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Perspective

The Current State of Research on PET Hydrolyzing Enzymes Available for Biorecycling

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Abstract: This short paper reviews two groups of enzymes designated as polyethylene terephthalate (PET) hydrolases: one consists of thermophilic cutinases from thermophilic microorganisms (actinomycetes and a fungus) and the other consists of mesophilic cutinases, the representative of which is *Is*PETase from a mesophilic bacterium. From the viewpoint that PET hydrolysis requires a high temperature close to the glass transition temperature (65–70 °C in water) of PET, mesophilic cutinases are not suitable for use in the enzymatic recycling of PET since their degradation level is one to three orders of magnitude lower than that of thermophilic cutinases. Many studies have attempted to increase the thermostability of *Is*PETase by introducing mutations, but even with these modifications, the mesophilic cutinase does not reach the same level of degradation as thermophilic cutinases. In addition, this kind of trial contradicts the claim that *Is*PETase works at ambient temperature. As plastic pollution is an urgent environmental issue, scientists must focus on feasible thermophilic enzymes for the enzymatic processing of disposed PET, rather than on mesophilic cutinases. Thermophilic and mesophilic cutinases must be evaluated precisely and comparatively, based on their features that enable them to hydrolyze PET, with the aim of enzymatic PET disposal. The level of thermophilic cutinases has already reached their optimal level in PET biorecycling. The optimal level may be reached through the processing of PET waste, by amorphization and micronization into readily hydrolysable forms and the improvement of PET hydrolases by engineering higher degradation ability and low-cost production. Here I summarize the critical points in the evaluation of PET hydrolases and discuss the biorecycling of PET.

Keywords: PET; PET degradation; PET hydrolase; cutinases; thermophilic PET hydrolase; mesophilic PET hydrolase

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1. Introduction

Plastic pollution is becoming increasingly obvious and, together with other environmental matters such as global warming, air and water pollution, deforestation, is attracting serious concern globally. The cumulative output of plastic is 8300 million tons (1950–2015) [1], and the annual production of plastic continues to increase every year (359 million tons in 2018) [2]. It is an emerging crisis that the total amount of plastic debris in the ocean will exceed the total amount of fish (800 million tons) by 2050 [3]. Another important issue in plastic pollution is the formation of microplastics, which enter the stomach of birds and fishes and circulate via the food chain until they are ingested by humans. The major plastics are high- and low-density polyethylene, polypropylene, polystyrene, polyvinylchloride, polyurethane and polyethylene terephthalate (PET). PET accounts for approximately 5% of the total plastic production. Among the major plastics, polyurethane (ester and urethane bonds) and PET have hydrolysable chemical structures; however, other types of plastic have carbon-chain backbone structures that are resistant to hydrolysis. Ester bonds are readily cleaved by only one enzyme, leading to fragmentation into small molecules. In contrast, carbon-chain structures require several enzymatic steps for fragmentation. Therefore, biological recycling is expected to be achievable for PET.

