Biodegradation of Polyethylene Microplastic using Culturable Coral-Associated Bacteria Isolated from Corals of Karimunjawa National Park

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Abstract

Polyethylene is a plastic material that was globally produced and is well known as a non-degradable pollutant product. Plastic pollution, primarily microplastics, have been distributed to coral reef ecosystems, where these areas are ecosystems with high productivity. Karimunjawa National Park in Indonesia is one of the protected areas for coral reef ecosystem habitat in Central Java, threatened by microplastic contamination. Recent studies have shown that coral-associated bacteria have an adequate ability to degrade marine pollutant materials. No one has reported that the use of indigenous coral-associated bacteria has the potential for microplastic biodegradation, especially low-density polyethylene microplastic materials. Hence, the objective of this study was to find the potential of microplastic biodegradation agents derived from coral-associated bacteria in Karimunjawa National Park area. Various coral life-forms were isolated in July 2020 from conservation areas and areas with anthropogenic influences. Bacterial isolates were screened using tributyrin and polycaprolactone as substrates to reveal potential microplastic degradation enzymes. The total isolation results obtained 92 bacterial isolates, and then from the result of enzyme screening, there were 7 active bacteria and only 1 bacteria that potential to degrade polyethylene. LBC 1 showed that strain could degrade by 2.25±0.0684 % low-density polyethylene microplastic pellet by incubating bacterial growth until the stationary phase. Identification of LBC 1 strain was carried out by extracting DNA and bacterial 16S rRNA sequences. Bacterial gene identification refers to Bacillus paramycoides with a similarity level in the National Center Biotechnology Information database of 99.44%. These results prove that hard coral association bacteria can degrade low-density polyethylene microplastics.

Keywords: *Low-Density Polyethylene microplastics, biodegradation, associated-bacteria, coral, Karimunjawa*

Introduction

Plastic waste is one of the worst pollutions in the Asia Pacific region, at least tens of billions of plastic items are estimated to be entangled in coral reefs (Lamb *et al*., 2018), one of them is microplastic. The spread of microplastics has been distributed to coral reef ecosystems (Connors, 2017; Cordova *et al*., 2018; Saliu *et al*., 2019). In Indonesia, at least 22.7 and 48.3 ± 13.98 microplastic particles per kilogram sample were found in coral reef ecosystem areas, in Karimunjawa and Sekotong National Parks, Lombok (Lie *et al*., 2018; Cordova *et al*., 2018). The impact of microplastic contamination on coral animals is reported to cause disturbances to corals such as bleaching and necrosis (Reichert *et al*., 2018; Syakti *et al*., 2019), energy reduction (Reichert *et al*., 2019), the danger of particle associations such as toxins (Lee *et al*., 2014) and opportunistic coral pathogens such as *Vibrio* sp. (Zettler *et al*., 2013), therefore microplastic contamination is a severe threat from anthropogenic impacts. However, in survival, coral animals have a unique mechanism, especially in communication with coral holobionts (associating organisms), one of them was bacteria (Rohwer *et al*., 2002). Under stress conditions, corals will increase the number of bacteria and mucus (transparent exopolymer) to maintain the abundance of the bacterial community to be relatively more stable (Garren and Azam, 2012). Coral association bacteria also contribute to the cycling of biogeochemical elements and the recycling of important and limiting elements in the coral reef environment (Bythell and Wild, 2011). In addition, various research reports

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state that the potential for coral association bacteria is also effective in degrading agents of contaminants in the sea, such as crude oil (93.5% effective from the genus *Cobetia*) (Ansari *et al*., 2021), urea (effective by the bacterial genus *Halomonas*) (Zhou *et al*., 2020), and organophosphate pesticides (effectively degraded from the bacterial genus *Bacillus*) (Sabdono and Radjasa, 2008). No one has reported the potential of coral association bacteria in degrading plastic contamination, especially microplastics. The aim of this study was to find the potential of microplastic biodegradation agents derived from coral-associated bacteria in the Karimunjawa National Park area. The utilization of indigenous bacteria in the pollutant degradation process also proves the availability and resilience of in-situ resources in tackling the presence of pollutants (Okafor *et al*., 2021).

