Aliphatic polyester biodegradation by coral-associated bacteria from Karimunjawa Marine National Park, Java Sea

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Abstract. Plastic waste is one of the environmental pollutants that is difficult to degrade. The spread of plastic waste is almost everywhere even in the ocean, especially in coral reef ecosystem. Non-degradable plastic like polyethylene, polypropylene, and polystyrene begins to be partially replaced with biodegradable plastic materials (i.e polycaprolactone) as a strategy to reduce non-degradable polymer materials. Hence, this study aims to find the potential of polycaprolactone biodegradation from coral associated-bacteria from Karimunjawa National Park. Coral samples were isolated in July 2020 from areas with influence by anthropogenic. Bacterial isolates were screened using tributyrin and polycaprolactone as substrates to reveal potential polyester degradation enzymes. The result obtained only one active bacterial isolate that potential to degrade polycaprolactone from a total of 18 isolates bacteria. LBB 2 showed that strain can degrade polycaprolactone by 8 days incubation period with 4 days in room temperature and 4 days in a 4°C incubation room. Bacterial identification by 16S rRNA sequences showed that strain LBB 2 refers to the bacteria Bacillus subtilis. The similarity level in the database of National Center Biotechnology Information by 99.45%. These results prove that associated bacteria from stony coral might play a role in degrading aliphatic polyesters.

Keywords: aliphatic polyester; associated-bacteria; biodegradation; Karimunjawa; stony coral

1. Introduction

Plastic waste is the major pollution at urban coastal area, estuaries, and especially in the ocean. The composition of plastic wastes are mostly from non-degradable synthetic material that is made by polymerizing molecules of monomer [1]. Due to the solid particle that they have, the conventional plastic (aromatic polyester) have been blended or replaced with aliphatic polyesters because of their degradability in the natural environment [2]. Biodegradable plastic from both aromatic and aliphatic polyesters (polycaprolactone) have been developed and produced during last decades [3, 4]. However, biodegradable plastic was disposed in the right way just only 60 % from more than 1 million production per year and 40 % approximately remains to the environment annually [5]. Over 80 % total marine debris were from land-based sources and remains from the sea-based activities [6], and between 60 – 80 % of marine debris are plastic [7]. Based on these facts, it gives a clear picture that waste pollution will end up in the oceans which can threaten marine life, especially for coral reef ecosystems. The entry of
plastic (i.e. microplastic) is a potential disturbance in the coral reef ecosystem, especially in coral animal [8, 9].

The potential spreading of plastic pollution to the coral animals is highly occur. In Indonesia, plastic waste is the most common waste in the waterways of coastal cities such as Jakarta, Semarang, Surabaya, etc. [10]. A city like this produces at least 30% of plastic waste from the total waste dumped into the ocean or an estimated 400,000 tons/year [10, 11]. These facts show that plastic waste might be threatened coral reef ecosystem areas especially in marine protected area such as Seribu Islands National Park (Jakarta) and Karimunjawa National Park (North Semarang) [12]. Reichert [9] and Syakti [13] explained that the impact of high exposure plastic (i.e microplastic) tend to be harmful the coral animals such as bleaching and necrosis on coral tissue. However, corals had several good mechanisms to prevent the bad impact of plastic exposure, so the corals could survive in those stressful conditions [8]. This mechanism cannot be separated from the role of coral association microorganism. Before ingestion, microplastics tend to accumulate in the coral mucus layer and cover coral tissue [8, 14]. The Surface Mucopolysaccharide Layer (SML) of corals provides a layer for biofilm formation, and this layer provides an abundant source of carbon and nutrients for microbes [15]. Microorganisms that colonize the plastic polymer stimulate biodegradation by forming a biofilm on the surface of the plastic polymer [16] and play an important role in the biodegradation of microplastics [17]. These microorganisms sometimes secrete extracellular enzymes that play a role in the degradation of polymeric materials [18]. Therefore, the aim of this study was to reveal the potential of coral-associated bacteria to degrade aliphatic polyester.

2. Methods
2.1. Study area
The research area includes an area with anthropogenic influences and marine protected area at Karimunjawa National Park, Central Java, Indonesia. Three areas with anthropogenic influences such as Legon Boyo (residential area), Taka Sendok and Cemara Kecil Island (Tourism), and then Bengkoang Island which the marine protected area.

