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The Potential of *Bacillus altitudinis* B538 and *Alcaligenes faecalis* B947 in PET and PCL Plastic Degradation

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ABSTRACT

Polyethylene terephthalate (PET) plastic is the most widely used type of plastic that produces waste and causes various environmental and health problems. The treatment of PET plastic waste with chemically and mechanically recycling approaches still has shortcomings, so biological processing using microorganisms or enzymes has new potential. Two bacterial isolates from the Indonesian Culture Collection of National Research and Innovation Agency (InaCC, BRIN), namely isolate InaCC B538 and InaCC B947, were further observed for their potential in PET plastic degradation. Firstly, both isolates were determined by the molecular marker 16S rDNA. The potential of both isolates was measured with following method: 10 days of degradation using PET and PCL substrates, esterase enzyme activity assay, and observation of the PET plastic surface using Scanning Electron Microscope (SEM). Species identification was performed using DNA sequencing of 16S rDNA. InaCC B538 and InaCC B947 were closely related to *Bacillus altitudinis* TBMAX41 and *Alcaligenes faecalis* AN-13, respectively. InaCC B947 isolate has a better potential in degrading PET plastic and PCL with a degradation percentage of 0.32% for PET plastic and 3.22% for PCL film for 10 days, respectively, and esterase activity of 0.06 U/ml; while InaCC B538 did not cause weight loss of PET and 2.49% for PCL, respectively, with esterase activity of 0.04 U/ml. The degradation of PET plastic by the isolates InaCC B947 was able to cause damage to the plastic surface leading to the degradation of PET plastic.

1. Introduction

Plastic is a material formed from the polymerization process between carbon and hydrogen. Plastics are usually used as daily necessities because of their unbreakable, strong, lightweight, and rust-proof characteristics. Plastic has a long carbon chain, so the natural degradation of plastic takes a very long time. Based on Ratnawati (2020), plastic in general takes up to 100 years to decompose completely. From 1950 to 2019, 6.3 trillion tons of plastic have been produced, however, only 9% has been recycled and 12% of plastic waste has been incinerated (Alabi *et al.* 2019). Polyethylene terephthalate (PET) is one of the most widely produced plastics. Global PET production reached 33 million metric tons in 2015 (Urbanek *et al.* 2021). The treatment of PET plastic waste has been carried out using chemical and mechanical methods. Chemical processing of PET plastic can cause the release of harmful compounds, complex processes, and requires certain reagents with high temperatures (Ragaert *et al.* 2017). Meanwhile, mechanical processing requires certain technologies with high costs and varied conditions. Therefore, the biological treatment of PET plastic has high potential because it is relatively low-cost, simpler yet efficient, and also utilizes natural decomposition.

Biological treatment of PET plastic waste by utilizing enzymatic degradation has been widely researched. Although PET is non-biodegradable plastic, many studies have found the ability of

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microorganisms to degrade PET plastic by utilizing enzymatic hydrolysis to produce simple monomers. The species *Ideonella sakaiensis* has PETase and MHETase enzymes that are capable of degrading PET film within 6 weeks (Yoshida *et al.* 2016). *Pseudomonas pseudoalcaligenes* species was found to secrete esterase enzymes and degrade ionic phthalic acid-based polyesters (Haernval *et al.* 2017).

Based on the bacterial collection from the Indonesian Culture Collection of National Research and Innovation Agency (InaCC, BRIN), two isolates, InaCC B538 and InaCC B947, are tested for their ability to degrade PET and PCL plastic material. The objective of this research was to identify and determine the potential of InaCC B538 and InaCC B947 isolates in degrading PET plastic material.

2. Materials and Methods

2.1. Bacterial Isolates Preparation

Both isolate cultures were obtained from the Indonesian Culture Collection of the National Research and Innovation Agency (InaCC-BRIN). InaCC B538 was isolated from rice rhizosphere soil in Patimban, Subang, West Java, while InaCC B947 was isolated from rice field soil in Indramayu, West Java, Indonesia. Both isolates were prepared by refreshing glycerol stock cultures of InaCC B538 and InaCC B947 isolates. Two percent (v/v) of each isolate was inoculated into 5 ml of Nutrient Broth (NB) medium pH 6.8 and incubated overnight or 16 hours. The overnight culture of 3% was inoculated into 20 ml of NB media and incubated to reach an OD of 0.7-0.8 at 37°C for 2 to 4 hours.