Materials and Methods

Study area

Sampling was conducted in July 2020. Sampling locations include Bengkoang Island which conservation area and location with anthropogenic influences (tourism and settlements) at Cemara Kecil Island, Menjangan Kecil Island, and Karimun Island (Legon Boyo and Taka Sendok) (Figure 1.).

Materials

Polyethylene granule (low density melt index 190°C/2.16 kg) produced by Sigma Aldrich Chemical Co. (USA) is commonly used to produce plastics. Tributyrin produced by Sigma Aldrich Chemical Co. (USA) and Polycaprolactone produced by China Industry were used as enzyme test substrates. Microplastics were obtained by weighing plastic pellet (<5 mm in diameter), weighed using an analytical balance OHAUS PX124 with an accuracy of 0.0001 mg, then sterilized using 70% ethanol, exposed to UV light for 20 minutes, and autoclaved for 105°C for 20 minutes (Harshvardhan, 2013; Kumari *et al*., 2018).

Sample collection and isolation

A purposive random sampling technique was used to sample of corals from three types of life forms, namely massive corals, branching corals, and foliose corals;. A tiny part of coral colonies was taken using a chisel and hammer. Then the sample was put in a zip lock bag and stored in a cool box. Bacterial isolation was carried out by the spread plate method (Madigan *et al.,* 2014). Previously, the coral

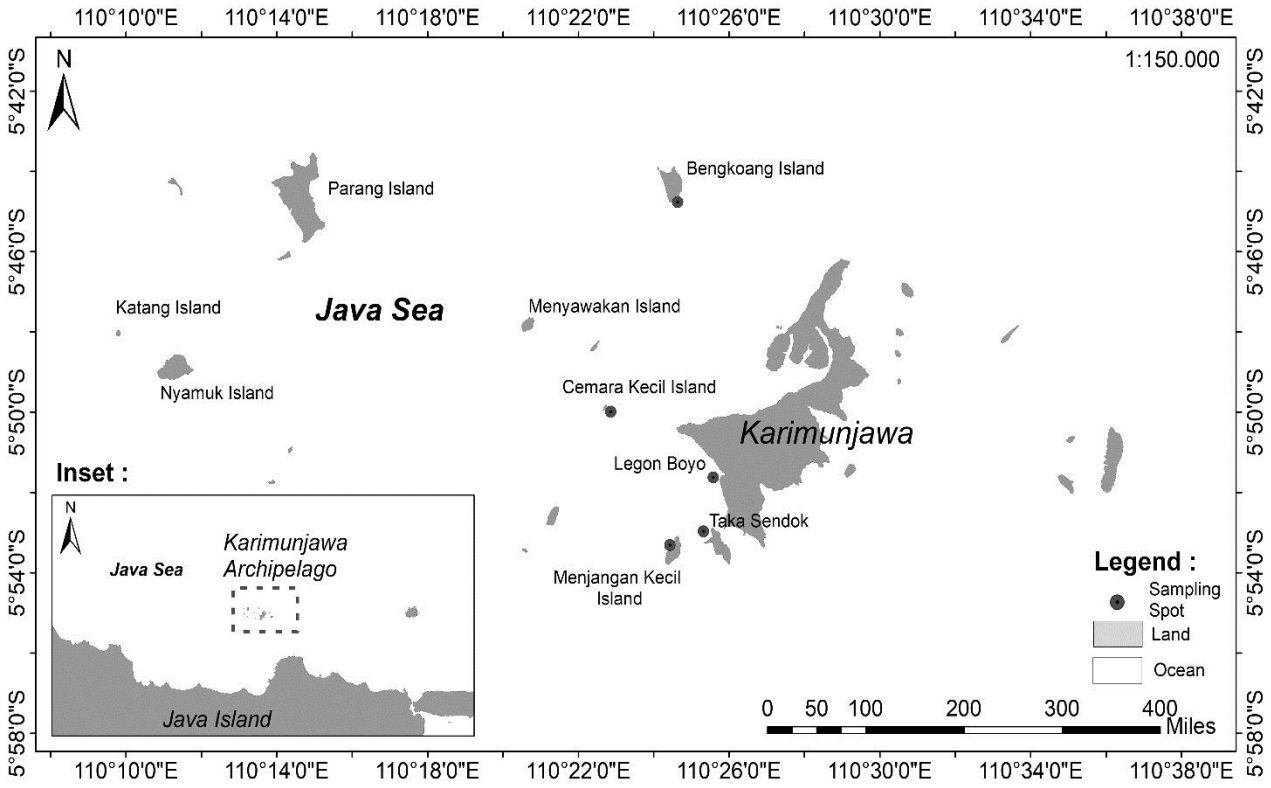

Figure 1. Sampling Location in Karimunjawa National Park (Source : Geospatial Information Agency Map)