2.2. Materials
As a enzym substrates, trybutyrin collected from Sigma Aldrich Chemical Co. (USA) and Polycaprolactone as a aliphatic polyester, produced by China Industry. Each of them is dissolved in two different places with gum arabic powder for optimal homogenization process [19].

2.3. Sample collection and bacteria isolation
Coral sample was the massive coral life form. The samples are collected from four different location. Small pieces of coral colonies were put in a sterile double zipper lock bag and stored into cooler box. The isolation of bacteria was performed by the pour plate method [20]. From each series of dilutions (from $10^{-1}$ to $10^{-3}$), about 5 to 10 µl of aliquot was taken to sterile petri dish and poured by liquid Zobell 2216E agar medium, after that incubated at room temperature for 2 days.

2.4. Enzyme test
Liquid tributyrin (Sigma-aldrich, USA) as much as 2% (v/v) was prepared in sterile distilled water, agar powder and 50 g/L of gum arabic powder [19]. Then the isolates were inoculated into agar plate containing the trybutyrin substrate. Furthermore, for the next step, the results of the screening tributyrin substrate was tested with a polycaprolactone substrate (the preparation media as same as tributyrin substrate). This test was conducted to reveal of the polyesterase enzyme. This protocol adopted from Jarett et al., [21] and then modified by Molitor [19] with slight additions.

2.5 Molecular identification
Extraction of isolate bacteria was conducted using procedure of Chelex 10%. Amplification based on 16S rRNA procedure with primers 27 F (5'AGAGTTTTGATCCTGGTGAGCGAG-3') [22] and 1492R (5'GGTTACCTGTGTCACCTGTACGACCT-3') [23]. The mixture of PCR reagent using 25 µl PCR cocktail that contained 1 µl of each primer (10 mM), 1.5 µl of DNA template, 9 µl of ddH2O, and 12.5 µl of
EconoTaq Plus Green Master Mix, Lucigen Corp, USA. Thermal cycler condition was set up for initial denaturation 94°C (2 min), followed by 35 cycles of denaturation 94°C (30 sec), annealing at 50.7°C for 30 sec, extension at 72°C for 1 min, and final extension 72°C for 5 min. The last was sent to DNA sequencing facility to be purified and get sequenced analysis using sanger methods. Similarity analysis conducted in-silico using BLAST gene database of National Centre for Biotechnology Information.

3. Results
The sampling location separated into two type of location (figure 1). The consideration of choosing this place refers to the activities in the environment around the coral reefs. The place where the influence of anthropogenic is high and another place was a marine protected area, both might be affecting for corals microbe’s inhabitant. Coral microbial communities are very specific for each host species [24, 25], these specific traits are related to coral health conditions. In addition, the microbial interactions of coral associations are not always the same in one coral colony [24, 26]. They are influenced by the physical environment [25].

Identification of bacterial colony morphology refers to the book Microbiology Laboratory Theory & Application [27]. Total of 18 bacterial isolates that were successfully obtained from massive corals from four different location. All those isolates were purified and preserve at Tropical Marine Biotechnology Laboratory (figure 2). The use of massive corals as research objects for bacterial isolation sources has certain reasons [28], explained that there are indications that massive corals have more estimates of associated bacterial diversity compared to other corals. Even so, each coral has its own preferences in choosing the bacterial associations that inhabit it [29]. At the coral substructural scale, coral tissue has a higher diversity than coral mucus [30]. Therefore, in this study, the coral mucus was cleaned to obtain endolytic coral bacterial associations.

Besides playing a role in the survival of the host, coral association microbes also play a role in ecological functions [31, 32]. Under conditions of stress (organic enrichment) and increasing the number of association bacteria in the water column, corals will increase the number of bacteria and mucus (Transparent Exopolymer) to maintain the abundance of bacterial communities on their surface to be relatively more stable [33]. Mucus has an ecological role as an energy carrier and particle trap,
thereby establishing metabolic communication with other reef organisms, especially microbes, and initiating cycling of biogeochemical elements that contribute to the conservation and rapid recycling of critical and limiting elements in the reef environment [34].

