schubert et al., 2023). By contrast, carboxylesterases exhibit poor activity towards polymeric PET but seem to be more accommodating of shorter PET fragments (Bååth et al., 2021). The water insoluble 3PET and the soluble compound BHET have been commonly employed as model substrates to assess PET hydrolase activity (Djapovic et al., 2021). During its characterization, TfCa was demonstrated to degrade DEP, a widely used phthalate-ester-based plasticizer, thus expanding the substrate scope of this carboxylesterase (von Haugwitz et al., 2022). Molecular docking of BHET, 3PET, and DEP onto MarCE demonstrates putative binding whereby each substrate fits comfortably into pocket regions on the enzyme's surface (Figure 3). Based on the binding energies obtained, 3PET appears to have the highest affinity for MarCE. This may be influenced by the fact that 3PET is a longer chain that thereby interacts with more amino acid residues overall and that has three aromatic rings, one of which sits into the same pocket as observed for BHET and DEP while the other two rings extend into neighbouring pockets (Figure 3C). In biochemical assays, both TfCa and BsEstB have previously been shown to prefer BHET as a substrate over MHET and 3PET, potentially due to an ideal size match between the BHET substrate and its binding pocket (Bååth et al., 2021; von Haugwitz et al., 2022).

The residues of the main substrate binding pocket of MarCE were deduced from the BHET docking output and are listed in Table 2. The nucleophilic Ser198, the oxyanion hole residue Gly113, and the His432 (proton acceptor/donor), are present for catalysis, where the ester bond is hydolyzed in a two-step process initiated by acylation of the catalytic serine by the substrate which involves proton transfer from serine to histidine and subsequent serine attack of the substrate carbonyl group (resulting in a temporary tetrahedral intermediate) and followed by deacylation where a second proton transfer occurs from an active site water molecule to histidine which then attacks the acylated serine intermediate and breaks its bond to the substrate (Jerves et al., 2021; Pinto et al., 2021). While comparison of MarCE pocket residues with the corresponding residues in TfCa revealed a number of similarities between the two enzymes which may contribute to substrate binding and catalysis, there are several additional residues found in MarCE in this region that are not present in TfCa and differences in some key residues at the same position (e.g., Phe73, Gly113, Ile202, and Phe277 in MarCE equates to Ile69, Ala108, Met189, and Leu282 in TfCa, respectively). In general, the functionality and substrate range of each CEH will depend on the structural configuration of their catalytic centers and the individual amino acids contributing to substrate binding and catalysis, with minor differences in sequence often having a major effect on activity for highly similar enzymes and variants (Ribitsch et al., 2017; Leitão and Enguita, 2021). Given that MarCE is derived from a marine mesophilic organism and TfCa is a thermophilic enzyme of terrestrial origin, differences in activity and substrate scope are likely to be observed upon functional comparison of the two enzymes (Crnjar et al., 2023).

MarCE was detected by SDS-PAGE and confirmed as active by clearing on PCD (1%) agar from both supernatant and purified enzyme preparations (Figure 4 and Supplementary Figure S4). The negative controls verify that this activity is not associated with the *E. coli* host and MarCE activity was only observed from the BL21 RIPL-(pET20b:MarCE) clone. The activity-based temperature profile suggested a temperature optimum of 20-30°C for MarCE and is in agreement with the growth preferences previously reported for a seashore isolated *Maribacter litoralis* strain under varying temperature conditions (Lee et al., 2018). PCD is a waxy polycaprolactone derivative and polyurethane precursor that has been employed as a polyester model substrate (Maafi et al., 2010; Molitor et al., 2020). The activity data generated here forms the basis of more detailed biochemical characterization, which is currently underway. Taking into consideration the true marine origin of MarCE, combined with its overall homology to functionally characterized CEs and supported by the docking of PET and phthalate oligomers,

we believe that our *Maribacter* sp. derived carboxylesterase represents a novel addition to the current selection of microbial CEHs of interest for synthetic polyester degradation and the sustainable catalysis of organic synthesis reactions. Furthermore, the identification of MarCE offers an opportunity to gain new insights into the degradation of chemical compounds in the marine environment, which currently remains rather poorly understood (Zhang et al., 2023).

6. Concluding Remarks

Carboxylic ester hydrolases with the capacity to degrade polyesters are currently sought after for their potential use in the biological degradation of PET and other chemically synthesized polymers. Given that plastics are a relatively recent invention and environmental contaminant, such activities are likely indicative of polyesterase promiscuity where the enzyme will accept both native and xenobiotic substrates. In a broader sense, polyester hydrolases therefore make useful biocatalysts for a range of chemical transformations whose potential has yet to be fully uncovered. The study of marine polyesterases, which are underrepresented compared to their terrestrial equivalents, may reveal novel biocatalysts with unique properties and functional plasticity resulting from their adaptation to complex and changeable marine environments.

There work here describes a synthetic polyester-degrading carboxylesterase MarCE, that was discovered by genome mining of a *Maribacter* sp. isolate of the marine sponge *Stelligera stuposa*, sampled from Lough Hyne in West Cork, Ireland. MarCE is an enzyme belonging to the α/β hydrolase superfamily and carboxylesterase family VII, whose closest relatives are carboxylesterase family proteins from a variety of *Maribacter* species, followed by similar enzymes from other marine-associated genera such as *Saonia* and *Euzebyella* from the Cytophaga-Flavobacterium-Bacteroides taxonomic group. The *Maribacter* sp. carboxylesterase shares an overall level of homology with its functionally characterized counterparts but appears to be evolutionary distinct, likely due to its marine origin. Based on its structural similarity to the promiscuous (tere)phthalate ester-degrading carboxylesterase TfCa, several synthetic oligomers were docked onto MarCE and shown to bind its active site pocket. Several key residues were identified for the hydrolysis of BHET by MarCE, while some variation in pocket residues between MarCE and TfCa suggest functional differences between the two enzymes. MarCE was successfully produced in an *E. coli* expression host and its hydrolytic activity was confirmed on the polycaprolactone derivative, PCD.