fragments were sprayed with sterile seawater, pounded with a mortar and pestle, then 1 gram of fine coral sample was put into a falcon tube containing 9 mL of sterile seawater. The dilution was carried out from 10^{-1} to 10^{-3}. From each series of dilutions, 10 µl of the liquid sample was taken and put into sterile petri dishes containing Zobell 2216E agar medium, then spread with an L-spreader. The petri dishes were then incubated at 30°C (room temperature) for 2 days.

Screening for Lipolytic and Polyesterase enzyme

An emulsion of 2% (v/v) tributyrin (Sigma-Aldrich, USA) was prepared in sterile distilled water and added 50 g/L of gum arabic (Carl Roth) (Jaeger and Kovacic, 2014). Gum arabic powder is used as an emulsifying agent for triglycerides. Homogenize the mixture for at least 1 mins to produce a stable emulsion on a hot magnetic stirrer. Tritributyrin substrate is a lipolytic enzyme trigger (Molitor *et al*., 2019). Then the test bacteria were inoculated into a petri dish containing the substrate. Furthermore, the results of the screening of the tributyrin substrate were tested with a polycaprolactone substrate. This test was carried out as a trigger test for the polyesterase enzyme, the protocol adopted from Jarett *et al*. (1984) and then modified by Molitor *et al*. (2019) with slight additions. The positive result showed a clear zone around the bacteria. The clear zone was measured by equation (1), the equation means of enzymatic index calculation (EI) (Florencio *et al*., 2012). All experiments were conducted with two replicates tests.

$$Enzymatic\ Index\ (EI) = \frac{diameter\ of\ clear\ zone}{diameter\ of\ colony}$$

Hydrophobicity assay

Microbial Adhesion To Hydrocarbon (MATH) / hydrophobicity test is based on the determination of the hydrophobicity of microbes by differential partitioning between the water-hydrocarbon surface. The potential bacterial isolates were grown as a pure culture on marine zobell agar 2216E media. Then, the bacteria were inoculated into marine zobell broth 2216E with a volume of 5 mL in a test tube and incubated with a shaker at room temperature until the culture reached a cell density of 3.8 x 108 CFU/mL (absorbance 1.09 at 600 nm) or in the log phase bacterial growth (Harshvardhan and Jha 2013). Then 1 mL of bacteria was taken (log phase and growth stationary) into a test tube, then separated by centrifugation and washed twice with PUM buffer solution (buffer containing per liter: 17 g K_2HPO_4, 7.26 g KH_2PO_4, 1.8 g urea, and 0.2 g $MgSO_4.7H_2O$). The bacterial precipitate (natan) was resuspended in 1 mL PUM buffer solution and given a hydrocarbon solution (decane) with different volumes (0.025 mL, 0.05 mL, 0.1 mL, 0.15 mL, 0.2 mL, and 0.25 mL) (Harshvardhan and Jha, 2013). Each test tube with a hydrocarbon solution and bacterial precipitate (natan) was homogenized with a vortex for 2 minutes and then allowed to stand for 30 minutes to form a two-phase solution (Saini, 2010). The hydrocarbon solution with the bacterial suspension will form a layer (two-phase solution). The bottom layer was carefully removed and then analyzed with a UV-Vis Spectrometer with OD600 / 600 nm wavelength. The PUM buffer solution was used as a blank, the control solution was bacteria suspended in the PUM buffer (A_0), and the hydrocarbon solution with bacterial suspension was A_1. Then the results are entered into the formula as in equation (2) (Saini, 2010).

