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Bioprospecting of polyhydroxyalkanoates-producing bacteria from Indonesian marine environment

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Abstract. *Tan WA, Wijaya I, Purwadaria T. 2019. Bioprospecting of polyhydroxyalkanoates-producing bacteria from Indonesian marine environment. Biodiversitas 20: 1309-1315.* Polyhydroxyalkanoates (PHA) are potential alternates to conventional synthetic plastics. PHA production in bacteria involves PHA synthase gene encoded by *phaC*. In this study, we isolated PHA-producing bacteria from the coastline and 1 mile from the coastline of three beaches in Indonesia. Further *phaC* detection and characterization of PHA production were conducted. The isolates were subjected to phylogenetic analysis based on 16S rDNA. Red Nile staining on minimal agar revealed that twenty-three isolates showed orange fluorescent, which indicated that they accumulated PHA in their cells. PCR detection showed the presence of PHA synthase class I-encoding gene *phaC* in twelve isolates. One representative amplicon was sequenced to verify its identity, in which it shared 86% similarity with the PHA synthase class I-encoding gene from an uncultured bacterium. Interestingly, the production of PHA in isolate ST.PA.75, which was closely related to *Vibrio* sp., was 2.1-fold higher than that in the *Ralstonia eutropha* JMP134 control. Three isolates showed similarity with bacterial genera and/or species for which PHA producing phenotypes had never been described before TP.SWC.20, which was closely related to *Microbacterium arborescens*, as well as TP.SWC.33 and TP.SWC.85, which were similar to *Psychrobacter* spp. Phylogenetic analysis showed that the PHA producing isolates were clustered into three phyla: γ-Proteobacteria, Actinomycetes, and Bacilli. A majority of the isolates (75%) were related to γ-Proteobacteria. In this study, we uncovered diverse novel promising strains for use in the production of PHA as a more environmentally-friendly alternative to hydrocarbon-based plastics.

Keywords*:* Biodegradable plastics, marine bacteria, polyhydroxyalkanoates

INTRODUCTION

Fossil hydrocarbon-based plastics, which constitute a majority of plastics used in the world, are not degradable. For more than 50 years, global production and consumption of plastics have continued to rise (Geyer et al. 2017). Despite recycling efforts, such extensive use lead to plastic accumulation in landfills and natural environment (Al-Salem et al. 2009). Some of the plastic wastes also made their way to the ocean, and it was estimated that the amount will rise from approximately 9 million metric tons in 2015 to 16 million in 2015 (Dauvergne 2018). This poses various ecological impact on organisms living within the marine environment (Browne et al. 2015; Wilcox et al. 2016; Victor 2018). Furthermore, slow deterioration of conventional plastics may also result in microplastics, which have been reported to accumulate in animals of commercial interests that are typically used for human consumption, such as fish, oysters, and clams (Cole et al. 2011; Bessa et al. 2018). Such occurrence raises some concerns on the ingestion of microplastics by human via microplastic-contaminated marine species.

One of the approaches being pursued to overcome the above issues is the search for biodegradable plastics derived from natural ingredients, also known as bioplastics. Polyhydroxyalkanoate (PHA)-based plastics derived from prokaryotic microorganisms is a promising option for this purpose. Some prokaryotes accumulate PHA granules as a response to poor nutrition and environmental stress (Obruca et al. 2018). Similar to synthetic hydrocarbon-based plastics, PHA polymers are resistant to high temperature, with a melting temperature of around 180 ºC (Madison and Huisman 1999). PHAs are generally classified into two groups based on the number of carbon atoms that build up their monomeric unit, in which short-chain-length PHAs consist of 3-5 carbon atoms, while medium-chain-length PHAs consist of 6-14 carbon atoms (Koller et al. 2017). Although its high production cost currently hinders the mass application of PHA, the global PHA market is expected to reach US$93.5 million by 2021, particularly for biomedical, packaging and food applications (Kourmentza et al. 2017; Koller et al. 2018).

