Rational protein engineering of PETase from Ideonella sakaiensis to stimulate the bioremediation of polyethylene terephthalate (PET) pollution

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Abstract

Despite vigorous recycling efforts, nearly 350 million tons of polyethylene terephthalate (PET)—plastic commonly used to package foods and beverages—accumulate each year, posing a serious threat to the global ecosystem. To combat this bioaccumulation of plastic pollution, the biological treatment of plastic waste has become an important research focus. This process involves the use of microorganism-derived enzymes to breakdown PET into its individual monomers, terephthalate and ethylene glycol, so that new plastic polymers can be reassembled in an environmentally friendly manner. Though many carboxylic acid hydrolases can depolymerize plastic, PETase from the bacterium *Ideonella sakaiensis* (*Is*PETase) is the most promising because of its remarkably high degradation efficiency at moderate temperatures. However, *Is*PETase's durability and efficiency must be improved before its performance in practical applications can be realized.

We hypothesized that by utilizing the novel Protein Repair One-Stop Shop (PROSS) computational algorithm, we would be able to develop *Is*PETase variants with a higher thermal stability than wild-type *Is*PETase. Thus, we generated four *Is*PETase and performed numerous activity tests to determine if the algorithm could effectively optimize *Is*PETase for thermal stability. In the end, the results indicate that PROSS is an effective tool for designing mutations for *Is*PETase. We generated variants with some of the highest melting point temperatures ever reported, and we were able to generate huge improvements with an incredibly efficient process. The insight gathered about protein structures that enhance the thermal properties of *Is*PETase will be utilized to develop a sustainable, cost-effective solution to plastic waste reduction.

Introduction

Nearly 4.9 billion tons of plastic waste have accumulated in oceans and landfills (Muñoz Meneses et al., 2022), placing a substantial burden on the global ecosystem (Geyer et al., 2016). Even with concerted recycling efforts, the annual mass of plastic waste is expected to double by 2050 (Lebreton & Andrady, 2019; Geyer et al., 2017). Therefore, new technologies are urgently needed to mitigate the effects of plastic pollution.

Polyethylene terephthalate (PET)—with a manufacturing capacity of over 30 million tons ("Polyethylene terephthalate," 2020)—is one of the most widely used synthetic plastics and is therefore a primary contributor to plastic waste. Over the past decade, the enzymatic hydrolysis of PET has become an important research focus, as several ester hydrolase enzymes, including cutinases, esterases, and lipases, have exhibited the ability to degrade PET (George & Kurian, 2014). However, this subset of enzymes can only operate at extremely high reaction temperatures of around 70℃ (Lu et al., 2022). This greatly limits their potential to be harnessed as a viable recycling strategy, as around 40% of plastic waste resides in natural environments with moderate temperatures (14℃) (Worm et al., 2017). Additionally, these enzymes demonstrate a low degradation efficiency towards highly crystallized PET (Wei & Zimmerman, 2017), the most abundant type of postconsumer PET waste, which further limits their potential environmental applications.

However, in 2016, Yoshida et al. isolated a new bacterium, *Ideonella sakaiensis*, which evolved naturally to degrade PET by secreting the enzyme PETase. What's more is PETase from *Ideonella sakaiensis* (*Is*PETase) demonstrates both the highest degradation efficiency at moderate temperatures of the known PET hydrolase enzymes (Yoshida et al., 2016) and the ability to degrade highly crystallized PET (Chen et al., 2020). Simply, *Is*PETase overcomes the limitations of the other known PET hydrolase enzymes. With that said, *Is*PETase still does not have the required efficiency and stability to form part of an economically viable plastic recycling process. This is problematic and must be addressed before the environmental potential of *Is*PETase can be realized. Therefore, *Is*PETase variants with characteristics of high thermal stability must be developed to consequently allow *Is*PETase to retain enzymatic activity for long periods of time at moderate, practical temperatures.

By changing the protein sequence of *Is*PETase with structure-based bioinformatics tools, researchers have reported several *Is*PETase variants with higher thermal stability than wild-type *Is*PETase. Namely, in 2021, Rennison et al. were the first to utilize the novel Protein Repair One-Stop Shop (PROSS) bioinformatics algorithm to introduce mutations to *Is*PETase. Rennison et al. successfully engineered *Is*PETase^{S136E}—mutation at location 136 of *Is*PETase's amino acid sequence from Serine (S) to Glutamic Acid (E)—to have a melting point temperature 1.1℃ higher than wild-type *Is*PETase. However, their study only partially relied on PROSS, combining the tool with the more traditional

rational design protein engineering approach; thus, the effect of solely using PROSS as a bioinformatics tool for *Is*PETase remains unknown. This drives our study. It is of utmost importance to utilize PROSS alone to understand its efficacy and potential as a route to improving *Is*PETase's stability and activity.

Our study is only the second to employ the PROSS algorithm for *Is*PETase developments and is the first study to solely rely on PROSS to select mutations to improve *Is*PETase's thermal stability. We developed and assessed four original and unique *Is*PETase variants using PROSS. The results of this study reveal the promise, efficiency, and practicality of structure-based bioinformatics tools for the stability optimization of proteins. Moreover, this study provides unique insights into the mechanisms of PET degradation, knowledge that is currently lacking yet promises to yield insights into molecular biology. The implications of our study are wide-reaching, as they provide a valuable step towards the development of an effective treatment for PET pollution.

Literature Review

Impact of Plastic Pollution

Although the first reports of plastic pollution in the 1970s (Fowler, 1987) raised minimal concern, growing reports of the harmful ecological effects of plastic debris in the following decades eventually drew heavy attention to the issue (Gregory & Andrady, 2003). To give an illustration, ingested plastic has been recorded in turtles (Tomas & Guitart, 2002) and in many species of birds (Mallory, 2008; Cadee, 2002), as over 44% of marine bird species have noticeable internal levels of plastic debris (Rios & Moore, 2007). In addition to disturbing marine life, human health is also put at risk from both the harmful chemicals released during plastic manufacturing (Rustagi et al., 2011) and from the ingestion of plastic particles by eating fish. Hence, plastic pollution is a major disturbance to the global ecosystem.

Polyethylene terephthalate (PET)

PET, the leading contributor to plastic pollution, is a synthetic aromatic plastic composed of repeated units of its monomers: terephthalic acid (TPA) and ethylene glycol (EG) (Son et al., 2019). Although its primary industrial application is in food and beverage packaging (Andrady & Neal, 2009), PET is also frequently used to manufacture textiles. Due to its high durability and strong resistance to degradation (Andrady & Neal, 2009), PET is highly regarded in industry. Its unwavering resistance to degradation, however, raises a serious environmental concern, especially as an estimated 583.5 billion PET bottles were produced in 2021 (Tiseo, 2021). As such, it is important to address the heavy impact PET has from both an economical and environmental standpoint (Rennison et al., 2021).

Current PET Recycling Strategies

As PET production has reached unprecedented levels, a large emphasis has been placed on PET recycling. Having said that, a majority of post-consumer PET remains in landfills for hundreds of years and is never recycled. Additionally, the primary recycling process—sorting, mechanical rinsing, grinding,

melting, and extrusion—is highly dysfunctional, as it reproduces plastic with appreciably lower product quality (Taniguchi et al., 2019). Thus, in order to guarantee that high-quality PET is regenerated, a new recycling strategy that can depolymerize post-consumer PET into its monomers and repolymerize it into high quality PET must be developed (Rennison et al., 2021).

Bioremediation of PET Pollution

To that end, many bacterial enzymes, including cutinases, esterases, and lipases (Inderthal & Harrison, 2021) have demonstrated the ability to convert PET into its monomers (Jenkins et al., 2019), which can then be reassembled into high crystallinity PET. Not only does this approach overcome current obstacles in recycling, but it also offers a sustainable and scalable treatment for plastic pollution. However, these PET-degrading enzymes are unable to be harnessed as a widespread treatment for PET pollution for a multitude of reasons. First, the PET-degrading enzymes only demonstrate degradation activity at remarkably high reaction temperatures that exceed the PET glass transition temperature of 70℃ (Lu et al., 2022). As most PET waste residues in natural environments with temperatures far below 70℃, this group of enzymes cannot be utilized as a practical treatment for PET waste. Additionally, Wei and Zimmerman verified that cutinases, esterases, and lipases can only degrade highly processed PET, or PET with a low crystallinity (2017), which is rarely found in the environment. For example, an engineered leaf-branch compose cutinase (LCC) can only degrade PET that is pretreated to lower its crystallinity and is found in extremely high temperatures. To be precise, LCC can degrade 90% of pretreated PET in 10 hours at 72℃ (Tournier et al., 2020).

