Evaluation of parameters affecting recombinant PETase and MHETase activity in PET degradation

To Lan Anh[1*], Dinh Thi Hoa[1], Le Thi Thu Hong[2], Pham Kien Cuong[1], Le Huy Hoang[1], Le Duy Khanh[1], Dang Phuong Nam[1], Nguyen Thi Xuan[1], Duong Thi Thanh Loan[1]

[1]Institute of Materials, Biology and Environment, Academy Military of Science and Technology, 17 Hoang Sam, Nghia Do, Ha Noi, Vietnam;
[2]Institute of Biology, Vietnam Academy of Science and Technology, 18 Hoang Quoc Viet, Nghia Do, Ha Noi, Vietnam.
[*]Corresponding author: lananh9422@gmail.com
Received 6 Sep. 2025; Revised 12 Nov. 2025; Accepted 10 Dec. 2025; Published 25 Dec. 2025.
DOI: https://doi.org/10.54939/1859-1043.j.mst.108.2025.98-104

ABSTRACT

PETase and MHETase are two enzymes catalyzing the degradation of polyethylene terephthalate (PET) into terephthalic acid (TPA) and ethylene glycol (EG), which are environmentally friendly and non-toxic. The application of these enzymes holds significant potential for treating plastic waste and advancing green technologies in environmental protection. In this study, several factors affecting enzyme activity, including buffer composition, temperature, and pH, were investigated. The optimal conditions for PETase and MHETase activity were identified as a temperature range of 30 - 40 °C, pH 8 - 9, and 100 mM sodium phosphate buffer. Under these conditions, the recombinant enzymes hydrolyzed commercial PET film within 7 days of treatment, resulting in a significant change in the PET surface morphology and thereby demonstrating the effectiveness of PETase and MHETase in PET film cleavage. These results highlight the potential of two recombinant enzymes for plastic waste treatment.

Keywords: PETase; MHETase; Polyethylene terephthalate (PET); Biodegradation; Synthetic polymer.

1. INTRODUCTION

Plastic waste pollution in terrestrial and marine environments has emerged as a critical global challenge, exerting severe impacts on ecosystems, environmental integrity, and human health. Current research efforts are focused on the development of green and sustainable strategies for plastic waste management. Among these, the application of microorganisms and their associated enzymes capable of degrading synthetic polymers represents a promising approach, with initial studies demonstrating encouraging outcomes. Several hydrolytic enzymes, including esterases, lipases, cutinases, and carboxylesterases, have been reported to exhibit polyethylene terephthalate (PET)-degrading activity [1]. Notably, PETase and MHETase, identified in *Ideonella sakaiensis* 201-F6, display remarkable PET-degrading capabilities under mesophilic conditions (~30 °C). These enzymes synergized to hydrolyze PET into environmentally benign products, terephthalic acid (TPA) and ethylene glycol (EG), with efficiencies reported to be 5.5–120-fold higher than those of previously characterized thermophilic PET-degrading enzymes [2].

PETase is a member of the α/β-hydrolase family, which shares similar structural features with lipases and esterases. It possesses a molecular weight of ~ 27 - 30 kDa and an isoelectric point (pI) of 9. Its core architecture consists of a central β-sheet composed of nine β-strands (β1–β9) flanked by seven α-helices, stabilized by two disulfide bonds located adjacent to the active site, which contribute to structural stability [3, 4]. In contrast, MHETase (65 kDa, pI 5) adopts a two-domain architecture, consisting of an α/β-hydrolase core domain that shares structural homology with the feruloyl esterase FaeB from *Aspergillus oryzae*, and an extended cap domain characterized by multiple loop regions. While PETase presents a highly polar surface, MHETase is characterized by an uneven distribution of surface charges and overall acidic properties. In earlier studies, both enzymes were heterologously expressed in *Escherichia coli* BL21(DE3) to improve yield and

stability for potential biotechnological applications [7, 8]. The present study aims to determine optimal conditions for PETase and MHETase activity and to evaluate their combined efficacy in degrading commercial PET substrates, thereby assessing their potential for practical application.

2. MATERIALS AND METHODS

2.1. Microorganisms

Escherichia coli BL21(DE3) strains carrying pET22b(+)-PETase and pET22b(+)-MHETase were obtained from the collection of the Department of Biotechnology, Institute of Materials, Biology and Environment.

