

EVALUATION OF PET SHEET DEGRADATION USING THERMOPHILIC *GEOBACILLUS* SP. PT206

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Abstract

This research aimed to evaluate the degradation of Polyethylene terephthalate (PET) sheet using the thermophilic *Geobacillus* sp. PT206 isolated from the landfill site at Saraburi province. Strain PT206 gave the highest Hydrolytic Capacity on MSM with BHET, at 29.1 ± 14.0 . PET degradation was evaluated in biometer flasks containing Bushnell Haas Broth with PT206 at 55°C for 1 month. Optical density was measured every 7 days to monitor the bacterial growth, and carbon dioxide evolution was performed in three conditions: PT206 with PET, Control without PT206, and Control without PET. During incubation, the bacteria were able to grow, and the cumulative carbon dioxide increased. However, these changes were not significantly different from the control. The water contact angle of the treated plastic decreased from $67.12 \pm 1.02^\circ$ to $61.19 \pm 3.31^\circ$. Furthermore, the infrared spectrum revealed a change in all PET peaks in the plastic incubated with bacteria and SEM showed abundant biofilm on the treated plastic surface, which was not observed in the control. These results indicate that the incubation of PT206 with PET for 1 month led to changes in the PET surface.

Keywords: Biodegradation; *Geobacillus*; Plastic degradation; Polyethylene terephthalate; Thermophilic bacteria

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Introduction

Plastic is a widely used synthetic material, but its usage has led to a surge in plastic waste, as reported by the Organization for Economic Development and Cooperation. Plastic usage and production doubled from 2000 to 2019, reaching 460 million tons. Global plastic waste production also doubled, reaching 353 million tons in 2019. The report indicates that nearly two-thirds of plastic waste, 40% from packaging and 12% from consumer products is generated. 19% is incinerated, 50% up to landfills, and 22% ends up in uncontrolled dumping sites, and only 15% collected for recycling. Out of this 15%, only 9% is recyclable, leaving 6% as waste. This substantial plastic waste impacts communities and the environment, leading to health, economic, and environmental issues.

Bioremediation, using microorganisms to break down plastic into carbon dioxide and water, offers a solution to plastic contamination. Some microorganisms produce effective plastic-degrading enzymes. For instance, *Ideonella sakaiensis* 201-F6 can degrade PET into eco-friendly byproducts (Yoshida *et al.*, 2016). Enzymes like PETases from *Ideonella sakaiensis*, cutinases from *Thermobifida alba* AHK119, and lipases from *Thermomyces lanuginosus* play important roles in plastic degradation (Zhou *et al.*, 2022). However, there are limitations in using microorganisms for plastic bioremediation. They can efficiently degrade plastics from renewable sources like PHA, PLA, and PBS, but their capability to break down fossil fuel-based plastics (PP, PE, and PET) is limited. This highlights the necessity for further research in this field.

This research recognized the importance of isolating and identifying thermophilic bacteria capable of plastic degradation in plastic-contaminated sites like landfills. These findings could facilitate the use of thermophilic bacteria or enzymes for plastic waste reduction, aiding in environmentally sustainable rehabilitation.

Materials and Methods

Isolation of thermophilic bacteria from landfill sites

Soil samples from Phra Phutthabat (PT1 and PT2, GPS = 14.731408, 100.764965) and Nong Khae (NK1 and NK2, GPS = 14.323697, 100.885538) landfills in Saraburi province were collected at a 20 cm. depth and stored at 4°C in sterile ziplock bags. Each flask containing 20 g of soil samples and 100 ml of Minimal Salts Medium (MSM) was enriched with each plastic sheets; PS

(Foam food box, Sitex Foam K-500 brand), PVC (Document folder, Bennon brand), or PET (ES30-FM-000145, Goodfellow, England) as the sole carbon source. Incubation occurred at 55°C and 150 rpm for one month. Afterward, the culture broth from each flask was ten-fold serial dilution and spread on MSM agar supplemented with PS (Sigma-Aldrich, USA), PVC (Sigma-Aldrich, USA), or BHET (Sigma-Aldrich, USA) emulsion and incubated at 55°C for 48 hours. Colonies growing on the plates were selected for plastic degradation tests on MSM-emulsified plastic agar.

