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Identification and Screening of Biofilm-Forming Bacteria Isolated from Mangrove Sediment for Plastic Degradation

Nur Fitriah Afianti[1*], Flora Kirana Febrianti[2], Ariani Hatmanti[1], Edy Endrotjahyo[1], Helena Manik[1], Lies Indah Sutiknowati[1]

[1] Research Center for Oceanography, National Research and Innovation Agency, Jakarta, Indonesia
[2] Faculty of Agricultural Technology, Department of Food Science and Biotechnology, University of Brawijaya, Malang, Indonesia
* Corresponding author's e-mail: nurfitriahafianti@gmail.com

ABSTRACT

Plastic waste is particularly harmful to human life and the environment since it is difficult to degrade. This plastic waste contributes to seawater pollution, which can disrupt the food chain and harm biodiversity in the environment, particularly the mangrove ecosystem. Mangroves act as a sediment barrier and help to decrease coastal abrasion. In the natural environment, indigenous bacteria are crucial to the bioremediation of environmental pollution, including plastic waste pollution. Bioremediation is considered environmentally friendly and can accelerate the degradation time of waste containing toxic compounds. Biofilm-forming bacteria also play an important role in the biodegradation of plastics. This research was conducted to isolate bacteria from mangrove sediment and characterize their potential as a candidate to degrade polyethylene plastic. We have found that 25 of 53 PEG-degrading bacteria could form biofilms on plastic surfaces. Seven bacterial isolates showed the ability to produce clear zones during the degradation of PEG and biofilm formation. The seven potential bacterial isolates identified using 16s rRNA gene as *Bacillus sporotermodurans, Cytobacillus firmus, Rossellomorea vietnamensis, Stutzerimonas stutzeri, Dyadobacter jejuensis, Rhodococcus* sp., and *Achromobacter* sp.

Keywords: bacteria, biofilm, clear zone, biodegradation, mangrove, polyethylene, plastic, sediment.

INTRODUCTION

Land-based sources reportedly dump over 4.8 million tons of plastic waste into the ocean annually, contributing significantly to anthropogenic litter in the aquatic environment (Jambeck et al., 2015). The Ministry of Environment and Forestry of Indonesia (2021) reports that industrial and household activities generate a total of 68.5 million tons of waste. Household waste primarily contributes 17.3% of the plastic-type waste. Wind and runoff transport the majority of plastic waste from these activities into the water environment, whether discarded on land, along shorelines, or in landfills.

Bintan Island's coastline is one of the coastal ecosystems known to be polluted by plastic debris due to the cross-trade routes, ports, tourism, industry, and domestic activities (Al Hamra and Patria, 2019; Hidayati et al., 2023; Idris et al., 2022; Syakti et al., 2018, 2019). Macro, meso, and micro-sized marine debris can be found along the shores of Tanjung Pinang and Bintan districts. Plastic waste is the most prevalent macro and meso garbage at each research site, with many variations of plastic types. Syakti et al. (2019) found that plastic waste on the coast of Bintan Island consists of 22.9% low-density polyethylene (LDPE), 19.5% polystyrene, 16.6% polypropylene (PP), 10.4% polyethylene-terephthalate (PET), and 9.2% high-density polyethylene (HDPE). The accumulation of plastic pollution affects coastal ecosystems, including mangrove ecosystems. Plastic waste will be retained by mangrove roots and float on the water's surface. Plastic debris can alter mangroves' structure and

water retention, thereby inhibiting root development. Plastic pollution in mangrove ecosystems also poses a significant threat to associated biota. Large-scale plastic accumulation can impact the biota's reproductive, secretory, and digestive systems (Hidayati et al., 2023).

