Received: 9 October 2021 | Revised: 25 November 2021 | Accepted: 26 November 2021

DOI: 10.1049/enb2.12018

Engineering Biology

The Institution of Engineering and Technology WILEY

REVIEW

The influences of substrates' physical properties on enzymatic PET hydrolysis: Implications for PET hydrolase engineering

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Funding information

National Research Foundation Singapore, Grant/Award Number: NRF-CRP22-2019-0005

Abstract

Plastic pollution in diverse terrestrial and marine environments is a widely recognised and growing problem. Bio-recycling and upcycling of plastic waste is a potential solution to plastic pollution, as these processes convert plastic waste into useful materials. Polyethylene terephthalate (PET) is the most abundant plastic waste, and this material can be degraded by a class of recently discovered bacterial esterase enzymes known as PET hydrolases (PETase). Investigations of the enzymatic hydrolysis of diverse PET molecules have clearly revealed that the biodegradability of various PET substrates depends on both their chemical structure and physical properties, including polymer length, crystallinity, glass transition temperature, surface area, and surface charge. This review summarises the known impacts of crystallinity and other physical properties on enzymatic PET hydrolysis.

KEYWORDS

biodegradability, crystallinity, *Is*PETase, PET hydrolase, polyethylene terephthalate

The ever-increasing demand, indiscriminate production, and widespread use of single-use plastic items have created a worldwide pollution problem, with growing amounts of plastic waste accumulating in landfills and in natural environments including marine and terrestrial ecosystems. Whilst resistance to degradation is an advantage in certain applications, it is a major cause of environment pollution as plastic wastes persist and accumulate. Studies have estimated that around 33 billion tons of discarded plastics will have accumulated on Earth by 2050 [1]. Polyethylene terephthalate (PET) is the most abundant plastic with an estimated global production of 70 million tons annually [2], and is widely used in disposable containers for varieties of soft drinks, juices, and drinking water. Like other plastics, PET is highly resistant to degradation, and the accumulation of PET waste in the environment is understood as a global problem with overriding implications that requires immediate and large-scale interventions. Accordingly, substantial efforts have been focussed on recycling PET waste. Thermal, chemical, and mechanical processes have been developed for this purpose, each with its pros and cons.

PET biodegradation has been proposed in recent years as an eco-friendly recycling opportunity, particularly in light of the exciting discoveries of PET hydrolase enzymes [3–6]. Viable green industrial bioprocesses for plastic recycling are closer to realisation, with rapid advancement of research and development on enzymatic PET hydrolysis [2, 7–9]. Enzyme engineering has been employed to overcome the limited catalytic efficiency of native PET hydrolase enzymes [7, 10–13]. To date, most reported studies have focussed on the discovery of new PET hydrolase enzymes, understanding the enzyme structure and reaction mechanisms, enzyme engineering efforts, and bioprocess optimisation (for both native and engineered enzymes). Compared to the relatively extensive research attention given to the protein structures and reaction mechanisms of PET hydrolase enzymes, considerably fewer studies have investigated how the physical properties of PET substrates impact the degradation process. The few reports that have investigated the effects of pre-treatment and alteration of substrates' physical properties show promising results. Table 1 summarises PET hydrolysis reactions by PET hydrolases (PETase) and cutinase enzymes. This report reviews the literatures investigating the influence of physical parameters of PET substrates—specifically crystallinity and particle size—on PET hydrolase performance. It also discusses pre-treatments