The identification of MarCE contributes to our overall knowledge of marine carboxylic ester hydrolases and highlights *Maribacter* species as a potential new source of polyesterases.

Following on from this work, there is plenty of scope to further characterize MarCE in terms of substrate scope and catalytic efficiency, and to investigate the activities of its closest marine relatives. Furthermore, questions emerge regarding the role of MarCE in its natural environment, in particular whether it is uniquely adapted to the Lough Hyne ecosystem and/or to its sponge host. Finally, MarCE may find use as a versatile biocatalyst for use in green chemistry applications or as part of a bioremediation system for the decontamination of marine waters.

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8. Supplementary Material

Table S1: Confirmed hydrolytic activities and 16S rRNA gene-based identification for 12 marine-derived cultures, where isolates coded B129-B98 are from deep-sea sponges (collected at depths > 1000 m), those coded J2 and J9 are from Lough Hyne (collected at 15-20 m), while ANHOS11 was isolated from *A. nodosum* seaweed.

Isolate ID	**Isolation Source**	**Classification (16S rRNA Gene)**	**Trib.**	**PCD**	**PCL**
ANHOS11	*Ascophyllum nodosum*	*Bacillus firmis*	+	+	+
B129SM11	*Pheronema* sp.	*Brachybacterium ginsengisoli*	+	+	+
B226M5	*Inflatella pellicula*	*Agreia* sp.	+	+	-
B226SK6	*Inflatella pellicula*	*Micrococcus* sp.	+	+	+
B98C26	Unidentified Sponge	*Jiella aquimaris*	+	+	-
B98SN116	Unidentified Sponge	*Tsukamurella pseudospumae*	+	-	-
J2009s	*Stelligera stuposa*	*Bacillus firmis*	+	+	+
J2022s	*Stelligera stuposa*	*Owenweeksia hongkongensis*	+	-	-
J2115m	*Stelligera stuposa*	*Joostella* sp.	+	+	-
J2146c	*Stelligera stuposa*	*Maribacter* sp.	+	+	+
J2163c	*Stelligera stuposa*	*Cobetia* sp.	+	+	-
J938L	*Eurypon major*	*Bacillus badius*	+	+	+

Table S2: Protein analysis tools and their respective outputs, used for the computational classification of MarCE.

Search Tool	Outputs											
	Top Hit #1				Top Hit #2				Top Hit #3			
	Description	Acc.	E-val.	Id. %	Description	Acc.	E-val.	Id. %	Description	Acc.	E-val.	Id. %
BLASTP (nr)	Carboxyl esterase family protein [*Maribacter litoralis*]	WP_116771751.1	0	97.7	Para-nitrobenzyl esterase [*Maribacter caenipelagi*]	TDS19070.1	0	97.1	Carboxyl esterase family protein [*Maribacter caenipelagi*]	WP_133671667.1	0	97.1
BLASTP swiss prot, default	Liver carboxylesterase 1 [*Oryctolagus cuniculus*]	P12337.3	5e-69	34.1	Acylcarnitine hydrolase [*Mus musculus*]	Q91WG0.1	8e-66	32.7	Pyrethroid hydrolase [*Rattus norvegicus*]	G3V7J5.2	1e-65	32.5
BLASTP swiss prot bact. only	Fumonisin B1 esterase [*Sphingopyxis macrogoltabida*]	D2D3B6.1	7e-63	32.1	Para-nitro benzyl esterase [*Bacillus subtilis*]	P37967.2	3e-57	31.3	Carboxyl-esterase [*Thermobifida fusca*]	P86325.1	2e-47	28.9

	Superfamily	Family	Conserved Sites
InterPro Scan	Alpha/beta hydrolase	Carboxylesterase family domain Carboxylesterase type B domain	Carboxylesterase type B, signature 2 Carboxylesterase type B, active site
Pfam	Alpha/beta hydrolase	Carboxylesterase (E-value = 6.4e-110)	
SUPFAM	Alpha/beta hydrolase (E-value = 6.20e-193)	Acetylcholinesterase-like (E-value = 0.012)	

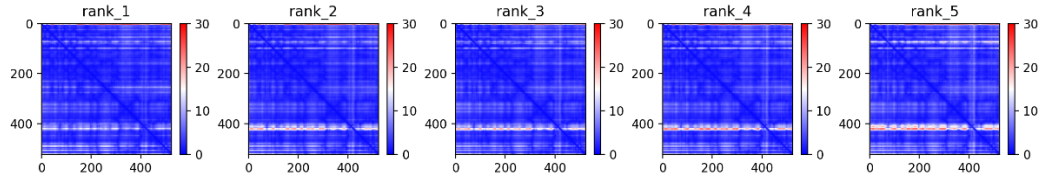
Table S3: Overall genome properties of *Maribacter* sp. isolate (ID: J2146c).

Genome feature	Value
Size (bp)	4797576
Number of contigs	15
Largest contig (bp)	2393481
N50	1014215
L50	2
GC content (%)	36.04
Completeness (%)	99.67
Contamination (%)	0.44

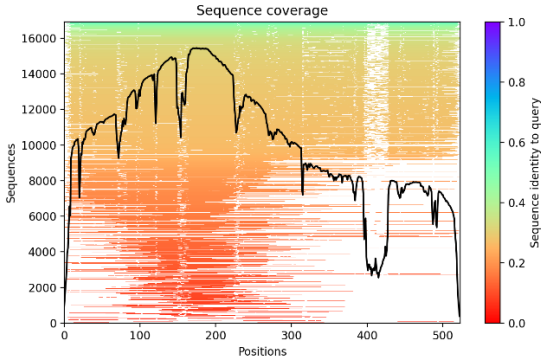
Table S4: Carboxylesterase family dataset compiled for phylogenetic analysis of 46 protein sequences homologous to MarCE, selected based on BLASTP search of MarCE against NCBI non-redundant, metagenomic, RefSeq, Swissprot, and PDB databases.