Ideonella sakaiensis PETase (IsPETase)

In 2016, a PET-assimilating bacteria was discovered in a recycling facility in Kyoto, Japan that consumes PET as its primary source of energy and carbon (Yoshida et al.). This bacteria, *Ideonella sakaiensis*, depolymerizes PET by secreting *Ideonella sakaiensis* PETase (*Is*PETase) (Austin et al., 2018). In fact, the discovery of *Is*PETase completely transformed the field of enzymatic PET degradation, as *Is*PETase does not demonstrate the same limitations as the other known PET hydrolase enzymes. First and foremost, *Is*PETase can depolymerize PET at moderate temperatures, indicating its environmental applicability (Yoshida et al., 2016). Secondly, *Is*PETase exhibits the ability to degrade highly crystallized PET (Chen et al., 2021). For these reasons, *Is*PETase is widely recognized as the most promising PET hydrolase enzyme.

Catalytic Mechanism of PET Degradation by *Is*PETase. A PET polymer first binds to the active site of *Is*PETase, an enzyme in the cutinase family. After initial binding, *Is*PETase breaks down PET into mono(1-hydroxyethyl)terephthalic acid (MHET) and bis(2-hydroxyethyl)terephthalic acid (BHET), the intermediate degradation products (Cui et al., 2021). Then *Ideonella sakaiensis* secretes MHETase (*Is*MHETase), an enzyme in the tannase family, to further break down MHET into terephthalic

acid (TPA) and ethylene glycol (EG) (Chen et al., 2021). Hence, *Is*PETase and *Is*MHETase work synergistically to convert PET into its final degradation products that aid bacterial growth (Figure 1).

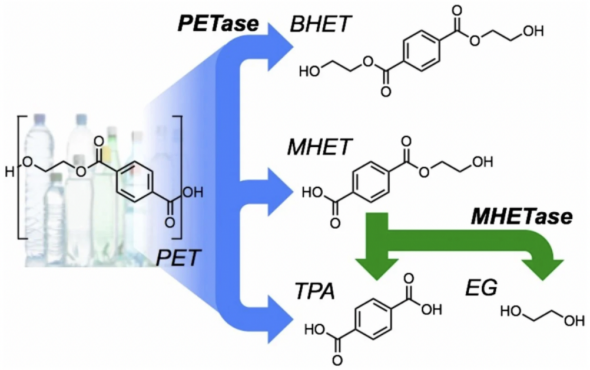

Figure 1. Through the release of PETase and MHETase, PET polymers are broken down into their final degradation products: TPA and EG (Austin et al., 2018).

Structure of *Is*PETase. *Is*PETase has the canonical α/β hydrolase fold (Han et al., 2017), meaning that the enzyme contains eight β strands and 6 α helices. The enzyme's active site is the specified region where PET substrate molecules bind to *Is*PETase and undergo a chemical reaction to depolymerize PET. In particular, the binding long, shallow L-shaped binding cleft is the exact location on the active site which binds to PET substrate—the binding cleft is mainly hydrophobic and has a length of approximately 40 Å (Joo et al., 2018). Additionally, the center of *Is*PETase's enzymatic activity is located at the catalytic triad: a collection of three amino acids located on *Is*PETase's active site (Figure 1). *Is*PETase's active site is composed of Serine-131 (S-131), Histidine-208 (H-208), and Aspartic Acid-177 (D-177) (Han et al., 2017).

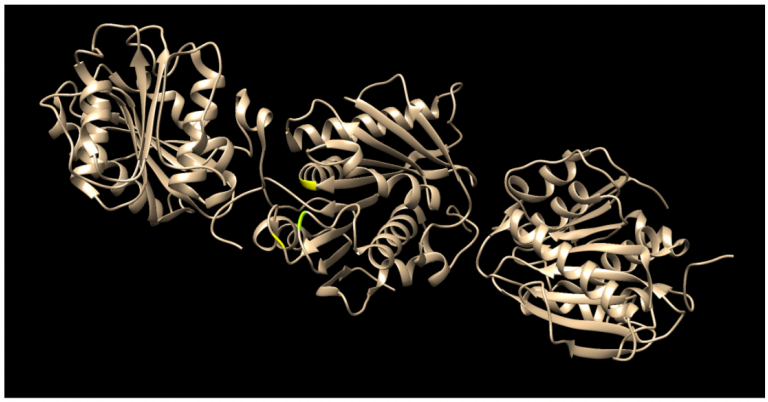

Figure 2. 3-dimensional model of *Is*PETase. The three regions highlighted in yellow represent the catalytic triad: S-131, D-177, H-208

***Is*PETase's Limitations.** Although *Is*PETase has the capacity to degrade PET in ambient conditions, its current efficiency and stability are insufficient and are preventing *Is*PETase from reaching its full potential. Specifically, *Is*PETase has a low thermal stability: at high reaction temperatures, the enzyme begins to denature and loses its ability to depolymerize PET. As such, *Is*PETase is unable to maintain enzymatic activity for extended periods of time: in its present state, *Is*PETase is labile and loses activity within 24 hours of incubation at 37℃ (Son et al., 2019). Additionally, *Is*PETase's low thermal stability corresponds to a slower PET degradation process (Rennison et al., 2021). Interestingly, *Is*PETase's low thermal stability is largely credited to the fact that *Ideonella sakaiensis* only grows under mild conditions (Joo et al., 2018). In accordance, *Is*PETase variants with high thermal stability and durability must be designed so as to support *Is*PETase's degradation activity for extended periods of time at moderate temperatures.

Rational Design Protein Engineering of IsPETase. In order to develop *Is*PETase variants with enhanced properties, researchers have used a myriad of approaches, the most popular being rational design protein engineering. By studying crystallized models of *Is*PETase (Palm et al., 2019; Knott et al., 2020), researchers such as Austin et al. (2019) and Joo et al. (2018) introduced mutations that increased *Is*PETase's efficiency. Austin et al. were able to achieve their enhanced variant by substituting Histidine (H) for Tryptophan (W) at the 159th location on the amino acid sequence and Phenylalanine (F) for Serine (S) at the 238th location on the amino acid sequence. Their ultimate goal was to narrow *Is*PETase's active site binding cleft. On the other hand, Joo et al. redesigned *Is*PETase's enzyme-substrate binding pocket by substituting Alanine (A) for Arginine (R) at the 280th location in the amino acid sequence. Son et al. introduced mutations that improved *Is*PETase's thermal stability. Son et al. hypothesized that the Proline-181 (P-181) amino acid located in the middle of the β6 strand of *Is*PETase blocked the continuity of the β sheet and therefore decreased the enzyme's thermal stability (2019). Therefore, Son et al. designed and developed an *Is*PETase variant that replaced Proline (P) with Alanine (A) at the 181st location in the amino acid sequence (*Is*PETase^{P181A}). They found that the new variant successfully displayed improved thermal stability, though the improvement was slight. Son et al. also developed variant *Is*PETase$^{S121E/D186H/R280A}$, where amino acid substitutions were introduced at three different locations in the sequence. They experimentally compared the melting point temperature of *Is*PETase$^{S121E/D186H/R280A}$ to that of wild-type *Is*PETase and found the melting point temperatures to be 57.67℃ and 48.81℃, respectively, thus indicating the higher thermal stability of the variant. Even with these substantial improvements to the enzyme's thermal stability, *Is*PETase has not yet been optimized to a point where it can be used to effectively recycle PET. Therefore, more research is needed to determine how *Is*PETase's thermal stability can be better optimized. Considering how long and extensive rational design protein engineering is, a more efficient protein engineering strategy must be generated in order to optimize *Is*PETase for thermal stability.

Protein Repair One-Stop Shop (PROSS). PROSS is a recently-developed, automated bioinformatics protein engineering algorithm for stabilization that is more efficient than traditional rational design in that it requires less experimental testing. The first group to utilize PROSS for *Is*PETase developments was Rennison et al. Although PROSS proposed 53 mutations, Rennison et al. rationally selected one mutation to introduce to each of the variants they produced. As Rennison et al. found that PROSS is an effective assisting tool to optimize for thermal stability, this study was designed as a follow-up. Our study is the first designed to fully rely on PROSS to develop *Is*PETase with improved thermal stability and overall activity so as to determine how effective PROSS is alone.