$$Hydrophobicity\ (\%) = \frac{(Ao-A1)}{Ao} X\ 100$$

Biodegradation assay

The potential bacterial isolate was grown as pure culture on marine zobell agar 2216E before being inoculated into marine zobell broth 2216E with a volume of 5 mL in test tubes and allowed to grow in a shaker at 30°C until the culture reached a cell density of 3.8 x 108 CFU/mL (1.09 absorbance at 600 nm). Then 1% culture of the total volume of the growing medium was inoculated into an erlenmeyer containing 100 mL Busnell-Haus liquid medium and 0.2% sterile polyethylene pellet (low-density melt index 190°C/2.16 kg), afterwards incubated in a shaker at 30°C and 150 RPM for 7 days (Bhatia *et al*., 2014). Growth observations were carried out through optical density (OD) at a wavelength of 600 nm. All experiments were repeated twice (Kapri *et al*., 2010). Observation on the seventh day was carried out by weighing the final weight of polyethylene pellet (low-density melt index 190°C/2.16 kg) by previously cleaning with 2% (w/v) Sodium Dodecyl Sulfate (SDS) for 2 hours and washing with distilled water as much as three times. The lost weight was calculated using equation (3) (Kumari *et al*., 2018).

$$Weight\ loss\ (\%) = \frac{(Wo-W1)}{Wo} X\ 100$$

Note : W_0: Initial Weight, W_1: Final Weight

Identification of Bacteria

DNA extraction was carried out using the Chelex 10% extraction procedure. Amplification using the 16S rRNA procedure with universal primers 27 F (5'AGAGTTTGATCCTGGCTCAG-3') (Weisburg *et al*., 1991) and 1492R (5'GGTTACCTTGTTACGACTT-3') (Reysenbach *et al*., 1992). The reaction mixture for 25 µl PCR cocktail contained 1 µl of each primer at 10 mM, 1 µl DNA template, 9.5 µl double distilled

water, and 12.5 µl of EconoTaq Plus Green Master Mix, Lucigen Corp, USA. The setting of thermal cycler condition including initial denaturation 94°C for 2 min, followed by 35 cycles of 94°C for 30 sec, 50.7°C for 30 sec, 72°C for 1 min, and final extension 72°C for 5 min. The PCR product was sent to DNA sequencing facility to be purified and get sequenced analysis using sanger methods. Contig sequence analysis using Mega X (Kumar *et al*., 2018) and similarity analysis conducted in-silico using BLAST gene database of National Center for Biotechnology Information.

Result and Discussion

A total of fifteen coral samples from growth forms such as massive corals, branching corals, and foliose corals (Figure 2.) were collected in this study. The selection of coral sample types is based on coral morphology because it has important involvement across scales such as differences in physiology, habitat, and the role of controlling ecosystems (Zawada *et al*., 2019). At the habitat scale, the diversity of coral colony morphology contributes to the structural complexity of the habitat, the availability of complex habitat structures that affect association organisms, food webs, and ecosystem functions (Richardson *et al*., 2017). Another report, there was a clear relationship between the high abundance of association bacteria related with coral morphology (life form) and the coral's tolerance to environmental stresses (Liang *et al*., 2017).

The results of bacterial isolation from fifteen coral samples obtained as many as 92 isolates of association bacteria. All isolates were screened for the potential biodegradation of low-density polyethylene based on enzyme activity. Rapid detection test using agar plates is a method that is often used in screening based on enzyme activity in samples, especially lipolytic enzymes (Popovic *et al*., 2017; Sulaiman *et al*., 2012). This method is also almost always used in tests that emphasize the detection of pollutant-degrading enzymes (Ufarté *et al*., 2015). As a universal substrate, tributyrin can also be used to detect the activity of esterases, true lipases, phospholipases, or even peptidases and acyl-transferases (Molitor *et al*., 2020). In this study, seven bacterial isolates indicated the activity of the esterase enzyme with a positive sign, it shown by a clear zone around the bacteria (Figure 3.), in line with the statement of Popovic *et al*. (2017), the clear zone on the tributyrin agar plate indicates esterase activity. In nature, especially in the marine environment, the activity of the esterase enzyme was detected in marine fish. The enzyme esterase (Cholinesterase) plays an important role in detoxification of organophosphate compounds (chlorpyrifos) in neurological pathways, if this activity is reduced, it will threaten the survival of fish (Martínez-Morcillo *et al*., 2019). Its capacity for detoxification such as the ability to hydrolyze xenobiotic compounds. In line with Sabdono and Radjasa (2008), this study explains that there is the ability of coral association bacteria to degrade organophosphate compounds, namely chlorpyrifos. Esterase, depolymerase, and dehydratase are the enzymes that have significant potential in the plastic biodegradation process because they can hydrolyze polyester (Urbanek *et al*., 2018). Ribitsch and Guebitz (2018), added that hydrolase enzymes such as cutinase, esterase, and lipase had been recognized as powerful tools in hydrolyzing synthetic polymers. However, esterase is a broad-spectrum enzyme defined as having the catalytic ability to hydrolyze a carboxylate ester bond (Casas-Godoy *et al*., 2012).