Enzyme Name or Description	Source	NCBI Accession
Carboxylesterase family protein	*Maribacter litoralis*	WP_116771751.1
Carboxylesterase family protein	*Maribacter caenipelagi*	WP_133671667.1
Carboxylesterase family protein	*Maribacter dokdonensis*	WP_058104101.1
Carboxylesterase family protein	*Maribacter confluentis*	WP_304436225.1
Carboxylesterase family protein	*Maribacter hydrothermalis*	WP_068481130.1
Carboxylesterase family protein	*Maribacter orientalis*	WP_091623870.1
Carboxylesterase family protein	*Maribacter stanieri*	WP_091902013.1
Carboxylesterase family protein	*Maribacter arcticus*	WP_079511831.1
Carboxylesterase family protein	*Maribacter sedimenticola*	WP_089259023.1
Carboxylesterase family protein	*Maribacter dokdonensis*	WP_091607293.1
Carboxylesterase family protein	*Maribacter algicola*	WP_125221195.1
Carboxylesterase family protein	*Maribacter thermophilus*	WP_047246338.1
Carboxylesterase family protein	*Maribacter flavus*	WP_154917126.1
Carboxylesterase family protein	*Maribacter aurantiacus*	WP_240042744.1
Carboxylesterase family protein	*Saonia flava*	WP_167960787.1
Carboxylesterase family protein	*Euzebyella marina*	WP_121848703.1
Carboxylesterase family protein	*Fulvivirga sedimenti*	WP_225697154.1
Carboxylesterase family protein	*Eudoraea adriatica*	WP_019670332.1
Carboxylesterase family protein	*Pricia antarctica*	WP_091867311.1
Carboxylesterase family protein	*Maribacter arenosus*	WP_188314939.1
Carboxylesterase family protein	*Euzebyella saccharophila*	WP_192461917.1
Carboxylesterase family protein	*Emticicia agri*	WP_130019672.1
Carboxylesterase family protein	*Arcicella aurantiaca*	WP_109744822.1
Carboxylesterase family protein	*Runella aurantiaca*	WP_114461372.1
Carboxylesterase family protein	*Emticicia aquatica*	WP_238806310.1
Carboxylesterase family protein	*Runella limosa*	WP_028524560.1
Carboxylesterase family protein	*Runella salmonicolor*	WP_253524793.1
Carboxylesterase family protein	*Eudoraea chungangensis*	WP_276391471.1
Carboxylesterase family protein	*Runella rosea*	WP_114069162.1
Carboxylesterase family protein	*Maribacter polysiphoniae*	WP_109655086.1
Carboxylesterase family protein	*Emticicia aquatilis*	WP_188769089.1
Carboxylesterase family protein	*Runella defluvii*	WP_183976921.1
Hypothetical protein	Marine sediment metagenome	KKN45957.1
Fumonisin B1 esterase	Compost metagenome	MNF21373.1
Fumonisin B1 esterase	Mine drainage metagenome	OIR18939.1
Hypothetical protein	Marine sediment metagenome	KKM98201.1
FumD	*Sphingopyxis macrogoltabida*	D2D3B6.1
Para-nitrobenzyl esterase	*Bacillus subtilis* subsp. *subtilis*	P37967.2
Carboxylesterase	*Thermobifida fusca* YX	Q47M62.1
Phenmedipham hydrolase	*Pseudarthrobacter oxydans*	Q01470.1
pNB-E	*Bacillus subtilis*	1QE3_A
Est50	*Geobacillus stearothermophilus*	2OGT_A
TfCa	*Thermobifida fusca*	7W1J_A
AqdA2	*Rhodococcus erythropolis*	A0A0E4AET8.1
E93	*Altericroceibacterium indicum*	7X8L_A
Chath_Est1	*Hungatella hathewayi*	5A2G_A

A



B



C

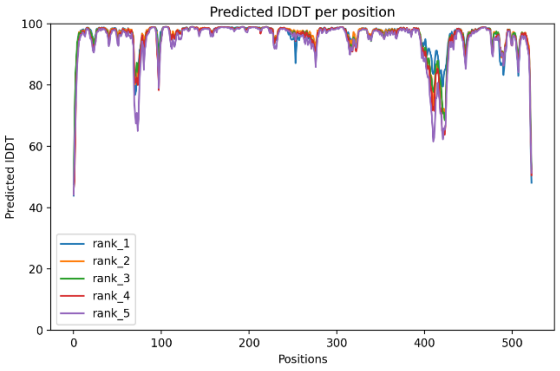


Figure S1: Alpha-Fold quality plots for the top five ranked models; (A) Predicted Aligned Error (PAE) matrices, (B) sequence coverage, and (C) per-residue confidence measure.