Project Overview

The goal of this study is to develop four *Is*PETase variants with improved thermal stability in order to increase the possibility of achieving the complete biodegradation of PET under mild conditions. While numerous studies have developed *Is*PETase variants with improved thermal stability, further improvements are necessary before *Is*PETase's implementation as a recycling strategy. In other words, there is a lack of understanding of how to most effectively optimize *Is*PETase's stability in a timely manner. Hence, this design is novel in the fact that this is the first study to solely use the efficient PROSS algorithm to generate the specified arrays of mutations to increase *Is*PETase's thermal stability so as to fill this gap in the literature. Ultimately, this study provides new insight into how information on protein structure can be utilized to develop enhanced thermal properties of *Is*PETase. Additionally, this study explores the benefits of utilizing a quick and efficient protein engineering method over an extended rational design approach. If we gain insight into the most effective way to optimize *Is*PETase's stability, then we can ultimately work to implement *Is*PETase in some capacity for PET recycling.

Project Objectives

1. To develop four unique *Is*PETase variants that will be designed and created using PROSS
2. To develop four *Is*PETase variants that result in highly expressed and active protein
3. To determine the melting point temperature (T_m) of wild-type *Is*PETase and related variants to provide insight into thermal stability
4. To analyze the degradation activity of *Is*PETase and related variants towards a soluble substrate after a thermal challenge at 30℃, 40℃, and 50℃
5. To analyze the activity of *Is*PETase and related variants towards commercial PET substrates to quantify degradation over 24 and 48 hours

Hypotheses

H_1: The *Is*PETase variants have a higher melting point temperature than wild-type *Is*PETase and therefore exhibit a higher thermal stability.

H_2: *Is*PETase variants will demonstrate higher activity at room temperature following incubation for 15 minutes at 30℃, 40℃, and 50℃ than wild-type *Is*PETase.

H_3: *Is*PETase variants will be active on commercial PET substrates and will demonstrate a higher degradation rate after 24 and 48 hours of incubation with PET film at 42℃.

H_4: The mutation at 214 from Serine to Histidine (S214H) will negatively impact the catalytic activity of variant 3, and this can be alleviated by reverting that change to the original residue.

Methods

My Role in the Study

After reading over 25 journal articles in the field of enzymatic depolymerization, I formally reached out to Dr. Danielle Tullman-Ercek at Northwestern University in March of 2021 for mentorship. In my first email to Dr. Tullman-Ercek, I shared my precise research questions and objectives. Because my extensive research was noticeable in this first email exchange, Dr. Tullman-Ercek excitedly announced that I would be the first high school student she would mentor—not the first student who had asked her for mentorship, but the first student who she felt was ready for this type of project. Over the next year and a half, I maintained my relationship with Dr. Tullman-Ercek via frequent Zoom meetings and weekly email contact. As Dr. Tullman-Ercek saw my passion and deep knowledge of protein engineering, she shared various lectures with me from the Synthetic Biology course she teaches at Northwestern. Further, Dr. Tullman-Ercek excitedly shared my project with Dr. Carolyn Mills, who generously offered to not only assist in answering any specific questions I had regarding *Is*PETase but also to guide me through my learning of cloning, expression, and purification.

From the spring of 2021 to the spring of 2022, I independently worked to computationally design the *Is*PETase variants that I would engineer in the lab in the spring and summer of 2022. After using the Protein Repair One-Stop Shop (PROSS) algorithm to develop *Is*PETase variants, I independently modeled each variant using the 3-dimensional molecular viewer Chimera to determine suitability, and I encoded the *Is*PETase mutations into each variant. I then strategically selected four variants to develop in the lab, after which I presented the variants and received approval from both Dr. Mills and Dr. Tullman-Ercek to proceed.

Once I arrived in the lab, I worked independently on all aspects of my project—any work done by a supervising postdoctoral researcher was done at my request for either a safety reason or for assistance with unfamiliar equipment. I independently selected all methods used in this study, and I conducted extensive background research on protein expression and purification so that I would be able to lead the procedures in the lab. In my first visit to the lab in May of 2022, I conducted molecular cloning using the Golden Gate method to create expression vectors for each variant. Upon my return in July of 2022, I ran bacterial cell culture protein expression and protein purification. Most importantly, though, I had complete control over my research goals and objectives in that I designed the protocols for the specific activity and stability tests I would perform on *Is*PETase and related variants. I designed and performed the Bicinchoninic acid (BCA) assay to determine the accurate protein concentration of each variant generated. I also independently carried out the SYPRO orange assay to determine the melting temperature of *Is*PETase and related variants, though I did receive help from Dr. Mills when setting up the qPCR instrument. I then designed and carried out my PET hydrolysis assay. Lastly, I designed and performed a

unique soluble substrate activity assay for *Is*PEase, though I did receive assistance from a graduate student with the final multichannel pipette run and inserting plate into the microplate reader spectrophotometer. Once all assays were completed, I independently interpreted all findings, which Dr. Tullman-Ercek later confirmed. As the leader of this project which I conceived, I was able to improve both my laboratory and data analysis skills.

Computational Design

PROSS

The online PROSS computational algorithm was utilized to design mutations to increase the thermal stability of *Is*PETase. In essence, this algorithm received the crystal structure and sequence of wild-type *Is*PETase (PDB code: 6EQD) taken from Austin et al. (2018) as an input and provided as output several mutated sequences that were expected to be more stable. During mutation design, the amino acid residues forming *Is*PETase's active site were fixed so as to prevent them from being altered—mutations to these specified regions risked halting *Is*PETase's enzymatic activity altogether. PROSS then used multiple sequence alignment (MSA) to compare the DNA sequence of *Is*PETase to that of other homologous proteins so as to effectively recommend residue substitutions for *Is*PETase. The preset MSA parameters for PROSS were used: a maximum of 4000 sequences were included in the alignments, and only proteins with a minimum sequence identity of 35% and a minimum coverage of 75%, generated from a BLAST search, were added. The distributions of sequence identity and coverage in MSA are shown in Figure 3. Furthermore, the energy function used in the Rosetta calculations was talaris 2014. Upon completion, PROSS generated nine *Is*PETase variants, though variants 4 through 9 respectively proposed 12 or more mutations. Therefore, in order to limit the complexity of the *Is*PETase variants created, only variants 1 through 3 were further analyzed.

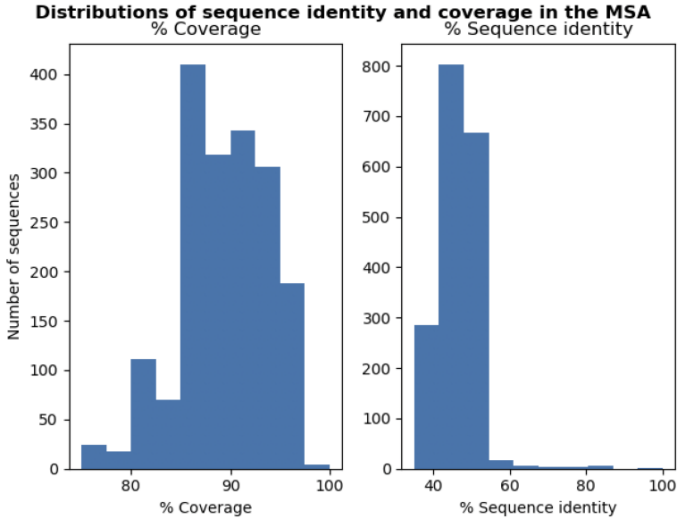

Figure 3. Sequence identity and coverage in the MSA.

Chimera

In order to determine the suitability of variants 1 through 3, each variant was visually examined using Chimera, a computational molecular viewer. Specifically, the variants were analyzed to determine if the proposed mutations interfered with *Is*PETase's catalytic triad—S-131, H-208, D-177—as disturbances

to the catalytic triad put the enzyme's functionality at risk. Variants 1, 2, and 3 appeared to be structurally viable, but the proposed residue substitution at location 214 from Serine to Histidine (S214H) was marginally close to the catalytic triad, as shown in Figure 4. Consequently, we elected to generate a fourth variant that was not proposed by PROSS: this variant proposed the same mutations as variant 3 but excluded the S214H mutation. The amino acid sequences of *Is*PETase and variants 1-4 were back-translated into DNA sequence and optimized for *Escherichia coli* expression. Full amino sequences of wild type *Is*PETase and related variants are available in Figure 5, and full DNA sequences are available in Figure 6.

