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Exploring plastic-degrading enzyme producers from Ascidian-associated bacteria in Karimunjawa Islands, Indonesia

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Abstract. *Ayuningrum D, Shukor MY, Patria MP, Pasaribu B, Sulistiowati S. 2025. Exploring plastic-degrading enzyme producers from Ascidian-associated bacteria in Karimunjawa Islands, Indonesia. Biodiversitas 26: 1050-1060.* Ascidians are sessile, benthic invertebrate chordates that attach to various substrates, including hard corals, ship decks, buoys, and marine debris such as wood or plastic waste. This study aimed to isolate ascidian-associated bacteria from Karimunjawa National Park, explore their potential as PETase enzyme producers, and identify the bacteria using morphological and molecular approaches via the 16S rRNA gene. The research employed an exploratory descriptive and experimental approach. A total of nine ascidian samples were used as inoculum sources, specifically *Eusynstyela* sp., *Didemnum* sp., *Clavelina arafurensis*, *Pseudodistoma fragile*, *Lissoclinum* sp., *Rhopalaea crassa*, *Phallusia* sp., *Rhopalaea macrothorax*, and *Aplidium breveriventer*. From these samples, 45 pure bacterial isolates were obtained. Screening for plastic degradation activity revealed that 33% of the isolates exhibited enzymatic activity, with two isolates (13%) demonstrating plastic-degrading activity as many as ≥5%. These two potential isolates, KJ12-01 Z $^{(-4)}$/3 and KJ07-01 $^{(-2)}$/1, were identified as Gram-positive cocci and Gram-negative rods, respectively. Sequence alignment and BLAST analysis from 16S rRNA gene amplification using PCR technique on the NCBI database showed that bacterial isolate of KJ12-01 Z $^{(-4)}$/3 had 99.16% similarity with *Staphylococcus condimenti*, while KJ07-01 $^{(-2)}$/1 had 99.36% similarity with *Alloalcanivorax dieselolei*. These findings indicate that ascidian-associated bacteria can serve as a promising source of PETase enzyme-producing inoculum.

Keywords: Bacteria, ecology, PETase, plastic, settlement

INTRODUCTION

Plastic pollution is one of today's most pressing environmental challenges, with millions tons of plastic waste entering ecosystems annually. Plastic pollution in ocean has reached alarming levels, with concentrations of 580,000 pieces per km^2, exacerbated by the rapid expansion of manufacturing (Rahman et al. 2023). The majority of primary microplastic losses (98%) stem from land-based activities, while only 2% originate from marine activities (Boucher and Friot 2017). Due to its persistent nature, plastic takes hundreds of years to decompose, adversely affecting marine organisms and human health over time. When marine species ingest plastic debris, toxins enter their digestive systems and eventually accumulate in the food chain, posing health risks to humans through seafood consumption (Rahman et al. 2023).

There is substantial evidence that entanglement or ingestion of plastics causes injury and death to marine organisms, including commercially significant fish and shellfish (Thompson 2017). Recent research in ascidian showed that microplastic has a significant effect on decreasing the fertilization rate of *Microcosmus exasperatus* (Heller, 1878) (Anderson and Shenkar 2021). Besides harming animals, plastic pollution also damages and threatens health of mangrove and coral reef ecosystems (Sabdono et al. 2022; Tekman et al. 2022). Ecologically, plastic pollution facilitates dispersal, organism rafting, habitat provision, and invasive species introduction, all of which threaten biodiversity and trophic relationships (Thushari and Senevirathna 2020).

Bioplastics may sound like a more environmentally friendly solution to plastic utilization. But in fact, bioplastics are composed of various polymers that are physically similar to conventional plastics (Albuquerque and Malafia 2018). Bioplastics are only able to degrade into compost under specific environmental conditions such as temperatures above 50°C for 180 days (van den Oever et al. 2017). This temperature can be achieved in terrestrial ecosystems, but in marine ecosystems it is impossible to achieve because it will threaten the lives of other living things (Siracusa et al. 2008). Therefore, very few studies have reported the degradation of bioplastics in both marine and fresh waters (Anderson and Shenkar 2021). Scientists are trying to find effective and efficient ways to reduce plastic waste. Mechanical recycling and incineration methods are currently

only partial solutions (Schyns and Shaver 2021; Shen et al. 2021; Bertocchini and Arias 2023). Biological methods are being investigated as an alternative to breaking down plastic waste in environment, i.e. utilize microbes to biologically break down Polyethylene (PE) plastic (Mohanan et al. 2020; Cai et al. 2023).

Researchers are increasingly investigating plastic-degrading bacteria as a biological solution to global plastic waste management (Dhali et al. 2024), as some finding suggests that microbial communities able to utilize plastics as carbon source (Mohanan et al. 2020). Extremophilic bacteria have demonstrated plastic degradation capabilities under both natural and laboratory conditions (Atanasova et al. 2021). These microorganisms produce enzymes that break down plastic polymers, such as laccases, proteases, cutinases, PETase and MHETase, (Mohanan et al. 2020; Dhali et al. 2024). Notably, enzymes like PETase and MHETase, identified in *Ideonella sakaiensis*, can degrade PET into monomers, facilitating environmentally friendly recycling (Yoshida et al. 2016).

In this study, we tried to explore the ability of ascidian symbiont bacteria to produce plastic-degrading enzymes. Ascidians are marine benthic filter feeders that inhabit depths ranging from the surface (0 m) to the deep sea. These benthic organisms can attach to various substrates, including sand, mud, coral reefs, marine debris and even plastic. Many ascidian species can colonize plastic surfaces, making them a promising source of plastic-degrading bacteria. To the best of the author's knowledge, no similar studies have been conducted. Existing research on plastic-degrading enzymes, specifically PETase and MHETase, relies on cloning methods, where genes encoding these enzymes from *I. sakaiensis* are extracted from the gene bank and expressed in *Escherichia coli* (Shi et al. 2021; Hong et al. 2023). However, this cloning approach is costly compared to direct enzyme production from the source bacteria. Additionally, PETase and MHETase from *I. sakaiensis* are not halophilic, as the bacterium was originally isolated from PET bottle recycling facility in Japan rather than a marine environment (Yoshida et al. 2016). Other bacteria found to be able to metabolize plastics are from genera *Neodevriesia* and *Lachnellula*, both isolated from Arctic terrestrial environment (Rüthi et al. 2023). Therefore, this study aimed to: i) isolate and purify ascidian-symbionts bacteria collected from the Karimunjawa Waters; ii) assess the plastic-degrading activity of ascidian-symionts isolates; and iii) identify potential plastic-degrading bacteria using morphological and molecular 16S rDNA methods. The findings of this study are valuable in expanding knowledge on bacteria with similar plastic-degrading abilities that can function effectively in marine ecosystems.

