Fusion of Chitin-Binding Domain From *Chitinolyticbacter meiyuanensis* SYBC-H1 to the Leaf-Branch Compost Cutinase for Enhanced PET Hydrolysis

Rui Xue¹, Yinping Chen¹, Huan Rong¹, Ren Wei², Zhongli Cui³, Jie Zhou¹*, Weiliang Dong¹* and Min Jiang¹

¹State Key Laboratory of Materials-Oriented Chemical Engineering, College of Biotechnology and Pharmaceutical Engineering, Nanjing Tech University, Nanjing, China, ²Junior Research Group Plastic Biodegradation, Department of Biotechnology and Enzyme Catalysis, Institute of Biochemistry, University of Greifswald, Greifswald, Germany, ³Key Laboratory of Agricultural Environmental Microbiology, College of Life Science, Nanjing Agriculture University, Nanjing, China

Polyethylene terephthalate (PET) is a mass-produced petroleum-based non-biodegradable plastic that contributes to the global plastic pollution. Recently, biocatalytic degradation has emerged as a viable recycling approach for PET waste, especially with thermophilic polyester hydrolases such as a cutinase (LCC) isolated from a leaf-branch compost metagenome and its variants. To improve the enzymatic PET hydrolysis performance, we fused a chitin-binding domain (ChBD) from *Chitinolyticbacter meiyuanensis* SYBC-H1 to the C-terminus of the previously reported LCCICCG variant, demonstrating higher adsorption to PET substrates and, as a result, improved degradation performance by up to 19.6% compared to its precursor enzyme without the binding module. For compare hydrolysis with different binding module, the catalytic activity of LCCICCG-ChBD, LCCICCG-CBM, LCCICCG-PBM and LCCICCG-HFB4 were further investigated with PET substrates of various crystallinity and it showed measurable activity on high crystalline PET with 40% crystallinity. These results indicated that fusing a polymer-binding module to LCCICCG is a promising method stimulating the enzymatic hydrolysis of PET.

**Keywords:** chitin-binding domain, polyethylene terephthalate, hydrolysis, leaf-branch compost, hydrophobicity

**INTRODUCTION**

Plastics are being used in an increasing number of applications in our society. As a result, improperly disposed waste plastics have resulted in environmental pollution, which has garnered increasing attention in recent decades. According to recent data, global plastics production has reached nearly 368 million tons in 2019 (PlasticsEurope, 2020). However, 70% of plastic waste is landfilled or discarded carelessly, 11% is incinerated, and only 19% is recycled (Kaza et al., 2018). Polyethylene terephthalate (PET) is a thermoplastic polyester synthesized with the monomers terephthalic acid (TPA) and ethylene glycol (EG). PET has been widely used in the production of beverage bottles and synthetic fibers because of its excellent mechanical and thermal properties (Wei and Zimmermann, 2017; Kawai et al., 2019; Chen et al., 2020). Chemical and mechanical PET involve harsh chemicals and energy-intensive physicochemical treatments (Ragaert et al., 2017). Enzymatic hydrolysis under mild reaction conditions has recently been recognized as an eco-friendly...
alternative recycling process for PET (Wei et al., 2020). PET-hydrolyzing enzymes, which belong to different subclasses of the alpha-beta hydrolase family, are the most extensively studied plastic-degrading enzymes (Chen et al., 2020). The first reported PET hydrolase was TTH from Thermobifida fusca which can degrade pretreated PET waste at 55 °C (Müller et al., 2005). A synergy between different hydrolase subclasses, for example a polyester degrading IsPETase and an oligomer degrading IsMEHTase, has been reported in the bacterium Ideonella sakaitensis, which is capable of metabolizing amorphous PET (Yoshida et al., 2016). Tournier et al. recently demonstrated the use of an engineered PET hydrolase variant in the degradation of pre-treated post-consumer PET bottles, resulting in a >90% depolymerization of PET waste in less than 10 h at an industrially relevant scale. This equates to a mean productivity of 16.7 g L\(^{-1}\) h\(^{-1}\) of TPA which was then recovered to synthesize virgin polymers, thereby closing the recycling loop (Tournier et al., 2020). This ground-breaking innovation developed by the French biotech company Carbios used the highly efficient wild-type the leaf-branch compost cutinase (LCC) (Sulaiman et al., 2012) for protein engineering, and thermomechanical pretreatment to reduce the crystallinity of the real-world PET waste. This procedure successfully showcased a closed-loop bio-recycling PET that holds great promise as a foundation for future applications (Wei et al., 2020).