In the natural environment, indigenous bacteria are crucial to the bioremediation of environmental pollution, including plastic waste pollution. Bioremediation is considered environmentally friendly and can accelerate the degradation time of waste containing toxic compounds. Several microorganisms known to degrade plastic include bacteria, fungi, and actinomycetes (Zeenat et al., 2021). Bacteria that are capable of degrading plastic particles can be isolated from water, sediment and marine plastic debris (Afianti et al., 2022; Auta et al., 2018; Sekiguchi et al., 2011). *Citrobacter fruendii, Enterobacter agglomerans, Pseudomonas aeruginosa*, and *Arizona* spp. can break down polyethylene plastic using the Winogradsky column method. These bacteria were found in mangrove sediments and were grown on a mineral salt medium for 12 days, with a degradation rate of 30.55% (Marquez, 2018). Furthermore, Novitasari et al. (2023) discovered that after 40 days of incubation, bacterial isolates in the mangrove sediments of Pasir Putih Wonorejo Beach degraded 8.73% of PET plastic. In aquatic ecosystems, plastic will become a new habitat for microorganisms, forming biofilms on the plastic's surface. Microbial biofilms also play an important role in degrading plastics because of their ability to do so (Afianti et al., 2022; Pinto et al., 2019). Biofilms might utilize plastics as their only carbon and energy source, in addition to being able to biodegrade complex biopolymeric molecules such as polyaromatic hydrocarbons, lignin and its derivatives. We conducted this research to isolate bacteria from mangrove sediments on Bintan Island, screening their potential to degrade plastic and form biofilm as a candidate for plastic bioremediation.

METHODOLOGY

Sampling site

Sediment was collected in Lagoi (L) and Kawal (K) mangrove forests, Bintan Island, Indonesia. Based on the dominant mangrove species at the site, we collected mangrove sediment samples from four different species: *Rhizopora apiculata, Xylocarpus granatum, Ceriops tagals*, and *Lumlitzera littorea.* Each zone is divided into three sized quadrant areas 10×10 m and sediment samples were taken using a pipe from three quadrants, homogenized, and placed aseptically into a 15-ml Falcon tube. The details of the sample locations were mentioned (Table 1).

Bacterial isolation and purification

Isolation was carried out using two methods: directly and through enrichment methods. Enrichment methods were carried out to obtain plastic-degrading bacteria using a synthetic medium containing 0.1% polyethylene powder as a carbon source (Ingavale and Raut, 2018). One gram of sediment sample was inoculated into the enrichment medium (1 g NH_4NO_3; 2 g $MgSO_4 \cdot 7H_2O$; 1 g K_2HPO_4; 1 g $CaCl_2 \cdot 2H_2O$; 0.15 g KCl; 1 g yeast extract; 1 mg $FeSO_4 \cdot 6H_2O$; 1.0 mg $ZnSO_4 \cdot 7H_2O$; 1 mg $MnSO_4$; 1 g PE powder) and incubated with 120 rpm agitation for four weeks. After incubation, 1 ml of the enriched sample was inoculated and purified using the same solid medium until a single culture of bacteria was obtained. With another method, bacteria were directly isolated from mangrove sediment samples using Zobell marine agar medium to obtain indigenous bacteria. One gram of sediment sample was vortexed for 10 minutes in sterile filtered seawater, and serial dilution was carried out. Then, 1 ml dilution was inoculated in ZMA using pour plate methods and incubated at 28 °C for 48 hours. Further purification is carried out with the same media.

Screening of plastic degrading bacteria

Screening of plastic-degrading bacteria was carried out using paper disc and clear zone methods (Skariyachan et al., 2015). Overnight broth cultures of the isolates were inoculated to solid mineral salt medium (4.5 g K_2HPO_4; 0.2 g $MgSO_4 \cdot 7H_2O$; 0.1 g NaCl; 0.002 g $FeCl \cdot 6H_2O$; 0.1 g $(NH_4)_2SO_4$; 0.1 g $CaCl_2 \cdot 2H_2O$; 15 g bacteriological agar), supplemented with polyethylene glycol (PEG) 1 g/L. After 7 days of incubation at 28 °C, the sample was stained using 1% coomassie brilliant blue (Nademo et al., 2023). The formation of a clear zone signifies positive results. The clear zone's diameter was then measured using an automatic colony counter (Interscience Scan 400, USA).