Three major pathways for bacterial PHA biosynthesis have been reported to date. Pathway I, which is the most common, was first reported in *Cupriavidus necator (*previously known as *Wauterisia eutropha* or *Ralstonia eutrophus*), in which the condensation of two acetyl-CoA molecules derived for sugar metabolism was used to produce short-chain-length PHA monomers (Reinecke and Steinbuchel 2009). In the second pathway, the fatty acid β-oxidation pathway generated substrates that can be polymerized into medium-chain-length PHA by *Pseudomonas olevorans* from various alkanes, alkenes and alkanoates (Lageveen et al. 1992). Lastly, in Pathway III the PHA monomers are generated from simple sugars such as glucose, sucrose, and fructose via the fatty acid de novo

biosynthesis pathway, such as in *P. putida* (Huijberts et al. 1992). The production of PHA in all three pathways involves the activity of PHA synthase (*phaC*). There are four major groups of this enzyme: class I, III, and IV favors the synthesis of short-chain-length PHA, while class II PhaC favors the synthesis of medium-chain-length PHA (Potter and Steinbüchel 2005; Tsuge et al. 2015; Koller 2018). PHA synthase class I accounts for the majority of PHA producing bacteria isolated to date (Yang et al. 2013).

PHA-producing bacteria have been isolated from wastewater and soil since the early 90s (Shrivastav et al. 2010; Teeka et al. 2010). In recent time, more attention has been directed towards the prospect of halophilic bacteria as low-cost PHA producers (López-Cortés et al. 2008; Quillaguamán et al. 2010; Martínez-Gutiérrez et al. 2018). In this study, we screened for PHA-producing bacteria from the marine environment in Indonesia. The PHA production in each isolate was further quantified and the genetic diversity among all isolates was determined based on 16S rDNA. Present work is of importance in exploring the potential diversity of bacteria that may be used for scaled-up PHA production in the future, in effort to search for a more environmentally-friendly alternative to non-degradable plastics.

MATERIALS AND METHODS

Sample collection

Seawater samples were collected from the coastline and 1 mile from the coastline of three beaches in Indonesia: Ancol Beach, Jakarta; Panjang Beach, Bengkulu; and Serang Beach, Banten. Samples were stored in sterile conical tubes at -20ºC prior to further analysis.

Bacterial Isolation and PHA Screening

Seawater samples were serially diluted (10^{-1}-10^{-4}) using 0.85% sodium chloride and spread on Pseudomonas agar (PA; Difco) and Sea Water Complex agar (SWC; 5 g peptone, 1 g year extract, 3 mL glycerol, 30 g sodium chloride, 20 g bacteriological agar per liter medium), followed by incubation at 30ºC for 24-48 hours. Bacterial colonies with various morphological characteristics were subsequently transferred to PA and SWC several times to obtain pure colonies. The morphology of all isolates was observed under the microscope, followed by Gram staining and spore staining. The presence of PHA granules within bacterial cells were screened using Nile Red staining (Spiekermann et al. 1999). *Ralstonia eutropha* JMP134 (Setiadi et al. 2015) and *Escherichia coli* DH5α were used as positive and negative controls for PHA accumulation, respectively.

Detection of *phaC*

All isolates were grown on Luria Broth medium (5 g yeast extract, 10 g peptone, 5 g sodium chloride per liter medium) overnight and subjected to genomic extraction using the CTAB method (Minas et al. 2011). The presence of class I *phaC* in all isolates was detected through PCR amplification using three specific primers: GD (5'-GTGCCGCC (GC) (CT) (AG) (GC)ATCAACAAGT-3'), G1R (5'-GTTCCAG (AT)ACAG (GC)A (GT) (AG)TCGAA-3'), dan G2R (5'-GTAGTTCCA (GC)A (CT)CAGGTCGTT-3') (Romo et al. 2007). PCR reactions were done using the GoTaq® Green Master Mix (Promega, AS). The PCR cycles were as follow: predenaturation at 94ºC for 10 min; 35 cycles of denaturation at 94ºC for 1 min, annealing at 56ºC for 45 secs, extension at 72ºC for 1 min; and post-extension at 72ºC for 5 mins. The presence of amplified bands was verified on 1% agarose gel with ethidium bromide staining. Thermo Scientific™ GeneRuler 100 bp Plus DNA Ladder has used a marker. One amplified fragment was randomly selected and sent out for sequencing, followed by confirmation by BLASTN (www.ncbi.nlm.nih.gov).