TABLE 1 Summary of selected enzymatic PET hydrolysis reactions

References	Enzyme	Mutations	Reaction condition	Substrate			
			pH/Temp/Time	Crystallinity (%)	Physical form	Pre-treatment	PET degradation (%) or comments
Austin *et al.* 2018 [7]	*Is*PETase	S238F/W159H	pH 7.2/30°C/96 h	13.3%	Film	-	Crystallinity decreased.Surface erosion observed in SEM.
Cui *et al.* 2021 [14]	DuraPETase	S214H/I168R/W159H/S188Q/R280A/A180I/G165A/Q119Y/L117F/T140D	pH 9.0/37°C/10 days	30%	Film	-	15%
			pH 9.0/37°C/1 h	ND	Nanoparticle (50–100 nm)	-	100%
			pH 9.0/37°C/2 weeks	ND	Microparticle (0.1–1 mm)	-	100%
Furukawa *et al.* 2018 [15]	*Is*PETase	-	pH 9.0/30°C/36 h	3%–5%	Film	Surfactant	Surfactant treatment improved *Is*PETase activity 120-fold. 22% decrease of film thickness
Furukawa *et al.* 2019 [16]	TfCut-2	-	pH 9.0/65°C/48 h	3%–5%	Film	Surfactant	13-fold faster reaction in the presence of cationic surfactant.
Meng *et al.* 2021 [17]	*Is*PETase	W159H/F229Y	pH 9.5/40°C/24 h	Amorphous	Amorphised Coca Cola bottle	Dissolved in 1,1,1,3,3,3-hexafluoro-2-propanol	40-fold increase of degradation activity compared to WT
				10%	Coca cola film	Melted with alcohol lamp and cooled down	16.5%.
Puspitarasari *et al.* 2021 [18]	*Is*PETase	-	pH 8.0/30°C/5 days	38.8%	Semi-crystalline PET fibre	Class I hydrophobins	18% without pre-treatment; Up to 34.6% with pre-treatment
				64.8%	High-crystalline PET bottle powder	-	17.3% without pre-treatment; Up to 29.2% with pre-treatment
Ronkvist *et al.* 2009 [19]	HiC, PmC, FsC		pH 8.0/80°C/96 h for HiCpH 8.0/50°C/96 h for PmCpH 8.0/40°C/96 hors for FsC	7%	Low-crystalline PET film	-	HiC, PmC and FsC showed 25-, 10-, and 6-fold higher initial activities, respectively, on the lcPET substrate than that on the boPET
				35%	Biaxially oriented PET film		
Shi *et al.* 2021 [20]	*Is*PETase	Secreted	pH 9.0/30°C/48 h	ND	PET powder	-	1117 μM degradation product obtained in 1 ml reaction using 5 mg PET powder
			pH 9.0/30°C/7 days	Amorphous	PET film		Surface erosion observed in SEM
Shirke *et al.* 2018 [21]	LCC	Glycosylated	pH 8.0/70°C/48 h	7%	lcPET film	-	95%
Son et. al. 2019 [22]	*Is*PETase	S121E/D186H/R280A	pH 9.0/40°C/72 h	41%	PET film	-	14-fold higher activity compared to wild type
Son *et al.* 2020 [23]	*Is*PETase	S121E/D186H/S242T/N246D	pH 9.0/37°C/20 days	-	PET bottle film	-	58-fold higher activity compared to wild type
Tournier *et al.*	LCC	WCCG	pH 8.0/72°C/15 h	Amorphous	Micronised PET waste	-	85%
		F243I/D238C/S283C/Y127G	pH 8.0/72°C/20 h				82%

TABLE 1 (Continued)

References	Enzyme	Mutations	Reaction condition	Substrate			PET degradation (%) or comments
			pH/Temp/Time	Crystallinity (%)	Physical form	Pre-treatment	
2020 [2]		F243W/D238C/S283C/Y127G	pH 8.0/72°C/20 h				53%
		-	pH 8.0/65°C/3 days	10%	Bottle-grade PET powder		31%
Vertommen *et al.* 2005 [24]	FsC	-	pH 8.0/30°C/5 days	4.1%	Amorphous PET film	-	10-fold more degradation product was obtained from the amorphous PET than that from the granular PET
				13.7%	Granular PET		
				48.2%	Biaxially oriented PET film		Very small amount of degradation products was detected.

Abbreviations: FsC, *Fusarium solani Cutinase*; HiC, *Humilica insolens Cutinase*; PET, polyethylene terephthalate; PmC, *Pseudomonas mendocina Cutinase*.

for PET substrates and considers their impacts on enzymatic PET degradation.