As a surface erosion process, enzymatic PET hydrolysis can be significantly accelerated when more biocatalysts can adsorb to the hydrophobic polymer surface (Kawai et al., 2019). Hydrophobic binding modules have been fused to selective PET hydrolyzing enzymes in order to improve the enzyme sorption at water-solid interface and thus facilitate the hydrolysis of insoluble polymer substrates (Din et al., 1991; Ribitsch et al., 2013). Ribitsch et al. (2015) engineered the PET cutinase Thc_Cut1 by fusing it with two Trichoderma hydrophobins HB4 and HB7, resulting in a more than 16-fold increase in PET hydrolysis efficiency with the fusion variants. Similarly, they were able to modify the same enzyme by fusing two other binding domains: the carbohydrate binding modules (CBM) from Hypocre a jecorina and the polyhydroxyalkanoate binding modules (PBM) from Alcaligenes faecalis, resulting in an enhanced hydrolytic activity on amorphous PET of up to 3.8 times (Ribitsch et al., 2013).

Plastic polymers and plants (such as cellulose and chitin) share chemical properties and polymer structures, including partially crystalline regions, surface hydrophobicity and backbones linked by hydrolysable bonds (Daly et al., 2021). The carbohydrate binding modules (CBM), which includes the chitin-binding domain (ChBD), is a type of noncatalytic domain found extensively in glycoside hydrolases facilitating their adsorption to the insoluble substrate (Boraston et al., 2004). CBMs have been developed as useful affinity tags for protein immobilization due to their high adsorbing capacity to solid materials. For example, Zhou et al. (2020) used chitin-binding domain as an affinity tag and achieved over 95% immobilization efficiency with their target protein. Furthermore, CBMs have a broad range of hydrophobic binding affinity with many polymer substrates including cellulose, chitin, xylan, and starch (Oliveira et al., 2015).

In this study, protein mutants were constructed based on the PET hydrolase variant LCC\(^{CcCGG}\), which has been shown by Tournier et al. as the most active PET hydrolase to date, with C-terminally fused binding modules including a ChBD from Chitinolyticibacter meiyuanensis SYBC-H1 (Zhang et al., 2018), the carbohydrate binding modules (CBM) from Hypocre a jecorina, the polyhydroxyalkanoate binding modules (PBM) from Alcaligenes faecalis and Trichoderma hydrophobins. While the latter three binding modules have been used to engineer other PET hydrolases, the ChBD was introduced to PET hydrolases for the first time due to its similar hydrophobic nature to CBM, which is mediated by tryptophan residues and can promote the enzyme adsorption and, as a result, the hydrolytic activity on PET (Ribitsch et al., 2013). To investigate the effect of the binding module on the PET-hydrolyzing cutinase, the degradation efficiency with LCC\(^{CcCGG}\) and its fusion variants on various PET materials were evaluated in terms of the surface adsorption, release of the degradation products and kinetic analysis (Figure 1).