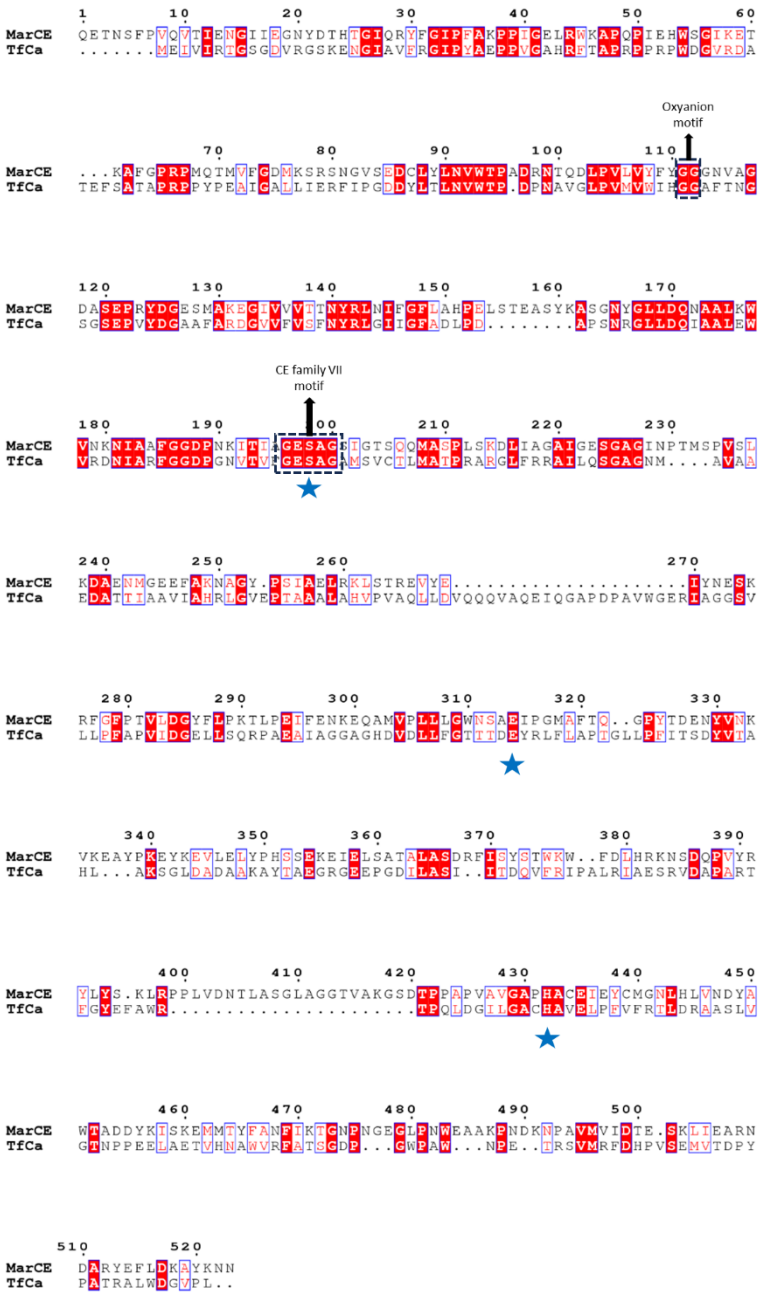


Figure S2: Structural alignment of MarCE and its thermophilic homolog TfCa (PDB code: 7W1J) generated by T-COFFEE in Expresso mode. Amino acid residues that are strictly conserved between the two sequences are shaded in red, while those outlined in blue share similar biochemical properties. The catalytic triad residues (S198-E314-H432) are marked with blue stars. Two conserved motifs are outlined and labelled, the first involved in oxyanion hole formation and the second a signifying feature of the carboxylesterase (CE) family VII.