2.2. Plastic Substrate Preparation

The materials used as degradation substrates were amorphous PET plastic (Goodfellow) and polycaprolactone (PCL) (Sigma-Aldrich). The amorphous PET substrate was cut to 1.5 × 1.5 cm and weighed. The PET plastic was sterilized using 96% alcohol and aerated in a Laminar Air Flow (LAF). The 1.5 grams of PCL was dissolved into 60 ml of acetone at 50°C with continuous stirring. Each 10 ml of PCL solution was poured on a petri dish and aerated to form a PCL film. The PCL film was cut into 3 × 3 cm and weighed. PCL film was also sterilized to prevent contamination.

2.3. Biodegradation Assay

The sterilized substrate was then subjected to a biodegradation assay by each InaCC isolate, respectively. A 10% starter culture of InaCC isolates B538 and InaCC B947 was inoculated into 20 ml minimum media (0.05% yeast extract, 0.2% ammonium sulfate, 1% trace elements (0.1% FeSO4•$7H_2O$, 0.1% $MgSO_4$•$7H_2O$, 0.01% $CuSO_4$•$5H_2O$, 0.01% $MnSO_4$•$5H_2O$, 0.01% $ZnSO_4$•$7H_2O$), and 10 mM phosphate buffer pH 7.0). Each sterile 1.5 × 1.5 cm PET plastic and 3 × 3 cm PCL film substrate was added to the isolate culture and incubated at 37°C with a 100 rpm agitation speed for 10 days, respectively. The substrates were removed and washed using 1% SDS, water, and 96% alcohol respectively. The final weight of the substrate was measured, and the percentage of degradation was calculated using the following formula:

$$\text{Weight loss (\%)} = \frac{\text{initial weight (Wo) - final weight (Wt)}}{\text{initial weight (Wo)}} \times 100\%$$

2.4. Standard Curve Preparation

Standard solutions were prepared by dilution of p-Nitrophenol (pNP) at concentration of 0 mg/ml to 6.25×10^{-3} mg/ml with range 6.25×10^{-4} mg/ml using 0.02 M phosphate buffer pH 8. The absorbance of pNP solution was measured at a wavelength of 405 nm. The absorbances were used for pNP standard curve.

2.5. Esterase Activity Assay

The biodegradation assay media of isolates InaCC B538 and B947 were measured for esterase activity using the method of Tekincanli *et al.* (2015) and Shin *et al.* (2021). The biodegradation assay medium was centrifuged at 6,000 rpm for 5 min to obtain the enzyme sample in the supernatant. 10 µl of enzyme sample was mixed into a solution containing 200 µl of 0.1 M p-Nitrophenyl acetate (pNPA) substrate and 890 µl of 0.02 M phosphate buffer pH 8. The solution was incubated at 37°C for 5 minutes, and the absorbance of p-nitrophenol was measured at a wavelength of 405 nm. Esterase enzyme activity was measured using the following formula:

$$\text{Esterase activity (U/ml)} = \frac{\text{pNP concentration (mg/ml)} \times \text{dillution factor} \times 1{,}000}{\text{molecule weight of pNP} \times \text{incubation time (minute)} \times \text{volume of enzyme (ml)}}$$

One unit activity is defined as the amount of enzymes that produce 1 µmol of p-nitrophenol (pNP) per min.

2.6. Determination of Structure Surface Using Scanning Electron Microscope (SEM)

The surface structure of PET plastic substrate and PCL film was observed using SEM. Sample was prepared by sterilization using alcohol 96% and dried. The sample was positioned at the bottom of the electron column and the electrons on the sample are captured by an electron detector. SEM observations were performed by Laboratorium of Mineral, National Research and Innovation Agency (BRIN) Tanjung Bintang, Lampung, Indonesia.

2.7. Species Identification Using 16S rDNA Sequencing

The species of InaCC B538 and InaCC B947 isolates were identified using the 16S rDNA PCR. Genomic DNA of both isolates was isolated using a genomic DNA isolation kit (Thermoscientific). The materials used in the 16S rDNA PCR stage consisted of genomic DNA of each isolate as DNA template, 27F and 1525 R primers, Taq HS DNA polymerase (Meridian Bioscience), 5x reaction buffer, and ddH_2O. PCR conditions were adjusted to the Taq HS DNA polymerase protocol with an initial denaturation of 95°C for 1 minute, denaturation of 95°C for 15 seconds, annealing of 55°C for 15 seconds, extension of 72°C for 10 seconds, and final extension of 72°C for 10 minutes. The amplification results were then subjected to DNA sequencing by Genetika Science. The DNA sequences obtained were identified with the Basic Local Alignment Search Tool (BLAST) feature of the National Center for Biotechnology Information (NCBI) website.