PHA production

PHA production was done using a method described by Katırcıoğlu et al. (2003). All isolates were grown on MS medium containing 10 g l^{-1} fructose at 30°C and 120 rpm for 24 hours and transferred to 50 mL fresh MS medium (10%) containing 40 g l^{-1} fructose, followed by incubation at 30°C and 120 rpm for 60 hours. Cells were harvested by centrifugation at 6000 xg for 45 minutes, suspended in 5 mL sterile distilled water, and lysed through sonication. The cell suspension (2 mL) was mixed to equal parts of 2 N HCl and incubated on boiling water for 2 hours. The suspension was further mixed with 5 mL chloroform, incubated at room temperature with 150 rpm shaking for 2 hours, and centrifuged at 6000 xg for 20 minutes. The lower liquid phase was collected (1 mL) and dried at 40ºC. The resulting pellet was mixed with 5 mL of concentrated H_2SO_4 by vortexing and incubated at 100°C for 20 minutes. The mixture was let cool to room temperature and PHA content was quantified based on its absorbance at 235 nm (Kunioka et al. 1988). *R. eutropha* JMP134 (Setiadi et al. 2015) and *E. coli* DH5α were used as positive and negative controls for PHA production, respectively.

Bacterial identification and diversity analysis

Genomic DNA was extracted using Wizard Genomic DNA Purification Kit (Promega) following the manufacturer's instruction. The identity of each isolate was determined based on 16S rDNA, as amplified using the primer pair 63f (5′-CAG GCC TAA CAC ATG CAA GTC-3′) and 1387r (5′-GGG CGG WGT GTA CAA GGC-3′) (Marchesi et al. 1998). Sequencing was carried out at Macrogen, Korea. The sequences were compared to Genbank database using BLASTN (www.ncbi.nlm.nih.gov/blast), aligned using Clustal W (Thompson et al. 1994) and subjected to phylogenetic analysis using MEGA7 (Kumar et al. 2016) based on a neighbor-joining algorithm (Saitou and Nei 1987).

RESULTS AND DISCUSSION

Bacterial isolation and PHA screening

A total of 95 pure bacterial isolates were obtained from Pseudomonas agar and SWC agar. Upon Nile Red

screening, 23 isolates showed orange fluorescence, in which TP.SWC.2, TP.SWC.33, ST.SWC.66 and ST.SWC.85 showed the brightest fluorescence (Table 1). Twelve of these isolates were obtained from the coastline, while eleven isolates were isolated from the 1 mL from coastline point. Nile Red specifically stains the PHA inclusion bodies in the cytoplasm (Spiekermann et al. 1998), yet it does not attach to other inclusion bodies, such as glycogen and polyphosphate (Anderson dan Dawes 1991). Therefore, the presence of orange fluorescence in the presence of the dye indicates that these 23 isolates may accumulate PHA granules within their cells. As false negative results may be obtained due to the presence of wax esters that also lead to positive fluorescence with Nile Red (Koller and Rodríguez-Contreras 2015), further analyses were conducted to confirm PHA production in these isolates. All isolates were subjected to morphological observation and Gram staining, as presented in Table 1.

Detection of *phaC*

Twelve bacterial isolates showed the presence of amplified bands of 551 bp and 491 bp upon PCR detection with the nested primer pairs GD-G1R and GD-G2R, respectively (Figure 1). This indicated that these isolates carried *phaC* that encoded for PHA synthase class I. Further sequence analysis on one randomly selected PCR product confirmed that the band shared 85% similarity and 76% identity to *phaC* and PHA synthase class I of an uncultured bacterium, respectively. Other isolates that did not show any amplicon might carry other classes of *phaC*, or they might be PHA degraders that accumulated PHA granules as energy storage for further catabolism.