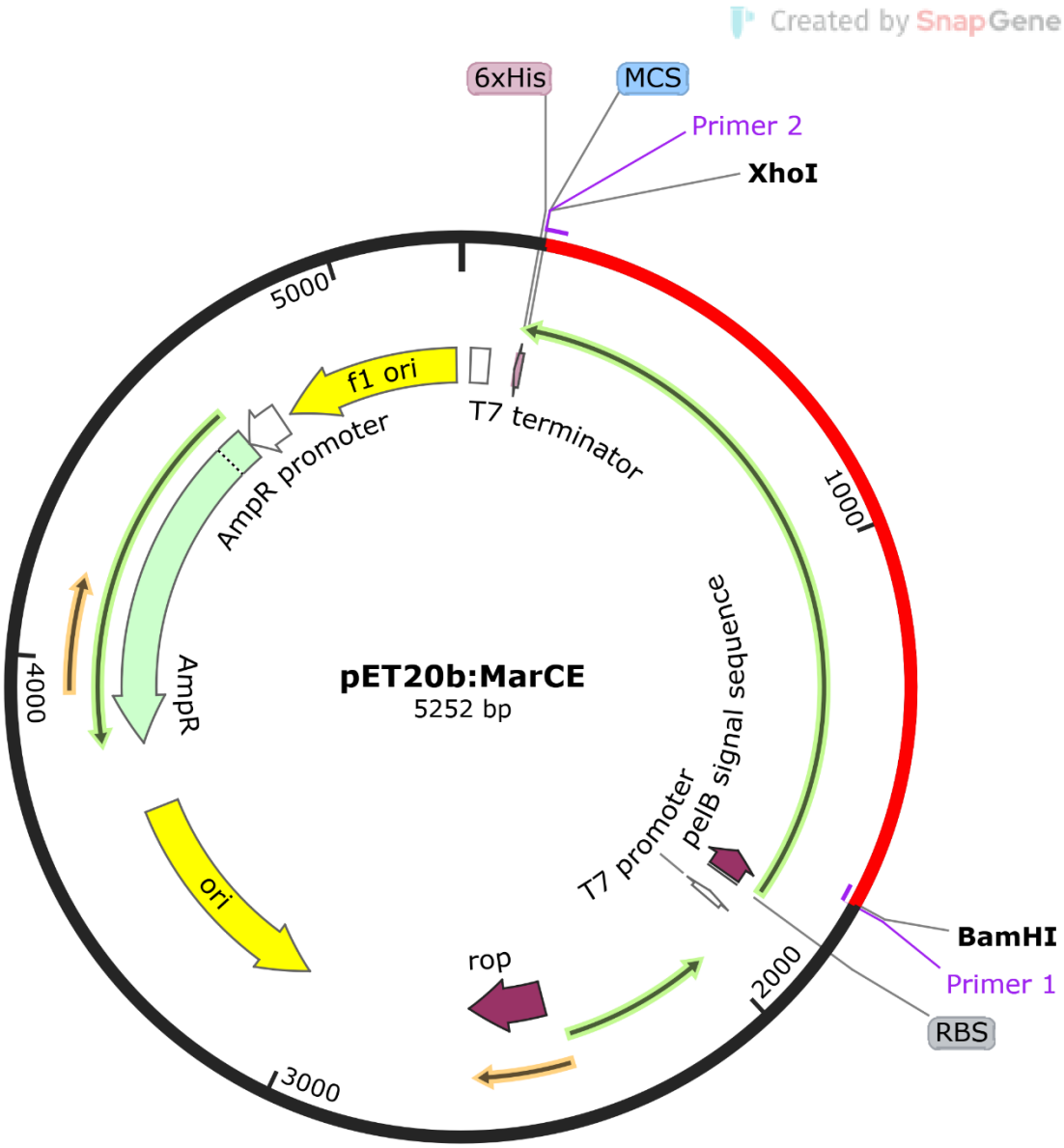


Figure S3: Vector map representing the pET20b:MarCE plasmid construct used for heterologous expression. The MarCE insert is shown in red and key features, including the T7 promoter, pelB signal sequence, histidine tag, restriction sites are displayed. Open reading frames to be translated are indicated by black arrows highlighted in green and orange.

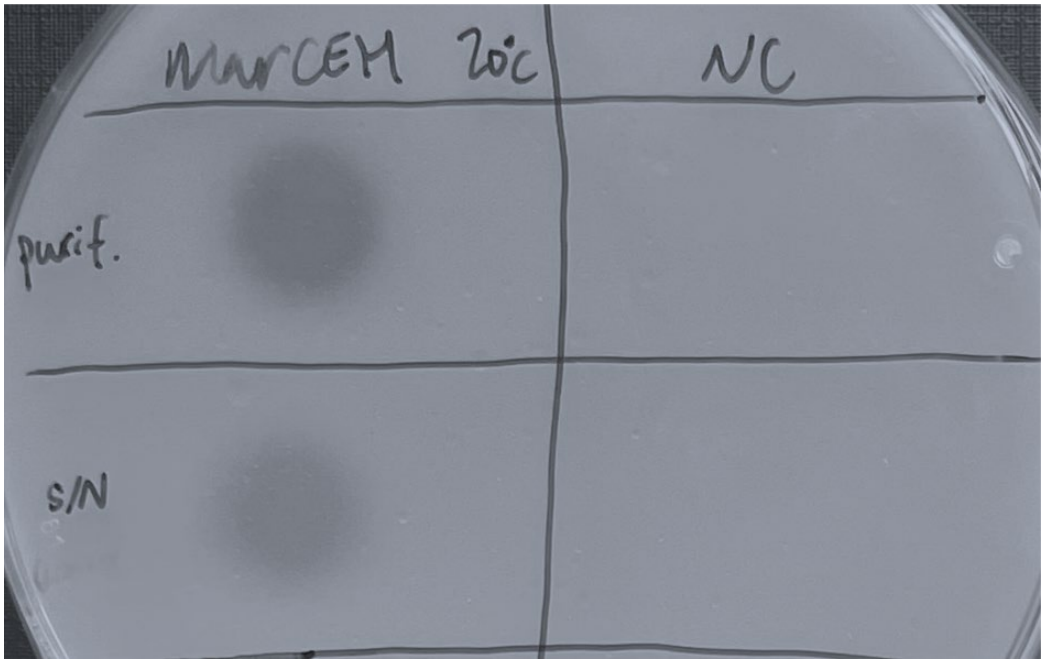


Figure S4: Confirmation of MarCE hydrolytic activity on polycaprolactone diol-based agar (1%) as indicated by substrate clearing. Induced culture supernatants and purified preparations from BL21 RIPL-(pET20b:MarCE) (left) and its negative control BL21 RIPL-(pET20b) (right) were plated (10 µL) and incubated at 20°C for 24 h.

Chapter 5

General Discussion and Concluding Remarks

1. Discussion

The field of enzymatic PET hydrolysis has seen extensive research efforts over the last few decades, with the most recent developments including the engineering of the highly efficient FAST-PETase, the molecular quantum dynamics supporting its mechanism, and the pinpointing of the N233K mutation attributed to its enhanced activity (García-Meseguer et al., 2023). Other advances have included the structure-based rational design of new LCC^{ICCG} variants exhibiting improved depolymerization of both amorphous and crystalline PET (Zeng et al., 2022) and the complete depolymerization of untreated post-consumer PET waste varying in weight, thickness, and crystallinity by DepoPETase at 50°C (Shi et al., 2023). The discovery and rational engineering of a *Ca*PETase from *Cryptosporangium aurantiacum* exhibiting mesophilic and thermophilic properties that result in remarkable PET hydrolysis at ambient temperatures (Hong et al., 2023) together with the degradation of pretreated commercial plastic bottles by LCC, Est119, and BhrPETase variants designed by a directional-path modification strategy for substrate groove remodeling and positive surface charge introduction also constitute important progress made recently in the field (Chen et al., 2022). Apart from the identification and improvement of PET hydrolase enzymes, novel strategies have been employed to further facilitate PET depolymerization, such as the use of affinity-boosting fusion chaperones, whole-cell surface co-display of FAST-PETase and MHETase, or the elevation of PETase system temperatures by solar-to-thermal energy conversion (Puspitasari et al., 2021; Li et al., 2022; Han et al., 2023; Tian and Sun, 2023).