Screening of biofilm-forming bacteria

Biofilm-forming bacteria were screened using a microplate plate assay (Kavitha and Raghavan, 2018). Overnight cultures grown in liquid media were transferred to a 96-well microplate (Iwaki)

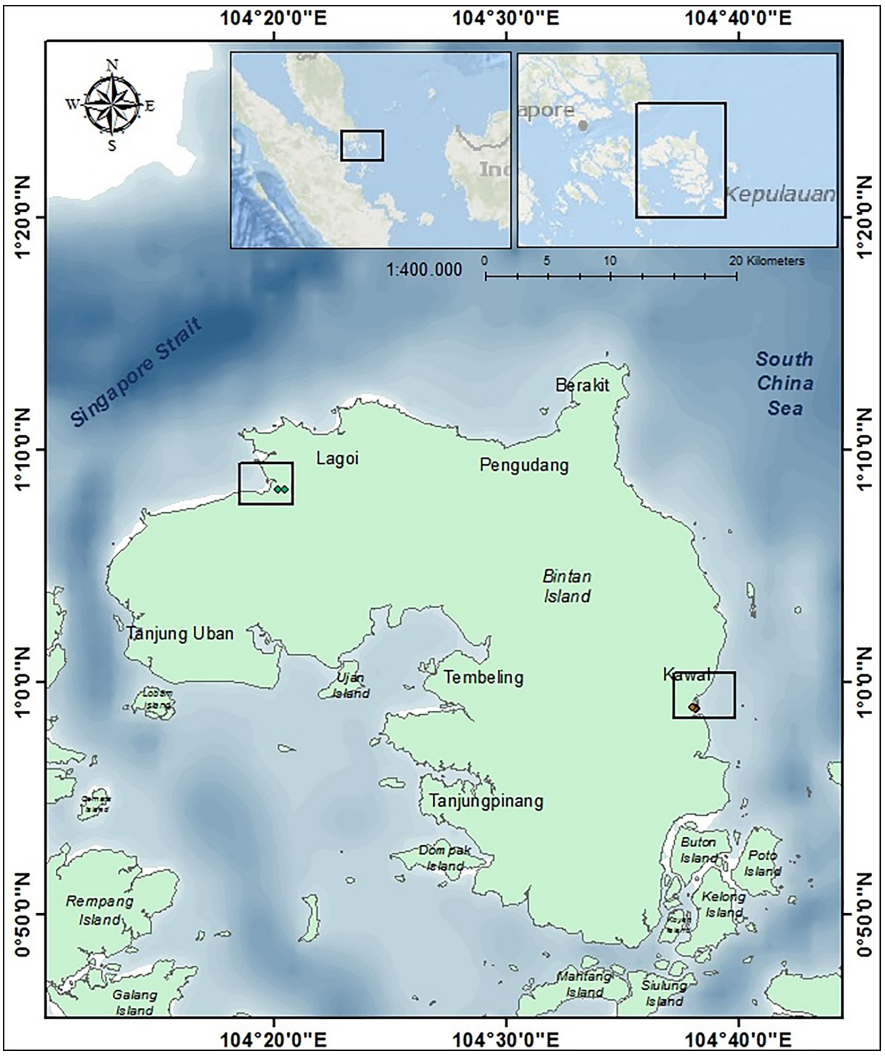

Figure 1. Map of sampling sites in Bintan Island

Table 1. Sampling location of mangrove sediment

Location	Zone	Coordinate	Mangrove type
Lagoi (L)	Seaward (A)	1.13971, 104.331	*Rhizopora apiculata*
	Landward (B)	1.13892, 104.332	*Ceriops tagal*
Kawal (K)	Seaward (A)	0.9815, 104.636	*Rhizopora apiculata*
	Landward (B)	0.98202, 104.634	*Lumlitzera littorea*

in triplicate and incubated for three days at room temperature. After incubation, the contents of the wells were discarded, and the wells were washed with phosphate-buffered saline (PBS) pH 7.4 to eliminate planktonic cells. After that, the microplate was stained with 0.1% crystal violet. The CV residue was then washed with 70% ethanol and allowed to dry. The optical density (OD) of the well was determined using a Multiskan Sky High Microplate Spectrophotometer at 570 nm. The OD value of ≥ 0.01 is considered an index of biofilm formation on the surface of the well.