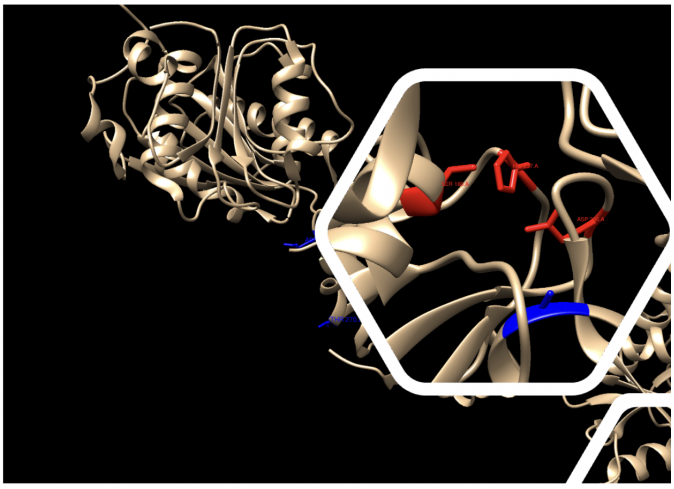

Figure 4. 3-dimensional model of *Is*PETase variant 3. The three red amino acids represents the catalytic triad, and the blue amino acid is the histidine mutation at location 214 (S214H)

A

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001 MNFPRASRLM QAAVLGGLMA VSAAATAQTN PYARGPNPTA ASLEASAGPF TVRSFTVSRP SGYGAGTVYY
071 PTNAGGTVGA IAIVPGYTAR QSSIKWWGPR LASHGFVVIT IDTNSTLDQP SSRSSQQMAA LRQVASLNGT
141 SSSPIYGKVD TARMGVMGWS MGGGGSLISA ANNPSLKAAA PQAPWDSSTN FSSVTVPTLI FACENDSIAP
211 VNSSALPIYD SMSRNAKQFL EINGGSHSCA NSGNSNQALI GKKGVAWMKR FMDNDTRYST FACENPNSTR
291 VSDFRTANCS LEHHHHHH
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B

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001 MNFPRASRLM QAAVLGGLMA VSAAATAQTN PYARGPNPTA ASLEASRGPF TVRSFTVSRP SGYGAGTVYY
071 PTNAGGTVGA IAIVPGYTAR QSSIKWWGPR LASHGFVVIT IDTNSTLDQP SSRSSQQMAA LDQVASLNGT
141 SSSPIYGKVD TSRMGVMGWS MGGGGSLISA ANNPSLKAAA PQAPWDSSTN FSSVTVPTLI FACENDSIAP
211 VNSHALPIYD SMSRNAKQFL EINGGSHSCA NSGNSNQALI GKKGVAWMKR FMDNDTRYST FACENPNSTR
291 VSDFRTANCS LEHHHHHH
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C

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001 MNFPRASRLM QAAVLGGLMA VSAAATAQTN PYARGPDPTA ASLEASRGPF TVRSFTVSRP SGYGAGTVYY
071 PTNAGGTVGA IAIVPGYTAR QSSIKWWGPR LASHGFVVIT IDTNSTLDQP SSRSSQQMAA LDQVASLNNT
141 SSSPIYGKVD TSRMGVMGWS MGGGGSLISA ANNPSLKAAA PQAPWDSSTN FSSVTVPTLI FACENDSIAP
211 VNSHALPIYD SMSRNAKQFL EINGGSHSCA NSGNSNQALI GKKGVAWMKR FMDNDTRYSQ FACENPNSTR
291 VSDFRTANCS LEHHHHHH
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D

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001 MNFPRASRLM QAAVLGGLMA VSAAATAQTN PYARGPDPTA ASLEASRGPF TVRSFTVSRP SGYGAGTVYY
071 PTNAGGTVGA IAIVPGYTAR QSSIKWWGPR LASHGFVVIT IDTNSTLDQP SSRSRQQMAA LDQVASLNNT
141 SSSPIYGKVD TSRMGVMGWS MGGGGSLISA ANNPSLKAAA PQAPWDSSTN FSSVTVPTLI FACENDSIAP
211 VNSHALPIYD SMSRNAKQFL EINGGSHSCA NSGNSNQALI GKKGVAWMKR FMDNDTRYSQ FACENPNSTR
291 VSDFRTANCS LEHHHHHH
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E

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001 MNFPRASRLM QAAVLGGLMA VSAAATAQTN PYARGPDPTA ASLEASRGPF TVRSFTVSRP SGYGAGTVYY
071 PTNAGGTVGA IAIVPGYTAR QSSIKWWGPR LASHGFVVIT IDTNSTLDQP SSRSRQQMAA LDQVASLNNT
141 SSSPIYGKVD TSRMGVMGWS MGGGGSLISA ANNPSLKAAA PQAPWDSSTN FSSVTVPTLI FACENDSIAP
211 VNSSALPIYD SMSRNAKQFL EINGGSHSCA NSGNSNQALI GKKGVAWMKR FMDNDTRYSQ FACENPNSTR
291 VSDFRTANCS LEHHHHHH
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Figure 5. (A) Amino acid sequence of wild-type *Is*PETase. (B) Amino acid sequence of *Is*PETase variant 1. (C) Amino acid sequence of *Is*PETase variant 2. (D) Amino acid sequence of *Is*PETase variant 3. (E) Amino acid sequence of *Is*PETase variant 4. Amino acids that are bolded and highlighted in yellow indicate mutations that are shared by all four variants (with the exception of one omitted in variant 4). Amino acids that are highlighted in green indicate mutations that are shared by variants 2-4. Amino acids highlighted in blue indicate mutations that are shared by variants 3-4. The amino acid in red text indicates the omitted mutation in variant 4. The amino acids highlighted in orange indicate the polyhistidine-tag.

Figure 6. (A) DNA sequence of wild-type *Is*PETase. (B) DNA sequence of *Is*PETase variant 1. (C) DNA sequence of *Is*PETase variant 2. (D) DNA sequence of *Is*PETase variant 3. (E) DNA sequence of *Is*PETase variant 4. Codons that are bolded and highlighted in yellow indicate mutations that are shared by all four variants (with the exception of one omitted in variant 4). Codons that are highlighted in green indicate mutations that are shared by variants 2-4. Codons highlighted in blue indicate mutations that are shared by variants 3-4. The codon in red text indicates the omitted mutation in variant 4. The amino acids highlighted in orange indicate the polyhistidine-tag.

Plasmid Construction

After deciding which amino acid mutations to make based on the PROSS software, we incorporated these mutations into the DNA sequence of *Is*PETase, selecting codons for mutations based on codon usage in *E. coli*. The gene blocks for *Is*PETase and variants were then ordered from Twist Biosciences as double-stranded DNA with BsaI sites compatible with a pET21b parent vector (p15A origin of replication, carbenicillin resistance). Full amino sequences of wild type *Is*PETase as well as PETase variants are available in Figure 3, and full DNA sequences are available in Figure 4.

The Golden Gate reactions were then used to incorporate the gene of interest (*Is*PETase and related variants) into the pET21b parent vector (Engler et al., 2019). These reactions were performed using the FastDigest Eco31U and T4 DNA ligase and transformed into *E. coli* DH10b cells. Individual transformants were then grown overnight in lysogeny broth, Miller formulation (LB-M) supplemented with carbenicillin (50 µg/mL) and plasmid DNA was extracted using a Zyppy plasmid miniprep kit. Successful incorporation of *Is*PETase and related variants into the pET21b vector was confirmed by Sanger sequencing (Genewiz). The variants were then transformed into *E. coli* C41(DE3), the protein expression line used in previous studies producing *Is*PETase (Urbanek et al., 2021).