**MATERIALS AND METHODS**

Reagents and Strains

Amorphous PET film (GF-PET, crystalline 6.7%) was purchased from Goodfellow Ltd. (Shanghai, China, thickness of 250 µm). Post-consumer PET waste (PCW-PET, crystalline 16%) samples were prepared from PET flakes following the pretreatment method described previously (Tournier et al., 2020). High-crystalline PET (Hc-PET, crystalline 40%) was purchased from Dopont Ltd. (Shanghai, China). All PET samples were cut into pieces then immersed in liquid nitrogen for 1 min before micronization. The percentage crystallinity of the sample is measured by the DSC method and calculated (Tournier et al., 2020). MHET is synthesized based a previously described method (Kaabel et al., 2021). All other chemicals and reagents were of analytical grade and purchased from Sahn chemical technology Co. Ltd. (Shanghai, China). Restriction enzymes, DNA polymerase were purchased from TaKaRa Co., Ltd. (Dalian, China). Escherichia coli strains were routinely cultivated in LB medium at 37°C. All constructs in this study were generated with the expression vector pET-29a for recombinant expression in E. coli BL21 (DE3). The gene encoding LCC\(^{CcCGG}\) cutinase, CBM, PBM and HB4 were synthesized based on previous studies (Ribitsch et al., 2013; Ribitsch et al., 2015; Tournier et al., 2020) and that for ChBD was derived from the genome of Chitinolyticibacter meiyuanensis SYBCH1 (Zhang et al., 2018). LCC\(^{CcCGG}\) cutinase and ChBD expression strain E. coli BL21 (pET29a-LCC\(^{CcCGG}\)) and E. coli BL21 (pET29a-ChBD) were constructed in previous experiments (Zhou et al., 2020).

**Construction of Chimeric Genes and Recombinant Plasmids**

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The genes were constructed using the method of splicing-by-overlap extension (Su et al., 2006). Synthetic oligonucleotides
encoding sequences of peptide linkers was introduced between the lcc and binding module genes. To express the fusion enzymes, the cleavage sites for the restriction enzymes Nde I and Xho I were introduce into the 5’ end of lcc and the 3’ end of binding module genes, respectively (Figure 2A). The gene fragments encoding GFP was then inserted into the multi clone site (MCS) by one step cloning for further investigations.

Enzyme Expression and Puriﬁcation

E. coli BL21 (DE3) harboring individual plasmids including pET29a-LCCICCG, pET29a-LCCICCG-ChBD, pET29a-LCCICCG-CBM, pET29a-LCCICCG-PBM, pET29a-LCCICCG-HFB4 and pET29a-LCCICCG-ChBD-GFP was used to inoculate 5 ml LB medium containing kanamycin (50 μg/ml) for incubation at 37°C and 200 rpm for 12 h. Then, a 2 ml seed culture solution was removed to inoculate 200 ml LB medium with kanamycin (50 μg/ml) for incubation at 37°C and 200 rpm for 4 h. When the OD value reached 0.6, IPTG was added at a final concentration of 1 mM to induce the recombinant protein expression by further incubating at 18°C for 24 h. The bacterial cells were harvested by centrifugation at 12,000 rpm at 4°C for 10 min. The obtained cell pellets were washed twice by phosphoric buffer (50 mM, pH 8.0) and resuspended with 50 mM phosphoric buffer (pH 8.0). Then, the cells were disrupted by a scientz-II D ultrasonic generator and cell debris was removed by centrifugation at 12,000 rpm and 4°C for 20 min. The recombinant enzyme with a C-terminal His6-tag was puriﬁed using Ni2+-NTA resin with a Biomolecular Liquid Chromatography System (AKTA design, GE Healthcare, US). The target proteins were eluted with buffer NPI-250 (50 mM NaH2PO4, 300 mM NaCl, 250 mM imidazole, pH 8.0). The puriﬁed protein was collected and concentrated by ultraﬁltration tube to remove imidazole. Esterase activity was determined using a slightly modiﬁed version of a previously described method (Alisch et al., 2004). In a cuvette, 980 μL of 50 mM PBS buffer (K2HPO4/NaH2PO4, pH 8.0) was mixed with 10 μL p-nitrophenyl butyrate (pNPB, 10 mM) dissolved in isopropanol. The hydrolysis reaction was started by adding 10 μL of appropriately diluted enzyme solution and stopped by
adding 20 μl of 1% sodium dodecyl sulphate, after which the absorbance was measured at 410 nm.