Plastic degrading properties test on MSM-emulsified plastic agar

Two grams of BHET powder were dissolved in 60 ml of dimethyl sulfoxide (Thermo Fisher Scientific, USA), heated at 55°C, and stirred with a magnetic stirrer. The BHET solution was then adjusted to a volume of 400 mL with distilled water to obtain a 0.5% BHET plastic emulsion. The volume of MSM-BHET plastic agar can be referenced from previous research (Chaimusik *et al.*, 2024).

A bacterial single colony was tested on MSM agar supplemented with emulsified BHET plastic and incubated at 55 °C for 24 hours. The presence of a clear zone around the bacterial colonies was observed, and the Hydrolysis Capacity (HC) was calculated as follows:

$$\text{HC value} = \frac{\text{diameter of the clear zone around the bacterial colony}}{\text{diameter of the bacterial colony}}$$

16S rRNA gene amplification and analysis

Genomic DNA was extracted from bacteria using the heat treatment method (Dashti *et al.*, 2009). Amplification reactions were prepared to a final volume of 20 µl containing 2X PCR Master mix solutions (i-Taq™, iNtRON Biotechnology, Korea), 10 pmols of primer, and template DNA. The 16S rRNA region was PCR amplified using the primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTACGACTT-3'). The positive control was *Rhodococcus ruber* DSM 45332. The PCR conditions were as follows: initial denaturation at 95°C for 5 min, followed by 30 cycles of denaturation at 95°C for 30 sec, annealing at 52°C for 30 sec, and extension at 72°C for 30 sec, and a final extension at 72°C for 5 min. The PCR products were verified by agarose gel electrophoresis and purified using the AccuPrep PCR Purification Kit. The purified PCR products were sequenced by U2Bio, Thailand. The full-

length 16S rRNA, approximately 1500 bp in length, the molecular bacterial identification was analyzed using EzBiocloud.

Growth investigation and carbon dioxide evolution test

PET degradation was evaluated in biometer flasks containing of 100 ml Bushnell Haas Broth (BHB), inoculated with one full loop of *Geobacillus* strain PT206 and five 1.5 cm by 1.5 cm PET sheets from water bottles, Crystal brand. Incubation at 55°C and 150 rpm for one month. A biometer flask was connected to a tube containing 15 ml of 0.1 N sodium hydroxide. Growth was monitored in three conditions: PT206 with PET, control without PT206, and control without PET. Absorbance at 600 nm measured bacterial growth, and carbon dioxide produced was assessed by titration using 0.1 N hydrochloric acid, with phenolphthalein as the indicator.

Measuring the weight loss of PET plastic

After one month of incubation, the PET sheet was cleaned with 75% ethanol for an hour, followed by three times rinses with distilled water. Subsequently, it was soaked in a 2% Sodium Dodecyl Sulfate (SDS) solution for 2 hours to remove attached bacterial cells. After three additional rinses with distilled water, the PET sheet was dried at 50°C overnight. The weight of each plastic was recorded using a 4-digital analytical balance to compare changes before and after bacterial degradation. This experiment was performed in triplicate.

Determination of the water contact angle on the PET surface

PET sheets from all conditions and virgin PET (untreated PET) have been cleaned following the steps above and observed the water contact angle on the plastic surface using the KYOWA interFace Measurement and Analysis System FAMAS. The degree of water contact angle on the PET sheet was compared with the virgin PET sheet. The experiment was performed in triplicate.

FT-IR analysis

Virgin PET sheets and all tested PET sheets were cleaned as previous experiment and subjected to a Fourier Transform Infrared Spectrometer (FT-IR). The FT-IR spectral changes in chemical groups on the plastic surface were compared between virgin and tested PET sheets using the SpectraGryph 1.2 program.

