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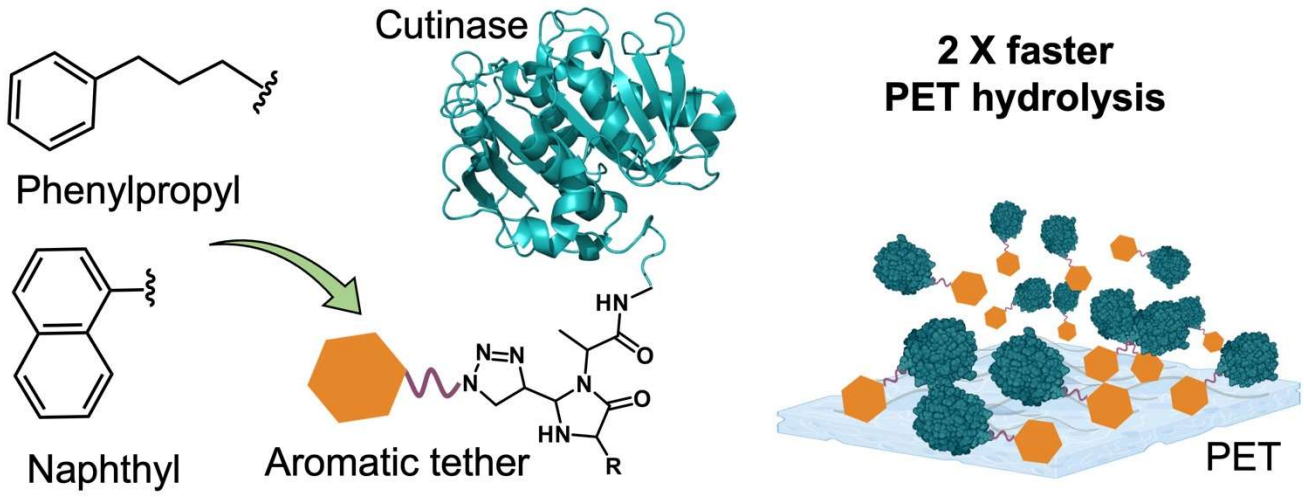
N-Terminal Modification of Aromatic Tether in Cutinase Enhances Degradation of Polyethylene Terephthalate Powder and Fabrics

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Abstract
Cutinase from *Thermobifida cellulosilytica* was site specifically conjugated with aromatic tether containing TA4C at the N-terminus. Both phenylpropyl-TA4C and naphthyl-TA4C conjugated cutinase (PP-Cut and NP-Cut) release more than two times higher terephthalic acid than unconjugated cutinase after 24 h. The aromatic tether conjugated cutinases also exhibits higher hydrolysis activity against less degradable PET fabrics. Scanning electron microscopy confirmed that conjugated variants show extensive surface erosion which is accelerated by enhanced enzyme-polymer interactions.

Keywords: N-Terminal modification, Polyethylene terephthalate, Biodegradation

Graphical Abstract



Plastic pollution is the top global environmental issue, with polyethylene terephthalate (PET) being a major contributor due to its extensive use and high resistance to degradation.[1–3] Compared to chemical recycling of PET, the enzymatic approach has emerged as a promising and eco-friendly alternative.[4,5] PETase, MHETase, and cutinase are widely studied esterases that hydrolyze PET.[6–8] Hydrophobic PET has less access to the enzymes in an aqueous solution. Alongside the mutational development of the PET-hydrolyzing enzyme to improve efficiency and thermal stability, notable approaches include anchoring with a hydrophobic amino acid-rich protein domain, charged peptide conjugation, and coating the PET surface with hydrophobic moieties, which have been shown to enhance the adsorption and hydrolysis of PET.[9–11]

Bioconjugation is a powerful tool for incorporating chemical substances into the specific sites of a protein, which is faster and more diverse than any genetic engineering approach for altering the desired properties