As outlined in chapter 1, the primary focus of microbial PET hydrolase research has been on the identification and optimization of cutinase-like polyesterases such as those homologous to either thermophilic LCC or mesophilic IsPETase, followed by the elucidation of their structures and reaction mechanisms. There is a gap in the current literature with regards to the exploration of marine microorganisms as a source of polyesterases, with the majority of functionally characterized PET-hydrolyzing enzymes coming from terrestrial environments to date. In chapter 2, BgP, a cutinase-type polyesterase derived from a deep-sea marine sponge-associated *Brachybacterium* sp. isolate was identified by genome mining and investigated by comparative protein analyses. In chapter 3, the PET-hydrolyzing activity of SM14est, a marine *Streptomyces* sp. polyesterase from the coastal sponge *Haliclona simulans* that had previously been reported by our group, was functionally verified using a suspension-based PET hydrolase assay and its activity was found to be enhanced by the addition of salt with a 5-fold increase in the initial reaction rate upon addition of 500 mM sodium chloride. In chapter 4, the genome mining of a

Maribacter sp. isolate derived from a *Stelligera stuposa* sponge sampled at the Lough Hyne Marine Nature Reserve revealed a novel marine-specific carboxylesterase-type polyesterase, which was confirmed as active on polycaprolactone diol and with putative binding of the PET intermediate oligomer BHET being indicated by molecular docking.

Prior to the commencement of this project, PET-hydrolyzing activity had been reported for a number of marine-associated enzymes, namely PET5 from *Oleispira antarctica*, PET6 from *Vibrio gazogenes*, PE-H from *Pseudomonas aestusnigri*, and the metagenome-derived PET2 (according to pazy.eu, accessed September 2023). In chapter 2, the activity of the cutinase-like polyesterase BgP was confirmed on the synthetic polyester substrates PCL and PCD, with PET-hydrolyzing potential suggested by its similarity to the well-characterized PET hydrolases LCC, TfCut2, and Cut190 (>55% amino acid identity) and the identification of key residues (e.g., Trp155 and Tyr62) of relevance for PET binding and/or hydrolysis. In chapter 3, the PCL-degrading SM14est was confirmed to hydrolyze micro-PET powder of high crystallinity (>40%) at 10, 28, 37, and 45°C. In chapter 4, MarCE was also found to hydrolyze PCD, with preliminary biochemical data indicating its activity towards BHET and to some extent MHET, the oligomeric intermediates of PET hydrolysis, under ambient conditions (30°C) (Bornscheuer group, personal communication). Thus, the work carried out here has contributed new knowledge on three marine-derived enzymes of interest for the degradation of synthetic polyesters like PET and importantly, further supports the continued exploration of marine microorganisms for polyester-degrading activities. In general, the research surrounding plastic-degrading enzymes and in particular, their application in biorecycling, bioupcycling, and bioremediation processes remains in its infancy (Chow et al., 2023). Moreover, the transferability of marine biocatalytic activities from the laboratory to meaningful industrial-scale biotechnological application may be challenged, at various stages, by an incomplete understanding of the physiological, biochemical, and metabolic characteristics of the marine microorganisms from which these enzymes originate (Rodrigues and de Carvalho, 2022). Following on from this project, a significant amount of research needs to be undertaken to help ensure the complete integration of marine polyesterases into the area of PET biodegradation on a practical level.

During the project, several technical difficulties were encountered, that in some cases, may require further troubleshooting. For example, with respect to BgP, we observed “leaky” expression from the T7 promoter which appeared to result in low overall levels of BgP production by the *E. coli* BL21-Codon Plus (DE3)-RIPL host. The level of protein production

was insufficient to enable purification of BgP and biochemical characterization of the enzyme in terms of PET hydrolysis. The *bgp* gene was subsequently codon optimized for expression in *Bacillus subtilis*, an approach which was successfully implemented for SM14est. However, the BgP expression levels remained low, and the enzyme could not be purified. MarCE expression was successfully optimized based on a general protocol developed for the production of a broad range of targets, with a low temperature (20°C), overnight induction yielding an abundance of protein for purification (Francis and Page, 2010). This approach was also tested for BgP expression with little improvement observed, suggesting that changes to construct design might be more beneficial in this case. In order to obtain a more BgP productive clone, it may be necessary to build a protein expression "toolbox" consisting of plasmids constructed with a number of interchangeable genetic parts and trialing different combinations of promoter-regulator pairs, antibiotic markers, and origins of replication (Schuster and Reisch, 2022). More recently developed cloning methods such as Golden Gate assembly could be employed to facilitate such a combinatorial approach and the efficient generation multiple plasmid variants (Tong et al., 2021).

While BgP was isolated from a marine environment, the *Brachybacterium* sp. from which it is derived has also been reported from terrestrial environments (Hoang et al., 2014). Therefore, this enzyme may not necessarily display any unique marine-adapted properties. Nonetheless, it remains interesting to consider the role of BgP in its native deep-sea marine sponge ecosystem. Sponge-microbial symbioses have been modelled to reconstruct their complex interactions and sponges have also been used as model organisms in assessing the consequences of marine pollution (Zahn et al., 1981; Engelberts et al., 2023). On this basis, an in-vitro model system could be developed to mimic the natural conditions encountered by the *Brachybacterium* sp. symbiont and its cutinase-like enzyme, BgP. Another approach could be to complement sponge sampling with DNA-stable isotope probing to identify metabolically active symbionts and/or the analytical detection of organic compounds from the sponge tissues, for example, by liquid chromatography-mass spectrometry and sensor technologies, to help determine the native substrates of BgP (da Costa Filho et al., 2022). Recently, a novel marine *Brachybacterium atlanticum* was isolated from the Atlantic Ocean (de Castro et al., 2023). This may represent an opportunity to compare marine and terrestrial members of *Brachybacterium* genus in terms of their polyester-degrading activities and any new polyesterase homologs that may be encoded in their genomes.