Bacteria adhesion on PET surface

Bacterial cells on virgin and treated PET sheets were fixed in 2% Glutaraldehyde in 0.1 M phosphate buffer at pH 7.2 for 2 hours. The cells were dehydrated by soaking in ethanol at different concentrations (30%, 50%, 70%, and 100%) for 30 minutes in each at 4°C. PET sheets were dried in a hot air oven at 30°C, overnight. Afterward, PET sheets were coated with gold and observed microbial adhesion on the plastic surface using an Axia™ ChemiSEM™ Scanning Electron Microscope (SEM) on virgin and tested PET sheets.

Results and Discussion

Isolation of thermophilic bacteria from soil samples

The bacteria enrichment was performed using MSM medium and enriched by PS, PVC, or PET sheet as a sole carbon source. The supernatant was spread plated on MSM supplemented with PS, PVC and BHET plastic emulsions. The result showed that, no bacterium from PT1 site. In contrast, 14, 12, and 12 isolates were shown in PT2, NK1, and NK2 site, respectively in enriched by PE. However, no bacteria were observed in the enrichment by PS and PVC. The soil sample characteristics at PT1 were clayey. However, the diversity of microbial communities residing in the deep terrestrial subsurface environment remain poorly understood. Clay rocks have been demonstrated to inhibit microbial growth. This is primarily due to the small pore size and limited interconnectivity, which restrict the availability of water and nutrients. Hence, microbial activity is expected to be low or communities in clay-rich environments (Stroes-Gascoyne *et al.*, 2007). In this study, none of the bacteria were isolated in the PS and PVC enrichment. PS and PVC are types of petroleum-based plastic, which are resistant to biodegradation.

Evaluation of plastic degradation on MSM-emulsified plastic agar

A total of 38 bacterial isolates were tested plastic degradation on MSM-BHET agar and incubated at 55 °C for 48 hours. The clear zone around the bacterial colonies was measured to calculate the Hydrolysis Capacity (HC) value. It was found that, at PT2 site, isolate PT206, PT207, and PT213 had the highest HC value at 29.1 ± 14.0 , 27.2 ± 15.3 , and 27.3 ± 5.0 , respectively. For NK1 site, isolate NK103, NK112, and NK102, had the highest HC values of 26.7 ± 14.5 , 19.3 ± 13.6 , and $18.2 \pm$

13.7, respectively, and NK2 site, isolate NK201, NK203, and NK206, had the highest HC values of 11 ± 2.6 , 8.5 ± 1.3 , and 7.6 ± 0.4 , respectively, as shown in Figure 1. This study demonstrated the capability to degrade plastic by cultivating thermophilic bacteria on MSM-BHET agar plates.

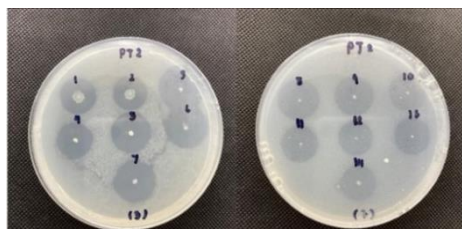


Figure 1. The clear zone of 14 thermophilic bacterial isolates at the PT2 site on MSM-BHET after incubated at 55°C for 48 hours

Identification of thermophilic bacterial isolates

Based on the identification by 16S rRNA region analysis using EZBiocloud. Thirty-one thermophilic bacteria isolates (isolates NK102, NK103, NK105, NK106, NK107, NK108, NK109, NK110, NK111, NK112, NK201, NK202, NK204, NK205, NK206, NK207, NK209, NK211, PT202, PT203, PT204, PT205, PT206, PT207, PT208, PT209, PT210, PT211, PT212, PT213 and PT214) exhibited highest 99-100% similarity to *Geobacillus thermodenitrificans* subsp. *calidus*. Isolates NK101 and NK210 displayed highest similarities of 99.93% and 100% to *Anoxybacillus rupiensis* and *Anoxybacillus caldiproteolyticus*, respectively. Isolates NK104 and NK212 had 99.57% and 99.48% similarity to *Geobacillus stearothermophilus*, respectively, and isolate NK203 exhibited a 99.80% similarity to the *Geobacillus thermodenitrificans* subsp. *thermodenitrificans*. Additionally, isolate PT201 belong to with 100% similarity to *Parageobacillus toebii*.