Protein Expression

Single clones of *E. coli* C41(DE3) cells containing relevant plasmids were inoculated into 6 mL of LB-M medium supplemented with 50 μg/mL carbenicillin, and the cultures were shaken at 37 ℃, 225 rpm for about 16 hours. The overnight culture was then inoculated into 500 mL LB-M medium supplemented with 50 μg/mL carbenicillin in 2.5 L flasks. Cultures were grown at 37 ℃, 225 rpm to an optical density at 600 nm (OD_{600}) of 0.5-0.6. Cultures were then placed on ice for 20 minutes. Expression was then induced with 0.5 mM of isopropyl β-D-1-thiogalactopyranoside (IPTG). The cultures were then shaken at 225 rpm, 16 ℃ for approximately 20 hours. The cells were harvested by centrifugation for 15 minutes at 4600 xg, and the supernatant was discarded. In order to resuspend the cell pellets, 15 mL of lysis binding buffer (20 mM sodium phosphate, 0.5 M NaCl, 20-40 mM imidazole, pH 7.4) was added to each pellet. The suspended pellets were then frozen at -20 °C until purification began.

Protein Purification

Resuspended cell pellets were thawed and were rocked at room temperature with 1 mg/mL lysozyme for 1 hour. The six pellets were then sonicated at 50% amplitude, pulsing 2 seconds on and 4 seconds off for a total of 10 minutes of "on" time (Fisher Scientific, catalog no. FB120A220, probe CL-18). Insoluble cell debris was removed by centrifugation for 15 minutes at 15,000 xg, and the supernatant was retained. 2.5 mL of 50% His-pur-Ni-NTA agarose resin slurry was added to each column assembly, and each column was equilibrated with 12.5 mL of lysis binding buffer (20 mM sodium phosphate, 0.5 M NaCl, 20-40 mM imidazole, pH 7.4). The resin was then resuspended in 5 mL of binding buffer and was mixed with the clarified lysate at 4℃ to allow the His-tagged proteins to bind to the Ni-NTA resin. Subsequently, the NiNTA/lysate mixtures were applied to a column assembly and allowed to flow through— this process is known as the flow through fraction. Each column was washed twice with 7.5 mL of binding buffer. The protein of interest, *Is*PETase and related variants, were eluted across 4 fractions by adding 1 mL of elution buffer (20 mM sodium phosphate, 0.5 M NaCl, 500 mM imidazole, pH 7.4) to each column, and each elution was collected in a separate tube. Protein content of elution fractions was assessed using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) with coomassie brilliant blue staining. Elution fractions containing the target PETase variants were combined and buffer exchanged into 50 mM sodium phosphate buffer, pH 7.4 using PD-10 desalting columns packed with Sephadex G-25

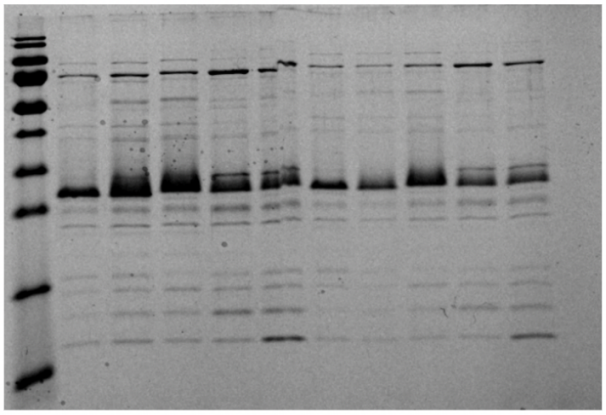

Figure 7. Coomassie blue staining on SDS-PAGE. The last five lanes represent wild-type *Is*PETase and related variants.

resin (Cytiva, 17085101). Aggregates formed after desalting were removed by centrifugation at 13,000xg for 5 minutes. Final protein purity was assessed using coomassie blue staining on SDS-PAGE, as shown in Figure 7, and final protein concentration was measured by bicinchoninic acid assay.

Bicinchoninic Acid (BCA) Assay for Colorimetric Detection and Quantification of Total Protein

Bicinchoninic acid (BCA) interacts with the amines in *Is*PETase's backbone—this interaction allows *Is*PETase and related variants to bind to copper and change the copper valence, resulting in a color change. Thus, the color change of *Is*PETase and related variants was measured in a plate reader to determine the respective protein concentrations. The thermo scientific BCA Protein Assay Kit procedure was followed with 9 standards in triplicate for the 5 samples.

Assessment of Melting Point Temperature (T_m) by SYPRO Orange Protein Thermal Shift Assay

A 200X SYPRO orange concentrate was prepared from a thawed 5000X stock. Each protein sample (including the buffer) was run in triplicate, so 18 samples were respectively prepared in 0.1 mL low-profile qPCR tubes with 2.5 µL 200X SYPRO orange concentrate, varying µL protein solution such that each final protein concentration was 0.5 mg/mL, and varying µL 50 mM sodium phosphate buffer (pH 7.2) such that the final volume in each qPCR tube was 25 µL. The qPCR tubes were capped with optically clear cap strips and spun down in the mini centrifuge. The 18 samples were then run in the qPCR machine, where all channels were tracked while heating from 25℃ to 80℃, with a ramp rate of 0.5℃ per minute. The Jupyer notebook determined the melting temperature of each variant.

Assessment of PET Hydrolysis

For simplicity, we chose an assay modeled by Sinskey et al. (2020) where a 33.8 fluid ounce Smartwater bottle was used as the substrate. 36 1'' discs with a surface area of 0.64 cm^2 were cut with a standard office hold punch from the Smartwater bottle, and the mass of each disk was calculated. The plastic disks were then submerged in ethanol to be cleaned. Once dried, each plastic disk was placed in a 2 mL eppendorf tube with 100 nM of enzyme and 50 mM sodium phosphate buffer (pH 7.2). Short pasteur polyethylene terephthalate pipette lengths were placed inside each tube to prevent the plastic disks from rising out of the liquid due to the high surface tension. Figure 8 includes an image of the assay setup. All of the tubes were then placed in a water bath at 42℃, and after 24 hours, the first 18 tubes were removed from the water bath so that the degradation activity of the enzyme variants towards PET could be analyzed. Each plastic disk was remassed, and 150 µL samples were taken from each of the 18 tubes to determine the concentration of degradation products by high-performance liquid chromatography

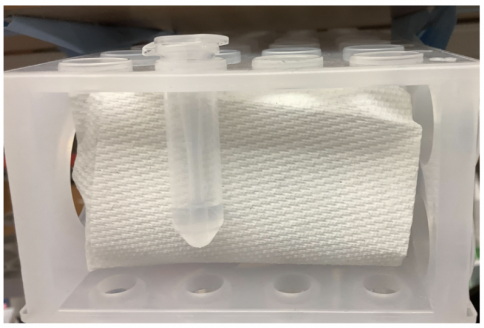

Figure 8. Setup of the PET hydrolysis assay. The 100 nM of enzyme and 50 mM sodium phosphate buffer (pH 7.2) are inside the eppendorf tube with the PET disc and PET pipette length.

(HPLC). After 48 hours, the other 18 tubes were removed—the plastic disks were remassed, and 150 µL samples were collected again.

Assessment of *Is*PETase esterase activity against colorimetric substrate p-nitrophenyl acetate to probe enzyme activity after a thermal challenge

Samples of *Is*PETase and related variants were prepared in triplicate for incubation at 30℃, 40℃, 50℃, and 4℃ (control). 50 µL of 10 µg/mL of *Is*PETase and related variants (including buffer) were allotted into PCR strips in triplicate. The PCR strips were spun down in the mini centrifuge, incubated in the thermal cycler at the temperatures of interest for 15 minutes, and spun down again to ensure that the solution was homogeneous. The assay plate was prepared by adding 20 µL of enzyme solution from the PCR strips to each well. The Agilent BioTek Gen5 Microplate reader was then set up to monitor absorbance at 347 nm for 20 minutes, measuring as frequently as possible. The assay solution—100 µL 100n mM p-nitrophenyl acetate solution in ethanol and 14.9 mL 50 mM pH 7.2 sodium phosphate buffer—was prepared in a trough immediately before the assay, and 180 µL of p-nitrophenyl acetate solution was added to each well using a multichannel pipette. The trough was then input into the microplate reader.

Results and Discussion

Objective 1: Variant Design

Effective optimization of IsPETase for increased thermal stability utilizing the PROSS algorithm

To develop *Is*PETase variants with improved thermal stability, the crystal structure (PDB code: 6EQD) was uploaded to the PROSS computational algorithm with parameters set. An extensive list of 48 amino acid substitutions was generated, as shown in Table 1, and different subsets of the mutations were assigned to the 9 generated variants.