Agar plates is a method that usually used for screening activity lipolytic enzymes [35]. This procedure is almost always used for detection of pollutant-degrading enzymes [36]. Tributyrin is a triglyceride obtained by formal acylation of three hydroxyl groups of glycerol by butyric acid. In general, the application of inexpensive and easily available triglycerides such as tributyrin and coconut oil for screening is very useful for selecting esteroletic organisms prior to specific polyesterase assays.

**Figure 2.** Isolate bacteria of LBB 2

**Figure 3.** Clear zone on polycaprolactone test. (a) and (c) 1st repeats, time observation at 94-hours and 192-hour, respectively, (b) and (d) 2nd repeats, time observation at 94-hours and 192-hour, respectively.
[19]. The clear zone that appears on this result, indicates positive activity on esterase enzymes [35], explained that the clear zone on the tributyrin agar plate-based test was esterase activity. Esterase is an important enzyme for degradation process because of its potential for plastic biodegradation [37]. The screening result was only one isolates from eighteen isolates of coral-associated bacteria that had positive results in metabolizing tributyrin substrate.

After knowing that only one isolates had activity on the esterase enzyme, the next step was to carry out for polyesterase activity. As previously noted, polyesterase is a lipolytic enzyme [19], a lipolytic enzyme that has activity on polyesters will be very important for the future in the treatment of plastic waste and microplastic degradation [38]. Several scientific studies on the production of polyesterase enzymes in bacteria, rely on the first screening process using a simple aliphatic polyester substrate, namely polycaprolactone or other low molecular weight polyester, Impranil DLN [39, 40]. The results of the test showed very clear activity on agar plates (Figure 3), it is indicate that bacteria could hydrolyse polycaprolactone. Therefore, the hydrolytic activity of esterases on polyester substrates (i.e., aliphatic polyesters) makes this enzyme important for further investigation because of its potential for plastic degradation [35].

There was an expansion of the clear zone at the 192-hour observation (figure 3). The expansion was due to the incubated treatment of bacterial isolates after 4 days at room temperature, transferred to incubator at 4°C temperature. the presence of this activity indicates that LBB 2 isolates have adaptations in cold environments. Sekiguchi [41] also found the bacteria that could degraded polycaprolactone from deep sea sediments which this area was cold. Yu [42] added, there is lipase activity produced in psychrophilic organisms by 20-40% and psychrotolerant organisms by 10-30%. The ability to live in cold conditions and secrete enzymes is a unique feature, these things might also provide numerous opportunities for biotechnological exploitation in the future. Based on the molecular identification using 16S rRNA, LBB 2 strain closely related to Bacillus subtilis. The similarity level in the database of National Centre Biotechnology Information was 99.45%.

4. Conclusion
In the present study, there was only one successfully coral-associated bacteria that could degrade aliphatic polyester (i.e., polycaprolactone). This strain also has cold adaptive activity which might be useful for future exploitation of enzyme. The strain closely related to Bacillus subtilis with 99.45 % similarity based on database of National Centre Biotechnology Information. Future aspects of this study, strain might have several abilities to degrade other aliphatic polyester such as polyethylene succinate (PES), poly butylene succinate (PBS) and poly lactic acid (PLA), etc. Interestingly to search the ability of the LBB 2 strain to degrade aliphatic polyester for a wider scope

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Reference
[1] Pawar P R, Shirgaonkar S S, and Patil R B 2016 Plastic marine debris: Sources, distribution and impacts on coastal and ocean biodiversity Publication of Biological Sciences 3 40–54.
[2] Shah A A, Kato S, Shhintani N, Kamini N A, and Nakajima–Kambe T 2014 Microbial degradation of aliphatic and aliphatic-aromatic co-polysters Applied Microbiology and Biotechnology, 98 3437–3447.
[3] Narayan R 2001 Drivers for biodegradable/compostable plastics & role of composting waste management & sustainable agriculture. Proc. ORBIT 2001 Conference. Seville, Spain: Spanish waste club p 158
[4] Okada M 2002 Chemical syntheses of biodegradable polymers Progress in Polymer Science (Oxford) 27 87–133.
[5] Scharathow R 2009 BIoplastics: the framework for market introduction in Europe, Presented at the 2nd International Science and Technology Conference, “The Future of Biodegradable Packaging”, Warsaw, Poland, Sept 29. COBRO, Warsaw, pp 34–52

[6] Sheavly, S.B. 2005 Sixth Meeting of the UN Open-ended Informal Consultative Processes on Oceans & the Law of the Sea. Marine debris – an overview of a critical issue for our oceans. June 6–10.