Rapid detection test treatment using polycaprolactone as a substrate is a validation or shrinking process for bacteria with the potential for the enzyme polyesterase. The application of low molecular weight polyesters such as polycaprolactone

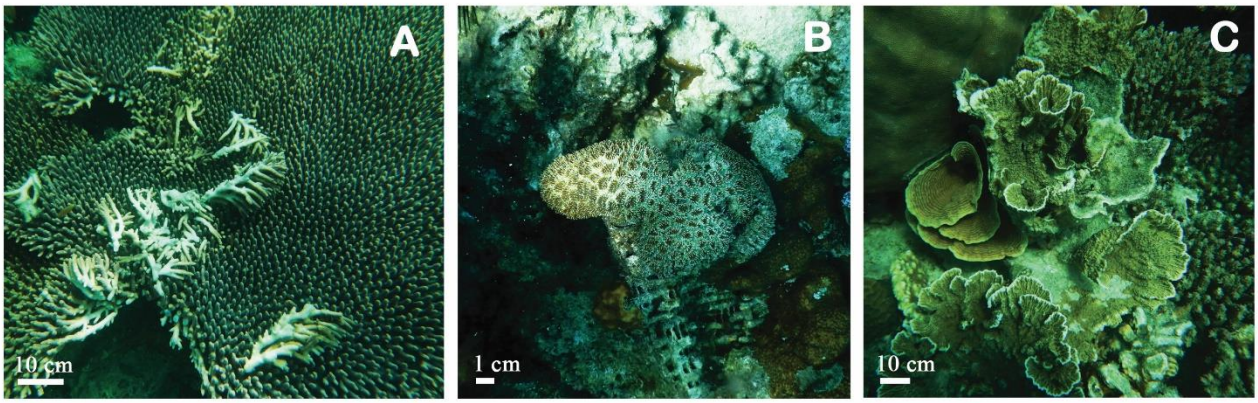

Figure 2. Three Different Life Form of Coral. A) Coral Branching, B) Coral Massive, C) Coral Foliose.