Enzymatic hydrolysis of post-consumer PET materials is extremely difficult due to their semi-crystalline nature. Like other polymers, PET has complex structures comprising both crystalline and amorphous regions. PET can exist in an amorphous or semi-crystalline state but not in a 100% crystalline form [25] (Figure 1). The amorphous regions are relatively flexible due to a disordered array of the polymer chains whilst the crystalline regions have extremely rigid chemical structures made up of closely packed parallel polymer chains [26]. However, chain mobility increases dramatically above glass transition temperature (Tg), a temperature where the polymer experiences transition from a rigid state to a more flexible state. Above the Tg, PET substrates are more susceptible to enzymatic hydrolysis as they become more accessible to the PET hydrolase enzymes. The Tg of most post-consumer PET is ~70°C, and performing PET hydrolysis above this temperature could potentially be a game changer with respect to efficiency [21]. Significant research effort is focussed on finding thermostable enzymes for PET degradation at high temperatures, although energy efficiency and sustainability of such processes is yet to be evaluated [2, 3, 16, 22]. In addition, PET substrates have been pre-treated to reduce their crystallinity, which also enhances their accessibility to the enzyme [2, 14, 17].

Multiple studies report enzymatic hydrolysis using PET substrates with a wide range of crystallinities. However, direct comparison of the results is not straightforward, as these studies were carried out using different reaction conditions and reaction times (varying from hours to several weeks) [2, 14, 15, 22, 23]. The general trend suggests that enzymatic hydrolysis is enhanced with decreased crystallinity of PET substrates, concurring with the relatively flexible polymer chains in amorphous PET compared to the closely packed and extremely rigid chemical structure of the crystalline regions. To date, the vast majority of studies use PET films but the most efficient enzymatic hydrolyses are reported with PET microparticles and nanoparticles, which usually have little or no crystallinity [2, 14].

Tournier *et al*. have achieved the highest efficiency and the fastest depolymerisation of post-consumer PET waste reported to date. They have shown 90% conversion of amorphised and micronised (micro-size PET particles as a result of a process to which the plastic films/bottles are broken down) post-consumer PET over 10 h reaction at 72°C using an engineered cutinase enzyme. Although the engineered enzyme showed higher thermostability and superior activity over the wild-type enzyme, micronisation and amorphisation contributed to the results. Interestingly, the wild-type enzyme took 3 days to convert 31% of a PET substrate with only 10% crystallinity at 65°C. The authors also noted fast re-crystallisation of the amorphised PET at higher temperature, reaching up to 37.5% crystallinity within 6 h at 75°C. This could be a major consideration in designing PET degradation studies at high temperatures closer to the glass transition of PET. The authors also indicated that