Degradation test

Plastic degradation testing was carried out using the method according to (Joshi et al., 2022). A piece of LDPE plastic (2×2 cm) was placed in a bottle containing sterile MSM medium. 10% inoculum (OD 0.5) of broth culture bacteria (7 potential isolates, 1 consortium) was added, then incubated for 60 days with 120 rpm agitation at 28 °C. A medium without bacterial inoculum was used as a negative control. After the incubation period, biofilm on the surface of the polyethylene

sample was extracted. The polyethylene film was washed using PBS, placed in a sterile filtered seawater containing a bead, and vortexed for 10 minutes. Bacterial density from extracted biofilm was enumerated using Zobell marine agar.

The degradation percentage of plastic was determined using loss of dry weight. The plastic sample was washed with 2% SDS and 70% alcohol to remove the biofilm of bacteria, then dried overnight at room temperature (25 ± 2 °C), and weighed. The % weight reduction was measured by using the formula (Ali et al., 2023).

$$Weight\ Reduction = \frac{Wi-Wf}{Wf} \times 100\% \quad (1)$$

Bacterial identification

Bacterial isolates were identified using Sanger sequencing of the 16s rRNA gene. Genomic DNA was extracted using the FavorPrep™ Tissue Genomic DNA Extraction Kit and amplified using 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1429R (5'-TACGGYTACCTTGTTACGACTT3'). Polymerase chain reaction was performed using the Dreamtaq Green PCR Master Mix Kit (12.5 µL of DreamTaq Green PCR Master Mix, 1 µL of reverse and forward primers, 2 µL of DNA template, and 9.5 µL of ddH2O). The PCR conditions were set to an initial denaturation temperature of 96 °C for 1 minute, followed by 30 cycles of 96 °C denaturation for 1 minute, 55 °C annealing for 30 seconds, and 72 °C elongation for 30 seconds. Then, the final elongation stage was at 72 °C for 90 seconds (Afianti et al., 2019). The sequencing results were analyzed using BLAST to determine the isolate sequence's similarity to the NCBI genebank database sequence.

RESULTS AND DISCUSSION

Isolation of mangrove sediment bacteria

Bacterial isolation was performed as an initial step to obtain potential microbial isolates for plastic degradation from mangrove sediments. Isolation was carried out by two methods: isolation from enrichment samples and direct isolation. Results shown in Figure 2 showed that we have isolated 197 bacterial isolates from mangrove sediment, including 132 isolates from the direct isolation method and 65 isolates from the enrichment method. The most isolated microbes were found in the seaward zone in the Kawal area (KA) using the direct isolation method, with a total of 51 isolates. The difference in the number of isolates obtained was also influenced by the origin of the samples and the vegetated mangroves in the area. The number of isolates obtained from direct isolation is higher because the media used is a common medium for the isolation and enumeration of marine bacteria. However, in the enrichment isolation method using media with polyethylene powder as a specific carbon source, we predicted that the isolated bacteria were capable of degrading the plastic polymer. The presence of a bacterial population in mangrove sediments indicates that food and sufficient energy are available to support microbial growth (Ambeng et al., 2019). However, it can be influenced by organic and inorganic chemical composition, as well as other environmental factors.