2.2. Recombinant expression and enzyme activity assays

Recombinant PETase and MHETase were expressed in *E. coli* BL21(DE3) induction with isopropyl-β-D-thiogalactopyranoside (IPTG), as described previously [7, 8]. PETase activity was assayed using 4-nitrophenyl acetate (*p*NPA) as the substrate, with the release of p-nitrophenol (*p*NP) quantified spectrophotometrically at 405 nm [9,10]. MHETase activity was evaluated using MHET as the substrate, and the release of terephthalic acid (TPA) was quantified by high-performance liquid chromatography (HPLC; Agilent Technologies, USA) [6, 11]. One unit of enzyme activity (U) was defined as the amount of enzyme required to catalyze the formation of 1 μmol of product per minute under the assay conditions. All chemicals were purchased from Sigma-Aldrich (Germany).

2.3. Optimization of enzyme activity

2.3.1. Temperature

The effect of temperature on enzyme activity was assessed following previously described protocols [12-14]. Enzyme–substrate hydrolysis reactions were performed at 30, 40, 50, and 60 °C.

2.3.2. Buffer composition

The activities of PETase and MHETase were evaluated in three buffer systems: 100 mM Tris-HCl (pH 8.0), 100 mM sodium phosphate (pH 8.0), and 100 mM sodium acetate (pH 8.0), following the method described previously [15]. Enzymatic reactions were performed at the optimal temperature for each enzyme, using constant incubation time and substrate concentration. The effect of buffer composition on substrate hydrolysis efficiency was assessed based on product formation and enzyme activity.

2.3.3. pH value

The effect of pH on enzyme activity was assessed across the range of pH 5.0 - 10.0, as described previously [14, 16]. The pH of the selected buffer was adjusted to the desired values, while other reaction conditions (temperature, substrate concentration, and incubation time) were kept constant. Enzyme activity was measured at each pH value, and the results were used to determine the optimal pH for recombinant PETase and MHETase activity.

2.4. PET plastic degradation assay

The degradation of PET plastic by recombinant PETase and MHETase was assessed using pretreated PET films. Commercial PET sheets were cut into 1 × 1 cm pieces and sequentially washed in 1% SDS, 70% ethanol, and sterilized deionized water, each for 30 min [17]. The treated PET pieces were incubated in two methods: (1) 200 μL of extracellular fluid from the control strain, and (2) 200 μL of recombinant PETase and MHETase mixed at a 1:1 (v/v) ratio in the selected buffer. Incubations were performed at 40 °C for 7 days. Following incubation, PET pieces were collected, dried, and examined by field-emission scanning electron microscopy (FE-SEM; Hitachi S-4800, Hitachi, Japan).

2.5. Statistical analysis

Data were analyzed using SPSS software version 20 (SPSS Inc., Chicago, IL, USA).

Comparisons between experimental and control groups were performed using Student's t-test. Results are presented as mean ± standard deviation (SD) with three replicates, and differences were considered statistically significant at $p < 0,05$.

3. RESULTS AND DISCUSSION

3.1. Effect of temperature on enzyme activity

Temperature is a critical factor influencing both the stability of enzyme conformation and catalytic efficiency, thereby directly affecting substrate hydrolysis. In this study, the activities of PETase and MHETase were evaluated across a temperature range of 30 - 60 °C (table 1). Both enzymes exhibited the highest activity between 30 and 40 °C. Notably, MHETase activity increased markedly with rising temperature and reached its maximum at 40 °C, as reflected by the amount of TPA formed (figure 1). At temperatures above 40 °C, the activities of both PETase and MHETase declined sharply, with near-complete loss of activity at 60 °C. These reductions were statistically significant compared with the control reaction in which enzymes were added after termination of the reaction ($p < 0,05$).