Table 1. Morphology and Nile Red screening of suspected PHA-producing bacterial isolates

Isolate	Sample source	Cell morphology	Gram staining	Orange fluorescence*
PP.SWC.5	Panjang Beach, coastline	Cocci	Negative	+
TT.SWC.9	Panjang Beach, 1 mile from the coastline	Cocci	Negative	+
TP.SWC.20	Panjang Beach, coastline	Cocci	Negative	++
TP.SWC.21	Panjang Beach, coastline	Cocci	Negative	+++
TP.SWC.22	Panjang Beach, coastline	Cocci	Negative	++
TP.SWC.25	Panjang Beach, coastline	Cocci	Negative	++
TP.SWC.31	Panjang Beach, coastline	Rods	Negative	++
TP.SWC.32	Panjang Beach, coastline	Cocci	Positive	++
TP.SWC.33	Panjang Beach, coastline	Rods	Positive	+++
TP.SWC.36	Panjang Beach, coastline	Coccobacilli	Negative	++
TP.SWC.39	Panjang Beach, coastline	Cocci	Positive	++
TP.PA.45	Panjang Beach, coastline	Coccobacilli	Negative	++
ST.SWC.59	Serang Beach, 1 mile from the coastline	Cocci	Positive	+
ST.SWC.63	Serang Beach, 1 mile from the coastline	Cocci	Negative	+
ST.SWC.65	Serang Beach, 1 mile from the coastline	Cocci	Negative	+
ST.SWC.66	Serang Beach, 1 mile from the coastline	Cocci	Negative	+++
ST.SWC.68	Serang Beach, 1 mile from the coastline	Coccobacilli	Negative	++
ST.SWC.69	Serang Beach, 1 mile from the coastline	Coccobacilli	Negative	++
ST.PA.75	Serang Beach, 1 mile from the coastline	Cocci	Negative	++
ST.PA. 77	Serang Beach, 1 mile from the coastline	Cocci	Negative	++
ST.SWC.85	Serang Beach, 1 mile from the coastline	Cocci	Negative	+++
AT.PA.89	Ancol Beach, 1 mile from the coastline	Cocci	Negative	++
AP.PA.92	Ancol Beach, coastline	Cocci	Negative	++

Note: *Orange fluorescence indicated the presence of PHA granules within the cells as qualitatively categorized to brightest (+++), medium (++) and slight (+) fluorescence.

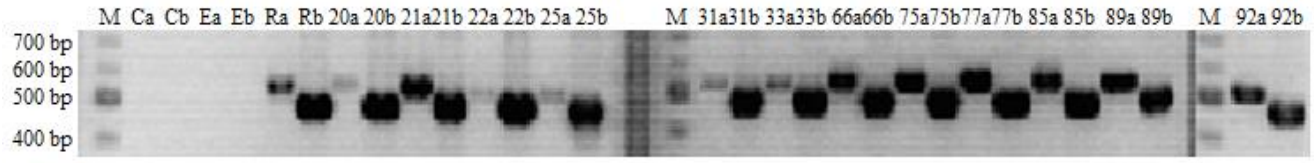

Figure 1. PCR detection of *phaC* using the nested primer pairs GD-G1R (a) and GD-G2R (b). C, PCR reactions in which sterile distilled water was used in place of DNA template served as contamination control; R, *Ralstonia eutropha* JMP134 served as positive control for *phaC*; E, *Escherichia coli* DH5α served as negative control for *phaC.* Thermo Scientific™ GeneRuler 100 bp Plus DNA Ladder (M) was used as a marker.

PHA production

A majority of the isolates produced PHA in a level that is relatively similar to that in the *R. eutropha* JMP134 control (Table 2, Figure 2). An exception was demonstrated by ST.SWC.66 and ST.PA.75, which PHA production was 1.5- and 2.1-fold higher than the control, respectively (Table 2). Fructose was selected as a substrate in this experiment as this carbon source led to maximum PHA production in *R. eutropha* (Khanna dan Srivastava 2005). However, it should be noted that the isolates may have preference for other carbon sources such as glucose and sucrose. For this reason, the brightness of fluorescence following Nile Red staining might not represent PHA production level using solely one type of carbon source.