MATERIALS AND METHODS

Sample collection

Ascidian samples were collected from the Karimunjawa Marine National Park, Indonesia, in October 2023 and August 2024. The coordinates for the 2023 collection were 5°47'3.39"S, 110°26'29.03"E, and 5°49'28.94"S, 110°23'15.91"E. In 2024, samples were collected at 5°52'44.75"S, 110°25'48.14"E. Samples were obtained from depths of 5-20 meters, placed in sterile plastic Ziplock bags with surrounding seawater, and stored in a cool box for laboratory processing.

Isolation and purification of Ascidian-associated bacteria

Bacteria were isolated using the serial dilution and spread plate method described by Ayuningrum et al. (2019a). The isolation of ascidian symbiont bacteria begins by thoroughly cleaning ascidian samples with sterile seawater to remove surface contaminants. For solitary ascidians, dissection is performed to isolate the zooid, whereas colonial ascidians, such as those from the *Didemnum* group, do not require dissection. After preparation, each ascidian sample is weighed (1 g), crushed, and homogenized with 9 mL of sterile seawater to create the initial (0^{th}) dilution. A serial dilution is then performed by transferring 1 mL of the initial solution into a new test tube containing 9 mL of sterile seawater. This process is repeated up to the fourth dilution. From the final dilution, 50 µL was taken and spread onto the pre-prepared Zobell agar 2216 (Himedia) medium. The isolated samples were then incubated at room temperature (29°C ± 2°C) and observed after 24 hours. Bacterial colonies exhibiting distinct morphological features were purified as individual isolates. Each isolate was maintained in duplicate: one for working stock and another for storage. Storage stocks were prepared by culturing isolates in 1.5 mL microtubes containing 750 µL of bacterial culture and glycerol in a 1:1 ratio (v/v).

Screening of plastic-degrading activity

The plastic degradation test using Ascidian-associated bacteria was performed following the bacterial identification process from the purification stage. The medium used for the degradation test was Minimal Salt Medium (MSM), which utilizes sterile seawater as the solvent. The composition of MSM followed the protocol established by (Kim et al. 2024). MSM is a bacterial growth medium that contains only the essential components required to support microorganism growth. It consists of mineral salts that are vital for microorganisms and classified as micronutrients needed in small quantities.

Next, to prepare the MSM, 400 mL was made by mixing all components in an Erlenmeyer flask. The mixture was homogenized using a hot plate magnetic stirrer at 70°C and 200 rpm until it was heated and free of clots. The MSM was then sterilized in an autoclave at 121°C for 15 minutes, along with the test tubes and gauze test tube covers, to eliminate unwanted microorganisms. After sterilization, the MSM was poured into test tubes (10 mL per tube) and allowed to stand at room temperature for 24 hours to ensure complete sterility and the absence of microbial growth.

Next, Low-Density Polyethylene (LDPE) plastic was introduced into each medium, followed by inoculation with a purified isolate using one stroke of an inoculating needle. The LDPE plastic used in this study was sourced from

Gajah-brand black plastic bags commonly available in supermarkets across Indonesia. The plastic bags were cut into 1 cm × 1 cm film pieces. This procedure was repeated until all isolates were inoculated into the test tubes containing MSM and test plastics. The test tubes were then incubated on a shaker at 130 rpm for 30 days (Mardalisa et al. 2021). After incubation, the weight of each plastic was measured, and the percentage of weight reduction was calculated using the degradation formula, as seen in Formula 1 (Riandi et al. 2017).

$$\text{Weight Loss Percentage} = \frac{Wi - Wf}{Wi} \; x \; 100\ \% \quad \ldots\ldots\ldots (1)$$

Where:

Wi : Initial dry weight before degradation (g)

Wf : Final dry weight after degradation (g)

After 30 days, the LDPE plastic film and control were examined for structural changes using a Scanning Electron Microscope (SEM). This analysis aimed to determine differences between the treated plastic (with added bacteria) and the control (without bacteria in the culture). The weight loss percentage of each bacterial was analyzed using the statistical test ANOVA to measure the most potential isolates to degrade the plastics.

Morphological identification of potential isolates

Microscopic observations were used at the Gram Stain Test stage on bacteria Gram staining begins with sterilizing a glass slide using alcohol and heat. A drop of distilled water is then added, and a bacterial isolate is spread evenly using an inoculating needle. The slide is dried carefully to prevent overheating. The staining process involves sequentially applying crystal violet (Gram A), iodine (Gram B), ethanol (Gram C) for decolorization, and safranin (Gram D) as a counterstain, with rinsing steps in between. The slide was allowed to air dry before being examined under a microscope with 1,000× magnification, using immersion oil to prevent damage to the preparation. Bacteria that retained the purple crystal violet stain are considered Gram-positive, while those that retained the red safranin stain are Gram-negative (Hendrawati et al. 2024).

Molecular identification of potential isolates

DNA extraction was performed to isolate the DNA from other cellular components, making it suitable for further analysis. The Chelex method was employed for DNA extraction, as it is relatively simple. The Chelex solution was diluted to a 10% concentration, consisting of 100 mg Chelex and 1,000 µL ddH_2O (Hendrawati et al. 2024). Two hundred microliters of the Chelex solution were added to a microtube, followed by inoculation with three strokes of the bacterial isolate using an inoculating needle. The sample was vortexed for 20 seconds and centrifuged at 13,000 rpm for 20 seconds to separate the particles by mass. The sample was then incubated in a heating block at 95°C for 45 minutes (Pratama et al. 2018). After incubation, the sample was vortexed again for 20 seconds and centrifuged for 2 minutes at 13,000 rpm. The supernatant, which is the liquid in the uppermost layer of the microtube, was stored at -20°C to preserve the DNA.