In the case of SM14est and MarCE, which appear to be more specific to the marine environment, certain biochemical properties may be of industrial relevance, for example, salt tolerance can often confer increased stability in the presence of organic solvents since water availability is lower in both high salt and aqueous-organic reaction conditions (Doukyu and Ogino, 2010; De Santi et al., 2016). Chemical recycling of PET waste by glycolysis has been optimized by the addition of the solvent xylene, with higher conversions to soluble products at lower temperatures while offering convenient recovery of pure monomers (Güçlü et al., 2003). For any application, an integrated chemo-biocatalytic approach that uses environmentally benign substitutes for each reaction component will be vital in the development of efficient, sustainable processes (Bering et al., 2022). Compared to biorecycling and biocatalysis, which are performed under a controlled reaction setup, the microbial bioremediation of marine pollution is complicated by fluctuating environmental conditions, the risk of interfering with the natural balance of the ecosystem, and low removal efficiencies in practice (Leung, 2004; Gavrilescu et al., 2015; Tan et al., 2023). Therefore, the implementation of marine enzymes like SM14est and MarCE for bioremediation purposes may be envisaged by the development of a semi-enclosed reactor system that could be placed *in situ* to capture pollutants for de-toxification by enzymes immobilized within its structure, where the feasibility of such a task would likely depend on a well-managed cross-disciplinary effort between microbiologists, biochemists, environmental and material scientists, and engineers (Gu, 2021).

Given that the wild-type catalytic activities of polyesterases are generally considered too low to address PET waste in a real-world setting, we anticipate that mutational analyses of BgP, SM14est, and MarCE would need to be conducted following their biochemical characterization to alter and potentially optimize their hydrolytic activities (Zhu et al., 2022). Directed evolution of newly identified enzymes has been successful in developing robust biocatalysts with desirable characteristics by numerous iterations of a multi-step process that involves randomly inserting mutations (e.g., by error-prone PCR), screening and selection of variants, and quantification of the enhanced traits, until genetic diversity is suitably expanded (Riaz et al., 2023). Structure-driven protein engineering methods, such as site-saturation mutagenesis, provide a more targeted approach based on key functional residues (Wang et al., 2023). Both directed evolution and rational design strategies have been deployed in the area of PET hydrolysis, in particular to improve the activity and stability of mesophilic polyesterases that are generally outcompeted by their thermophilic homologs since PET is more easily degraded around its glass transition temperature (~65°C) (Cui et al., 2021; Bell et al., 2022). However,

the major challenge associated with any protein engineering campaign is the highly intensive screening efforts required to identify a mutant that outperforms its wildtype and the detection of hydrolysis products from thousands of candidates. Recently, chimeric re-design of the Antarctic PET hydrolase Mors1 generated a shift in its optimal activity temperature from 25 to 45°C with a 5-fold enhancement in hydrolytic activity towards amorphous PET films, where Mors1 acted as a mesophilic scaffold and one of its loop regions was exchanged for the equivalent loop from LC-cutinase (Blázquez-Sánchez et al., 2023). The design of chimeras enables several mutations to be introduced at once with less disruption and the generation of functional mutants at a much higher frequency. The implementation of such an approach presents the possibility of a modular "building block" process for PET hydrolase engineering, thereby unlocking the full potential of mesophilic, marine-adapted enzymes like SM14est and MarCE. In addition to traditional modification of the protein, engineering techniques can also be applied to the enzyme's local microenvironment, for example, to enhance substrate binding and reaction rates, control substrate selectivity, and broaden the working pH range through the introduction of or encapsulation by polymer nanostructures (Lancaster et al., 2018).

The discovery of BgP, SM14est, and MarCE highlights the importance of the marine environment as a reservoir of polyesterases. Based on this work, various marine bioresources could be further explored for microbial polyester-degrading activities, including but not limited to marine sponges, macroalgae, corals, mollusks, and phytoplankton, in particular those which are found in marine plastic hotspots (Haygood et al., 1999; da Fonseca et al., 2022). That said, the ubiquity and promiscuity of carboxylic ester hydrolases helps to ensure that any search effort will result in the identification of some interesting or useful activities (Degli-Innocenti et al., 2023; Faber and Fritz, 2023). Culture-based, culture-independent, computational, and multi-omics approaches have all been used to survey the prevalence of PET hydrolase-encoding genes in the environment, predict the functional profiles of plastic-associated microbial communities, and identify novel PET-hydrolyzing enzymes (Danso et al., 2018; Viljakainen and Hug, 2021). Future bioprospecting ventures could attempt to characterize and compare different microbial or environmental sources in terms of their overall PET-degrading capacity and determine which can deliver the best activities for the practical hydrolysis of PET under the relevant set of conditions for each specific application, for example, biological recycling under ambient temperatures versus bioremediation of PET-contaminated marine waters. Such an approach is ambitious but as the quantification of PET hydrolysis becomes more standardized, facilitating a more straightforward comparison of activity data; we need to develop a large-scale framework

that facilitates the assessment of useful activities from nature as has been achieved to some extent for a few benchmark enzymes and their engineered variants (Wei et al., 2022). In any case, newly identified enzymes and variants would ideally feed into a well-defined panel of candidate biocatalysts that can be conveniently selected for an intended biotechnological purpose.