3. Results

3.1. Biodegradation Effectiveness

The biodegradation effectiveness of InaCC isolates B538 and B947 on PET plastic and PCL film was shown as the weight loss percentage of both substrates after incubation for 10 days. The weight loss of PET plastic was only found in InaCC B947 with a percentage of 0.32%, lower than other bacteria like *Stenotrophomonas malthopilia* with 0.5% weight loss on PET flakes degradation for 15 days. Degradation of PET plastic by InaCC B538 isolate did not cause any weight loss on PET plastic (Figure 1A).

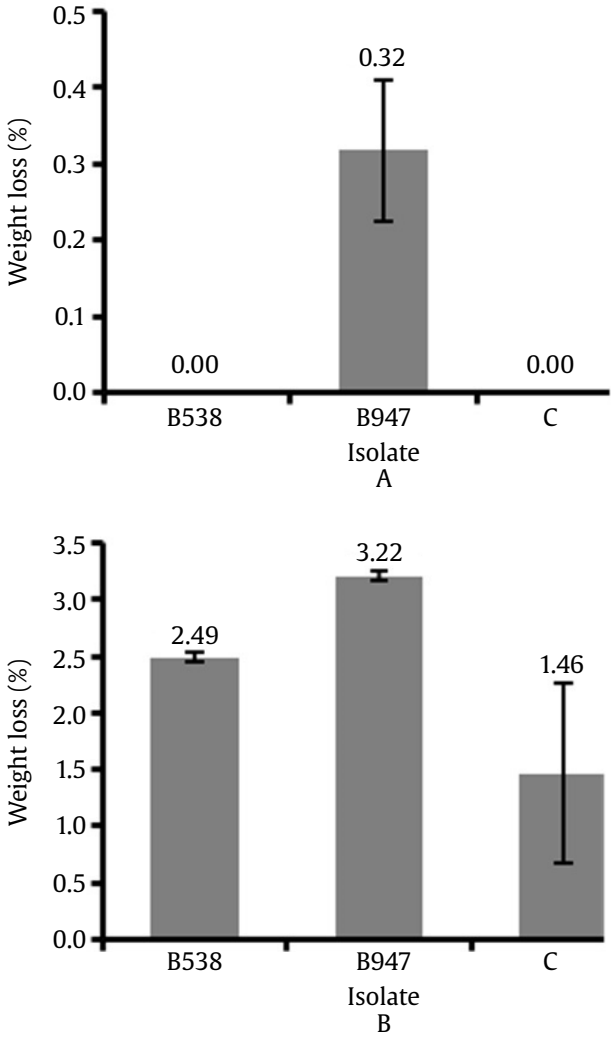


Figure 1. (A) Weight loss percentage of PET plastic for 10 days by isolates InaCC B538, InaCC B947, and negative control, (B) weight loss percentage of PCL film for 10 days by isolates InaCC B538, InaCC B947, and C

Meanwhile, the weight of PCL film was reduced after the biodegradation process by each isolate with the highest weight loss percentage of 3.22% obtained on PCL film degraded by InaCC B947 (Figure 1B).

3.2. Esterase Activity

Enzyme samples were obtained from the biodegradation assay of PET plastic and PCL film by InaCC B538 and InaCC B947. The highest esterase activity was obtained from the biodegradation medium of InaCC B947 on both types of substrates after the biodegradation for 10 days with a value of 0.06 U/ml on PET substrate and 0.08 U/ml on PCL substrate. The esterase activity of the enzyme InaCC B538 on the enzyme samples from the

biodegradation test of PET plastic and PCL film was 0.04 U/ml and 0.05 U/ml, respectively (Figure 2A and B).