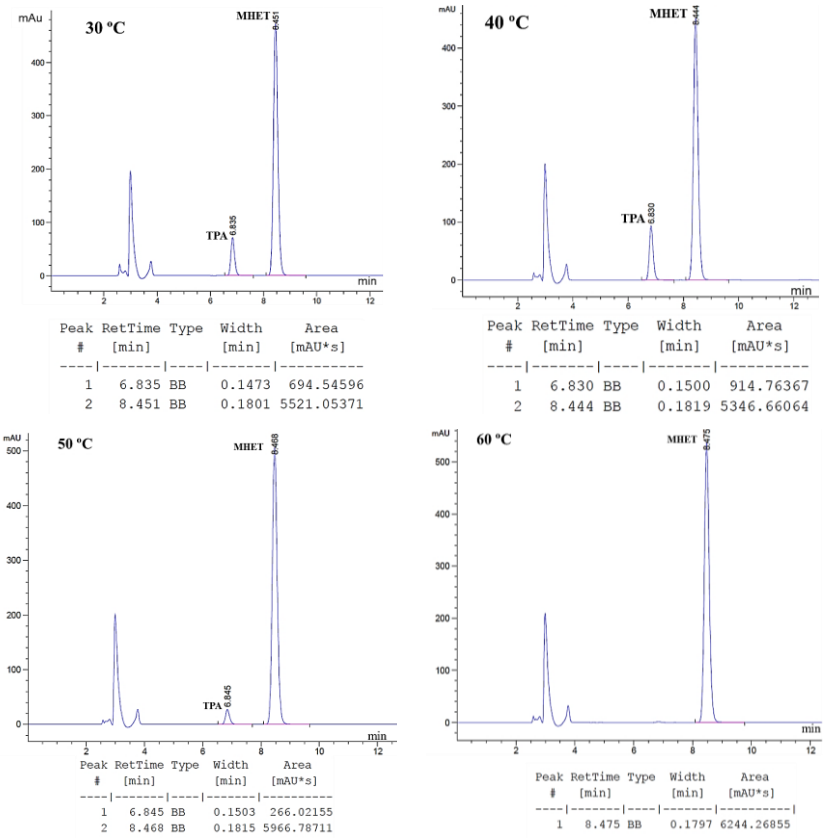

Figure 1. *Analysis of TPA produced from the MHETase-catalyzed hydrolysis of MHET substrate (the retention times of MHET and TPA were 8,4 and 6,8 minutes, respectively).*

Table 1. *PETase và MHETase activiy in different temperatures.*

Tem (°C)	PETase activity (U/ mL)	MHETase activity (U/ mL)
30	8,94 ± 0,007	28,49 ± 0,881
40	**9,21 ± 0,094**	**37,48 ± 0,662**
50	4,49 ± 0,083	11,03 ± 0,604
60	0,25 ± 0,069	0,15 ± 0,009

The observed optimal temperature range (30 - 40 °C) aligns with previously reported values for enzymes from *I. sakaiensis* [2, 16]. This range also corresponds to natural environmental conditions, highlighting the potential of PETase and MHETase for practical applications in plastic biodegradation. Additionally, Palm et al. (2019) reported that MHETase activity increased with temperature up to 44 °C but underwent rapid inactivation beyond this point [6, 12].

3.2. Effect of buffer composition on enzyme activity

The influence of buffer composition on PETase and MHETase activity was assessed in 100 mM Tris-HCl (pH 8.0), 100 mM sodium phosphate (pH 8.0), and 100 mM sodium acetate (pH 8.0). The results are presented in Figure 2. PETase exhibited the highest activity in phosphate buffer (7.55 ± 0,005 U/mL), followed by Tris-HCl buffer (4.12 ± 0,003 U/mL), and was nearly inactive in acetate buffer. Similarly, that was observed for MHETase, with the highest activity recorded in phosphate buffer (39.25 ± 0,742 U/mL). However, unlike PETase, MHETase retained considerable activity in both Tris-HCl buffer (37.12 ± 0,718 U/mL) and acetate buffer (35,02 ± 0,681 U/mL). These differences were statistically significant compared to the negative control ($p < 0,05$).

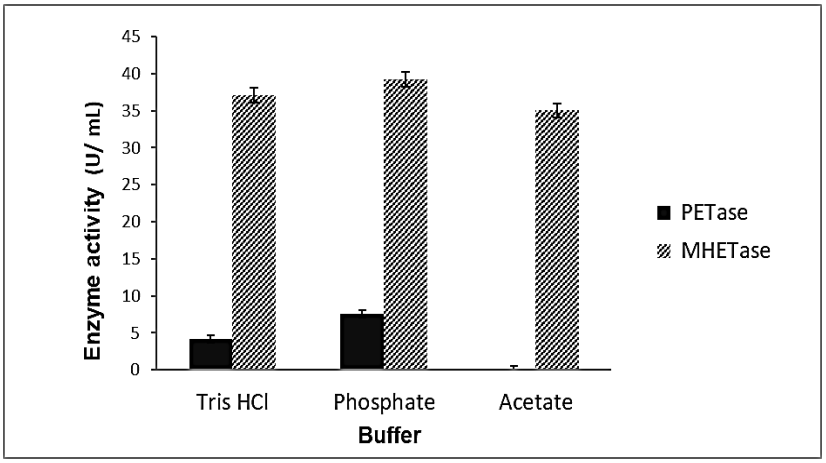

Figure 2. *Effect of buffer composition on the activity of two recombinant enzymes.*