The first report on a PET hydrolase, poly(butylene terephthalate-*co*-adipate) (BTA)-hydrolase from *Thermobifida fusca*, dates back approximately 15 years [4]. Since then, various thermostable PET hydrolases and their homologs from the cutinase group EC. 3.1.1.74 (a subgroup of carboxyl ester hydrolases [EC 3.1.1]), have been cloned from thermophilic actinomycetes such as *T. alba* [5], *T. cellulosilytica* [6], *Thermomonospora curvata* [7], and *Saccharomonospora viridis* (Cut190) [8], and from leaf compost (LC-cutinase), which consists of a mixture of various thermophilic actinomycetes. LC-cutinase was identified using a metagenomic approach [9]. A commercialized cutinase (HiC) from the thermophilic fungus, *Humicola insolens*, shows high activity as a PET hydrolase [10]. The search for PET hydrolases is based on the recognition that PET can be well hydrolyzed at temperatures higher than its glass transition temperature, which is approximately 70–80 °C in air, but approximately 10 °C lower in water, where the polymer chains become flexible, thereby making PET hydrolases thermophilic. However, Yoshida et al. [11] isolated a mesophilic bacterium (*Ideonella sakaiensis*) that can grow on an amorphous PET film as a major carbon source at 30 °C. The authors were of the opinion that the activity of *Is*PETase was highest at 30 °C, in contrast to previously described thermophilic PET hydrolases from actinomycetes, which potentially makes *Is*PETase the best enzyme for the decomposition of PET waste. If confirmed, this would be a substantial advantage that would potentially make *Is*PETase the preferred hydrolase for PET waste decomposition. This position has been widely accepted and disseminated through mass communication media, without any independent verification by scientists. Regarding *Is*PETase, some objections were submitted as e-letters to Science [12], but there has been no response from the authors or followers to date. Additionally, Wei et al. [13] compared the degradation rates of LC-cutinase and *Is*PETase at their respective optimal temperatures (24 h at 70 °C and 30 °C, respectively). The degradation rate of LC-cutinase was more than 40%, whereas that of *Is*PETase was only 1%. It is generally recognized that a 10 °C increase in temperature in chemical reactions results in twice the activity. However, as *Is*PETase is heat-labile, the enzyme loses its activity after 1 h at temperatures above 40 °C. Recently, Tournier et al. [14] reported the highest degradation rate to date (approximately 90% at pH 8.0 and 72 °C for 10 h) using variants of LC-cutinase. At the same time, they showed far lower activity of *Is*PETase at 40 °C (0.01 mg of terephthalic acid produced/h/mg of enzyme), compared with *Fusarium solani* cutinase (0.07) at 40 °C and LC-cutinase at 65 °C (93.2).

In this paper, I evaluate and compare thermophilic and mesophilic cutinases and discuss the enzymatic biorecycling of PET by PET-hydrolyzing enzymes.

2. Comparison of Thermophilic and Mesophilic Cutinases: Desirable Characteristics for the Enzymatic Recycling of Polyethylene Terephthalate (PET)

Here I focus on the definition of PET hydrolases. PET hydrolases have attracted research attention because of their feasible application in the functionalization of polyester textiles (practically PET textiles) by hydrophilization due to the production of carboxyl and hydroxyl groups [15]. For this purpose, only surface modification was expected, which would not damage the textile body. To date, versatile hydrolases have been reported to be useful for this purpose, as they do not require high temperatures and degradation is at trace levels, for example, much lower than 1%. These enzymes should be called as PET-modifying enzymes rather than PET-degrading enzymes. However, PET hydrolases used for biorecycling PET are expected to completely destroy the body of PET, by depolymerization to monomeric products such as bis(hydroxyethyl)terephthalate (BHET), monohydroxyethyl terephthalate (MHET) and terephthalate (TPA), thereby indicating that a higher degradation level is desirable, as seen with the LC-cutinase variants described above. A distinction between PET surface-modifying enzymes and PET body-degrading enzymes is needed to avoid confusion.

Taken together, the requirements for PET-hydrolyzing enzymes applicable to the biorecycling of PET are a greater quantity of degradation products in a shorter time (designated as the degradation level).

Thermophilic cutinases have been applied to the enzymatic recycling of PET, although these cutinases could also be used for surface modification at lower temperatures. The identification of *Is*PETase raised the possibility that mesophilic cutinase could be used for recycling PET [11]. Based on this illusion, there was an influx of publications on the X-ray crystallography of *Is*PETase at approximately the same time in 2017–2018, all of which were based on the belief that *Is*PETase is the best enzyme for PET hydrolysis [15,16]. Joo et al. [17] proposed a catalytic mechanism, suggesting that the docking conformation of 2-hydroxyethyl $(MHET)_4$ in the substrate binding cleft of *Is*PETase indicated its superiority over a representative thermophilic cutinase, TfCut2 from *Thermobifica fusca*, at 30 °C. However, the degradation level of *Is*PETase [11] was calculated to be only 0.23% [15], and its degradation ability was reproduced as 1% by two groups [13,14]. Moreover, this proposed mechanism at approximately 30 °C was disproved by Wei et al. [14]. However, some studies on *Is*PETase still do not compare its degradation levels with those of thermophilic cutinases and cite the mechanism proposed by Joo et al. [17].