3.3. Surface Morphology by Scanning Electron Microscope (SEM)

The surface structure of PET plastic after biodegradation by the control, InaCC B538, and InaCC B947 isolates is shown in Figure 3, respectively. The surface of PET plastic degraded by InaCC B538 and InaCC B947 was damaged as shown by the presence of flakes and white cracks on the plastic surface. PET plastic degraded by InaCC B947 has more damage as seen from the rough texture and various sizes of white flakes compared to PET plastic degraded by InaCC B538. The damage on the surface of PET plastic proves the enzymatic hydrolysis activity by both InaCC isolates (Figure 3).

Figure 4 shows the SEM results of the PCL film surface degraded by the control, InaCC isolate B538, and InaCC isolate B947, respectively. The PCL film degraded by InaCC B947 was damaged, as indicated by a rougher surface and the presence of flakes on the surface of the PCL film. The PCL film degraded by InaCC B538 showed an uneven surface with a certain pattern of fibers on the entire film surface (Figure 4).

3.4. Identification of Species

Based on the 16S rDNA sequencing results, it was found that the InaCC B538 isolate was related to the *Bacillus altitudinis* TBMAX41 species. This was indicated by the sequence identity similarity of 99.00%. Meanwhile, the InaCC B947 isolate has the closest relation with the *Alcaligenes faecalis* AN-13 species with a sequence identity similarity of 98.94% (Figure 5A and B).

4. Discussion

The species of InaCC B947 isolate, which was identified as belonging to the *Alcaligenes faecalis* species. From previous research of Nag *et al.* (2021), the same species had the ability to degrade plastic made from Polyethylene (PE) with a degradation percentage of 37.20-47.36% over 10 weeks. It was able to utilize polyethylene as a carbon source to survive and degrade it, resulting in a reduction in dry weight. Polymers with higher flexibility and a lower number of carbon atoms than PET, such as polyethylene, can be more easily utilized by bacterial cells for their metabolic processes. The weight loss of the PET plastic substrate and PCL film is due to the

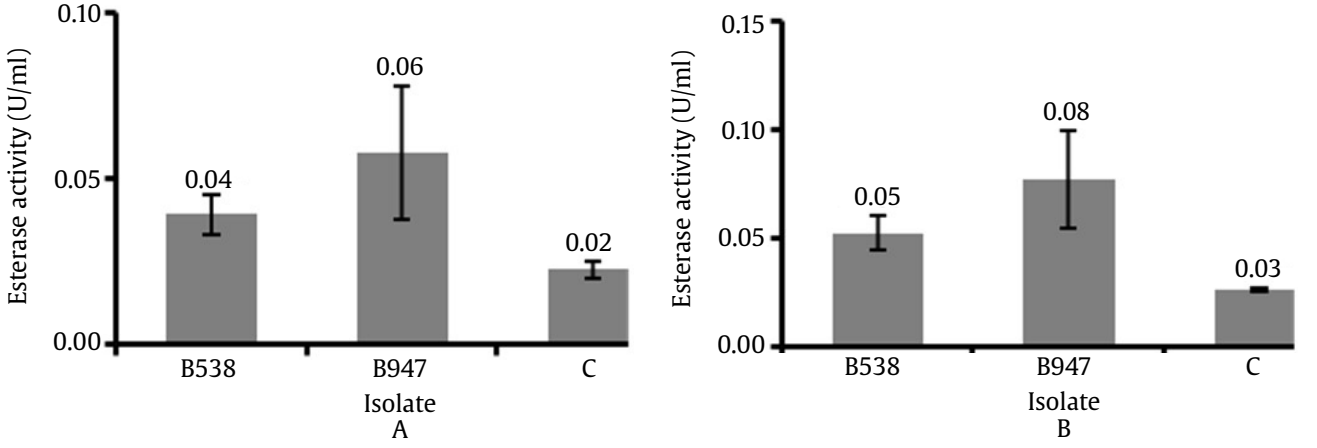
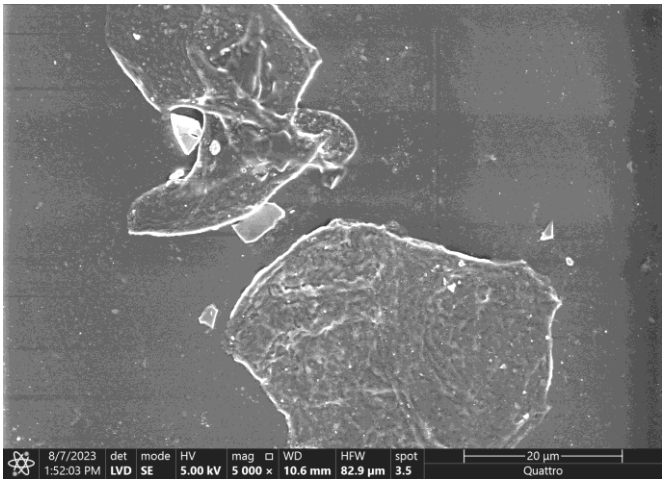
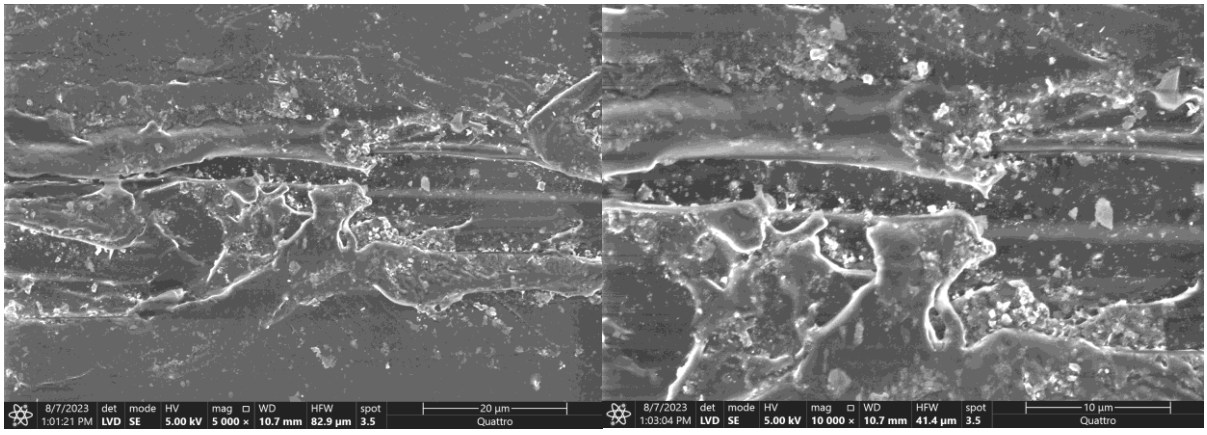