These results demonstrate that buffer composition markedly affects the catalytic efficiency of both enzymes, with phosphate buffer providing the most favorable environment for hydrolysis. This observation is consistent with previous work by Schmidt et al. (2016), who reported that phosphate buffer supported superior hydrolytic activity of polyester hydrolases compared with other buffer systems [15]. The enhanced activity in phosphate buffer may be attributed to improved stabilization of enzyme structure and substrate interactions, suggesting its suitability for enzymatic PET degradation assays.

3.3. Effect of pH on enzyme activity

The results of the activity of PETase and MHETase at different pH values showed that the enzymes reached their highest activity at pH 8 and pH 9, respectively. Subsequently, the activity significantly decreased under strongly alkaline conditions ($pH > 9$) or acidic conditions ($pH < 6$). Specifically, PETase showed almost no activity in acidic environments (figure 3). These differences were statistically significant compared to the control sample ($p < 0,05$).

This result aligns well with previous reports on PETase and MHETase derived from *I. sakaiensis*, which demonstrated the highest activity in mildly alkaline environments around pH ~ 8 [2, 16]. In another study, the optimal pH for MHET-like enzyme Mle046 activity from marine environments was evaluated at pH 8 - 9, and it was sensitivity to strong acid or alkaline conditions [14]. This similarity indicates that, despite their different origins, they share optimal activity in alkaline conditions that may support enzyme stability and enhance substrate hydrolysis.

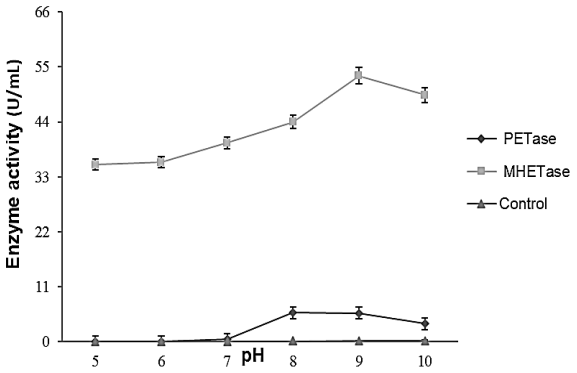

Figure 3. *Effect of pH on the activity of two recombinant enzymes.*

3.4. PET plastic degradation by recombinant enzymes

The degradative ability of recombinant PETase and MHETase was assessed by incubating commercial PET films in enzyme solutions at 40 °C for 7 days, followed by surface morphology analysis using scanning electron microscopy (SEM). Representative images are shown in figure 4. At a magnification of 50.000 X, PET film incubated with recombinant enzymes exhibited pronounced surface alterations, including the appearance of large holes, indicative of enzymatic degradation. In contrast, PET film incubated with extracellular fluid from the control strain showed little to no observable surface change at the same magnification.