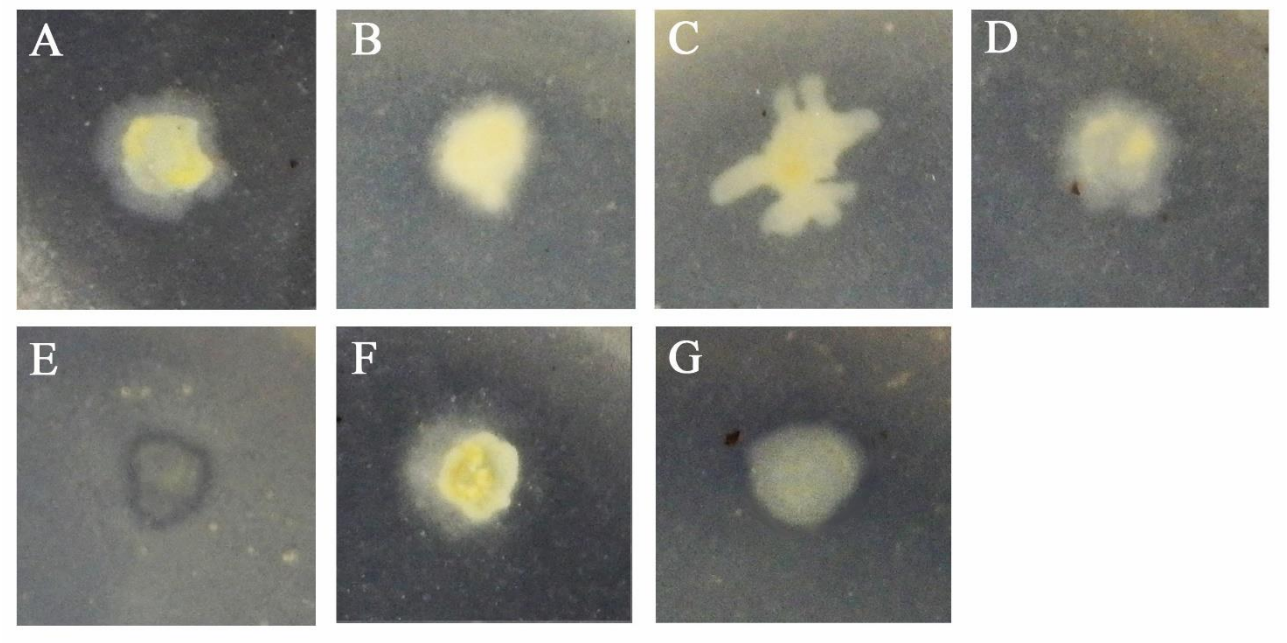

Figure 3. Positive result of Tributyrin Screening Assay**.** (A) CKC 6, (B) CKC 1, (C) LBC 1, (D) LBC .2, (E) TSA 4., (F) CBA 6. (G) CKA 8 ** CKC = Cemara Kecil Coral Foliose, CKA = Cemara Kecil Coral Branching, LBC = Legon Boyo Coral Foliose, TSA = Taka Sendok Coral Branching, CBA = Cemara Kecil Coral Branching.

or Impranil DLN as a test substrate is an effective procedure or method and directly targeted to determine the potential of the polyesterase enzyme (Molitor *et al*., 2020). However, polyesterase enzymes must be able to hydrolyze triglyceride substrates very well (Bollinger *et al*. 2020), like tributyrin. test results, isolate LBC 1 showed an apparent activity for the ability of bacteria to hydrolyze polycaprolactone (Figure 4.). The clear zone around the bacteria could be clearly observed at the 96 hours of observation, the zone was clearer than the clear zone during the initial screening with tributyrin substrate, with equation of Enzymatic Index, the value was 1.32±0.0272 . According to (Molitor *et al*., 2020), the different results on the two substrates was caused by different enzyme-specific activities or caused by the production and secretion of polyesterase, which is regulated differently by bacteria depending on the reaction to the test substrate. Therefore, the hydrolytic activity of esterases identified on polyester substrates makes the potential of this enzyme attractive for further studies in plastic depolymerization (Popovic *et al*., 2017). According to enzymatic index equation, the clear zone around the bacteria increased at 192 hours of observation from 1.32±0.0272 to 1.56± 0.0933, the increasing of the clear zone was due to the storage treatment of bacterial isolate at 4°C after 96 hours of observation (Figure 4.). Enzyme activity in marine microorganisms is strongly influenced by two environmental factors, such a temperature and osmolarity (Tchigvintsev *et al*., 2015). The activity formed, shown by increasing the area of the clear zone, indicated that the LBC 1 isolate was a bacterium that had the potential for cold-adapting enzymes. The unique specific adaptation and thermostability of proteins at low temperatures indicate that this type of enzyme is a cold-adaptive enzyme (Siddiqui and Cavicchioli, 2006). Furthermore, Urbanek *et al*. (2018) added that microbes resistant to cold temperatures show many unique features, one of them being the potential for cold-adapting enzymes produced due to adaptation to new carbon sources and can show significant halotolerance (Srimathi *et al*., 2007).

Hydrophobicity is one of the important limiting factors in the biodegradation process of synthetic polymers (Hajighasemi *et al*., 2018). Ribitsch *et al*. (2015) showed that the hydrophobic surface of the polymer limits the adsorption and activity of effective polymer-degrading enzymes. Therefore, hydrophobicity is a surface property (could be in microorganism/ polymer) that is important to observe in biodegradation studies because the relationship between surface hydrophobicity and microorganisms will determine colonization on polymer substrates (Restrepo-Flórez *et al*., 2014). The results obtained in the hydrophobicity test on LBC 1 bacterial isolates showed that in the early stages of bacterial growth (log phase) (Figure 5.), the absorbance value in the liquid phase ranged from 41-53%, which means that about 47-59% was partitioned in the hydrocarbon phase. (in this case decana). While in the stationary phase of bacterial growth, the absorbance value in