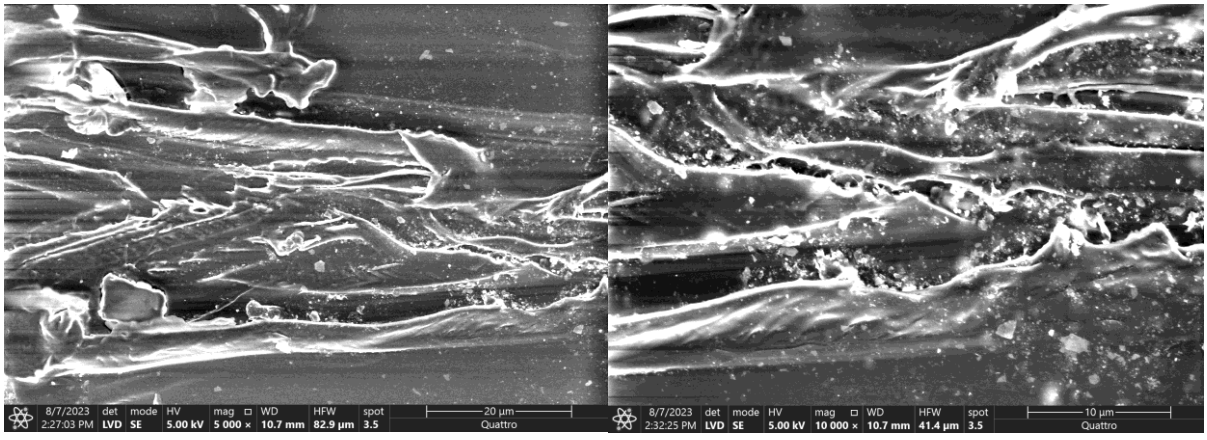
Figure 2. (A) Esterase activity of PET plastic biodegradation enzyme samples by InaCC B538, InaCC B947, and negative control, (B) esterase activity of PCL film biodegradation enzyme samples by InaCC B538, InaCC B947, and negative control