After DNA extraction, amplification was performed using the 16S rRNA gene. The amplification primers used were reverse primer 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and forward primer 1492R (5'-GGTTACCTTGTTACGACTT-3') (Weisburg et al. 1991). The reaction mixture consisted of 2 µL template DNA, 1 µL of each primer, 8.5 µL ddH_2O, and 12.5 µL PCR mix (MyTaq™ Red Mix-Bioline). The PCR tube was placed into a thermal cycler with the following protocol: initial denaturation at 95°C for 10 minutes, 34 cycles of denaturation at 95°C for 45 seconds, annealing at 50°C for 60 seconds, and extension at 72°C for 90 seconds, followed by a final extension at 72°C for 5 minutes (Ayuningrum et al. 2019b).

The next step, electrophoresis, separates charged molecules under the influence of an electric field. Electrophoresis was carried out using 1% agarose gel prepared with a TAE buffer solution (1:9 ratio of TAE to ddH_2O). To prepare the 1% agarose gel, 0.4 g of agarose powder was dissolved in 40 mL of TAE buffer. GelGreen, a non-toxic fluorescent dye, was added (3 µL) to the hot, liquid agarose gel to stain the DNA (Lafta and Shamran 2019). The agarose gel was poured into a well mold and allowed to solidify before being placed in the electrophoresis tank and submerged in a buffer solution. The DNA samples (2 µL) were mixed with 1 µL of loading dye, and 3 µL of DNA ladder was loaded into one well. The electrophoresis was run at 100 volts for 30 minutes. After electrophoresis, the DNA bands were visualized and analyzed under a UV transilluminator. Ideal DNA band lengths for bacteria range from 1,000 to 1,500 base pairs (bp). Samples that met the required band length were sent to the Apical Scientific laboratory via Genetics Science Indonesia for sequencing and further analysis.

Phylogenetic tree of potential bacteria isolates

The sequencing results from Apical Scientific provide the nitrogen base sequence of the test sample's DNA. These sequences were analyzed using the MEGA-11 software (Feng et al. 2016). The data were converted to FASTA format and subjected to the Basic Local Alignment Search Tool (BLAST) on the National Center for Biotechnology Information (NCBI) website (Hebert and Meglécz 2022). The results of the BLAST analysis indicated the percentage of DNA sequence similarity between the sample and known sequences in the NCBI database, enabling the identification of the sample species. A phylogenetic tree was constructed using the maximum likelihood method with the Tamura-Nei model.

RESULTS AND DISCUSSION

Sample identification

Ascidian samples collected from Karimunjawa waters consisted of nine species: *Eusynstyela* sp., *Didemnum* sp., *Clavelina arafurensis* (Tokioka, 1952), *Pseudodistoma fragile* (Tokioka, 1958), *Lissoclinum* sp., *Rhopalaea crassa* (Herdman, 1880), *Phallusia* sp., *Rhopalaea macrothorax* (Tokioka, 1953), and *Aplidium breveriventer* (Monniot F. & Monniot C., 2001). A purple and yellow coloration at the

center characterized the *Eusynstyela* samples. The genus *Eusynstyela* has also been previously reported in Singapore, along with *P. fragile* and *R. macrothorax* (Lee et al. 2016). *Aplidium breveriventer* has been identified in Western Australia (Monniot and Monniot 2001). The genus *Phallusia* is distributed across Singapore, the Philippines, and Western Australia (Lee et al. 2013). Additionally, species such as *Didemnum molle* have been reported in Indonesia (Hirose et al. 2014), alongside *Clavelina*, *Rhopalaea* sp., *R. crassa*, *Phallusia arabica* (Savigny, 1816), and many others (Leleran et al. 2022). The bacterial isolates were obtained from the samples mentioned above (Figure 1).

Isolation and purification of Ascidian-associated bacteria

Bacterial isolation yielded 45 pure isolates, distributed as follows: seven isolates from *Eusynstyela* sp., two from *Didemnum* sp., four from *C. arafurensis*, two from *P. fragile*, eight from *Lissoclinum* sp., eight from *R. crassa*, five from *A. breveriventer*, five from *R. macrothorax*, and four from *Phallusia* sp. All of the bacterial isolates from each ascidian sample are shown in Figure 2. Previous studies have also explored ascidian symbiont bacteria as sources of antibacterial inocula (Ayuningrum et al. 2019a, 2019b; Hendrawati et al. 2024). The morphological characteristics of each bacterial isolate are detailed in Table 1.

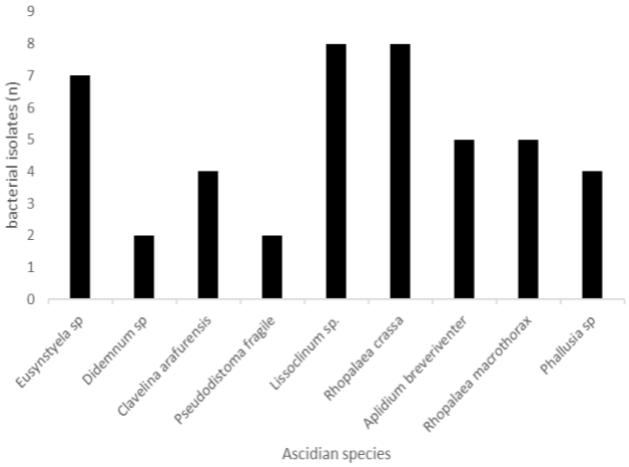

Figure 2. Bacterial isolation and purification results from nine Ascidian samples