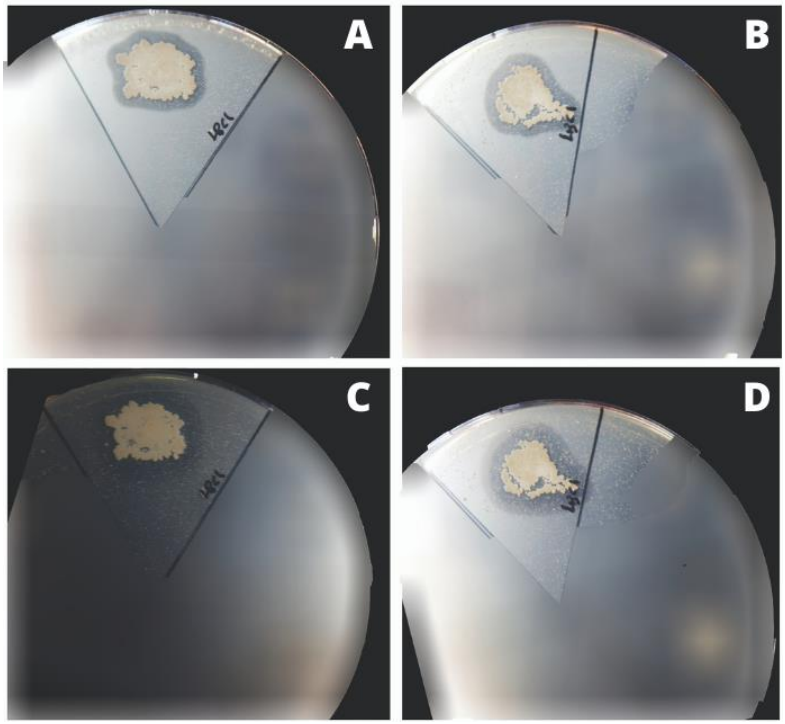

Figure 4. Clear zone around bacteria in LB Media + PCL Emulsion. (A) and (B) observations of 96 hours of incubation at room temperature. (C) and (D) observations 192 hours after incubation at temperature 4°C

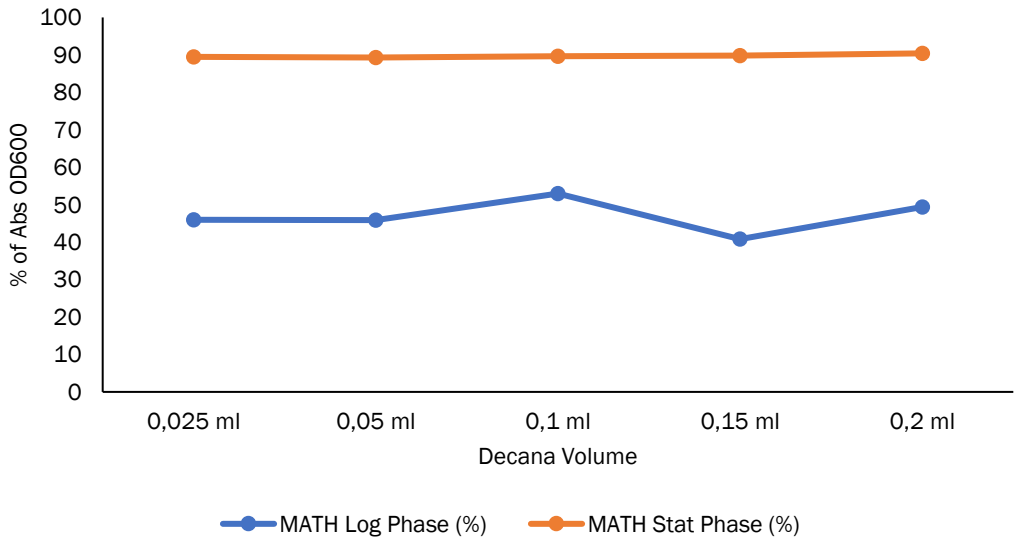

Figure 5. Hydrophobicity/MATH test for two growth phases. Log and stationary growth phase in LBC 1 bacteria.