Potential screening of isolated bacteria for plastic degradation

It was found that several bacteria have the potential to degrade plastic, indicated by clear zone formation on screening of degradation using

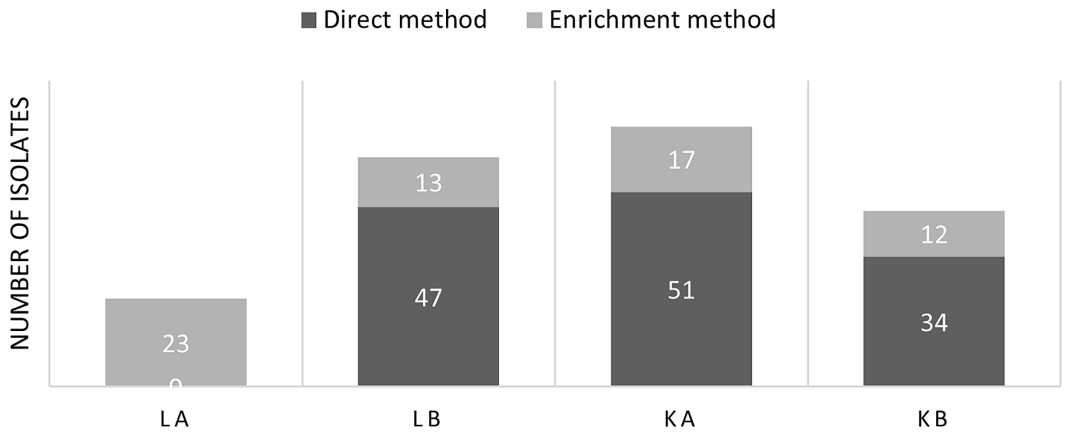

Figure 2. The number of bacteria isolated from mangrove sediment

PEG (Figure 3). There were 53 bacteria isolates from 197 total isolates, that can degrade PEG. Most bacteria that were capable of forming clear zones were found from bacteria isolated by the enrichment method. This can be caused by the use of polyethylene during the enrichment period, which leads to the bacteria being well-adapted to the plastic carbon source. The formation of a clear zone indicates that the bacteria can metabolize PEG as the only carbon source in the test medium. The structure of PEG has a shape similar to that of polyethylene with additional functional groups. PEG is easier to degrade because it has ether bonds and hydroxyl groups at the end of the chain (Wilkes and Aristilde, 2017).

In this study, it was found that 25 of 53 PEG-degrading bacteria could form biofilms on plastic surfaces (Figure 4). A biofilm test on a microplate is used to study the early stages of biofilm formation. This simple biofilm test allows the formation of biofilms on the walls or bottom of the microplate (O'Toole, 2011). Marine bacteria found in colonies on the plastic's surface release extracellular polymeric substances (EPS) to envelop the bacteria. EPS acts as a bioadhesive between the bacteria and the carrier, causing the bacteria to easily stick to the plastic surface (Ogonowski et al., 2018). EPS, a highly hydrated substance, can absorb large amounts of water into its structure through hydrogen bonds. In the early colonization stages of biofilms, marine bacteria prefer to attach to hydrophilic carrier interfaces. On the plastic surface, it can be seen that the abundance of hydrophilic groups, such as CO and C = O, increased during the initial process of biofilm formation on the PE surface. At the next stage of biofilm formation, hydrophobicity is positively related