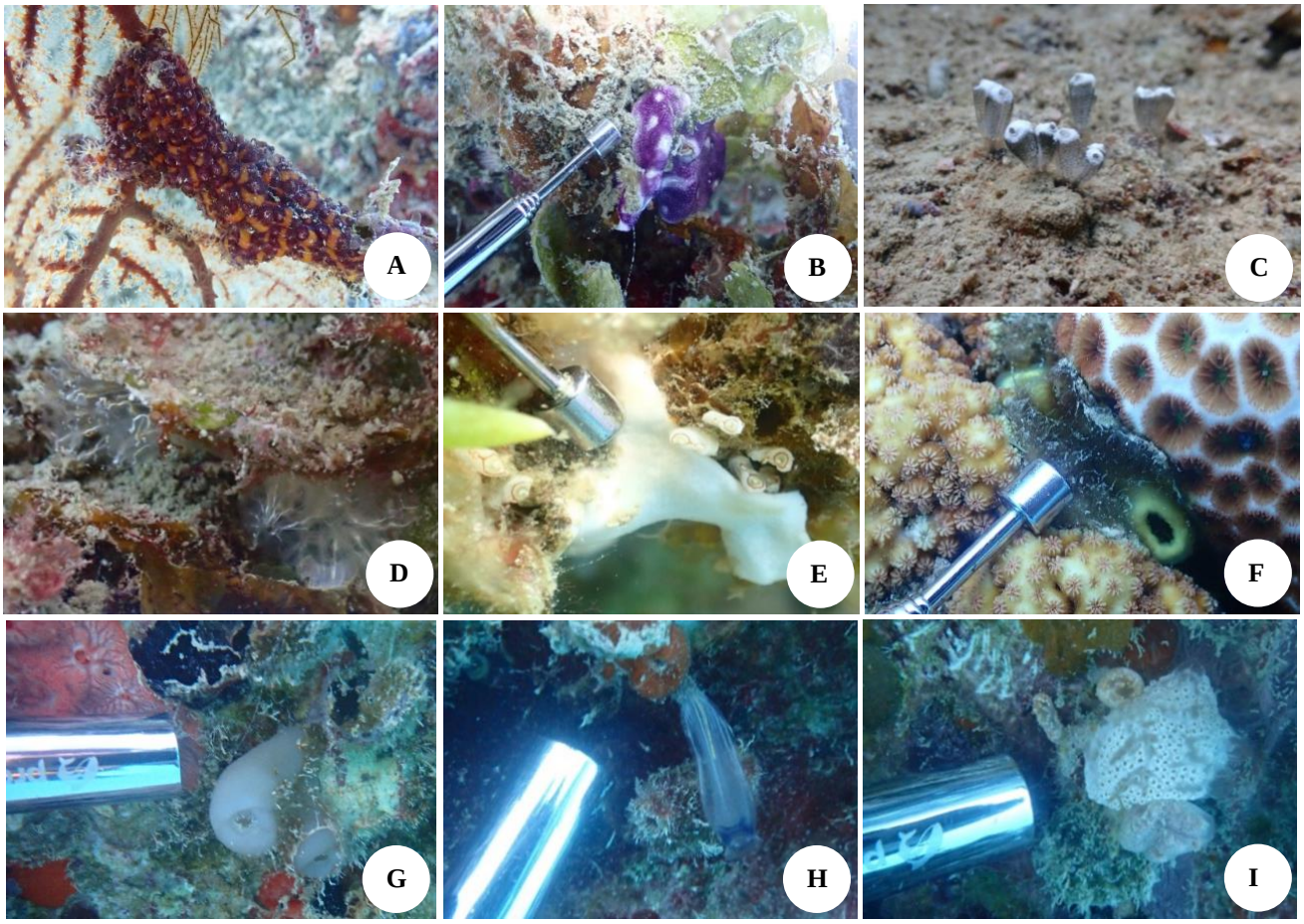

Figure 1. Ascidian samples isolated for bacterial analysis: A. *Eusynstyela* sp.; B. *Didemnum* sp.; C. *Clavelina arafurensis*; D. *Pseudodistoma fragile*; E. *Lissoclinum* sp.; F. *Rhopalaea crassa*; G. *Phallusia* sp.; H. *Rhopalaea macrothorax*; I. *Aplidium breveriventer*

Table 1. Morphological characteristics of bacteria associated with ascidian samples