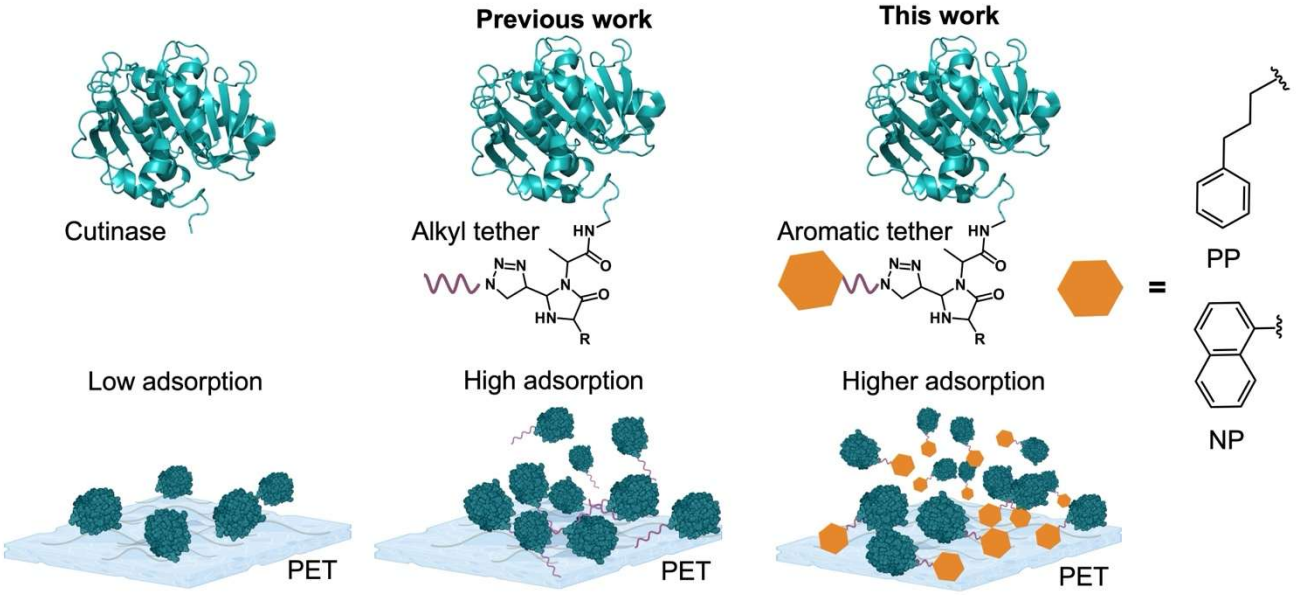


Fig. 1. Previous work showing the enhanced adsorption of alkyl tether conjugated cutinase to the PET film surface compared to Cut. This work explores increased hydrolysis of PET fabric and powder by cutinase conjugated with an aromatic tether.

of the protein.[12–14] The catalytic performance was improved by attaching different hydrophobic, hydrophilic, and cationic monomer units on the PETase enzyme by nonspecific conjugation.[15]

In our previous work, we found that conjugation of an alkyl tether to the N-terminus of cutinase from *Thermobifida cellulosilytica* (Thc_Cut1) improved adsorption of enzyme to the PET surface and improved hydrolysis thereby.[16] In this study, we report further enhanced PET hydrolysis by cutinase enzyme having an N-terminal aromatic tether of the derivatives of 1*H*-1,2,3-triazole-4-carbaldehyde (TA4C) (Fig. 1).

Phenylpropylamine and naphthylamine react with 1-(4-nitrophenyl)-1*H*-1,2,3-triazole-4-carbaldehyde to form 1-Phenylpropyl-1*H*-1,2,3-triazole-4carbaldehyde (PP-TA4C) and 1-naphthyl-1*H*-1,2,3-triazole-4carbaldehyde (NP-TA4C), respectively, by Dimroth rearrangement reaction.[17] PP-TA4C and NP-TA4C was conjugated to the N-terminus of the cutinase enzyme in a single-step reaction using mild reaction conditions (Supplementary Figs. S1 and S2).[18] Cutinase conjugated by PP-TA4C (PP-Cut) and NP-TA4C (NP-Cut) was purified by using a Hitrap SP HP column on the ÄKTA go™ protein purification system and characterized by LC-MS (Supplementary Fig. S3). Deconvoluted ESI-MS spectra of Cut, PP-Cut, and NP-Cut indicate masses of 29120 (calcd. 29121), 29319 (calcd. 29321), and 29325 (calcd. 29328), respectively. The purified PP-Cut and NP-Cut were used to hydrolyze PET powder with a particle size of less than 125 µm and PET fabric, along with unconjugated cutinase (Cut) and hexyl tether conjugated Cut (C_6-Cut), to evaluate the impact of hydrophobic moieties on PET hydrolysis. Enzyme kinetics were measured for Cut and