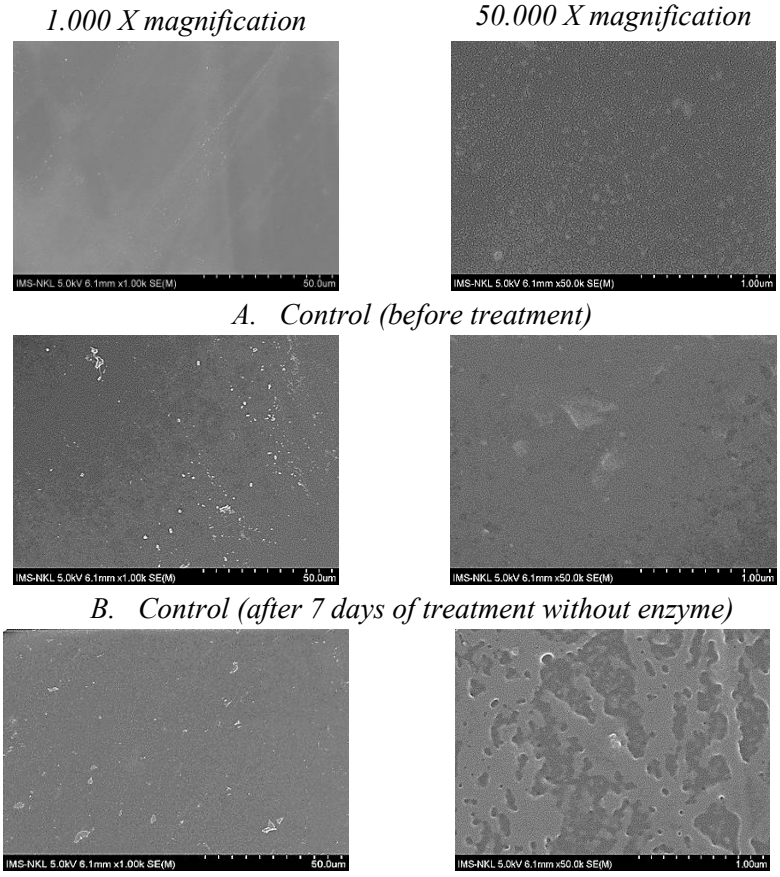

Figure 4. *SEM analysis of PET degradation by recombinant PETase and MHETase.*

These results provide visual evidence that recombinant PETase and MHETase acted synergistically to disrupt PET film integrity. While SEM analysis revealed clear surface erosion, further confirmation of enzymatic PET degradation in practical applications will require quantitative determination of intermediate and final products (MHET, BHET, and TPA), as well as long-term monitoring of film weight loss and chemical composition. Nevertheless, this initial assay highlights the potential of PETase and MHETase as biocatalysts for PET plastic waste degradation.

4. CONCLUSIONS

In this study, the effects of temperature, pH, and buffer composition on the activities of PETase and MHETase were systematically evaluated. The results demonstrated that both enzymes exhibited optimal activity within the temperature range of 30 - 40 °C, at alkaline pH values (8 - 9), and in 100 mM sodium phosphate buffer. Furthermore, SEM analysis of commercial PET films revealed substantial alterations in surface morphology after 7 days of incubation with recombinant PETase and MHETase, confirming their degradative capability. These findings highlight the potential of PETase and MHETase as promising biocatalysts for plastic waste treatment and provide a foundation for future research aimed at enhancing their activity and stability for practical applications.

Acknowledgments: *This research was supported by the Institute of Materials, Biology and Environment project. The authors also thank the Department of Omics Technology and Applications, Institute of Biology, Vietnam Academy of Science and Technology, for providing access to experimental facilities.*

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TÓM TẮT

Khảo sát một số yếu tố ảnh hưởng đến quá trình phân hủy nhựa polyethylene terephthalate của hệ 2 enzyme PETase và MHETase tái tổ hợp

PETase và MHETase là hai enzyme có nguồn gốc từ vi khuẩn được chứng minh có khả năng xúc tác thủy phân nhựa polyethylene terephthalate (PET) tạo sản phẩm cuối là TPA và EG thân thiện với môi trường. Việc sử dụng nguồn nguyên liệu sinh học này trong xử lý chất thải nhựa được coi là hướng nghiên cứu tiềm năng, theo xu hướng phát triển công nghệ xanh để bảo vệ môi trường. Trong nghiên cứu này, một số điều kiện thích hợp cho hoạt động của enzyme đã được khảo sát bao gồm nhiệt độ, pH, dung dịch đệm sử dụng. Hai enzyme PETase và MHETase tái tổ hợp thể hiện khả năng hoạt động hiệu quả nhất tại nhiệt độ 30-40 °C, dung dịch đệm sodium phosphate 100 mM và pH 8-9. Sau quá trình khảo sát, hệ hai enzyme được thử nghiệm khả năng phân hủy nhựa PET thương mại. Kết quả chụp ảnh SEM màng PET trước và sau phân hủy 7 ngày cho thấy, cấu trúc bề mặt nhựa PET đã có sự thay đổi rõ rệt dưới sự tác động của hai enzyme PETase và MHETase. Các kết quả bước đầu chứng minh khả năng ứng dụng thực tế đầy tiềm năng của 02 enzyme tái tổ hợp để xử lý chất thải nhựa bằng phương pháp sinh học.

Keywords: PETase; MHETase; Polyethylene terephthalate (PET); Phân hủy sinh học; Polyme tổng hợp.