Ascidian species (code)	Bacterial isolate code	Color	Colony shape	Margin	Elevation
Eusynstyella sp. (KJ6-01$^{(-2)}$)	KJ6-01$^{(-2)}$/1	White turbid	Circular	Entire	Raised
	KJ6-01$^{(-2)}$/2	White turbid	Irregular	Undulate	Raised
	KJ6-01$^{(-2)}$/3	White turbid	Irregular	Entire	Raised
Eusynstyella sp. (KJ6-01$^{(-3)}$)	KJ6-01$^{(-3)}$/1	Thin white	Circular	Entire	Flat
	KJ6-01$^{(-3)}$/2	Thin white	Irregular	Entire	Flat
	KJ6-01$^{(-3)}$/3	Thin white	Punctiform	Entire	Flat
	KJ6-01$^{(-3)}$/4	Thin white	Spindle	Entire	Flat
Didemnum sp. (KJ6-02$^{(-2)}$)	KJ6-02$^{(-2)}$/1	White turbid	Filamentous	Lobate	Flat
Didemnum sp. (KJ6-02$^{(-3)}$)	KJ6-02$^{(-3)}$/2	White turbid	Circular	Entire	Flat
Clavelina arafurensis (KJ6-07$^{(-2)}$)	KJ6-07$^{(-2)}$/1	Yellow	Circular	Entire	Flat
	KJ6-07$^{(-2)}$/2	Yellow	Irregular	Undulate	Flat
Clavelina arafurensis (KJ6-07$^{(-3)}$)	KJ6-07$^{(-3)}$/1	Brown	Spindle	Undulate	Raised
	KJ6-07$^{(-3)}$/2	Brown	Irregular	Entire	Flat
Pseudodistoma fragile (KJ6-10$^{(-2)}$)	KJ6-10$^{(-2)}$/1	Brown	Circular	Entire	Raised
Pseudodistoma fragile (KJ6-10$^{(-3)}$)	KJ6-10$^{(-3)}$/1	Thin white	Irregular	Undulate	Flat
Lissoclinum sp. (KJ6-11$^{(-2)}$)	KJ6-11$^{(-2)}$/1	White turbid	Circular	Undulate	Raised
	KJ6-11$^{(-2)}$/2	Thin white	Spindle	Undulate	Flat
	KJ6-11$^{(-2)}$/3	White turbid	Irregular	Entire	Flat
	KJ6-11$^{(-2)}$/4	White turbid	Circular	Entire	Flat
Lissoclinum sp. (KJ6-11$^{(-3)}$)	KJ6-11$^{(-3)}$/1	White turbid	Circular	Undulate	Raised
	KJ6-11$^{(-3)}$/2	White turbid	Irregular	Lobate	Raised
	KJ6-11$^{(-3)}$/3	White turbid	Circular	Entire	Raised
	KJ6-11$^{(-3)}$/4	Brown	Circular	Entire	Flat
Rhopalaea crassa (KJ7-01$^{(-2)}$)	KJ7-01$^{(-2)}$/1	Thin white	Circular	Undulate	Flat
	KJ7-01$^{(-2)}$/2	Thin white	Irregular	Undulate	Flat
	KJ7-01$^{(-2)}$/3	Thin white	Irregular	Circular	Flat
*Rhopalaea crssa (*KJ7-01$^{(-3)}$)	KJ7-01$^{(-3)}$/1	Thin white	Circular	Undulate	Flat
	KJ7-01$^{(-3)}$/2	Thin white	Irregular	Lobate	Flat
	KJ7-01$^{(-3)}$/3	Thin white	Circular	Entire	Flat
	KJ7-01$^{(-3)}$/4	Thin white	Circular	Undulate	Flat
	KJ7-01$^{(-3)}$/5	Thin white	Spindle	Entire	Flat
Phallusia sp. (KJ12-01)	KJ12-01 Z $^{(-3)}$/1	Milky white	Circular	Entire	Raised
	KJ12-01 Z $^{(-3)}$/2	Milky white	Irregular	Entire	Raised
	KJ12-01 Z $^{(-4)}$/3	Milky white	Circular	Entire	Raised
	KJ12-01 T $^{(-3)}$/1	Clear white	Circular	Entire	Raised
	KJ12-01 T $^{(-4)}$/2	Clear white	Circular	Entire	Raised
Rhopalaea macrothorax (KJ12-03)	KJ12-03 T $^{(-3)}$/1	Milky white	Circular	Entire	Raised
	KJ12-03 T $^{(-3)}$/2	Turbid white	Circular	Entire	Raised
	KJ12-03 Z $^{(-3)}$/1	Milky white	Circular	Entire	Raised
	KJ12-03 Z $^{(-3)}$/2	Milky white	Circular	Entire	Raised
	KJ12-03 Z $^{(-4)}$/3	White	Circular	Entire	Raised
Aplidium breviventer (KJ12-03)	KJ12-05 $^{(-3)}$/1	White turbid	Irregular	Entire	Raised
	KJ12-05 $^{(-3)}$/2	Yellow	Irregular	Entire	Raised
	KJ12-05 $^{(-4)}$/3	Milky white	Circular	Entire	Raised
	KJ12-05 $^{(-4)}$/4	Clear white	Circular	Entire	Raised

The morphological characteristics of each pure bacterial isolate are presented in Table 1. Each bacterial isolate has different characteristics ranging from color, colony shape, margin and elevation. Pure bacterial isolates obtained are dominated by white color, either cloudy white, thin white, milky white, and transparent white. Some bacterial isolates emit pigments such as brown and yellow. Based on the colony shape, the average pure bacterial isolate has a circular shape. Some pure isolates bacteria have irregular, punctiform, spindle, and filamentous forms. Based on the margin or edge of the bacterial colony, the average pure bacterial isolate has an entire edge. Some bacterial isolates have undulate, lobate, and circular margins. The last characteristic is elevation; there are only two categories that dominate in this character, namely raised and flat. Marine bacteria exhibit a variety of shapes, including rods, cocci, and bacilli. Colonies of marine bacteria can vary in appearance. Strain M44, a member of the genus *Sulfitobacter*, forms slightly yellowish, round, convex, and smooth colonies (Long et al. 2011). Strain JUB59^T forms butyrous, shiny, yellowish-orange colonies (Kannahi and Sivasankari 2014). The marine bacteria that have filamentous colony shapes mostly come from the sub-phylum actinobacteria with a well-known species, *Streptomyces* sp., as primary and secondary metabolite producers (Ayuningrum et al. 2022). Pure bacterial isolates that have been isolated from marine invertebrates have unique characteristics; recent research showed that bacteria isolated from hard-coral emit pigments

such as yellow, brown, red, orange, and many more (Ayuningrum et al. 2020). Recent studies related to ascidian-associated bacteria also revealed many unique morphological characteristics from its symbiont bacteria (Ayuningrum et al. 2019a, 2019b; Hendrawati et al. 2024).

Screening of plastic-degrading activity

A total of 45 ascidian symbiont bacterial isolates were tested for plastic-degrading activity over one month. Results indicated that 33% of the isolates exhibited enzymatic activity, with two isolates (13%) showing activity levels of ≥5%. The screening results are presented in Figure 3. The raw data on weight loss was provided in Supplementary Material 1. Although these values are relatively low compared to the plastic degradation abilities of insects (An et al. 2023; Yang et al. 2023; He and Liu 2024; Yang et al. 2024), this potential could be enhanced through optimization of incubation time, medium composition, rotation, temperature, pH, and other factors.

Plastic-degrading bacteria represent a promising solution to the global plastic crisis, particularly for contaminants like Polyethylene Terephthalate (PET), a material commonly used in bottles and packaging. *Ideonella sakaiensis* is a notable bacterium that produces the enzyme PETase, which is capable of converting PET into monomers. Discovered in 2016, PETase has since been extensively studied to enhance its efficiency. Recent advancements include the use of machine learning to optimize the PETase gene, achieving faster and more efficient plastic degradation. In addition, researchers discovered bacteria such as *Vibrio natriegens* capable of degrading microplastics like PET in the atmosphere, a significant step towards addressing plastic pollution in the atmosphere. However, there are several challenges, such as the high degradation rate of PET and the potential risk to the environment from large-scale genetic research. Bacteria from marine environments could serve as an inoculum source for many types of enzymes (Ayuningrum et al. 2021). The potential isolates with high plastic degradation activity are shown in Figure 4. The structural change of plastic film is shown in Figure 5.