Kinetische Studien

Die kinetischen Parameter für die enzymatische Hydolysereaktion von pNPB wurden berechnet auf der Grundlage der Reaktionsraten, die in der initialen Reaktionsphase während der ersten 3 min ermittelt wurden. Die kinetischen Parameter für die Hydolysereaktion von amorphen PET-Filmen, die durch Kühlung in flüssigen Stickstoff für 1 min und Mikronisierung erhalten wurden, wurden in der initialen Reaktionsphase während der ersten 3 min ermittelt, um die Reaktionsraten zu bestimmen. Weitere, diesmal aus dem berechneten Gesamtrag, wurden von der Summe der totalen löslichen Produkte (TPA, MHET und BHET) abgezogen. Die reziproke Kinetik-PARAMETER für die enzymatische Hydrolyse von amorphem PET wurden berechnet und mit denen, die ohne ChBD erhalten wurden, im Vergleich dargestellt (Tabelle 1).

Enzym-PET Bindungsassay

Die gereinigte Enzyme (LCCICCG-ChBD-GFP) und die vorbehandelten PET-Substrate wurden für 10 min bei 12.000 U/min und 4°C aufgestellt, nachdem die Adsorptionsrate der Bindungsmodule zum plastischen Substrat basierend auf der Proteinkonzentration in der initialen Phase während der ersten 3 min bestimmt wurde. Die Adsorptionsrate effizienz wurden an verschiedenen Inkubations Temperaturen (4, 20, 40, und 65°C) zu messen, um die maximale Adsorptionsrate von unterschiedlichen Enzymvarianten bei der Temperatur zu testen, bei der die plastische Degradation ermittelt wurde.

Proteinkonzentrationen wurden mit der Bradford-Methode (Campion et al., 2017) bestimmt. Die konzentriertere ChBD-GFP wurde bei 4°C in einem Flüssigphasen-Fluorimeter (Hitachi F7000) erfasst. Die Adsorptionsrate wurde mit der Formel berechnet:

\[
\text{Adsorption rate} = \left(1 - \frac{\text{GFP concentration in supernatant}}{\text{Initial GFP concentration}}\right) \times 100\%
\]

Hydolysereaktion der PET-Substrate

Die enzymatische Degradation von PET-Substraten wurde in einem 500 ml Reaktor durchgeführt. Die Reaktion begann mit der Zugabe von 0,5 μmol einer 200 ml Buffer (100 mM phosphat-basierend pHH 8) enthaltend 120 mg PET-Substrat, das dann bei 65°C in einer Zeit von 100 rpm bei 12 h gerührt wurde. Die enzymatische Depolymerisierung der Enzyme wurde durch die Summe der Konzentrationen von BHET, MHET und TPA in der Supranat ermittelt. Für diese Berechnung wurde der durchschnittliche molare Gewicht der PET-Polymer 192,2 g/mol (Yan et al., 2021).
As shown in Figure 3D, the adsorption time courses with LCCICCG-ChBD were investigated at various temperatures. At 40°C, the maximum adsorption efficiency of 30.2% was achieved after 70 min of incubation. This is the optimal binding temperature for the chitin binding domain used in this study according to previous publications (Zhou et al., 2020). With increasing incubation temperature to 65°C or 72°C, the maximum adsorption rate decreased slightly to 27% or 23.5%, which was achieved after 2 h incubation and considered beneficial for the enzymatic plastic degradation carried out at this temperature.