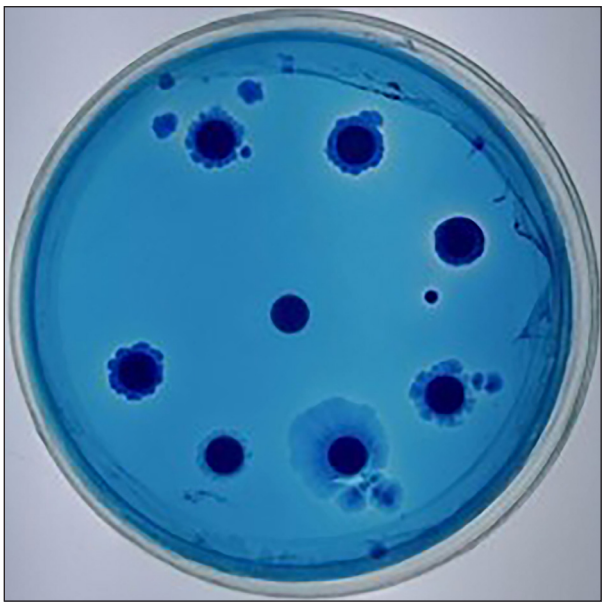

Figure 3. Clear zone formation on screening of PEG-degrading bacteria

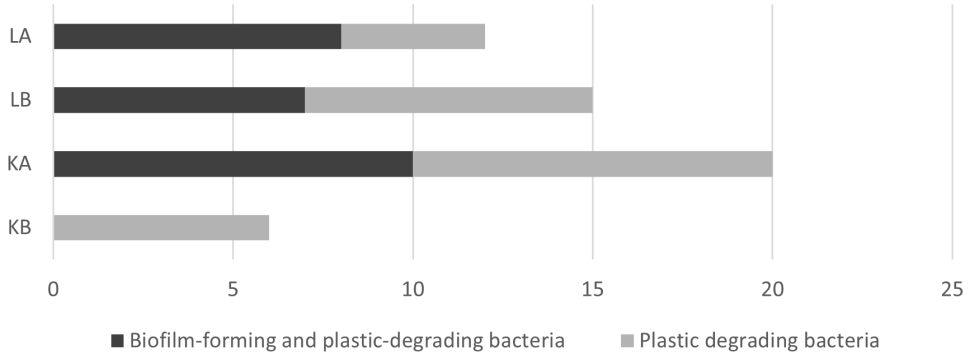

Figure 4. The number of plastic-degrading bacteria capable of forming biofilms

to the level of adhesion of the microbial community and the formation of biofilms on plastic surfaces (Sooriyakumar et al., 2022).

Seven potential bacterial isolates were selected based on the diameter of the clear zone in the PEG screening test and biofilm index formation (Table 2). The largest clear zone diameter was shown by bacterial isolate RCO.L3.26, which had a 2.2 mm clear zone diameter with a 0.0100 biofilm index. While the highest capability to form biofilm was shown by RCO.KE1.18 with a 0.1697 biofilm index. These selected potential bacteria were tested for plastic degradation on broth medium.

Plastic degradation

The degradation test aims to see the effectiveness of bacterial isolates in degrading polyethylene plastic in an incubation period time. After 60 days of incubation, there was a decrease in the dry weight of polyethylene plastic (Figure 5) and the formation of biofilm on the plastic surface, as indicated by the bacterial density value (Figure 6). The highest weight reduction was shown by a degradation test using a consortium of seven bacterial isolates, with a weight reduction value of 1.86%. Meanwhile, in the single culture polyethylene degradation test, the bacterial isolate RCO.KE1.14 showed the highest weight reduction (1.7%), with a bacterial biofilm density of 8.94×10^5 CFU/cm^2. However, in this study, the weight reduction is relatively small. Several factors can influence the degradation of plastic such as microbial biomass concentration, population diversity, enzyme activity, substrate physicochemical properties, substrate molecular structure, substrate concentration, and environmental factors, namely pH, temperature, water content, availability of electron acceptors, and carbon energy sources (Zubairu et al., 2018).

Table 2. Potential biofilm-forming bacteria for plastic degradation

Isolate	PEG Degradation test (Clear zone diameter; mm)	Biofilm index (OD 570nm)
RCO.LE1.8	2.0 ± 0.44	0.0728
RCO.L3.26	2.2 ± 0.84	0.0100
RCO.L3.32	2.0 ± 0.00	0.0742
RCO.K1.51	1.9 ± 0.60	0.0626
RCO.KE1.11	1.9 ± 0.42	0.0319
RCO.KE1.14	1.7 ± 0.38	0.0742
RCO.KE1.18	1.2 ± 0.26	0.1697