*Is*PETase has recently suggested the presence of mesophilic enzymes homologous to PET hydrolases in the ecosystem. Following the report on *Is*PETase [11], Danso et al. identified 504 possible candidate PET hydrolase genes from various databases [18]. They indicated that Actinobacteria in the terrestrial environment and *Bacteroides* in the marine environment were the main hosts of PET hydrolase genes and Proteobacteria were the second most common hosts in both terrestrial and marine environments. Many *Is*PETase homologs, with greater than 65% identity, exist in mesophilic bacteria. Among these, poly(tetramethylene succinate) depolymerase from *Acidovorax delafieldii* strain BS-3 [19] has the highest identity (82%) with *Is*PETase [15]. The actinomycetes PET hydrolase group and the *Is*PETase homologue group are phylogenetically different, as suggested in several reports [15,17,18]. The exploitation of either new enzymes or new sources is useful for the future application of PET-hydrolyzing enzymes for PET biorecycling. Mesophilic *Streptomyses* and *Pseudomonas* species have been isolated and shown to have mesophilic enzymes that are homologous to *Is*PETase [20,21]. However, we need to keep in mind that structural homologs have neither the same activity as PET hydrolase, nor the hydrolyzing ability itself. It is questionable whether mesophilic enzymes or their mesophilic microbial hosts contribute significantly to the hydrolysis of PET in the ecosystem, because the overall temperatures in both the marine and terrestrial environments are lower than 30 °C. We cannot expect the roles of thermophilic enzymes and hosts in the ecosystem, either, because high temperatures at 60–70 °C are not feasible. We should not expect too much enzymatic hydrolysis of PET in the ecosystem, as the increase in PET waste far surpasses any biodegradation that may occur in the ecosystem. The desirable scope of our research is the selection of the enzymes suitable for PET recycling and the strategies to put them in practice for PET recycling from various viewpoints described below.

Most recently, a Chinese group newly cloned a thermostable PET hydrolase (BhrPETase) gene [22], which was annotated by metagenomic approach as a PET hydrolase present in a bacterium HR29 belonging to a thermophilic bacterial phylum, *Chloroflexi*, which was obtained following collection of a microbial mat sample from a subsurface geothermal stream and long-term cultivation at 65 °C and 70 °C [23]. BhrPETase has 94% sequence identity with LC-cutinase and has PET degradation ability slightly superior to that of LC-cutinase, but its product level is at 6 mM with PET powder. The activities of BhrPETase, LC-cutinase and *Is*PETase at 30–80 °C were compared. Both of the thermophilic enzymes showed an optimal temperature at 70 °C, whereas the optimal temperature of *Is*PETase was 40 °C.

Several studies have suggested that mutations can improve the activity of *Is*PETase. The degradation products of an amorphous PET film reacted with *Is*PETase variants are at the micromolar level, for example, 30 μM [17] and 120 μM [24], while those of thermostable enzymes are on the order of 10 mM at their optimal temperatures (65–70 °C) [15,16]. Examples of these enzymes are listed in Table 1. It is not easy to transform mesophilic enzymes into thermophilic enzymes that catalyze significant PET hydrolysis, as indicated by investi-

gations of several thermophilic enzymes [15,16]. Cui et al. indicated that the production level by *Is*PETase is in the µM order and that by DuraPETase mutated systematically from *Is*PETase is in the mM order (at most 4.3 mM at 40 °C) [25]. Above all, the thermostabilization of *Is*PETase is contrary to the original notion that *Is*PETase could be used for waste PET treatment at ambient temperature.