Degradation Time Course of GF-PET Powder by Fusion Enzymes

Figure 4 showed the time course study of the GF PET degradation by the cutinase variant with and without various binding modules in terms of the release of HPLC detectable hydrolysis products. In all samples, mono (2-hydroxyethyl) terephthalate (MHET) is the most abundant product followed by terephthalic acid (TPA), and bis (2-hydroxyethyl) terephthalate (BHET). During the total incubation time up to 12 h, the levels of released TPA and MHET increased continuously. After 12 h reaction at 65°C, LCCICCG yielded 2.12 mM hydrolysis products composed of 0.76 mM TPA, 1.25 mM MHET and 0.11 mM BHET (Figure 4A). LCCICCG-ChBD showed a higher activity against the GF-PET, releasing 0.92 mM TPA, 1.5 mM MHET and 0.32 mM BHET after 12 h incubation, accounting for 29% more yield in total product amount than that without binding module (Figure 4A). In comparison, the highest amount of degradation products composed of 1.23 mM TPA, 1.56 mM MHET and 0.25 mM BHET was yielded with LCCICCG-CBM, and is 14% higher than with LCCICCG-ChBD (Figure 4A). The yield of degradation products with LCCICCG-PBM and LCCICCG-HFB4 was comparable or slightly lower than that with LCCICCG, indicating that the fusion of PBM and HFB4 to LCCICCG might have weakly impaired its catalytic activity. As shown in

(FIGURE 3C).
Figure 4B, depolymerization degrees of 87.5 and 98.5% were achieved with LCCICCG-ChBD and LCCICCG-CBM, respectively, which are 19.6 and 30.6% higher than that obtained with the enzyme without binding modules (Figure 4B).

Hydrolysis of PET Substrates With Different Crystallinity

PET substrates with different crystallinity, such as GF-PET (6.7%), Pcw-PET (16%) and Hc-PET (40%), were used to investigate the effect of crystallinity on the degradation performance catalyzed by various cutinase versions. Figure 5 indicated that the effectiveness of enzymatic hydrolysis decreased significantly as polymer crystallinity increased. LCCICCG, in particular, was nearly impossible to break down Hc-PET exhibiting only 0.029 mM hydrolysis products released after 12 h. Interestingly, the fusion enzymes LCCICCG-ChBD and LCCICCG-CBM showed markedly increased depolymerization performance against Hc-PET by releasing 0.335 and 0.215 mM hydrolysis products.
products, respectively, which are 11.6 and 7.1 times higher than without binding modules. The superior activity of LCCICCG-ChBD and LCCICCG-CBM was noticeable against all three PET substrates with different crystallinity. Furthermore, ChBD outperform other binding modules in facilitating the breakdown of Hc-PET materials.

**Scanning Electron Microscopy Analysis of GF-PET Film Exposed to LCCICCG-ChBD**

The degradation of GF-PET film with LCCICCG-ChBD was carried out at 65 °C for 6 h. As shown in Figure 6A, GF-PET is cut into 2*2 cm pieces, and Figure 6B shows the residual plastic fragments after enzymatic breakdown. Figure 6C shows a SEM image of a plastic sheet prior to the degradation, with the surface appearing smooth. A SEM image of a GF-PET film after degradation with increased surface roughness is shown in Figure 6D.

**DISCUSSION**

Carbohydrate-binding module (CBM) including ChBD have a high adsorption capacity to a variety of insoluble substrates. These characteristics enable the potential application CBM proteins function as adhesive in food industry, biomedicine, environmental protection and molecular biology. In this study, we employed ChBD to construct a novel fusion enzyme variant for enhanced hydrolysis of synthetic polymers. To that purpose, an engineered cutinase (LCCICCG) (Tournier et al., 2020) previously published for PET hydrolysis was fused with a C-terminal chitin-binding module from chitinase CmChi1 from *Chitinolyticbacter meiyuanensis* SYBCH1 (LCCICCG-ChBD). This ChBD belongs to carbohydrate-binding modules, which have a similar interaction mechanism with insoluble polymers that involves hydrophobic interactions through tryptophan residues. The ChBD is fused to LCCICCG via a linker region (S2), which has been described as an appropriate linker peptide that can be introduced between distinct enzymes to avoid folding interference from each other allowing the two moieties of a fusion enzyme to function as independently as possible (Lu and Feng, 2008).