This SEM image captures the degradation of a plastic film by marine bacteria isolate code KJ07-01 $^{(-2)}$/1, showcasing bacterial colonization, biofilm formation, and surface erosion (Figure 5.B). Compared to Figure 5.A, there is no significant change in the control plastic film. The bacteria have rod-shaped and actively colonizing the plastic film surface. The rough, eroded texture of the plastic suggests that bacterial activity has broken down the material. The presence of biofilms (visible as organic deposits) indicates that microbes are secreting enzymes to degrade the plastic (Zhai et al. 2023). Small debris or fragmented plastic particles may be evidence of microbial biodegradation. Marine bacteria are able to degrade plastic through several stages: (i) biofilm formation, (ii) secretion of enzymes, (iii) fragmentation into microplastic, (iv) complete mineralization (Urbanek et al. 2018).

Marine bacteria first attach to the plastic surface and form biofilms, which are protective layers of microbial communities. This helps them adhere to non-natural surfaces like plastic. Various marine bacteria, including *Alcanivorax*, *Pseudomonas*, and *Bacillus* species, adhere to the plastic surface, forming biofilms that help them survive in harsh marine conditions (Kumari et al. 2021). After that, they secret several plastic-degrading enzymes such as: Esterases and lipases (break down polyester-based plastics like PET), Oxidases and peroxidases (help degrade polyethylene and polypropylene by oxidation), Depolymerases (break long plastic polymer chains into smaller molecules). As bacteria consume plastic, they create microplastics, which are further broken down by microbial action or environmental factors (e.g., UV light, waves). Some bacteria can fully convert plastic fragments into carbon dioxide (CO_2), water (H_2O), or biomass, leading to true biodegradation. Understanding these mechanisms could also contribute to the development of biodegradable plastics designed for faster breakdown in marine environments, helping to mitigate the growing issue of plastic pollution in oceans.

Morphological identification of potential isolates

Morphological identification was conducted using Gram staining to observe the shape and size of bacterial cells from the potential isolates. Four potential isolates were identified morphologically, as shown in Figure 6. Isolate KJ12-01 Z was identified as Gram-positive cocci, isolate KJ07-01 as Gram-negative bacilli, and isolates KJ06-01 $^{(-2)}$/2 and KJ07-01 $^{(-2)}$/1 as Gram-negative cocci. The best isolate KJ07-01$^{(-2)}$/1 was characterized by bacilli cell shape and Gram-negative. Some of the Gram-negative marine bacteria that have the ability to secrete plastic-degrading enzymes are *Alcanivorax* and *Pseudomonas*, meanwhile, the Gram-positive marine bacteria that have the ability to secrete plastic-degrading enzymes are from genus *Bacillus* (Kumari et al. 2021). The SEM results from plastic film with and without bacteria incubation are shown in Figure 5.

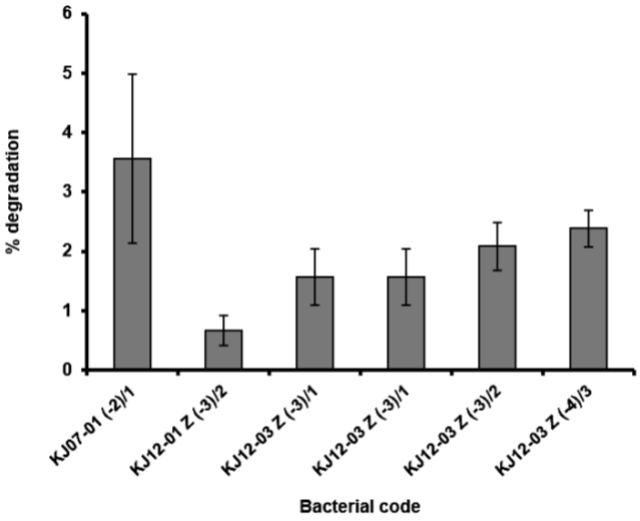

Figure 4. Highest potential isolates based on ANOVA test with p<0,005 is isolates; KJ07-01 $^{(-2)}$/1; KJ12-01 Z $^{(-3)}$/2; KJ12-03 Z $^{(-3)}$/1; KJ12-03 Z $^{(-3)}$/1; KJ12-03 Z $^{(-3)}$/2; and KJ12-03 Z $^{(-4)}$/3