[7] Environmental Protection Agency (EPA) 2011 Marine Debris in the North Pacific: A Summary of Existing Information and Identification of Data Gaps. EPA-909-R-11-006.

[8] Hall N M, Berry K L E, Rintoul L, and Hoogenboom M O 2015 Microplastic ingestion by scleractinian corals Marine Biology 162 725–732.

[9] Reichert J, Arnold A L, Hoogenboom M O, Schubert P, and Wilke T 2019 Impacts of microplastics on growth and health of hermatypic corals are species-specific Environmental Pollution 254 113074.

[10] World Bank Group 2018 Hotspot Sampah Laut Indonesia Public Disclosure Authorized (April) p 1–49.

[11] Jambeck J R, Geyer R, Wilcox C, Siegler T R, Perryman M, Andrady A, Narayan R, and Law K L 2015 Plastic Waste Inputs from Land into the Ocean Science 347 764–68.

[12] Purba N P, Faizal I, Abimanyu A, Zenyda K S, Jaelani A, Indriawan D, Priadhi M M, and Martasuganda M K 2020 Vulnerability of Java Sea marine protected areas affected by marine debris IOP Conference Series: Earth and Environmental Science 584.

[13] Syakti A D, Jaya J V, Rahman A, Hidayati N V, Raza’I T S, Idris F, and Trenggono M, Doumenq P, and Chou L M 2019 Bleaching and Necrosis of Staghorn Coral (Acropora Formosa) in Laboratory Assays: Immediate Impact of LDPE Microplastics Chemosphere 228 528–35.

[14] Reichert J, Johannes S, Schubert P, and Wilke T 2018 Responses of Reef Building Corals to Microplastic Exposure Environmental Pollution 237 955–60.

[15] Sweet M J, Croquer A and Bythell J C 2011 Development of bacterial biofilms on artificial corals in comparison to surface-associated microbes of hard corals PLoS ONE 6.

[16] Auta H S, Emenike C U, and Fauziah S H 2018 Growth kinetics and biodeterioration of polypropylene microplastics by Bacillus sp. and Rhodococcus sp. isolated from mangrove sediment Marine Pollution Bulletin 127 15–21.

[17] Eich A, Mildenberger T, Laforsch C, and Weber M 2015 Biofilm and diatom succession on polyethylene (PE) and biodegradable plastic bags in two marine habitats: Early signs of degradation in the pelagic and benthic zone? PLoS ONE 10 1–16.

[18] Sekhar V C, Nampoothiri K M, Mohan A J, Nair N R, Bhaskar T, and Pandey A 2016 icrobial degradation of high impact polystyrene (HIPS), an e-plastic with decabromodiphenyl oxide and antimony trioxide Journal of Hazardous Materials 318 347–354.

[19] Molitor R, Bollinger A, Kubicki S, Loeschcke A, Jaeger K E and Thies S 2020 Agar plate-based screening methods for the identification of polyester hydrolysis by Pseudomonas species Microbial Biotechnology 13 274–84.

[20] Michael T M 2015 Brock Biology of Microorganisms Harlow: Pearson Education p 239-247

[21] Jarrett P, Benedict C V, Bell J P, Cameron J A and Huang S J 1984 Mechanism of the Biodegradation of polycaprolactone. In Polymers as Biomaterials. Boston, MA: Springer US, pp. 181–192

[22] Weisburg W G, Barns S M, Pelletier D A and Lane D J, 1991 16S ribosomal DNA amplification for phylogenetic study Journal of Bacteriology 173 697–703.

[23] Reysenbach A L, Giver L J, Wickham G S and Pace N R 1992 Differential amplification of rRNA genes by polymerase chain reaction Applied and Environmental Microbiology 58 3417–3418.

[24] Bourne, David G., Kathleen M. Morrow and Webster N S 2016. Insights into the Coral Microbiome: Underpinning the Health and Resilience of Reef Ecosystems Annual Review of Microbiology 70 317–40.