Table 1. Comparison of thermophilic and mesophilic polyethylene terephthalate (PET) hydrolases and their homologs.

Enzyme	Source	PET Degradation	Product Level	PET Used	Reaction Conditions	Reference
			Thermophilic cutinase			
TfH (BTA-1)	*Thermobifida fusca* DSM43793	≈50%		amorphous PET film or amorphous bottle	55 °C, 3 weeks	[4]
TfCut2 variant	*T. fusca* KW3	42%	49 mM	PET-GF film [a]	65 °C, 50 h	[26]
LC-cutinase	Metagenome from leaf branch compost	≤25%	28 mM	PET package [b]	70 °C, 24 h	[27]
LC-cutinase variant	Metagenome from leaf branch compost	90%	936 mM	amorphized and micronized PET	72 °C, 10 h	[14]
BhrPETase	thermophilic bacterium strain HR29		6.3 mM	PET powder	70 °C, 20 h	[22]
Cut190 variant	*Saccharomonospora viridis* AHK190	34% 59%	15 mM 29 mM	PET-GF film [a] PET package [b]	70 °C, 3 days 60 °C, 3 days	[28]
HiC (Novo)	*Humicola insolens*	97%	135 mM	PET-GF film [a]	70 °C, 4 days	[10]
Est 1	*T. alba* AHK119	Not confirmed				[29]
Tha_Cut1	*T. alba* DSM43185	Not confirmed				[30]
Thc_Cut2 variant	*T. cellusilytica* DSM44535		0.45 mM	PET film (not specified)	50 °C, 2 days	[31]
Tcur1278	*T. curvata* DSM43183			PET nanoparticles	50–60 °C	[7]
Thh_Est	*T. halotolerans* DSM44931	Not confirmed				[32]
			Mesophilic cutinases			
*Is*PETase	*Ideonella sakaiensis*	0.23%	0.3 mM	amorphous PET film	30 °C, 48 h	[11]
*Is*PETase DuraPETase (*Is*PETase variant)	*Ideonella sakaiensis*		12 µM 1.8 mM 3.4 mM	PET film	37 °C, 12 h [d] 40 °C, 72 h 40 °C, 10 days	[25]
PBS depolymerase	*Acidovorax delafieldii* strain BS-3	Not confirmed			30 °C	[19]
SM14est [c]	*Streptomyces* sp. SM14 (marine)	Not confirmed			28 °C	[20]
PE-H PE-H/Y250S	*Pseudomonas aestusnigri* (marine)		21 µM 31 µM	PET-GF film [a]	30 °C, 48 h	[21]

[a] amorphous (commercially available from Goodfellow Ltd.); [b] amorphous; [c] no disulfide bond; [d] the enzyme activity was completely lost thereafter.

Taken together, it is reasonable to conclude that *Is*PETase and its mutants show only limited ability at temperatures lower than the desired temperatures (greater than 65 °C). Unfortunately, the feasibility of using mesophilic cutinases has often not been evaluated or has not been compared with the use of thermophilic cutinases.

Due to the serious issue of plastic pollution, many review articles regarding PET hydrolases have been published in the last few years. However, some studies have treated

every enzyme equally as a PET hydrolase applicable to PET biorecycling, even though the degradation product levels of these enzymes range from trace amounts (μM) to high amounts (10–100 mM order). Can this be justified? As described at the beginning of this section, the distinction of enzymes based on their degradation levels is important, and their degradation levels of different enzymes need to be evaluated precisely.