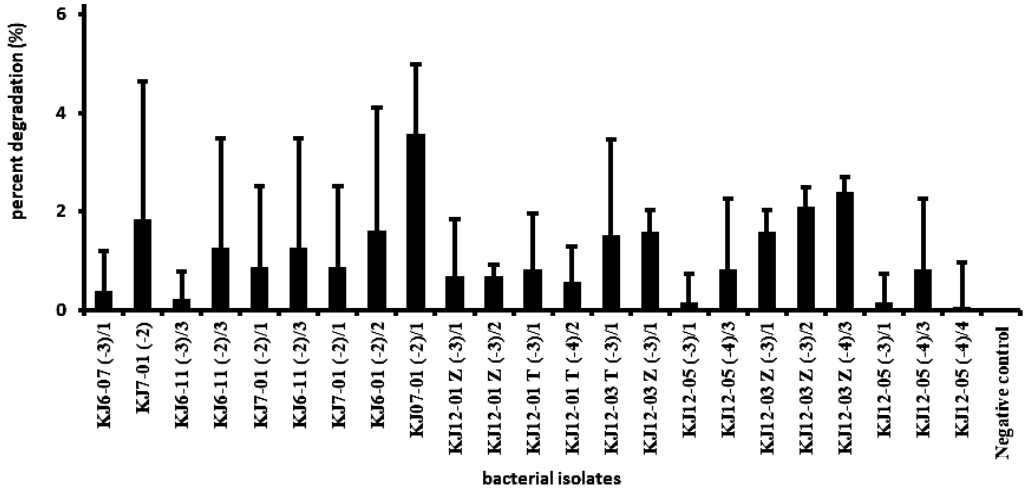

Figure 3. Screening results of plastic degradation activity of isolated bacteria

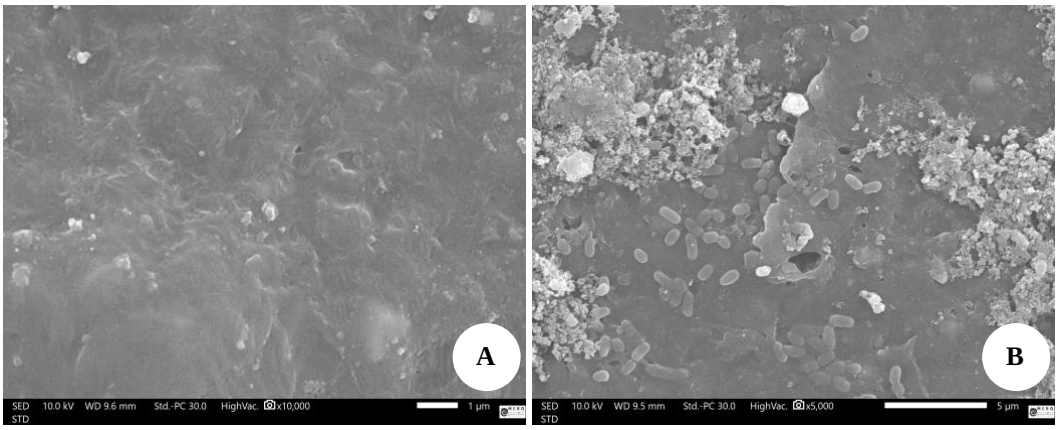

Figure 5. SEM observations from bacterial isolate KJ07-01 $^{(-2)}$/1 revealed that: A. The control plastic showed no visible changes, with an intact plastic film and no peeling at 5000x magnification; while B. The treated plastic exhibited bacterial adhesion and structural damage, breaking into small particles

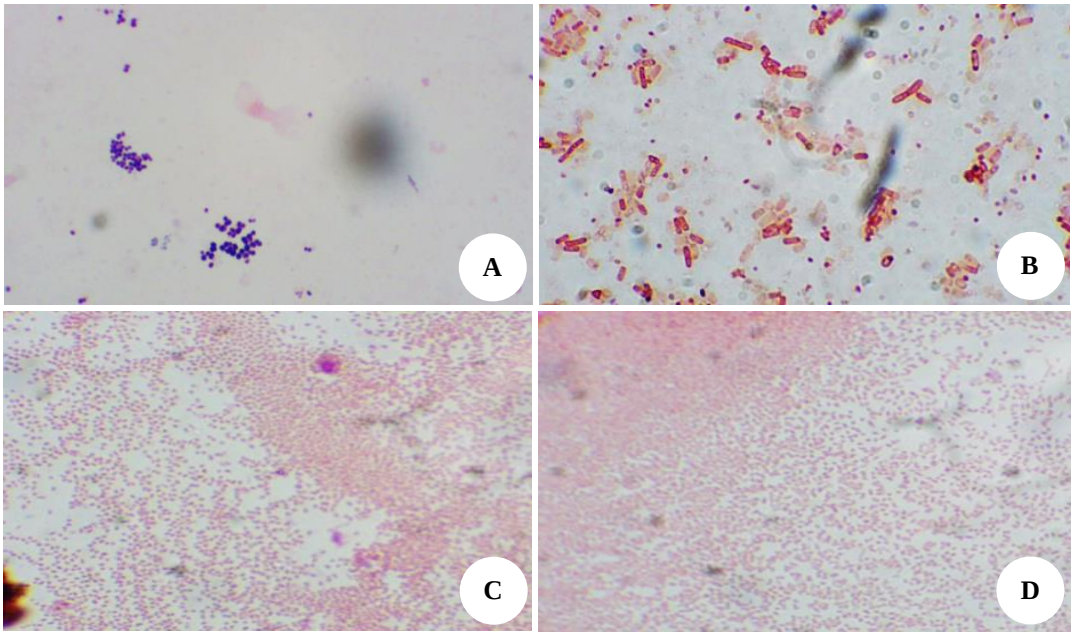

Figure 6. Gram staining results of potential plastic-degrading isolates: A. KJ12-01 Z $^{(-4)}$/3; B. KJ07-01 $^{(-2)}$; C. KJ06-01 $^{(-2)}$/2; D. KJ07-01 $^{(-2)}$/1

Molecular identification of potential isolates

The two most promising isolates were identified through 16S rRNA gene sequencing. The PCR products of isolates KJ12-01 Z $^{(-4)}$/3 and KJ07-01 $^{(-2)}$/1 were approximately 1,500 bp in length (Figure 7), consistent with findings from Ayuningrum et al. (2019b). The PCR products were sequenced by 1st Base Malaysia through PT. Genetika Science, Jakarta, and the resulting sequences were aligned and analyzed using MEGA 11 and BLAST on the NCBI website (Table 2). Isolate KJ12-01 Z $^{(-4)}$/3 exhibited 99.16% similarity to *Staphylococcus condimenti*, while isolate KJ07-01 $^{(-2)}$/1 showed 99.36% similarity to *Alcanivorax dieselolei*.

S. condimenti is a Gram-positive, coagulase-negative bacterium within the *Staphylococcus* genus. It is primarily recognized for its role in food fermentation, particularly in the production of traditional fermented foods such as soy sauce, miso, and various seasonings. The name *condimenti* is significant as it reflects its association with such applications. Morphologically, the cells are spherical (cocci) and often form clusters. Gram staining reveals a thick peptidoglycan layer, confirming its Gram-positive nature. The coagulase test is negative, distinguishing it from the pathogenic *Staphylococcus aureus*. Notably, *S. condimenti* exhibits halotolerance, thriving in high-salt environments typical of fermentation processes. Its enzymatic activity includes the production of proteolytic and lipolytic enzymes, which aid in the breakdown of proteins and fats during fermentation.

S. condimenti plays a non-pathogenic role in enhancing the flavor profile and safety of fermented foods. Its metabolic activities help reduce undesirable microorganisms while contributing to the development of amino acids and other flavor compounds. Unlike pathogenic species such as *S. aureus* or *S. epidermidis*, *S. condimenti* is not commonly associated with human infections and is generally regarded as safe in food contexts. Its coagulase-negative status and absence of key virulence factors set it apart from more harmful *Staphylococcus* species (Probst et al. 1998).