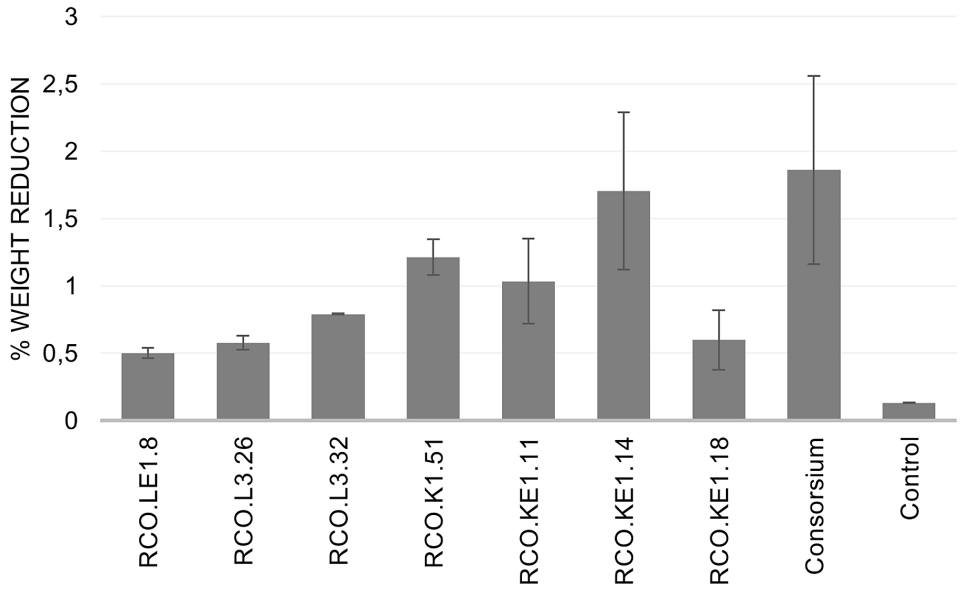

Figure 5. Weight reduction percentage of polyethylene after 60 days of incubation

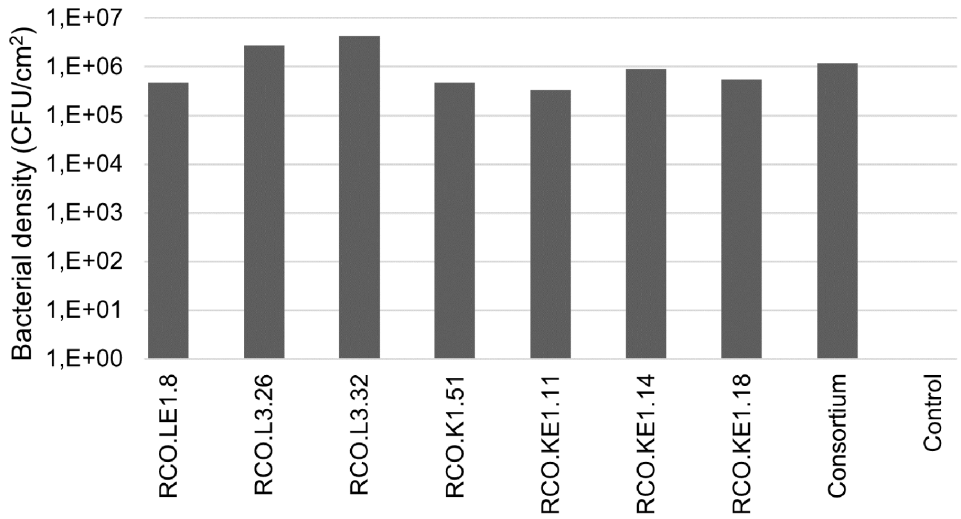

Figure 6. Bacterial density of biofilm on the polyethylene surface after 60 days of incubation