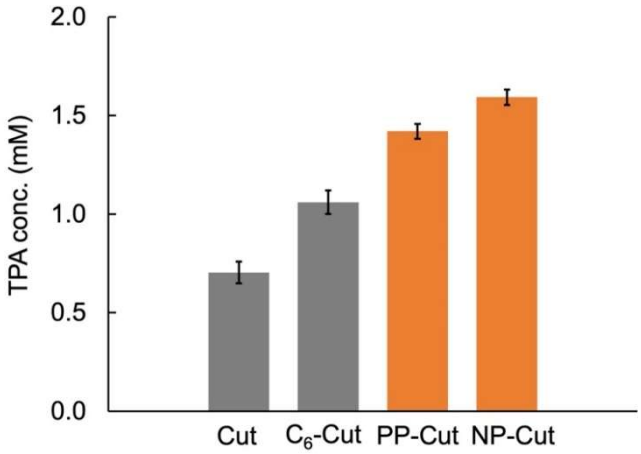


Fig. 2. TPA concentration of PET hydrolysis by Cut and aromatic tether conjugated Cut. PP-Cut and NP-Cut show higher TPA production from PET powder than unconjugated Cut and cutinase conjugate with a hexyl tether (C_6-Cut) after 24 h.

conjugated Cut for the substrate *p*-nitrophenylbutyrate. K_m values are 142.8 ± 32.8 µM, 115.2 ± 17.6 µM, and 135.4 ± 42.6 µM for Cut, PP-Cut, and NP-Cut, respectively. This indicates that conjugated Cut has slightly higher affinity for the substrate. V_{max} values of Cut, PP-Cut, and NP-Cut are 5.5 ± 0.5 µM/min, 9.78 ± 0.5 µM/min, and 8.38 ± 1.0 µM/min, respectively, for 1 nM of enzyme concentration.

Amorphous PET powder was hydrolyzed by cutinase for 24 hours. On the other hand, PET fabric was incubated for 7 days due to poor degradability. The hydrolysis product, terephthalic acid, was measured by HPLC using a reverse-phase InertSustain RP-C18 HP 3 µm column (GL Sciences) with a mobile phase of 20% acetonitrile, 70% water, and 10% formic acid (1%), and PDA detection was performed at 254 nm. The concentration of released TPA from PET powder is illustrated in Fig. 2. TPA released by unconjugated Cut is 0.70 ± 0.06 mM. On the contrary, PET powder hydrolyzed by PP-Cut and NP-Cut released TPA at 1.42 ± 0.05 mM and 1.60 ± 0.04 mM, respectively, after 24 h. C_6-Cut conjugated with the alkyl tether (hexyl-TA4C) released 1.1 ± 0.1 mM TPA. The PP-Cut and NP-Cut produced more than two times terephthalic acid than the unconjugated Cut.

In terms of PET fabric hydrolysis, a similar trend of TPA production is observed, where hydrophobic moiety conjugated cutinase has higher TPA production than the unconjugated cutinase. However, the amount is less than that of the amorphous PET powder (Supplementary Fig. S4). This difference is attributed to the higher surface area and lower crystallinity of powdered PET, which provide better enzyme accessibility and facilitate hydrolysis. In contrast, the woven structure and potentially higher crystallinity of PET fabric likely limit enzyme penetration and interaction, reducing overall degradation efficiency. TPA produced by Cut, PP-Cut and NP-Cut are 46.8 ± 1.3 µM, 70.5 ± 4.0 µM and 85.3 ± 6.0 µM, respectively, after 7 days. The N-terminus of the cutinase is distal from the catalytic site. However, the N-terminus is surrounded by hydrophilic residues. Incorporation of a highly hydrophobic tether at the flexible N-terminus reduces the overall hydrophilicity of cutinase. The difference between the Cut and the conjugated Cut is the presence of a hydrophobic tether at the N-terminus. The hydrophobic tether at the N-terminus increases the hydrophobic interaction of cutinase with the PET. PP-Cut and NP-Cut contain aromatic rings, which also facilitate π-π stacking interactions in addition to hydrophobic interactions with the aromatic backbone of PET.[19,20] All these phenomena enhance the adsorption of conjugated cutinase to the PET film than the unconjugated cutinase. The anchoring of a hydrophobic peptide anchor increased the force of adhesion compared to the unanchored and hydrophilic anchor, which also validates the application of hydrophobic conjugation to PET hydrolyzing enzymes.[21]