Additionally, *Geobacillus* sp. PT206 were selected for further study by the highest HC value. These bacteria were characterized by small, cloudy-white colonies at the center with a darker-white periphery. The colony edges exhibited slight waviness and formed a clear zone in the surrounding colony, as shown in Figure 2. According to prior research, *Geobacillus* sp. has the ability to generate a heat-resistant lipase enzyme that can endure temperatures of up to 85°C (Mahadevan and Neelagund, 2014).

The growth of PT206 during PET sheet evaluation

Geobacillus sp. PT206 was employed to test PET degradation in BHB broth under three

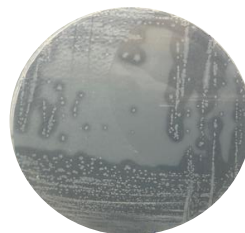


Figure 2. The morphology of *Geobacillus* sp. PT206 after incubation at 55°C, 48 hours

conditions; PT206 with PET, control without PT206, and control without PET. Optical density was measured every 7 days for a month to monitor bacterial growth. PT206 with PET was gradually grew since day 7 until reaching the highest at day 28; however, this growth was not significantly different from the control without PT206, as shown in Figure 3. Control PT206 exhibited very little growth at day 7 and decline.

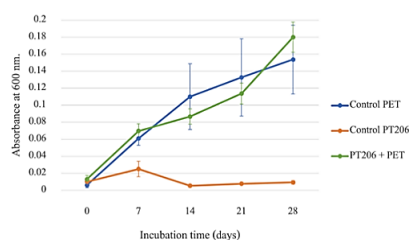


Figure 3. The growth of PT206 on different incubation time (days). The error bar represents the standard deviation of triplicate experiments

A spread plate technique was employed to examine the increased absorbance in the control PET and there were none of the contaminants. Additionally, the physical appearance of the control PET flask remained clear (data not shown).

Carbon dioxide evolution of *Geobacillus* sp. PT206

After the complete PET biodegradation, the end products were carbon dioxide and water. Carbon dioxide evolution evaluates the plastic biodegradation. The results indicated that as the incubation time increased, PT206 + PET exhibited an increase in carbon dioxide accumulation until reach 0.045 mg in 28 days. However, this increase was not significantly different from the control condition. Consequently, this result does not provide a clear conclusion regarding whether PT206 could degrade PET plastic sheets. Currently, no research reports PET degradation by *Geobacillus* sp.

The weight loss in PET plastic

After incubation for 1 month, three PET plastic sheets were washed and their weights were recorded comparing to virgin PET. The results showed that the percentage weight loss of plastic sheets in the control PET was 0.2821 ± 1.2972 , whereas in the PT206 + PET, it was 0.2067 ± 1.7422 . Notably, there was no significant difference between control and treated condition.

The water contact angle on PET surface

To determine PET surface hydrophobicity, the water contact angle on the PET plastic sheet were measured triplicate. The results revealed that the water contact angle on virgin PET sheets was $73.2 \pm 0.1^\circ$, while it measured $67.12 \pm 1.02^\circ$ for control PET and $61.19 \pm 3.31^\circ$ for PT206 + PET, as shown in Figure 4. The water contact angle of PT206 treatment (PT206 + PET) was lower than that of virgin PET sheets, indicating that *Geobacillus* sp. PT206 led to alterations on the surface of the PET sheets with increase of hydrophilicity properties on the PET surface. Biodegradation may reduce the hydrophobicity of PET sheet and increase its hydrophilicity, making it less resistant to bacterial degradation (Kumari *et al.*, 2021).

Examination of chemical groups on PET Surface by FT-IR

After one month of incubation, the chemical groups on the surface of PET sheets were analyzed using FT-IR. The infrared spectral graph was compared to the control. It is noteworthy that the

plastic sheets used in this study are authentic PET plastic, as evidenced by their distinctive infrared spectral PET peaks (1711 , 1240 , 1095 , and 722 cm^{-1}), which matches the standard peaks (1713 , 1241 , 1094 , and 720 cm^{-1}) (Jung *et al.*, 2018). The infrared spectrum clearly indicates a change in the intensity of all PET peaks in the plastic incubated with *Geobacillus* sp. PT206 (PT206 + PET), as shown in Figure 5. This finding demonstrates that incubation with *Geobacillus* sp. PT206 has induced alterations on the PET plastic surface. These alterations are likely attributed to biofilm formation and oxidation reactions, leading to an increase in hydrophilicity. This facilitates bacterial adhesion and biodegradation, which is consistent with a previous study with *Bacillus gottheilii* (Auta *et al.*, 2017).