3. Structural Insights into the Reaction Mechanism of PET-Hydrolyzing Enzymes

Crystallography of enzymes has contributed significantly to the understanding of enzymatic reaction mechanisms. The first crystallization of an actinomycetes cutinase was performed using *Streptomyces exfoliates* lipase [33]. Several crystal structures of aromatic polyesterases from actinomycetes have been solved, including Est119 of *T. alba* AHK119 [34], TfCut2 [35], LC-cutinase [27], Cut190 [36,37], and Thc_Cut1 and Cut2 [38]. These cutinases share approximately the same size and structure. Many research groups have also solved the crystal structure of *Is*PETase [15,16].

Interestingly, the overall crystal structures of actinomycetes cutinases and *Is*PETase overlap, with one of their major differences being the number of disulfide bonds they contain. While all PET hydrolases from actinomycetes and *Is*PETase share one disulfide bond at the same position, *Is*PETase has an additional disulfide bond, as shown in Figure 1. As *Is*PETase is heat-labile [11], the additional disulfide bond is not relevant to its thermostability. The thermostability of PET hydrolases from actinomycetes is reportedly increased by divalent cations such as Ca^{2+} and Mg^{2+} [5,27,39]. A mutant of Cut190, Cut190$^{S226P/R228S}$, designated Cut190*, has three Ca^{2+}-binding sites on the protein surface [28]. When a new disulfide bond is introduced at site 1, the T_m value of Cut190* increased by approximately 10 °C and the resulting mutant, Cut190*$^{Q138A/D250C-E296C}$, degrades amorphous PET considerably at 70 °C. This result is in accordance with the results of TfCut2 reported by Then et al. [40]. Tournier et al. [14] introduced a disulfide bond in the LC-cutinase at the same position as Cut 190* and TfCut2, which led to a higher T_m value. The binding of Ca^{2+} or the existence of a disulfide bond at site 1 contributes to the thermostabilization of PET hydrolases, which is different from the effect of a second disulfide bond in *Is*PETase (Figure 1). The following PET hydrolase complexes, with model substrates, have been solved: Cut190*complexes with ethyl succinate and ethyl adipate, which are partial structures of poly(butylene succinate-*co*-adipate) [37]; and Est119 complexes with butyl lactate and lactate [41]. The structures of the substrate-binding clefts of Cut190* and Est119 were found to be similar. Although the overall structures of actinomycetes cutinases are similar, their thermostabilities and degradation abilities (greatly relevant to thermostability) range from lower than 60 °C to higher than 70 °C and product levels from a few millimolar to over 100 mM. Therefore, subtle differences in their structures, due to differences in local amino acids such as polarity, hydrophobicity, and aromaticity differences, may have major effect on their properties.

Joo et al. [17], Austin et al. [42] and Chen et al. [43] found that *Is*PETase has a broader, more open active site cleft than actionomycetes cutinases, although their crystal structures overlapped well. Joo et al. [17] mutated *Is*PETase to match the sequence of actinomycetes cutinases (W159H and S238F), resulting in reduced activity toward an amorphous PET film. However, Liu et al. [44] reported an increased efficiency of the W159H mutant. Austin et al. [42] also introduced the same mutation as Joo et al. [17], resulting in a narrowed active cleft and improved PET degradation. Recently, Cui et al. [25] introduced many systematic mutations in *Is*PETase. The mutations were based on a novel computational strategy, that resulted in a redesigned variant (DuraPETase) with a melting temperature increased by 31 °C. Most of the mutation sites were directed at amino acids also found in actinomycetes PET hydrolases. In addition, based on a solid-state nuclear magnetic resonance analysis of amorphous PET at 30 °C, Wei et al. [13] found that the highly stiff polymer chain barely resembles the suggested docking conformation of 2-HE(MHET)$_4$ proposed by Joo et al. [17].