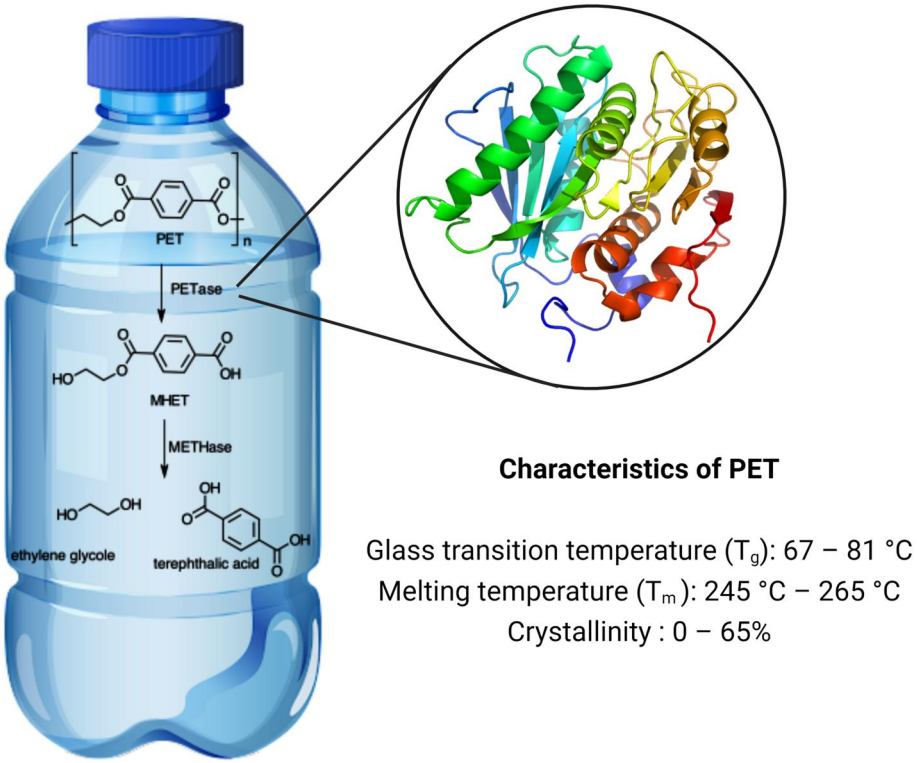

FIGURE 1 Polyethylene terephthalate (PET) biodegradation is catalyzed by the enzyme PETase (represented by PDB ID 6ILW). The activity is influenced by the physical characteristics of the PET substrates including glass transition temperature, crystallinity, and particle formats and sizes (e.g., bulk, films, microplastics, nanoplastics)

micronisation is useful for large-scale enzymatic PET recycling as it enhances the accessibility of substrate to the enzyme by increasing the surface area, thus accelerating the reaction rate.

In another interesting study, Cui *et al.* showed that decreased crystallinity and decreased particle size have been shown to enhance the efficiency of enzymatic PET hydrolysis. They reported 100% degradation of PET microplastics (0.1–1 mm diameter) and nanoplastics (50–100 nm diameter) using a thermostable *Is*PETase variant named 'DuraPETase' that was developed using a computation-based protein engineering tool [14]. The complete degradation of PET nanoplastics was achieved within one hour compared to 2 weeks for degrading the microplastics using the wild-type enzyme. However, the same enzyme hydrolysed only 15% of PET film (8 mm coupon diameter) with 30% crystallinity over a 10-day reaction. All reactions were carried out at 37°C, despite the DuraPETase variant retaining activity at much higher temperatures. The thermostability allows for longer reaction time at the milder temperature. The results clearly suggest that crystallinity and particle size play important roles in the efficiency of enzymatic PET hydrolysis. Another mutant *Is*PETase designed using a bioinformatic approach showed a 10.4°C higher melting temperature than that of the wild-type enzyme [17]. The mutant enzyme was 40-fold more active than the wild-type on the amorphised PET bottle, and it was able to degrade >16% of a pre-treated Coca Cola bottle film with crystallinity reduced from 29.2% to 10.7%. However, the authors did not mention degradation of untreated bottle by the enzyme, presumably due to inactivity of the enzyme on this substrate. Puspitasari *et al.* compared enzymatic degradation of semi-crystalline PET fibres and high-crystalline PET powder using the *Is*PETase enzyme with and without hydrophobin treatment. The results showed significant increase in enzymatic hydrolysis of both PET substrates when pre-treated with hydrophobins; a higher conversion was achieved for PET fibres compared to PET powder substrate [18]. Although the powder had a higher surface area, the authors attributed higher conversion of PET fibres to their lower crystallinity and concluded that enzymatic PET hydrolysis is influenced more by crystallinity than the surface area of the substrate.

The influence of crystallinity on cutinase-catalysed PET hydrolysis has been directly compared using low-crystallinity PET (lcPET, 7% crystallinity) and biaxially oriented PET (boPET, 35% crystallinity) substrates, and three different cutinases from *Humilica insolens* (HiC), *Pseudomonas mendocina* (PmC), and *Fusarium solani* (FsC) [19]. About 10-fold higher enzymatic hydrolysis was observed on the lcPET compared to the boPET substrate, although the optimum reaction conditions and catalytic efficiencies vary from one enzyme to another. HiC, PmC and FsC showed 25-, 10-, and 6-fold higher initial activities, respectively, on the lcPET substrate over boPET. In another study, higher hydrolytic activity of FsC towards amorphous PET was demonstrated using three PET substrates: amorphous PET film (4.1% crystallinity), granular PET (13.7% crystallinity) and biaxially oriented PET film (48.2% crystallinity) [24]. Very small amounts of

degradation products were detected from the biaxially oriented PET after a 5-day reaction at 30°C temperature and pH 8.0. In similar reaction settings, 10-fold more enzymatic hydrolysis of amorphous PET was achieved compared to granular PET despite the higher surface area of the latter. This observation also suggests that crystallinity plays a more important role in enzymatic PET hydrolysis than surface area.