Seven bacterial isolates showed the ability to produce clear zones during the degradation of PEG and biofilm formation. The seven potential bacterial isolates were identified using 16S rRNA gene as *Bacillus sporotermodurans, Cytobacillus firmus, Rossellomorea vietnamensis, Stutzerimonas stutzeri, Dyadobacter jejuensis, Rhodococcus* sp*., and Achromobacter* sp. (Table 3). *Bacillus sporothermodurans*, isolated from metal-habituated locations, were reported to have the highest toxicity tolerance and bioleaching potential (Thakur and Kumar, 2023). This bacteria also has hydrolase enzymes, such as esterase enzyme activity. Esterase enzymes were known can act on the biodegradation of plastic polymers (Haritash and Kaushik, 2009). Similarly, both *Cytobacillus firmus* and *Rosselomorea vietnamensis* have esterase enzyme activity, indicating that they can break down polymer chains (Dutta et al., 2023). *Achromobacter* sp was reported that capability to degrade polystyrene (El-Kurdi et al., 2024) and high-density polyethylene (Kowalczyk et al., 2016). *Stutzerimonas stutzeri* (*Pseudomonas stutzeri)* can degrade polyester (Howard et al., 2023) and PET monomer terephthalate (Liu et al., 2022). In this study, *Rhodococcus* sp. showed the highest weight reduction in plastic degradation as a single culture. *Rhodococcus* is reported to have the ability to degrade several types of plastic, such as low-density polyethylene (Rong et al., 2024; Tao et al., 2023), polycaprolactone (Zampolli et al., 2023), and PET (Guo et al., 2023). According to the research, the plastic weight loss from *Rhodococcus* sp. degradation can reach 1–36% (Tao et al., 2023). These seven isolates all have one thing in common: they are aerobic bacteria with an optimal growth temperature of 35–37 °C and the ability to grow in salt conditions ranging from 0% to

Table 3. Molecular identification and characterization of morphological colony of potential biofilm-forming bacteria for plastic degradation

Isolate	Closest related species	NCBI Acc. No.	Sequence length (bp)	%Similarity	Morphological colony						
					Color	Shape	Margin	Elevation	Size	Surface	Opacity
RCO.LE1.8	*Bacillus sporotermodurans*	LC588450.1	1405	96.80	White	Round	Entire	Flat	Small	Smooth	Opaque
RCO.L3.26	*Cytobacillus firmus*	OR116160.1	1405	100	Cream-White	Round	Entire	Flat	Small	Smooth	Opaque
RCO.L3.32	*Rossellomorea vietnamensis*	KX982769.1	1404	98.22	Orange	Round	Entire	Flat	Small	Smooth	Opaque
RCO.K1.51	*Stutzerimonas stutzeri*	MT356167.1	1384	100	White	Round	Entire	Rised	Small	Shiny	Transparant
RCO.KE1.11	*Dyadobacter jejuensis*	MH259923.1	1346	98.22	Yellowish Cream	Round	Entire	Convex	Small	Smooth	Opaque
RCO.KE1.14	*Rhodococcus* sp.	KY052180.1	1376	99.93	Pale Orange	Round	Entire	Flat	Small	Smooth	Opaque
RCO.KE1.18	*Achromobacter* sp.	MZ352114.1	1334	98.58	White	Round	Entire	Rised	Small	Smooth	Opaque

5% and at pH levels ranging from 5 to 9. This condition is ideal for the mangrove environment on the Indonesian coast, as it encourages the growth of a wide range of bacterial species

CONCLUSIONS

Biofilm-forming bacteria also play an important role in the biodegradation of plastics. In this study, 25 of 53 PEG-degrading bacteria from mangrove sediment could form biofilms on plastic surfaces. Seven bacterial isolates were selected for the biodegradation test which resulted in a 0.5–1.7% weight reduction of polyethylene after 60 days of incubation. *Rhodococcus* sp. showed a high capability to degrade polyethylene. Furthermore, a consortium of seven isolated bacteria can achieve the highest weight reduction of polyethylene. Though the weight reduction on the plastic degradation test by both single and consortium bacteria was relatively small, it can indicate the prospects for using such microorganisms in technologies for the bioremediation of plastic waste. Further investigation is still needed to ensure the degradation efficacy and persistence.

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