Several factors should be taken into account during PHA production. Upon the decline of growth substrate, bacteria will start producing depolymerase to hydrolyze PHA and use it as an energy source, typically by 60-62 hours post inoculation and growth on MS medium (Elbanna et al. 2004). Despite that currently known PHA producers are either obligate or facultative aerobic, PHA synthesis is promoted in oxygen- and nitrogen-limiting conditions (García-Torreiro et al. 2016; Blunt et al. 2018). In the presence of low oxygen, the NADH/NAD ratio increases and NADH concentrations inhibit citrate synthase and isocitrate dehydrogenase, leading to TCA cycle blockage. This allows for the accumulation of acetyl-CoA, therefore triggering PHA synthesis, which is then used as an alternative electron acceptor (Anderson and Dawes 1990; García-Torreiro et al. 2016). For that reason, we utilized minimal medium and slow shaking up to 60 hours for all PHA production experiments in this study.

Bacterial identification and diversity analysis

The identity of all PHA-producing isolates was revealed based on their 16S rDNA sequences (Table 2). Most isolates shared 99-100% similarities to various bacterial species recovered from the marine estuaries or other environment containing high salt concentration (Table 2). An exception was shown by isolate TP.SWC.33 and ST.SWC.66, which both showed relatively lower similarities to *Psychrobacter* sp. B-QPyG3 (86%) and bacterium enrichment culture clone NAP-24 (91%), respectively. Interestingly, despite that both ST.PA.75 and ST.PA.77 shared 100% similarity with the same reference sequence (Table 2), both isolates showed different PHA production capability (Figure 2). This indicates that PHA production may vary even up to the strain level.

All isolates were grouped into four major clusters: γ-Proteobacteria (ST.PA.75, ST.PA.77, TP.SWC.25, TP.SWC.21, TP.SWC.22, TP.SWC.33, ST.SWC.85, AP.PA.92 and AT.PA.89), Actinomycetes (TP.SWC.20), and Bacilli (ST.SWC.66 and TP.SWC.31) (Figure 3). A majority of the bacterial isolates (75%) belong to γ-Proteobacteria. Similarly, Martínez-Gutiérrez et al. (2018) reported the abundance of α-Proteobacteria and γ-Proteobacteria carrying PHA synthases class I-encoding genes in two hypersaline microbial mats in Mexico.

Halophiles are attractive candidates for low-cost PHA production. Of which, *Halomonas* spp. have been widely studied. Closely related to isolate TP.SWC.21, *H. hydrothermalis* accumulated 75.8% poly hydroxybutyrate (PHB), a type of PHA during its growth on residual glycerol as a sole carbon source (Shrivastav et al. 2010). *H. campaniensis* LS21 was able to grow in artificial seawater and mixed substrates that simulate kitchen-waste, indicating a great promise to generate PHA from low-cost substrates (Yue et al. 2014). Moreover, hypersaline conditions preferred by halophiles may inhibit the growth of non-halophiles, thus eliminating the need for substrate sterilization and therefore lowering production cost (Quillaguamán et al. 2010).

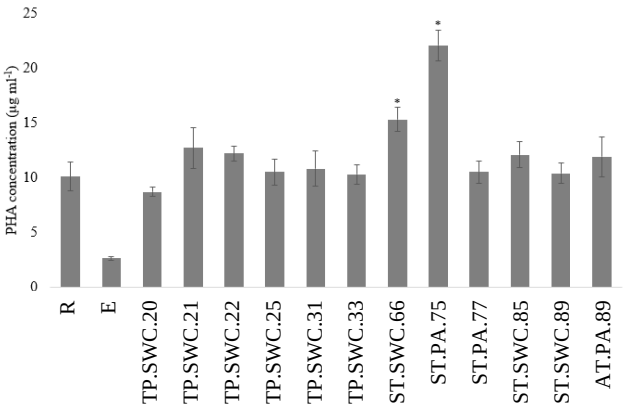

Figure 2. PHA production by marine bacterial isolates. Error bars represent standard deviation based on triplicates. Asterisks indicate significant difference at 95% degree of confidence