List of Mutations

	Wild Type	Design 1	Design 2	Design 3	Design 4	Design 5	Design 6	Design 7	Design 8	Design 9
135	A									I
40	A									E
137	L									E
172	N									D
173	N									R
190	N									D
192	Q									M
224	R									Q
53	R									S
186	S									T
193	S									N
213	S									T
238	S									T
140	T									R
151	T									P
159	W									H
33	A								Q	Q
117	L								Y	Y
169	S								A	A
118	T								P	P
279	T								S	S
65	A							Q	G	G
121	S							D	D	D
136	S							Q	Q	Q
166	S							A	A	A
77	T							P	P	P
186	D						H	H	H	H
220	D						N	N	N	N
124	S						A	A	A	A
307	S						T	T	T	T
236	S					D	D	D	D	
61	S						N	N	N	N
91	T						A	A	A	A
260	M					V	V	V	V	V
240	N					H	H	H	H	H
90	R					T	T	T	T	T
98	S					P	P	P	P	P
166	I				Q	Q	Q	Q	R	Q
95	K				A	A	A	A	A	A
167	S				T	T	T	T	T	T
134	Y				L	L	L	L	L	L
125	S			R	R	N	D	R	R	R
139	G		N	N	N	N	N	N	N	N
37	N		D	D	D	D	D	D	D	D
270	T		Q	Q	Q	Q	Q	Q	Q	Q
152	A	S	S	S	S	S	T	S	S	T
47	A	R	R	R	R	R	R	R	R	R
132	R	D	D	D	D	D	D	D	D	D
214	S	H	H	H	H	H	H	H	H	H

Table 1. *Is*PETase variant designs proposed by PROSS based on parameters set.

As 48 mutations is too many to study for the scope of this project, and designs 4 through 9 contain upwards of 4.56% mutations, only designs 1-3 were selected for engineering. Although the S214H substitution was included in DuraPETase (Cui et al., 2021), my computational modeling revealed that mutations to 214 might hinder enzymatic activity considering its close proximity to the catalytic triad. Therefore, a fourth design was generated that introduced the same amino acid substitutions as design 3 excluding S214H. The full list of each variant generated is displayed in Table 2.

V1	*Is* PETase A47R/R132D/A152S/S214H.
V2	*Is* PETase N37D/A47R/R132D/G139N/A152S/S214H/T270Q
V3	*Is* PETase N37D/A47R/S125R/R132D/G139N/A152S/S214H/T270Q
V4	*Is* PETase N37D/A47R/S125R/R132D/G139N/A152S/T270Q

Table 2. Full variant names.

Objective 2: Protein Expression

The four IsPETase variants resulted in highly expressed and active protein.

The results of the BCA assay, as shown in Table 3, are indicative of the substantial quantities of protein expressed by each variant. The introduction of certain mutations can affect RNA stability and reduce expression, but the subset of mutations suggested by PROSS did not hinder protein expression.

Samples		
samples	Concentration (ug/mL)	Abs-Blank
WT	1841.909091	2.217
v1	1667.363636	2.025
v2	1661	2.018
v3	345.5454545	0.571
v4	607.3636364	0.859

Table 3. Protein concentrations generated from the BCA assay of wild-type *Is*PETase and related variants.

Objective 3: Assessment of the thermal stability of *Is*PETase and related variants by analyzing melting point temperatures (T_m)

IsPETase variants demonstrated a higher thermal stability than wild-type IsPETase

To determine the melting temperature—the temperature at which 50% of an enzyme denatures—of wild-type *Is*PETase and related variants, a SYPRO orange protein thermal shift assay was performed. I hypothesized that the *Is*PETase variants have a higher melting point temperature than wild-type *Is*PETase and therefore exhibit a higher thermal stability. The results of the SYPRO orange assay show that each of the designed variants has a higher melting temperature than wild-type *Is*PETase, as exhibited in figure 9. Wild-type *Is*PETase demonstrated a melting temperature of 42 ℃. *Is*PETase N37D/A47R/S125R/R132D/G139N/A152S/S214H/T270Q (variant 3) demonstrated the highest melting temperature of the variants at 49.5 ℃—7.5 ℃ higher than the melting point temperature of wild-type

*Is*PETase. *Is*PETase A47R/R132D/A152S/S214H (variant 1) has the second highest melting point temperature at 48.5 ℃—6.5 ℃ higher than the melting temperature of wild-type *Is*PETase. Variants 2 and 4 have melting temperatures of 47 ℃ and 42.5 ℃, respectively.

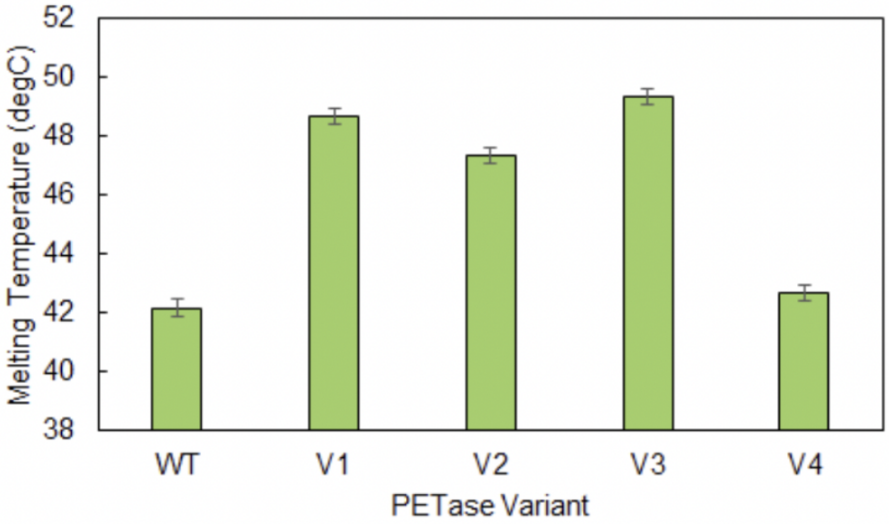

Figure 9. The melting point temperatures of wild-type *Is*PETase and related variants.

The PROSS computational algorithm is most effective when proposed designs are fully implemented

Rennison et al. did not develop any of the designs proposed by PROSS and instead selected single mutations to introduce based on *Is*PETase's alignment to other homologous enzymes (2021). In other words, Rennison et al. only partially utilized the PROSS algorithm when optimizing *Is*PETase for thermal stability. Their results only indicated slight changes in *Is*PETase's melting point temperature. They developed wild-type *Is*PETase with a melting temperature of 42.5 ℃ and two variants with melting temperatures of 43.1 ℃ and 43.6 ℃, respectively. They also developed a variant with a lower melting point temperature than their wild-type at 42.1 °C.

This is the first study to fully utilize the PROSS algorithm and engineer designs proposed by PROSS, and the reported melting temperatures of the variants in this study are substantially higher than those generated by Rennison et al. Thus, it can be concluded that the PROSS algorithm can generate *Is*PETase variants with improved thermal stability. It can also be concluded that the PROSS algorithm is most effective when proposed designs are fully implemented—not when a single point mutation is introduced. Additional evidence comes from *Is*PETase N37D/A47R/S125R/R132D/G139N/A152S/T270Q (variant 4), as this was the only variant generated in

this study not proposed by PROSS, and it exhibits the smallest increase in thermal stability of all of the variants tested in this study.

Objective 4: Analysis of the degradation activity of *Is*PETase and related variants after a thermal challenge at 30 ℃, 40 ℃, and 50 ℃

To determine how much degradation activity each *Is*PETase variant retains after incubation at increased temperatures, a p-nitrophenyl acetate (pNp) soluble substrate assay was performed. The results (figure 9) indicate that wild-type *Is*PETase exhibits little to no enzymatic activity at 50 ℃, and wild-type only retains 2.538% of its activity from 30 ℃ to 50 ℃, characteristic of *Is*PETase's low thermal stability. Variants 1 and 3 respectively retained 37.793% and 37.219% of their activity from 30 ℃ to 50 ℃, indicative of their higher thermal stability than wild-type *Is*PETase. It is important to note that this finding aligns with the recorded melting temperatures determined by the SYPRO orange assay, as both are indicative of the high thermal stability of variants 1 and 3. Variant 2 also demonstrated an increase in thermal stability, as it retained 21.723%, but this increase is not as distinguished as that of variants 1 and 3. Moreover, variant 4 only retained 3.872% of its activity. All in all, the results of this assay suggest that the mutations introduced to variants 1 and 3 increased their thermal stability, which allowed for increased enzymatic activity after a thermal challenge. This is the first study to perform a thermal challenge on *Is*PETase with incubation at high temperatures for only 15 minutes—most other research (Son et al., 2019) has assessed *Is*PETase's activity when incubated at a high temperature for anywhere from 24 to 72 hours.