A

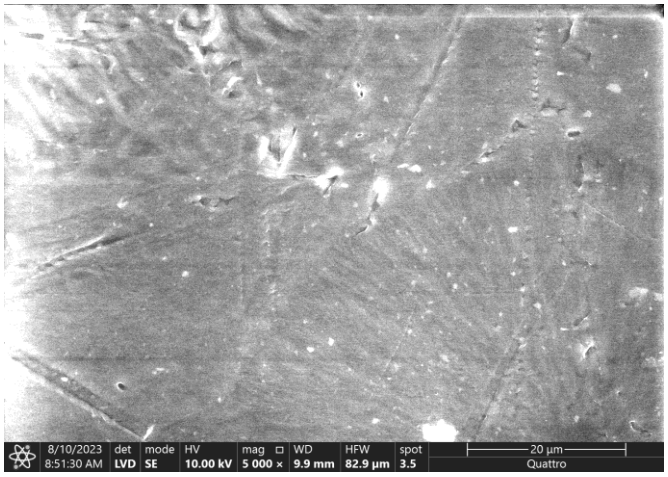


B C

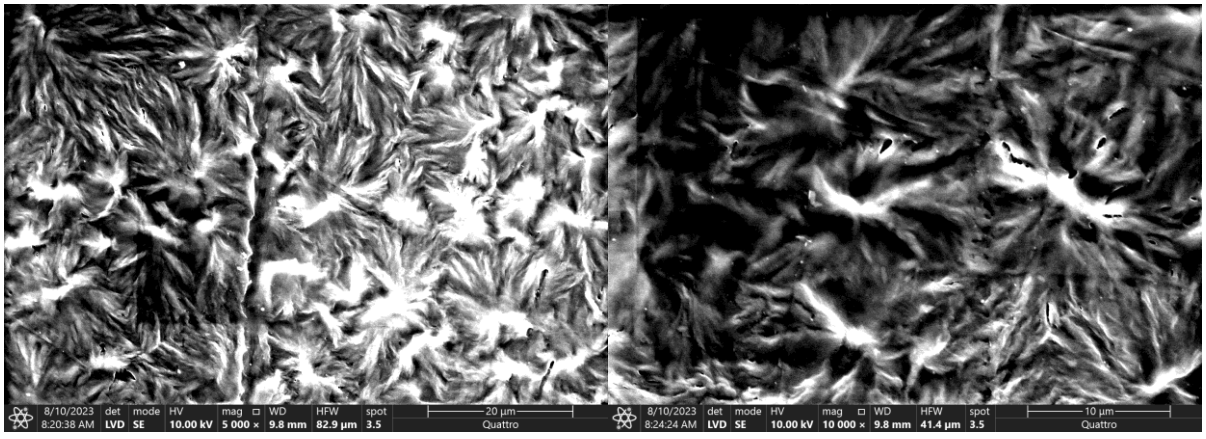


D E

Figure 3. (A) Scanning Electron Microscope (SEM) images of PET plastic surface after degradation for 10 days: control with 5,000x magnification, (B) degradation by InaCC B538 with 5,000x magnification, and (C) 10,000x magnification, (D) degradation by InaCC B947 with 5,000x magnification, and (E) 10,000x magnification