Table 2. Identity of PHA-producing isolates based on 16S rDNA analysis

Isolate code	Organism	Identity (%)	Source	Accession number
TP.SWC.20	*Microbacterium arborescens* strain 13635B	100	Marine sediment	EU741114.1
TP.SWC.21	*Halomonas hydrothermalis* strain SMP3M	100	Rocky shores	GU938192.1
TP.SWC.22	*Enterobacter cloacae* strain IBP-V001	99	South Cina Sea	HM021764.1
TP.SWC.25	*Vibrio campbellii* strain H12	100	Marine water	KY790451.1
TP.SWC.31	*Bacillus cereus* strain 1345	100	South Cina Sea	GU726854.1
TP.SWC.33	*Psychrobacter* sp. B-QPyG3	86	Marine environment	EU710703.1
ST.SWC.66	Bacterium enrichment culture clone NAP-24	91	Marine sediment	GU597355.1
ST.PA.75	*Vibrio* sp. strain E466-7	100	Marine sediment	MG563758.1
ST.PA.77	*Vibrio* sp. strain E466-7	100	Marine sediment	MG563758.1
ST.SWC.85	*Psychrobacter* sp. Z19	100	Seawater	HM059659.1
AT.PA.89	*Aeromonas* sp. BCCS 058	99	Salt lake	GQ352449.1

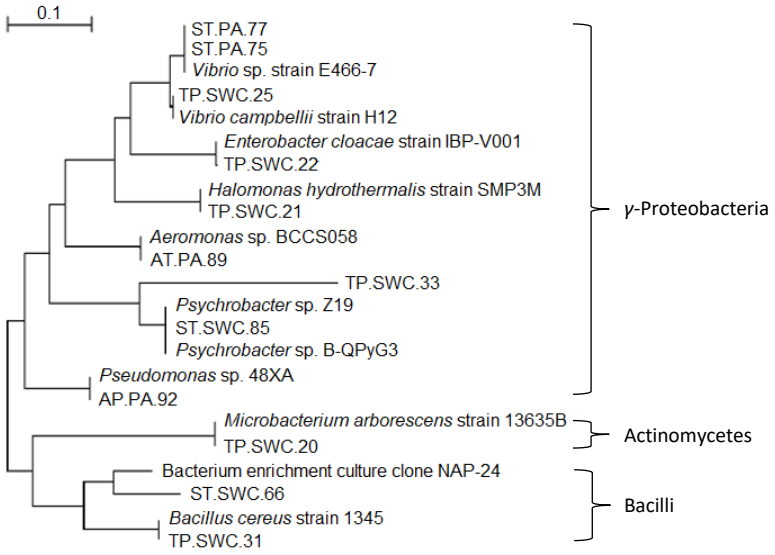

Figure 3. Diversity of PHA-producing bacterial isolates. Horizontal bar indicates genetic distance

Isolate TP.SWC.25, ST.PA.75 and ST.PA.77 were clustered with *Vibrio* sp. strain E466-7 and *Vibrio campbellii* strain H12 (Figure 3). *V. campbellii* is a marine bacterium commonly known as a pathogen in many commercially farmed marine organisms (Pande et al. 2013). As in other bioluminescent vibrios, pathogenesis is regulated by LuxR-mediated quorum sensing, which also plays a role in regulating the synthesis of PHB (Miyamoto et al. 1998; Wang et al. 2013). In a mixed microbial culture experiment, Cui et al. (2016) demonstrated the presence of *Vibrio*, along with *Oceanicella* and *Piscicoccus*, as PHA accumulating bacteria in glucose-enriched medium. Four *Vibrio* spp. strains (M11, M14, M20, and M31) closely related to *V. natriegens* were reported to synthesize PHB upon growth on diverse carbon source, including acetate, glycerol, succinate, glucose and sucrose (Chien et al. 2007).