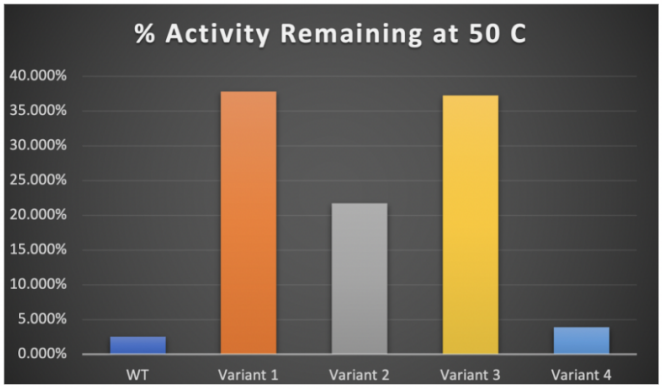

Figure 9. % activity retained from incubation at 30 °C to 50 °C

Objective 5: Analysis of the degradation activity of *Is*PETase and related variants towards commercial PET substrates

To model enzymatic activity using a PET substrate, *Is*PETase variants were incubated with 1" discs at room temperature for 24 hours, and the mass of the discs before and after incubation was determined. As exhibited in figure 10, variant 1 records substantial weight loss over 24 hours, indicative of the enzymatic degradation of PET film. Variants 2, 3, and 4 do not show any substantial signs of weight loss over 24 hours.

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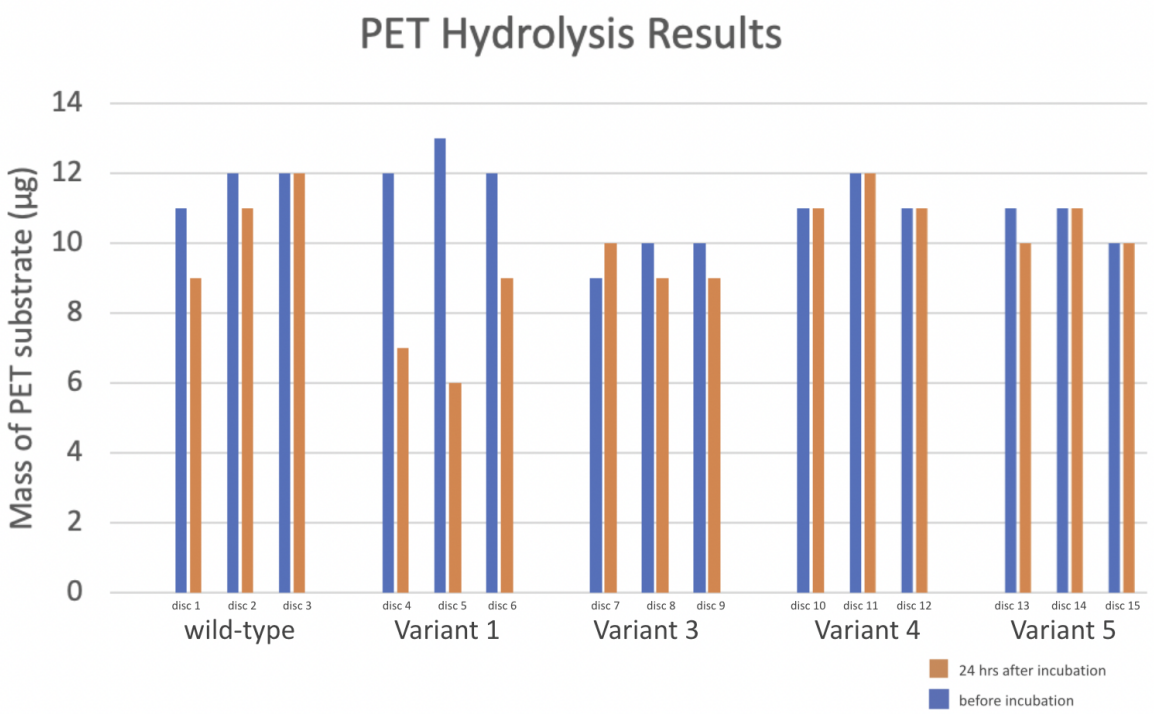

Figure 10. Mass (µg) of PET substrate before and after incubation with the *Is*PETase variants for 24 hours. Assay was completed in triplicate.

Our study used a novel computational algorithm to develop *Is*PETase variants with improved thermal stability so as to contribute towards improvements to plastic waste recovery strategies. First, using the Protein Repair One-Stop Shop, I was the first to develop unique *Is*PETase variants that incorporated all of the mutations proposed by PROSS. Moreover, this was the first study to verify that the PROSS algorithm is most effective when all mutations are introduced as opposed to a single point mutation. In particular, *Is*PETase variants 1 and 3 demonstrated substantially higher thermal stability and subsequently higher PET degradation activity in comparison with those of wild-type *Is*PETase.

Discussion

Although polyethylene terephthalate's high durability is a great asset from an industrial standpoint, its durability has proven to be a major environmental burden, placing an enormous strain on the ecosystem. Even after plastic is physically broken down by natural processes such as weathering and erosion, it continues to pose a threat to marine life at the microplastic level and eventually propagates up the food chain. Thus, the biodegradation of polyethylene terephthalate is an important research focus, especially for uncollectible microplastics. To this end, the seminal discovery of *Is*PETase inspired numerous research groups (Yoshida et al., 2019) to investigate the catalytic mechanisms behind PET degradation and ways to increase the stability of the enzyme. Although combining random mutagenesis with high-throughput screening has proven to be a successful strategy for the modification of enzyme properties, a long-sought alternative to screening-based approaches is reliable in silico design of

performance-enhancing mutations, especially for the degradation of insoluble solid synthetic polymers. Thus, The Protein Repair One-Stop Algorithm proposed in this study represents a huge step forward in the computational enzyme design because it standardizes mutation design with a speedy turnover time. This is the first study to implement all of the mutations proposed by PROSS, and the generated variants demonstrate a remarkably higher thermal stability and efficiency than those generated by Rennison et al. (2019)—the first group to partially implement this algorithm. Furthermore, variant 4, which did not introduce all of the mutations proposed by PROSS, exhibited the lowest melting temperature of the variants generated, further highlighting the effectiveness of implementing all mutations proposed by PROSS.

In summary, this work shows that a collection of subtle variations identified by in silico approaches with minimal experimental screening provides clues regarding how to design a PET hydrolase to improve its incorporation of semicrystalline aromatic polyesters. We believe that the proposed PROSS strategy constitutes a significant advance in enzyme design methodology that is complementary to traditional rational design strategies. The variants designed here by the PROSS strategy serve as a useful catalyst for efficient PET degradation at moderate temperatures and open up avenues for research in decreasing environmental plastic accumulation. Despite the aforementioned achievements, complete degradation of plastic waste still presents a number of challenges. There is an urgent need to continue running the PROSS software to analyze the outputs and develop a variant of *Is*PETase that is further optimized.

Conclusion

Additional measures to eliminate plastic waste are urgently needed. While the enzymatic depolymerization of plastic polymers offers a potential scalable solution, PET hydrolase enzymes must be further optimized for stability and activity before their widespread implementation. The Protein Repair One-Stop Shop (PROSS) algorithm offers an efficient and highly effective mutation design algorithm for thermal stability optimization. This approach is very promising, and this is the first study to verify the effectiveness of implementing multiple mutations proposed by PROSS. The *Is*PETase variants generated in this study demonstrate some of the highest thermal stabilities ever reported in this field. Overall, our novel research sparks much hope. It not only serves as a major breakthrough in the biochemistry field, but it also brings us one step closer to developing a widespread solution to the pressing issue of plastic waste.

References

Andrady A.L., Neal M.A. Applications and societal benefits of plastics. *Philos. Trans. R. Soc. B Biol. Sci.* 2009;364:1977–1984. doi: 10.1098/rstb.2008.0304.