Scanning electron microscopy (SEM) reveals the progressive degradation of PET powder treated with different cutinase variants (Fig. 3). The PET treated only with phosphate buffer exhibits a smooth surface, indicating no degradation. Cut-treated PET exhibits mild erosion, characterized by shallow pits, indicating limited enzymatic hydrolysis. In contrast, PP-Cut-treated PET displays deeper pits and increased roughness, suggesting that phenyl propyl conjugation

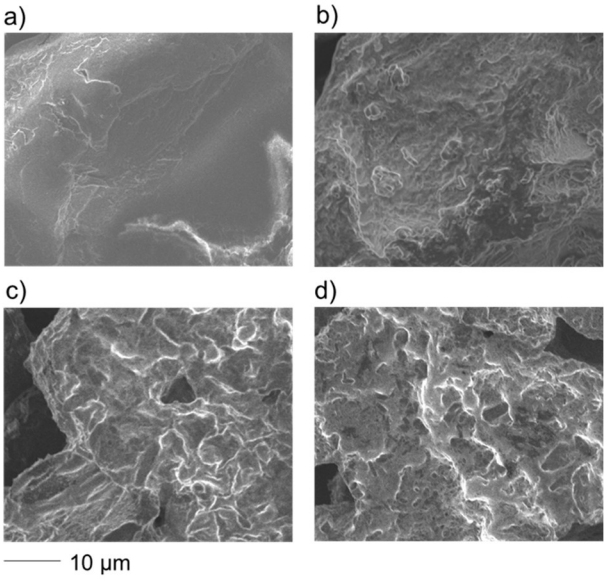


Fig. 3. Scanning electron microscopy of PET powder treated by (a) phosphate buffer, (b) Cut (c) PP-Cut, and (d) NP-Cut. PP-Cut and NP-Cut caused a more robust degradation of PET powder than Cut.

enhances enzyme-substrate interaction, likely through improved hydrophobic binding. The most acute degradation occurs with PET treated with NP-Cut, where extensive erosion and pit formation are observed, demonstrating that naphthyl conjugation maximizes PET breakdown due to stronger aromatic interactions with the polymer.[22]

The SEM images of PET fabric also illustrate a progression in enzymatic degradation efficiency, directly correlated with the type of cutinase modification (Supplementary Fig. S5). The fabric without Cut treatment exhibits smooth, intact fibers. Treatment with unconjugated cutinase results in modest surface erosion, appearing minor pitting and slight roughening, which reflects the inherent but limited hydrolytic capability of the enzyme. The fabrics treated with PP-Cut and NP-Cut exhibit significantly enhanced degradation, featuring pronounced fiber etching and increased surface roughness.

This improvement is attributable to stronger hydrophobic interactions between the phenylpropyl and naphthyl groups and the PET surface, which facilitate more effective enzymatic action. The degradation efficiency follows the order NP-Cut > PP-Cut > Cut > untreated, highlighting the impact of aromatic modifications on enzymatic performance. The naphthyl group appears to be most effective in promoting PET hydrolysis, while phenylpropyl provides intermediate enhancement. In fact, the amount of protein adsorbed of PET was 0.012 µg/cm^2 for Cut, 0.044 µg/cm^2 for PP-Cut, and 0.020 µg/cm^2 for NP-Cut. MD simulation suggests that hydrophobic tethers of PP and NP linked at the N-terminus are expected to be

flexible enabling higher affinity to the PET surface (Figs. 6, 7 and 8). These results highlight the potential of site-specific enzyme modifications to enhance biodegradation, with aromatic molecules demonstrating particularly promising for PET waste biodegradation.

In summary, we have conjugated phenylpropyl TA4C and naphthyl TA4C to the N-terminus of cutinase and applied to degrade PET. Compared to unconjugated cutinase, higher hydrolysis was observed by the conjugated cutinase based on terephthalic acid release amount. Further studies could explore the structural mechanisms behind these improvements for industrial applications.

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Supplementary data

Experimental methods, Conjugation of cutinase. Hydrolysis of PET fabric, SEM of PET fabric, MD analysis

Conflict of interest statement. None declared.

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