[25] Littman R A, Bette L W, Christian P and David G 2009 Diversities of coral-associated bacteria differ with location, but not species, for three Acroporid corals on the Great Barrier Reef FEMS
Microbiology Ecology 68 152–63.
[26] Glasl B, Gerhard J H and Pedro R F 2016 The microbiome of coral surface mucus has a key role in mediating holobiont health and survival upon disturbance ISME Journal 10 2280–92.
[27] Leboffe M J and Pierce B E 2010 Microbiology: Laboratory Theory and Application, Third Edition 3rd edition: Loose Leaf
[28] Li J, Chen Q, Zhang S, Huang H, Yang J, Tian X P and Long L J 2013 Highly heterogeneous bacterial communities associated with the South China Sea Reef Corals Porites lutea, Galaxea fascicularis and Acropora millepora PLoS ONE 8.
[29] Liang J, Yu K, Wang Y, Huang X, Huang W, Qin Z, Pan Z, Yao Q, Wang W and Wu Z 2017 Distinct bacterial communities associated with massive and branching scleractinian corals and potential linkages to coral susceptibility to thermal or cold stress Frontiers in Microbiology 8 1–10.
[30] Zhang Y Y, Ling J, Yang Q S, Wang Y S, Sun C C, Sun H Y, Feng J B, Jiang Y F, Zhang Y Z, Wu M L and Dong J D 2015 The diversity of coral associated bacteria and the environmental factors affect their community variation Ecotoxicology 24 1467–77.
[31] Pernice M, Raina J B, Radacker N, Cardenas S, Pogoreutz C and Voolstra C R 2020 Down to the bone: The role of overlooked endolithic microbiomes in reef coral health ISME Journal 14 325–34.
[32] Raina J B, Tapiolas D, Willis B L and Bourne D G 2009 Coral-associated bacteria and their role in the biogeochemical cycling of sulfur Applied and Environmental Microbiology 75 3492–3501.
[33] Garren M and Farooq A 2012 Corals shed bacteria as a potential mechanism of resilience to organic matter enrichment ISME Journal 6 1159–65.
[34] Bythell J C and Wild C 2011 Biology and ecology of coral mucus release Journal of Experimental Marine Biology and Ecology 408 88–93.
[35] Popovic A, Hai T, Tchigvinstev A, Hajigasemi M, Nocek B, Khusnutdinova A N, Brown G, Glinos J, Flick R, Skarina T, Chernikova T N, Yim V, Bruls T, Paslier D L, Yakimov M M, Joachimiak A, Ferrer M, Golyshina O V, Savchenko A, Golyshin P N and Yakunin A F 2017 Activity screening of environmental metagenomic libraries reveals novel carboxylesterase families Scientific Reports 7 1–15.
[36] Ufarté L, Laville E, Duquesne S and Veronese G V 2015 Metagenomics for the discovery of pollutant degrading enzymes Biotechnology Advances 33 1845–54.
[37] Urbanek A K, Rymowicz W and Mirończuk A M 2018 Degradation of plastics and plastic-degrading bacteria in cold marine habitats Applied Microbiology and Biotechnology 102 7669–78
[38] Wei R and Zimmermann W 2017 Microbial enzymes for the recycling of recalcitrant petroleum-based plastics: how far are we? Microbial Biotechnology 10 1308–22.
[39] Wei R, Oeser T, Then J, Kuhn N, Barth M, Schmidt J and Zimmerman W 2014 Functional characterization and structural modeling of synthetic polyester-degrading hydrolases from Thermononsopora curvata AMB Express 4 1–10.
[40] Bollinger A, Thies S, Grunhagen E K, Gertzien C, Kobus S, Hopchner A, Ferrer M, Gohlke H, Smits S H J and Jaeger K E 2020 A novel polyester hydrolase from the marine bacterium Pseudomonas Aestusngiri – structural and functional insights Frontiers in Microbiology 11 1–16
[41] Sekiguchi T, Sato T, Enoki M, Kanchiro H, Uematsu K and Kato C 2011 Isolation and characterization of biodegradable plastic degrading bacteria from deep-sea environments JAMSTEC Report of Research and Development 11 33–41.
[42] Yu Y, li H, Zeng Y and Chen B 2009 Extracellular enzymes of cold-adapted bacteria from arctic sea ice, Canada Basin Polar Biology 32 1539–1547.