Austin H.P., Allen M.D., Donohoe B.S., Rorrer N., Kearns F., Silveira R.L., Pollard B.C., Dominick G., Duman R., El Omari K., et al. Characterization and engineering of a plastic-degrading aromatic polyesterase. *Proc. Natl. Acad. Sci. USA.* 2018;115:E4350–E4357. doi: 10.1073/pnas.1718804115.

Cadee, G.C., 2002. Seabirds and floating plastic debris. Mar. Pollut. Bull. 44 (11), 1294-1295

Chen, C.C., Han, X., Ko, T.-P., Liu, W. and Guo, R.-T. (2018), Structural studies reveal the molecular mechanism of PETase. FEBS J, 285: 3717-3723. https://doi.org/10.1111/febs.14612

Chen, C.C., Han, X., Li, X. *et al.* General features to enhance enzymatic activity of poly(ethylene terephthalate) hydrolysis. *Nat Catal* 4, 425–430 (2021). https://doi.org/10.1038/s41929-021-00616-y

Chen, Z., Wang, Y., Cheng, Y., Wang, X., Tong, S., Yang, H., & Wang, Z. (2020). Efficient biodegradation of highly crystallized polyethylene terephthalate through cell surface display of bacterial PETase. *The Science of the total environment*, *709*, 136138. https://doi.org/10.1016/j.scitotenv.2019.136138

Cui, Y. et al. Computation redesign of a PETase for plastic biodegradation under ambient conditions by the GRAPE strategy. *ACS Catal.* https://doi.org/10.1021/acscatal. 0c05126 (2021).

Engler C, Gruetzner R, Kandzia R, Marillonnet S. 2009. Golden Gate Shuffling: A One-Pot DNA Shuffling Method Based on Type IIs Restriction Enzymes. PLoS ONE 4:e5553.

Fowler, C.W., 1987. Marine debris and northern fur seals: a case study. Mar. Pollut. Bull. 18 (6), 326-335.

George, N. & Kurian, T. Recent developments in the chemical recycling of postconsumer poly(ethylene terephthalate) waste. *Ind. Eng. Chem. Res.* 53, 14185–14198 (2014).

Geyer, B., Lorenz, G. & Kandelbauer, A. Recycling of poly(ethylene terephthalate)—a review focusing on chemical methods. *Express Polym. Lett.* 10, 559–586 (2016).

Geyer, R., Jambeck, J.R., Law, K.L. (2017). Production, use, and fate of all plastics ever made. *Sci. Adv.* 3, e1700782

Gregory, M.R., Andrady, A.L. Plastics in the marine environment. *Plastics and the Environment*, ed AL Andrady (Wiley, New York), pp. 379–402 (2003).

Han, X., Liu, W., Huang, JW. *et al.* Structural insight into catalytic mechanism of PET hydrolase. *Nat Commun* 8, 2106 (2017). https://doi.org/10.1038/s41467-017-02255-z

Inderthal,H., Tai, S.L & Harrison, S.T.L. Non-hydrolyzable plastics–an interdisciplinary look at plastic bio-oxidation. *Trends Biotechnol.* 39, 12-23 (2021).

Jenkins S., Quer A.M.I., Fonseca C., Varrone C. Microbial degradation of plastics: New plastic degraders, mixed cultures and engineering strategies. *Soil Microenviron. Bioremediation Polym. Prod.* 2019:213–238. doi: 10.1002/9781119592129.ch12.

Joo S., Cho I.J., Seo H., Son H.F., Sagong H.-Y., Shin T.J., Choi S.Y., Lee S.Y., Kim K.-J. Structural insight into molecular mechanism of poly(ethylene terephthalate) degradation. *Nat. Commun.* 2018;9:1–12. doi: 10.1038/s41467-018-02881-1.

Knott, Brandon C., Erickson, Erika, Allen, Mark D., Gado, Japheth E., Graham, Rosie, Kearns, Fiona L., Pardo, Isabel, Topuzlu, Ece, Anderson, Jared J., Austin, Harry P., Dominick, Graham, Johnson, Christopher W., Rorrer, Nicholas A., Szostkiewicz, Caralyn J., Copié, Valérie, Payne, Christina M., Woodcock, H. Lee, Donohoe, Bryon S., Beckham, Gregg T., & McGeehan, John E. *Characterization and engineering of a two-enzyme system for plastics depolymerization*. United States. https://doi.org/10.1073/pnas.2006753117

Lebreton, L., Andrady, A., (2019). Future scenarios of global plastic waste generation and disposal. *Palgrave Commun.* 5, 6

Lu, H., Diaz, D.J., Czarnecki, N.J. *et al.* Machine learning-aided engineering of hydrolases for PET depolymerization. *Nature* 604, 662–667 (2022). https://doi.org/10.1038/s41586-022-04599-z

Mallory, M.L., 2008. Marine plastic debris in northern fulmars from the Canadian high Arctic. Mar. Pollut. Bull. 56, 1501–1504.

Muñoz Meneses, R. A., Cabrera-Papamija, G., Machuca-Martínez, F., Rodríguez, L. A., Diosa, J. E., & Mosquera-Vargas, E. (2022). Plastic recycling and their use as raw material for the synthesis of carbonaceous materials. *Heliyon*, *8*(3), e09028. https://doi.org/10.1016/j.heliyon.2022.e09028

Palm, G.J., Reisky, L., Böttcher, D. *et al.* Structure of the plastic-degrading *Ideonella sakaiensis* MHETase bound to a substrate. *Nat Commun* 10, 1717 (2019). https://doi.org/10.1038/s41467-019-09326-3

Polyethylene Terephthalate (PET): Production, Price, Market and its Properties. Available at https://www.plasticsinsight.com/resin- intelligence/resin-prices/polyethylene-terephthalate/

Rennison A, Winther JR, Varrone C. Rational Protein Engineering to Increase the Activity and Stability of *Is*PETase Using the PROSS Algorithm. Polymers (Basel). 2021 Nov 10;13(22):3884. doi: 10.3390/polym13223884. PMID: 34833182; PMCID: PMC8621346.

Rios, L.M., Moore, C., 2007. Persistent organic pollutants carried by synthetic polymers in the ocean environment. Mar. Pollut. Bull. 54 (8), 1230– 1237.

Rustagi, N., Pradhan, S.K., Singh, R. Public health impact of plastics: An overview. Indian J Occup Environ Med. 2011
Sep;15(3):100-3. doi: 10.4103/0019-5278.93198. PMID: 22412286; PMCID: PMC3299092.

Son, H. F.; Cho, I. J.; Joo, S.; Seo, H.; Sagong, H. Y.; Choi, S. Y.; Lee, S. Y.; Kim, K. J. Rational Protein Engineering of Thermo-stable PETase from Ideonella Sakaiensis for Highly Efficient PET Degradation. ACS Catal. 2019, 9, 3519−3526.

Taniguchi I., Yoshida S., Hiraga K., Miyamoto K., Kimura Y., Oda K. Biodegradation of PET: Current status and application aspects. *ACS Catal.* 2019;9:4089–4105. doi: 10.1021/acscatal.8b05171

Tiseo I. Global PET bottle production 2004–2021. *Statista.* 2021

Tomas, J., Guitart, R., 2002. Marine debris ingestion in loggerhead sea turtles, Caretta caretta, from the Western Mediterranean. Mar. Pollut. Bull. 44 (3), 211– 216.

Tournier, V. et al. An engineered PET depolymerase to break down and recycle plastic bottles. *Nature* 580, 216-229 (2020).

Urbanek A.K., Kosiorowska K.E., Mirończuk A.M. Current Knowledge on Polyethylene Terephthalate Degradation

by Genetically Modified Microorganisms. Front Bioeng Biotechnol. 2021 Nov 30;9:771133. Doi: 10.3389/fbioe.2021.771133. PMID: 34917598; PMCID: PMC8669999.

Wei, T.; Zimmerman, W. Biocatalysis as a green route for recycling the recalcitrant plastic polyethylene terephthalate. Micro. Biotechnol. 2017, 10, 1302-1307.

Worm, B., Lotze, H.K., Jubinville, I., Wilcox, C. & Jambeck, J. Plastic as a persistent marine pollutant. *Ann. Rev. Env. Resourc.* https://doi.org/10.1146/annurev-environ-102016-060700 (2017).

Yoshida, S. et al. A bacterium that degrades and assimilates poly(ethylene terephthalate). *Science* 351, 1196–1199 (2016).