In the presence of lactose, *E. cloacae* SU-1, which species was similar to TP.SWC.22, was reported to accumulate up to 94% of PHA/dry weight of the organism in lactose medium (Samrot et al. 2011). In contrast, *Enterobacter cloacae* sp. IBP-V001 isolated from the South China Sea was found to degrade PHA instead (Volova et al. 2011). Another species, *Enterobacter aerogenes* 12Bi, was able to produce PHB from domestic wastewater (Ceyhan and Ozdemir 2011).

Aeromonas hydrophila 4AK4, which showed a match with isolate AT.PA.89, is a known PHA producer which whole genomic sequences have been completed (Gao et al. 2011). Such information will be useful for the improvement of PHA production in this strain via metabolic engineering approach. The crystal structure of phasin, a protein that plays a role in determining the amount of PHA accumulation and the size of PHA granules in the cells (Tian et al. 2005; Galán et al. 2011), has also been determined in this strain (Zhao et al. 2016).

Isolate TP.SWC.31 was similar to *B. cereus* 1345 (Table 2), and based on the primers we used for *phaC* detection, carried a gene that encoded for PHA synthase class I (Figure 1). This is a unique finding, as to our knowledge, all PHA-producing *Bacillus* spp. identified to date produce PHA synthase class IV (Tsuge et al. 2015; Kihara et al. 2017). Despite that, both classes of enzymes favor short-chain-length PHA polymerization (Potter and Steinbüchel 2005; Tsuge et al. 2015), PHA synthases belonging to Class I consist of one catalytic PhaC subunit, while those belonging to Class IV are composed of PhaC and PhaR subunits (Tsuge et al. 2015).

Three of our isolates showed similarity with bacterial genera and/or species for which PHA producing phenotypes had never been described before. Isolate TP.SWC.33 and ST.SWC.85 were closely related to *Psychrobacter* spp. (Table 2). Members of this genus are typically psychrophilic or psychrotolerant, halotolerant, and cohabitate in the ocean (Bowman et al. 1997). Although no specific PHA-producing *Psychrobacter* strain has been isolated to date, this genus was detected in abundance in an activated sludge in which PHA was accumulated (Liu et al. 2013). The ability of *Psychrobacter* to thrive in both warm and cold environment is a desirable characteristic for industrial purposes (Rodrigues et al. 2009). In addition, TP.SWC.20 shared 100% similarity with *Microbacterium arborescens.* To date, *Microbacterium barkeri* DSM 20145 is the only *Microbacterium* sp. reported with the ability to accumulate poly (3-hydroxybutyrate) (PHB) or any other types of PHA (Shivakumar 2012). The isolate accumulated 0.6 gL^{-1} PHB under nitrogen-deficient conditions in the presence of glucose and yeast extract (Shivakumar 2012).

López-Cortés et al. (2008) compared the PHA-producing bacterial composition in polluted versus pristine environment using 16S rDNA denaturing gradient gel

electrophoresis (DGGE), and showed that the bacterial community at the polluted site was more complex and diverse than at the pristine site, which might be due to a difference in organic and inorganic nutrient availability. Compared to Panjang Beach and Serang Beach, Ancol Beach is relatively more polluted, as it is exposed to more human activities, including tourism and fisheries. However, only two PHA-producing isolates from Ancol Beach were recovered from the site (Table 1). It is possible that such environment harbors many bacteria that cannot be cultivated artificially. A metagenomic approach based on 16S rDNA, such as DGGE, amplified ribosomal DNA restriction analysis, or high throughput sequencing will provide more thorough information on the bacterial community in marine environment (Tseng and Tang 2014). As the Indonesian marine estuaries harbor a diverse array of life, such information will complement our findings in effort to explore bacterial isolates that are promising for future production of PHA as a more environmentally-friendly substitute to conventional hydrocarbon-based plastics.

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