Geobacillus sp. PT206 adhesion on PET surface by SEM

After one month of incubation, the PET sheets were examined for bacteria adhesion to the plastic surface using Scanning Electron Microscope, as showed in Table 1. The SEM images revealed that the virgin PET and control PET had smooth surface showing no formation of bacterial biofilm. In contrast, PET sheets from the PT206 treatment exhibited abundant biofilm adhesion to the treated plastic surface. Due to no significant surface damage e.g., hole, it indicated that the degradation occurs only at surface level. From previous study, some plasticizers are biodegradable by bacteria. For instance, *Pseudomonas* sp. V21b and

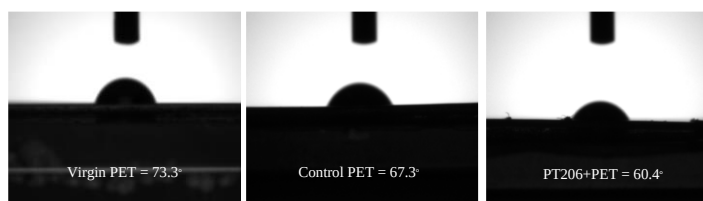


Figure 4. The water contact angles on the PET surface of virgin PET, Control PET, and PT206 + PET

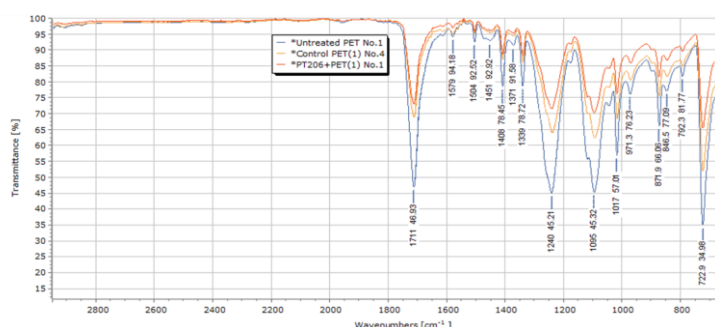
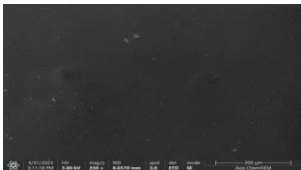
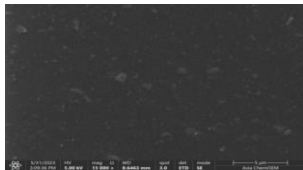
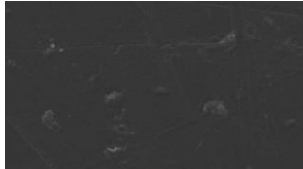
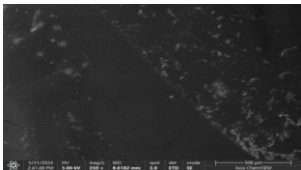
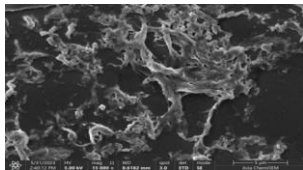


Figure 5. Comparison of the infrared spectrum of virgin PET plastic sheets, and treated PET (control PET and PT206 + PET)

Table 1. The PET plastic surface under Scanning Electron Microscope

Condition	Plastic surface under Scanning Electron Microscope	
	350X	15000X
Virgin PET		
Control PET		
PT206 + PET		

Comamonas sp. 51F isolated from Indian landfill areas degraded dibutyl phthalate (DBP) by 57% and 46%, respectively, in 192 hours, even at high concentrations (Kumar *et al.*, 2017). Thus, certain bacteria possess the capacity to degrade plasticizers, potentially leading to surface modifications on plastic sheets.