the liquid phase ranged from 89-90%, which means that about 10-11% was partitioned in the hydrocarbon phase. This percentage value indicates that in the log phase, LBC 1 bacteria have moderate to strong hydrophobicity. in the stationary phase, hydrophobicity decreases to a not hydrophobic level, the classification of the level of hydrophobicity according to the provisions of the gram-positive bacteria test (Krepsky *et al*., 2003). The decrease in hydrophobicity is influenced by nutrients available in the environment, in line with the results of the study of Saini *et al*. (2011) which explains that the decrease in hydrophobicity from the log phase to the stationary phase is influenced by short-term cell starvation (±7 days). Under starvation conditions, bacteria have the potential to have smaller cell sizes, reduced protein

Table 1. Percentage Weight Loss of Microplastic LDPE in Biodegradation Assay

Sample	Duplicate	Initial weight (mg)	Final weight (mg)	Weight Loss (mg)	Avg (mg)	St Dev	% Loss	% Avg	St Dev
LBC 1	1	28,17	27,55	0,62	0,625	0,0071	2,2009	2,2493	0,0684
	2	27,42	26,79	0,63			2,2976		

content and altered hydrophobicity, which may lead to their release from the surface and potential for compaction of biofilms (Saini *et al*., 2011). The results of the microplastic biodegradation test showed a mass reduction in low-density polyethylene ore with an incubation period of 7 days. The reduction was 0.625±0.0071 mg, or 2.25± 0.0684 % initial weight (Table 1.). The same results were also obtained in the low-density polyethylene degradation test by Pseudomonas sp. AKS2 strain with a weight reduction of 2% at an incubation period of 15 days (Tribedi and Sil 2013). In addition, the use of bacteria sourced from the sea is also capable of biodegrading low density polyethylene, among others, resulting in a weight reduction of 1.26±0.17% in an incubation period of 75 days (Kumari *et al*., 2019), 1–1,75% within the incubation period of 30 days (Harshvardhan and Jha, 2013). Although in a small percentage, the ability of the bacteria mentioned above can biodegrade low density polyethylene.

The results of molecular identification of bacteria showed 99.44% similarity with the BLAST database at the National Center for Biotechnology Information. The isolate was identified as *Bacillus paramycoides* (accession no. MZ373159). The genus of Bacillus has confirmed as an associate bacteria in corals. Chen *et al.* (2011) found that the genus of Bacillus was associated with *Isopora palifera*. In addition, Rohwer *et al.* (2002) explained Bacillus was detected at massive coral (*Diploria strigosa* and *Porites astreoides*), different from that, Wilson *et al* (2012) and Ceh *et al.* (2012) showed that Bacillus had proportion of associated-bacteria on *Acropora tenuis* and *Pocillopora damicornis,* respectively. The capacity for degradation of plastic polymer, similar results were also obtained by Kumari *et al*. (2019), explaining that the AIIW2 strain was identified as a bacterium from the genus *Bacillus,* which could degrade different plastic polymers, such as PE and PVC. Ribitsch *et al*. (2011) added their findings, the species of bacteria *Bacillus subtilis* has the enzyme p-Nitrobenzylesterase which can degrade polyethylene terephthalate (PET). In addition, Devi *et al*. (2019) explained, from a total of 10 marine bacteria that were isolated, as many as 7 bacteria came from the *Bacillus* genus and had a potential for biodegradation of High-Density Polyethylene. Another fact is that the bacteria which most frequently reported to form biofilms on polyethylene surfaces include the genera *Pseudomonas, Rhodococcus*, and *Bacillus*, which have shown great potential for biodegradation of plastic polymers (Montazer *et al*., 2020).

Conclusion

The LBC 1 bacterial isolate from hard corals (foliose life form) from Karimunjawa National Park was identified as *Bacillus paramycoides*. This isolate had the potential for biodegradation of low-density polyethylene microplastic polymers indicated by a reduction in the weight of microplastics by 2.25± 0.0684% during the incubation period for 7 days. The test results are based on tests and observations in the laboratory with the environmental conditions of bacterial growth being measured. With this capability, it is possible that the biodegradation of plastic polymers is a cooperative process in a real ecosystem. Therefore, identifying the role of microorganisms and also carrying out genetic engineering to modify enzymes are the goals of further relevant research.

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