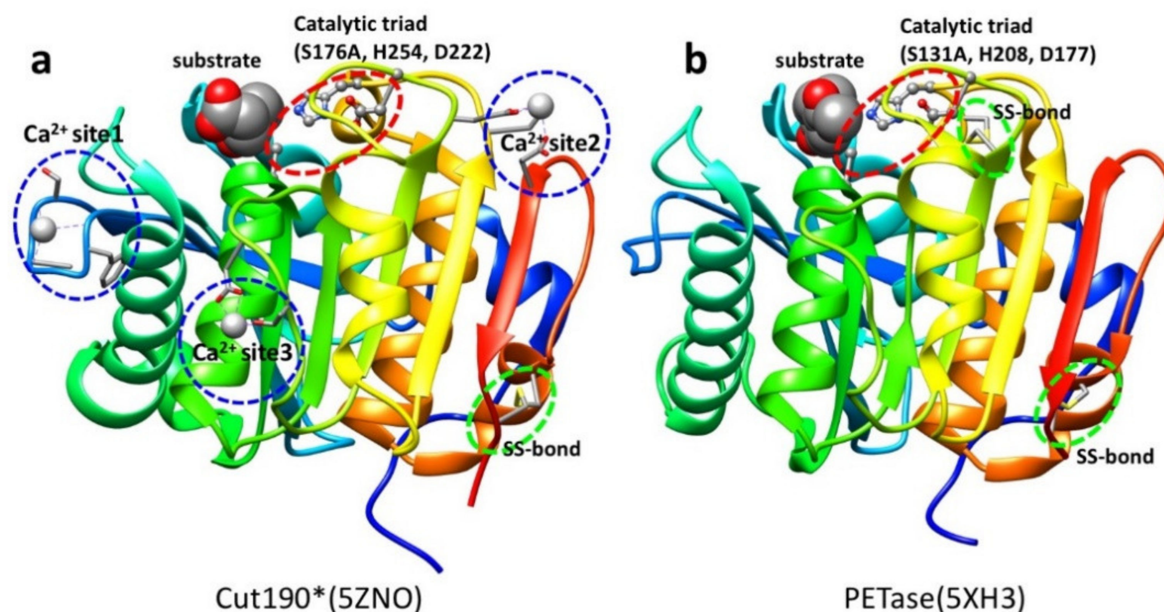

Figure 1. Comparison of overall 3D structures of Cut190* and PETase. The main chains are shown in ribbon models, the side chains of catalytic triads are shown in ball and stick models, and Ca^{2+}-binding sites and disulfide bonds are shown in stick models. Substrates are shown in space-filling models. Note that serine in the catalytic triad (S176 or S131) is replaced with alanine. (**a**) Cut190* (Protein Data Bank (PDB) ID: 5ZNO), which has three Ca^{2+} binding sites and one disulfide bond. The bound substrate (ethyl succinate) is taken from PDB ID: 5ZRR. (**b**) PETase (PDB ID: 5XH3), which has no Ca^{2+} binding site and two disulfide bonds. Reproduced from Kawai et al. *Appl Microbiol Biotechnol*. **2019,** *10*, 4253–4268 [15].

In general, scientists trust preceding research results and try to use them as a baseline for advancement. It can be reasonably expected that, in subsequent studies, researchers accepted the superficial superiority of *Is*PETas. However, at the same time, scientists are responsible for their results. The presence of a new model for PET hydrolyzing enzymes in the ecosystem and their utility in waste PET treatment are quite different concepts. As described above, some reports have denied the efficiency of *Is*PETase, but some scientists still assume that *Is*PETase is suitable for use in biorecycling. However, based on a fair evaluation of related enzymes, scientists can amicably fact-check each other and find the right way toward a common goal. As plastic pollution is assumed to increase on Earth, the treatment of plastic waste is an urgent global issue.

4. Perspectives for Biorecycling of PET

Polyester hydrolysis is the result of polymer chain flexibility and carboxylesterase structure (active site accessibility to the polymer surface), as suggested using BTA [45], which could be applicable to PET hydrolysis.