Conclusions

Our study successfully isolated thermophilic bacteria from the landfill site in Phra Phutthabat and Nong Khae, Saraburi province. These bacteria demonstrated the capability to degrade MSM-BHET agar. The bacterial *Geobacillus thermodenitrificans* subsp. *Calidus* strain PT206 exhibited the highest Hydrolysis Capacity (HC) value. Cultivation of *Geobacillus* sp. PT206 with PET sheets in BHB flask for one month led to observable changes on the surface of the PET sheets.

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References

Auta, H., Emenike, C., and Fauziah, S. (2017). Screening of *Bacillus* strains isolated from mangrove ecosystems in

- Peninsular Malaysia for microplastic degradation. *Environmental Pollution*, 231:1552-1559. <https://doi.org/10.1016/j.envpol.2017.09.043>
- Chaimusik, N., Sombuttra, N., Nakaramontri, Y., Sompongchaiyakul, P., Charoenpong, C., Intra, B., and Euanorasetr, J. (2024). The comparative plastisphere microbial community profile at Kung Wiman beach unveils potential plastic-specific degrading microorganisms. *PeerJ*, 12:e17165. <https://doi.org/10.7717/peerj.17165>
- Dashti, A.A., Jadaon, M.M., Abdulsamad, A.M., and Dashti, H.M. (2009). Heat treatment of bacteria: A simple method of DNA extraction for molecular techniques. *Kuwait Medical Journal*, 41(2):117-122.
- Jung, M.R., Horgen, F.D., Orski, S.V., Rodriguez, V., Beers, K.L., Balazs, G.H., Jones, T.T., Work, T.M., Brignac, K.C., and Royer, S.-J. (2018). Validation of ATR FT-IR to identify polymers of plastic marine debris, including those ingested by marine organisms. *Marine Pollution Bulletin*, 127:704-716. <https://doi.org/10.1016/j.marpolbul.2017.12.061>
- Kumar, V., Sharma, N., and Maitra, S. (2017). Comparative study on the degradation of dibutyl phthalate by two newly isolated *Pseudomonas* sp. V21b and *Comamonas* sp. 51F. *Biotechnology Reports*, 15:1-10. <https://doi.org/10.1016/j.btre.2017.04.002>
- Kumari, A., Bano, N., Chaudhary, D.R., and Jha, B. (2021). Draft genome sequence of plastic degrading *Bacillus* sp. AIIW2 isolated from the Arabian Ocean. *Journal of Basic Microbiology*, 61(1):37-44. <https://doi.org/10.1002/jobm.202000416>
- Mahadevan, G.D., and Neelagund, S.E. (2014). Thermostable lipase from *Geobacillus* sp. Iso5: Bioseparation, characterization and native structural studies. *Journal of Basic Microbiology*, 54(5):386-396. <https://doi.org/10.1002/jobm.201200656>
- Stroes-Gascoyne, S., Schippers, A., Schwyn, B., Poulain, S., Sergeant, C., Simonoff, M., Le Marrec, C., Altmann, S., Nagaoka, T., and Mauclaire, L. (2007). Microbial community analysis of Opalinus clay drill core samples from the Mont Terri underground research laboratory, Switzerland. *Geomicrobiology Journal*, 24(1):1-17. <https://doi.org/10.1080/01490450601134275>

- Yoshida, S., Hiraga, K., Takehana, T., Taniguchi, I., Yamaji, H., Maeda, Y., Toyohara, K., Miyamoto, K., Kimura, Y., and Oda, K. (2016). A bacterium that degrades and assimilates poly(ethylene terephthalate). *Science*, 351(6278):1196-1199. <https://doi.org/10.1126/science.aad6359>
- Zhou, Y., Kumar, V., Harirchi, S., Vigneswaran, V., Rajendran, K., Sharma, P., Tong, Y.W., Binod, P., Sindhu, R., and Sarsaiya, S. (2022). Recovery of value-added products from biowaste: A review. *Bioresource Technology*, 354:127565. <https://doi.org/10.1016/j.biortech.2022.127565>