A



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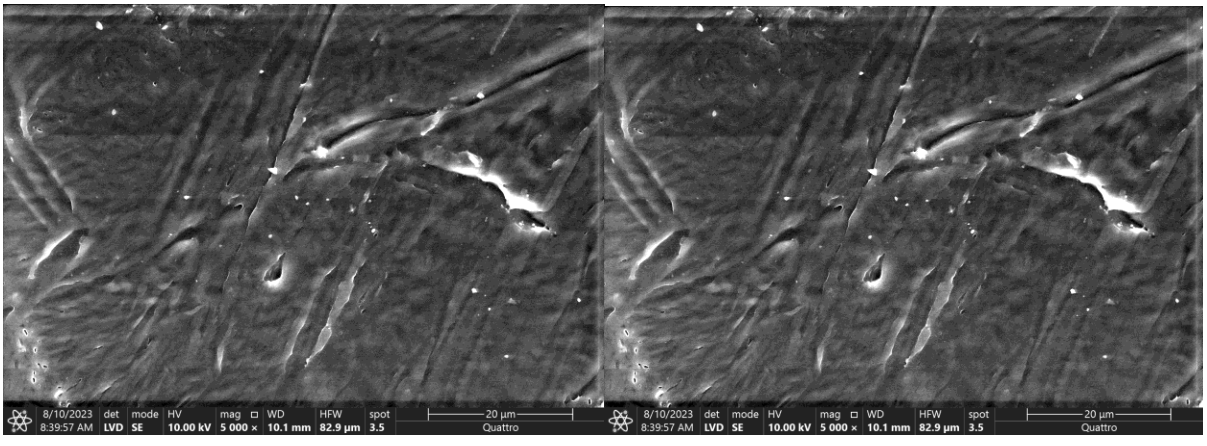
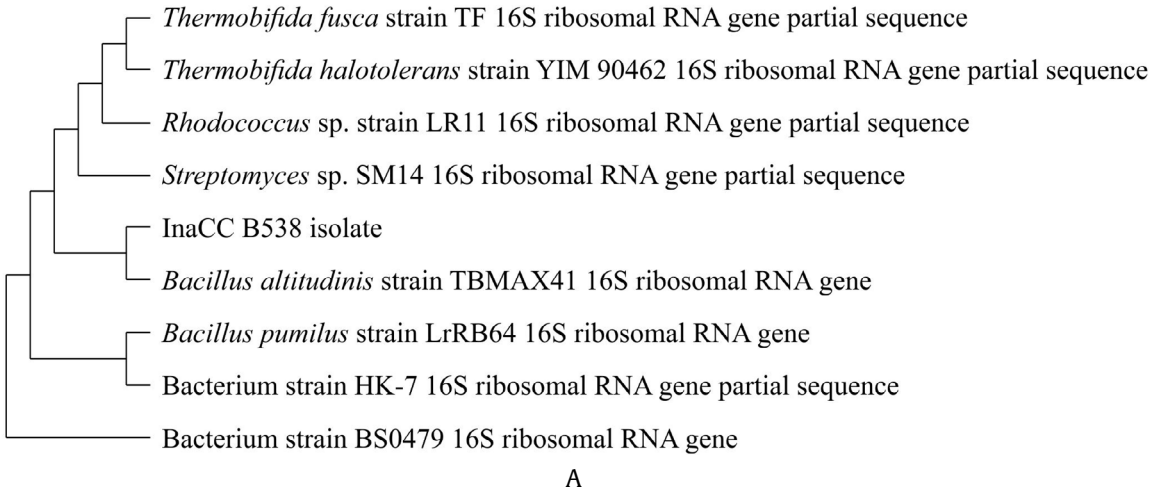
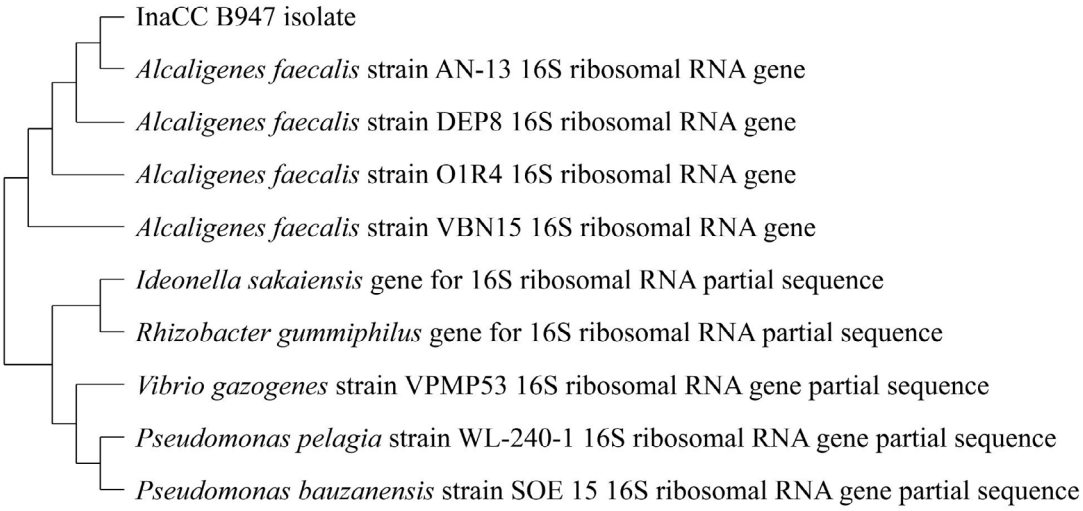