Wei et al. [46] reported that the microstructural changes, known as physical aging, in post-consumer PET films are caused by incubation at 70 °C for more than 72 h, leading to no further enzymatic weight loss. Tournier et al. [14] achieved a minimum of 90% PET depolymerization over 10 h, with a high level of terephthalate productivity, using amorphized and micronized post-consumer PET and LC-cutinase variants. They also reported that a temperature higher than 70 °C gradually caused crystallization, but at 65 °C, no crystallization was observed after 24 h. These studies shed light on the future direction of PET recycling and indicate that the required thermostability is less than 70 °C. Moreover, they indicate that amorphization and micronization (increase of the surface dimension accessible to the active site of enzymes) are required for the enzymatic recycling of PET.

Shirke et al. expressed LC-cutinase in *Pichia pastoris* ATCC76273 and reported elevated thermostability due to glycosylation [47]. Yan et al. engineered a thermophilic anaero-

bic bacterium, *Clostridium thermocellum* to express LC-cutinase extracellularly, and the engineered whole-cell biocatalyst degraded more than 60% of commercial PET films (approximately 50 mg) into soluble monomer feedstocks after 14 days at 60 °C [48]. Wei et al. expressed TfCut2 in *Bacillus subtilis* strain RH11496, which showed increased thermostability and activity compared to the enzyme expressed in *E. coli* [46]. These findings suggest that thermophilic microbes are a more promising microbial chassis than mesophilic species for thermophilic PET hydrolases. There are at least four candidate PET hydrolases that have feasible applications in the biorecycling of PET [15,16]; however, the common goal of realizing PET recycling by these enzymes has not yet been achieved. Further strategies are needed, for example, the engineering of enzyme genes and expression hosts, the immobilization of enzymes, the selection of the microbial chassis for enzymes to use as whole-cell-catalysts, and the use of additives, such as surfactants, metal ions, and stabilizers. TfCut2 activity is inhibited by BHET and MHET (product inhibition), but product inhibition is overcome by mutation (TfCut2G62A) [26]. LC-cutinase and Cut190* have no product inhibition, as the corresponding position of G62 in TfCut2 is alanine in both LC-cutinase and Cut190*, which facilitates their high production levels [14,28]. None of the four candidate PET hydrolases necessitate another enzyme, such as MHETase (requisite for the metabolism of PET into TPA, together with *Is*PETase in *Ideonella sakaiensis* [11]), as TPA is produced by the same enzymes. However, dual-enzyme systems have been used to accelerate the degradation of PET. For example, TfCut2 and LC-cutinase have been used together with carboxylesterase [49], and HiC has been used with lipase [50]. The removal of monomeric products by carboxylesterase and lipase avoids either product inhibition or an increase in acidity.

As described above, LC-cutinase variants are closest to practical applications for the enzymatic hydrolysis of PET, although the cost of the enzyme production is still high. The realization of PET biorecycling is dependent on the total cost of producing monomer materials (raw materials used for the resynthesis of PET) or the production of value-added products from PET waste.

5. Concluding Remarks

Some research groups are still working on *Is*PETase under the assumption that it is the best enzyme for PET hydrolysis. However, various studies have clearly demonstrated the superiority of thermophilic PET hydrolases (more than 65 °C) over mesophilic enzymes for use in PET biorecycling. The current state of PET hydrolases is approaching the goal of PET biorecycling. However, further improvements are required, based on the viewpoints of enzymatic and material approaches. First, the productivity of PET hydrolases requires improvement by the expression of an enzyme gene in an optimal host, expression as a whole-cell catalyst in a thermophilic microbial chassis, immobilization of enzymes and cells, and improved efficiency of an enzyme for PET hydrolysis. Second, it is important to process PET waste into forms that are readily biodegradable, for example, by amorphization and micronization, and partial depolymerization by physicochemical methods. Although economic viability is a key issue for PET biorecycling, the enzymatic recycling of PET is expected to be the best solution to close the circle from production to waste, as it is eco-friendly and has low energy consumption.

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