With respect to the parent strains from which our three polyesterases were identified, there is an opportunity to investigate their individual hydrolytic and metabolic profiles in more detail. Additional polyesterase homologs may be identified using less stringent search criteria, for example, two hypothetical proteins were detected in the genome of the *Maribacter* sp. isolate J2146c as hits against our custom polyesterase database by employing higher E-value cutoffs of 1e-10 and 1e-06, and even more remote homologs could potentially be pursued (Kinch and Grishin, 2002; Söding et al., 2005). The contribution of each enzyme to the polyester degradation process could be explored together with their synergistic interactions when combined, similar to earlier work that was undertaken on IsPETase and MHETase from *Ideonella sakaiensis* (Yoshida et al., 2021). Furthermore, strain-level engineering could be employed for the modification of wild-type bacteria to increase polyesterase production, with *Streptomyces* sp. SM14 representing a good candidate based on the frequent use of *Streptomyces* species as an industrial enzyme production platform (Sevillano et al., 2016). The enhanced production of xylanase by ribosome engineering of a marine-derived *Streptomyces viridochromogenes* demonstrates potential scope for improvement of polyesterase production by our marine *Streptomyces* sp. isolate.

While it has been suggested that microorganisms may have evolved specifically to metabolize PET in response to rising global levels of plastic pollution in natural environments, it remains unclear whether the relatively short time period that has elapsed since the commercial introduction of PET (in the late 1970s) would have been sufficient to have enabled such an adaptation (Gervet, 2007; Bornscheuer, 2016). Instead, it has been argued that the observed enzymatic activities towards PET are a result of the intrinsic promiscuity of hydrolases, enabling them to act on substrates other than those which they would preferentially target in the environment. Recently, PET40, a novel metagenome-derived esterase from soil was reported to hydrolyze a broad range of substrates including tributyrin, PCL, PET, BHET, *p*-nitrophenyl esters (carbon chain lengths C4-C18) and the polyester-polyurethane Impranil DLN, together with various δ-lactones and β-lactam antibiotics (Zhang et al., 2023). Future work could explore the biocatalytic versatility of marine-derived enzymes such as BgP, SM14est, and MarCE by

testing against a plethora of industrially relevant substrates. Significantly, an esterase designated 26D from a marine metagenomic library was characterized as an enantioselective biocatalyst for the transformation of sterically demanding substrates with bulky side groups, as exemplified by the resolution of a pharmaceutical intermediate for sertraline synthesis by this enzyme (Gavin et al., 2019). Considering that polyesterases have previously been highlighted as versatile biocatalysts and that marine environments have already yielded functionally diverse proteins, it seems a worthwhile endeavour to investigate the wider substrate scope of the marine-associated, polyester-degrading enzymes that have been reported on by our group, further extending their potential beyond PET waste management.

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Appendix I

Other Publications and Contributions

1. Howard, S.A., Carr, C.M., Sbahtu, H.I., Onwukwe, U., López, M.J., Dobson, A.D.W. et al. (2023) Enrichment of Native Plastic-Associated Biofilm Communities to Enhance Polyester Degrading Activity. *Environmental Microbiology,* 1–21. Available from: https://doi.org/10.1111/1462-2920.16466.

Abstract:

Plastic pollution is an increasing worldwide problem urgently requiring a solution. While recycling rates are increasing globally, only 9% of all plastic waste has been recycled, and with the cost and limited downstream uses of recycled plastic, an alternative is needed. Here, we found that expanded polystyrene (EPS) promoted high levels of bacterial biofilm formation and sought out environmental EPS waste to characterize these native communities. We demonstrated that the EPS attached communities had limited plastic degrading activity. We then performed a long-term enrichment experiment where we placed a robust selection pressure on these communities by limiting carbon availability such that the waste plastic was the only carbon source. Seven of the resulting enriched bacterial communities had increased plastic degrading activity compared to the starting bacterial communities. *Pseudomonas stutzeri* was predominantly identified in six of the seven enriched communities as the strongest polyester degrader. Sequencing of one isolate of *P. stutzeri* revealed two putative polyesterases and one putative MHETase. This indicates that waste plastic-associated biofilms are a source for bacteria that have plastic-degrading potential, and that this potential can be unlocked through selective pressure and further in vitro enrichment experiments, resulting in biodegradative communities that are better than nature.

Contribution:

CMC performed DNA extractions, genomic and metagenomic analyses, genome mining, and computational protein analyses.

2. de Oliveira, B.F.R., Carr, C.M., Dobson, A.D.W. et al. Harnessing the sponge microbiome for industrial biocatalysts. *Appl Microbiol Biotechnol* 104, 8131–8154 (2020). https://doi.org/10.1007/s00253-020-10817-3

Abstract:

Within the marine sphere, host-associated microbiomes are receiving growing attention as prolific sources of novel biocatalysts. Given the known biocatalytic potential of poriferan microbial inhabitants, this review focuses on enzymes from the sponge microbiome, with special attention on their relevant properties and the wide range of their potential biotechnological applications within various industries. Cultivable bacterial and filamentous fungal isolates account for the majority of the enzymatic sources. Hydrolases, mainly glycoside hydrolases and carboxylesterases, are the predominant reported group of enzymes, with varying degrees of tolerance to alkaline pH and growing salt concentrations being common. Prospective areas for the application of these microbial enzymes include biorefinery, detergent, food and effluent treatment industries. Finally, alternative strategies to identify novel biocatalysts from the sponge microbiome are addressed, with an emphasis on modern -omics-based approaches that are currently available in the enzyme research arena. By providing this current overview of the field, we hope to not only increase the appetite of researchers to instigate forthcoming studies but also to stress how basic and applied research can pave the way for new biocatalysts from these symbiotic microbial communities in a productive fashion.

Contribution:

CMC contributed to the writing of the manuscript and read and approved the final version.