Alcanivorax dieselolei is a hydrocarbon-degrading bacterium known for its significant contribution to the breakdown of petroleum hydrocarbons, particularly within marine ecosystems. Its proficiency in degrading alkanes makes it a crucial organism in bioremediation efforts, especially following oil spills. Research indicates that *A. dieselolei* metabolizes a range of hydrocarbons and possesses specialized enzymatic pathways that facilitate survival in oil-contaminated aquatic environments (Liu and Shao 2005).

Yakimov et al. (2007) demonstrate that *Alcanivorax* spp. tend to dominate in oil-polluted environments, enhancing their degradation efficiency under aerobic conditions. Additionally, a study in PLoS One reveals that *A. dieselolei* B-5 produces dehalogenase enzymes, which are critical for degrading hydrocarbons and neutralizing halogenated substances under optimal temperature and pH conditions.

Phylogenetic tree of potential bacteria isolates

A phylogenetic tree illustrates the evolutionary relationships among organisms, highlighting the diversity within bacterial life. *A. dieselolei* and *S.condimenti* belong to different phyla, reflecting distinct ecological roles and physiological traits. The phylogenetic tree of the two most promising isolates is shown in Figure 8. A phylogenetic tree illustrates the evolutionary relationships among organisms, highlighting the diversity within bacterial life. *A. dieselolei* and *S. condimenti* belong to different phyla, reflecting distinct ecological roles and physiological traits.

Classification of *A. dieselolei*: It belongs to the domain Bacteria, phylum Proteobacteria, class Gammaproteobacteria, order Oceanospirillales, family Alcanivoracaceae, genus *Alcanivorax*, and species *A. dieselolei*. Key characteristics include its ability to degrade hydrocarbons, its prevalence in marine ecosystems-particularly in oil-polluted areas-and its significant role in bioremediation by metabolizing petroleum hydrocarbons. Classification of *S.condimenti*: It belongs to the domain Bacteria, phylum Firmicutes, class Bacilli, order Bacillales, family Staphylococcaceae, genus *Staphylococcus*, and species *S. condimenti*. Key characteristics include its status as a non-pathogenic species within the *Staphylococcus* genus, its isolation from fermented products such as soy sauce, and its contributions to food fermentation and preservation.

Table 2. BLAST homology results for the two most promising isolates

Bacterial code	Species name	Similarity	Reference number
KJ12-01 Z $^{(-4)}$/3	*Staphylococcus condimenti*	99.16%	NR_116435.1
KJ07-01 $^{(-2)}$/1	*Alloalcanivorax dieselolei*	99.36%	NR_074734.1

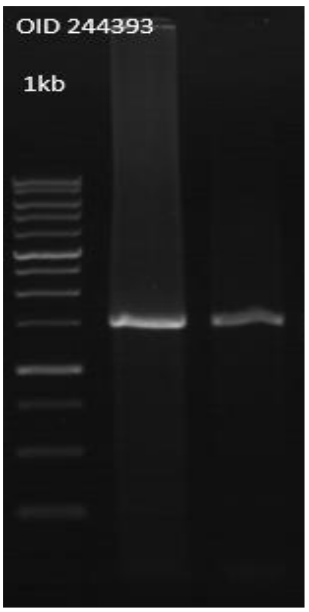

Figure 7. Electrophoresis results of potential isolates showing PCR products of ~1,500 bp

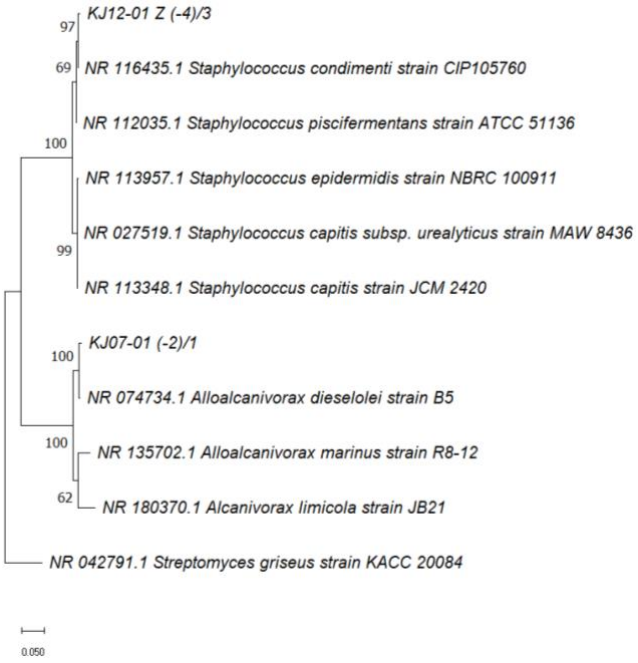

Figure 8. Phylogenetic tree of potential isolates (KJ12-01 Z (-4)/3) using maximum likelihood method (Tamura-Nei model) in MEGA XI

These two bacteria belong to separate phyla. *A. dieselolei*, classified under *Proteobacteria*, is part of one of the most extensive and diverse phyla (Liu and Shao 2005). In contrast, *S. condimenti* is classified under Firmicutes, a phylum characterized by Gram-positive bacteria with a low Guanine-Cytosine (GC) content.

In conclusion, the study utilized nine ascidian samples as bacterial inoculum sources: *Eusynstyela* sp., *Didemnum* sp., *Clavelina arafurensis*, *Pseudodistoma fragile*, *Lissoclinum* sp., *Rhopalaea crassa*, *Phallusia* sp., *Rhopalaea macrothorax*, and *Aplidium breveriventer*. A total of 45 pure bacterial isolates were obtained. Screening for plastic-degrading activity revealed that 33% of the isolates exhibited enzymatic activity, with 13% showing activity levels of 5%. The two most promising isolates were kj12-01 z (-4)/3, a Gram-positive coccus identified as *Staphylococcus condimenti* (99.16% similarity), and kj07-01 (-2)/1, a Gram-negative bacillus identified as *Alcanivorax dieselolei* (99.36% similarity).

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