The hydrolysis of GF-PET, an amorphous PET material commonly used to study other PET hydrolases, showed intriguing mechanistic insights based on varied ratios of hydrolysis products released by the fusion enzyme and its precursor (Wei et al., 2019; Tiso et al., 2021; Yan et al., 2021). When compared to LCCICCG-ChBD, the one without binding module produced lower amounts of hydrolysis products. LCCICCG initially released comparable amounts of all hydrolysis products, but the levels of increasing release of MHET and TPA were comparable later in the incubation stage. The ratios of MHET and TPA were nearly unchanged following hydrolysis, regardless of whether the fused protein or its precursor was used. This suggests that the inclusion of the chitin binding module may aid in the depolymerization of the oligomeric PET-related molecules. The results of kinetic characterization further support this view. LCCICCG-ChBD showed a lower $K_m$ value for the pNPB hydrolysis than that with its precursor enzyme, indicating a reduced substrate affinity to the soluble model compound. As a result, the catalytic efficiency ($k_{cat}/K_m$) of the enzyme carrying ChBD (0.404 s⁻¹/μM) was slightly lower than that measured for LCCICCG (0.534 s⁻¹/μM). LCCICCG-ChBD, on the other hand, has a higher catalytic efficiency ($k_{cat}/K_m$) and depolymerization rate on polymers with various crystallinity than the one without binding module. In general, the addition of ChBD did not appear to considerably improve the depolymerization of PET-related oligomers, although the enhanced depolymerization efficiency of PET polymers was more pronounced. This could be related to the mechanism of the binding module which can potentially destroy the structures of the polymers like celluloses, releasing more accessible bonds to the enzymatic degradation (Din et al., 1991). However, as for PET polymers, more experimental evidence in this context is still required.

In this study, we also compared the promoting effect of the ChBD *Chitinolyticbacter meiyuanensis* with three other binding domains: cellulose–binding domain (CBM) of cellobiohydrolase 1 from *Trichoderma reesei* (TrCBH), polyhydroxyalkanoate binding modules (PBM) from *Alcaligenes faecalis* and hydrophobin HFB4 from *T. reesei* QM6a, which have been previously reported to significantly improve PET degradation efficiency when fused to another PET hydrolase. By fusing to the hydrolyase variant LCCICCG, our results (Figures 4, 5) showed that CBM and ChBD can enhance the hydrolysis of PET samples with different crystallinity. When high-crystalline PET was used as the substrate, the enzyme with ChBD fusion released 11.6 times more degradation products, indicating its superiority in promoting the breakdown of Hc-PET materials. With amorphized PET sample, the increased degradation performance by 19.6% with the fused ChBD could be attributed to an increased adsorption capacity to PET by up to 27%. As a result, more research is needed to understand why ChBD improves PET hydrolase to different levels against PET with varying crystallinity. In addition, mutagenesis of ChBD by protein engineering approaches may facilitate to gain a better knowledge on its binding process to PET, similarly as previously reported with other CBMs (Weber et al., 2019). Because ChBD has an optimal temperature for PET binding at 40 °C, which is significantly lower than the optimal breakdown temperature of PET catalyzed by LCC variants, enhancing the thermal stability of ChBD may be another useful strategy for improving the overall degradation efficacy catalyzed by the fusion protein.

**CONCLUSION**

We have successfully fused a chitin binding domain to LCCICCG, a cutinase variant with outstanding PET hydrolytic activity. The fusion protein was functionally expressed in *E. coli* and showed an enhanced adsorption capacity to PET films by fluorescence detection. As a result, the fusion protein with ChBD demonstrated improved degradation performance on all investigated PET materials as indicated by the higher release of degradation products than that obtained with LCCICCG. The improved hydrolytic activity of LCCICCG-ChBD led toetectable
degradation performance on PET substrates with a high crystallinity of 40%.

DATA AVAILABILITY STATEMENT
The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding authors.

AUTHOR CONTRIBUTIONS
RX was responsible for experimental design and thesis writing. RX, YC and HR were responsible for experimental operation. RW, YG and WD contributed to data analysis and drafting and proofreading of the manuscript. All co-authors have approved the final manuscript version.

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