D E

Figure 4. (A) Scanning Electron Microscope (SEM) images of PCL film surface after degradation for 10 days: control with 5,000x magnification, (B) degradation by InaCC B538 with 5,000x magnification, and (C) 10,000x magnification, (D) degradation by InaCC B947 with 5,000x magnification, and (E) 10,000x magnification



A



B

Figure 5. Phylogenetic tree of InaCC isolate B538 (A) and InaCC isolate B947 (B) based on 16S rDNA sequencing results

attachment of bacterial cells on the polymer surface, which aids in the degradation mechanism.

The biodegradation process begins with cells colonizing and adhering to the polymer surface. The bacterial cells then use the polymer from the substrate as their only carbon source available in the environment, which continues for up to 10 days of biodegradation. The degradation is also supported by the presence of extracellular enzymes secreted by the bacterial cells, causing damage to the plastic surface and resulting in weight loss.

On the other hand, there was no visible degradation process observed by the InaCC B538 isolate on the PET substrate, as there was no decrease in PET plastic weight. The lower percentage of degradation observed towards the PET plastic substrate compared to the PCL film is due to the more rigid structure of PET plastic compared to PCL (Lu *et al.* 2022). This property makes the PCL film more easily degraded by both isolates, resulting in a higher percentage of degradation. The *A. faecalis* species has the esterase enzyme that works to catalyze the breaking of ester bonds. This is supported by the research of Poornima and Kasthuri (2016), who found that *A. faecalis* was able to degrade the biodegradable polymer PHBV or Poly (3-hydroxybutyrate-co-3-hydroxyvalerate) at 30°C at pH 8 and was able to achieve esterase activity of ch 0.22-0.26 U/ml (Poornima and Kasthuri 2016). Another study by Ramnath *et al.* (2017) mentioned that *A. faecalis* contains the esterase enzyme with esterase activity of 0.27 U/L. *A. faecalis* was able to degrade polyethylene (PE) plastic with the help of esterase enzymes that play a role in the biofragmentation stage (Ramnath *et*

al. 2017). The lower esterase activity in this study could be due to the use of suboptimal temperature and pH conditions. The optimal conditions found for esterase enzymes from the *Alcaligenes faecalis* species based on the study of Ramnath *et al.* (2017) were at 30°C and pH 8. The catalysis process of breaking ester bonds by esterase enzymes will result in the formation of products in the form of TPA (terephthalic acid), EG (ethylene glycol), and products that have not been completely hydrolyzed, namely BHET (Bis-(2-hydroxyethyl) terephthalate) and MHET (monohydroxylethyl terephthalate) (Salvador *et al.* 2019). The isolate InaCC B538, which was identified as belonging to the Bacillus altitudinis species in the research of Gonzalez *et al.* (2021), has an esterase enzyme that works on the synthesis, degradation, and modification of carbohydrate compounds or belongs to the carbohydrate-active enzyme group. This may be the reason why it does not work actively on other compounds such as PET polymers.

The surface of PET plastic and PCL film degraded by the InaCC B947 isolate has similar characteristics. The uneven surface with grainy material may represent some of the undecomposed plastic fragments. White flakes were thought to be plastic fragments resulting from decomposition due to the enzymatic activity of esterase enzymes secreted by microorganisms. An increase in the incubation time in the study of Nag *et al.* (2021) from 5 to 10 weeks can cause biofilm formation by *A. faecalis*, which plays a role in increasing degradation activity, causing more damage and cracks on the surface of the substrate made from Low-density Polyethylene (LDPE). Apparently, InaCC B538 was able to cause damage to the surface of the PET film even without a decrease in plastic weight. Meanwhile, the fiber-shaped pattern in Figure 3A is thought to be the texture of the PCL film.

It can be concluded that the InaCC B947 isolate has the potential to degrade PET plastic with a degradation percentage of 0.32% and esterase activity of 0.06 U/ml compared to the isolate InaCC B538, which did not reduce PET weight with esterase activity of 0.04 U/ml. PET plastic degradation by InaCC B947 and InaCC B538 isolates was able to cause damage to the surface of PET plastic.

Conflict of Interest

The authors declare that there is no conflict of interest.

Acknowledgements

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