Furukawa *et al*. demonstrated improved enzymatic hydrolysis of low-crystalline (3%–5% crystallinity) and high-crystalline (40% crystallinity) PET substrates after pre-treatment with surfactants [15, 16]. Binding of anionic (TfCut2) and cationic (*Is*PETase) enzymes on the PET film surface was improved by pre-treatment with cationic and anionic surfactants, respectively, which resulted in significant improvement of enzymatic PET hydrolysis. Improvement of *Is*PETase activity by 120-fold and 13-fold for TfCut2 was achieved for degradation of low-crystalline PET. Activities also improved for high-crystalline PET degradation but to a lower extent. For both enzymes, PET degradation rate for low-crystalline PET was >100-fold higher than high-crystalline PET irrespective of the surfactant treatment.

In contrast to the above-mentioned studies, many others have reported that enzymatic PET hydrolysis using commercial PET films without any significant pre-treatment results in very little degradation [7, 10, 12]. Austin *et al*. reported an *Is*PETase mutant (S238F/W159H) with a narrower cleft at its active site that showed increased substrate affinity and improved PET degradation compared to wild-type PETase [7]. However, the studies were carried out using a PET coupon with 14.8% crystallinity and the activity was expressed in terms of '% crystallinity change' and supported by SEM images. Notably, identical mutations have been reported in other studies, supporting the functional relevance of these mutations [10, 11]. Shirke *et al*. achieved 95% weight loss of a low-crystalline PET film (7% crystallinity) using a leaf and branch compost cutinase (LCC) variant while Sulaiman *et al*. (2014) reported ~ 30% weight loss of another PET film (crystallinity not mentioned) using the wild-type LCC enzyme. Shi *et al*. (2021) used a combination of SEM imaging of PET film and quantification of PET powder degradation products to characterise PET depolymerisation by a secreted recombinant *Is*PETase enzyme. Son *et al*. developed thermostable *Is*PETase variants and reported several mutant enzymes [22]. The S121E/D186H/R280A mutant showed an 8.81 °C higher Tm value and 14-fold higher activity towards 41% crystalline PET film over the wild-type enzyme. The quadruple mutant S121E/D186H/S242T/N246D showed 1 °C higher Tm over the triple mutant S121E/D186H/R280A and 58-fold higher activity for PET bottle hydrolysis compared to the wild-type enzyme.

PET hydrolysis is especially challenging owing to its extreme resistance to both biotic as well as abiotic degradation [27]. The resistance stems from its physical properties which can be abated by various pre-treatment processes that alter the physical properties of the material, making it more susceptible to enzymatic degradation. Micronisation, amorphisation, surface-coating, and/or partial depolymerisation by physicochemical pre-treatment are promising, but challenges associated with the pre-treatment process (including energy efficacy, cost effectiveness, and viability in large scale) need to be carefully assessed when considering the economic viability of PET bio-recycling. The quest to find the optimal PET degrading enzymes is rapidly evolving with primary focus on enzyme engineering for improved degradation of PET plastics. However, efforts towards identifying PET substrate morphologies and green pre-treatment processes are lacking. Current evidence suggests that particle properties such as crystallinity, surface area, and surface charge contribute significantly to PET degradation. The path towards the development of industrially viable biodegradation platforms will, therefore, require a unison between the highly efficient enzymes and optimized pre-processing steps to overcome the inherent recalcitrance of PET.

ACKNOWLEDGEMENT

This publication is supported by the National Research Foundation, Singapore, under its Competitive Research Programme (Award# NRF-CRP22-2019-0005).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

PERMISSION TO REPRODUCE MATERIALS FROM OTHER SOURCES

None.

DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this study as no new data were created or analysed in this study.

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How to cite this article: Pasula, R.R., et al.: The influences of substrates' physical properties on enzymatic PET hydrolysis: implications for PET hydrolase engineering. Eng. Biol. 6(1), 17–22 (2022). https://doi.org/10.1049/enb2.12018