MICROBIOLOGY INDONESIA
ISSN 1978-3477, eISSN 2087-8587
Vol.9, No.1, March 2015, p 9-16

Available online at
http://jurnal.permi.or.id/index.php/mionline
DOI: 10.5454/mi.9.1.2

Isolation of Oxo-Degradable Polyethylene Degrading-Bacteria of Benowo Landfill Soil Surabaya

MUKAMTO*[1], YUNI SRI RAHAYU[1], LISA LISDIANA[1], AND HARDANING PRANAMUDA[2]

[1]*Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Negeri Surabaya, Jalan Ketintang Baru, Wonokromo, Surabaya 60231, Indonesia;*
[2]*Center for Bioindustrial Technology, Gedung 611, LAPTIAB, PUSPIPTEK-Serpong, Banten, Indonesia*

The widespread consumption of oxo-degradable polyethylene plastics in Indonesia is potentially cause pollution. This problem can be overcome by utilizing plastic degrading-bacteria as degradation agent. The present study aims to isolate and characterize of oxo-degradable polyethylene degrading-bacteria from Benowo landfill soil Surabaya. Isolation and screening was done by the culture technique and clear zone method. Total abundance of bacteria was calculated based on the Total Plate Count method. Bacterial colonies screening was done based on morphological characteristics and the diameter of clear zone. Four isolates with the largest diameter of clear zone were futher characterized based on cell morphology and physiology biochemistry characters. The results showed the average of total abundance of oxo-degradable polyethylene degrading-bacteria of Benowo landfill was 1.28 x 10^4 CFU g^{-1}. The four of twelve isolates with the largest diameter of clear zone showing the highest degradation capability, that were isolates A221 (0.7 cm), A231 (1 cm), A232 (0.6 cm), and C231 (1.3 cm). Colony morphology characteristics of four isolates were generally shape of circular and irregular; margin of entire and lobate; elevation of flat; optics of opaque; and pigmentation of yellow, greenish and cream. These four isolates were Gram negative with the shape bacilli and cell size range of 3-4 µm. Physiological and biochemical characteristics among the four isolates were resistant to acidic conditions; non-motile; produce catalase enzyme except A231; capable to reduce glucose and mannitol; uncapable to reduce lactose; capable to produce acids, except A232; produce of 2.3-butanediol; unable reduce sodium malonate; and capable to reduce nitrate, except A232 and C231; as well as producing hydrogen sulfide except A221 and A232. The four isolates has similar properties with the genus *Mycobacterium*.

Key words: Benowo landfill, characterization, isolation, oxo-degradable polyethylene-degrading bacteria

Penggunaan plastik jenis polietilen *oxo-degradable* di Indonesia cukup tinggi sehingga berpotensi menimbulkan pencemaran. Salah satu metode untuk mengatasi pencemaran yang disebabkan oleh sampah plastik polietilen *oxo-degradable* adalah dengan memanfaatkan isolat bakteri yang mampu mendegradasi plastik tersebut. Tujuan penelitian ini adalah mengisolasi dan mengkarakterisasi bakteri pendegradasi polietilen *oxo-degradable* dari tanah TPA Benowo Surabaya. Isolasi dan skrining bakteri dilakukan menggunakan teknik kultur dan pengamatan zona bening. Kelimpahan total bakteri dihitung berdasarkan metode Angka Lempeng Total. Koloni bakteri diskrining berdasarkan karakteristik morfologi koloni dan zona bening yang terbentuk. Empat isolat bakteri dengan diameter zona bening terbesar dikarakterisasi morfologi sel dan fisiologi biokimia. Hasil rerata kelimpahan total bakteri sebesar 1,28 x 10^4 CFU g^{-1}. Empat dari duabelas isolat memiliki diameter *clear zone* terbesar yang menunjukkan kemampuan degradasi paling tinggi, yaitu isolat A221 (0,7 cm), A231 (1 cm), A232 (0,6 cm), dan C231 (1,3 cm). Karakteristik morfologi koloni keempat isolat tersebut adalah bentuk *circular* dan *irregular*; tepian *entire* dan *lobate;* elevasi *flat*; optik *opaque*; serta pigmentasi kuning, kehijauan dan *cream*. Keempat isolat bakteri merupakan bakteri Gram negatif berbentuk basil dengan ukuran sel berkisar 3-4 µm. Karakteristik fisiologi biokimia keempat isolat diantaranya tahan kondisi asam, non motil, menghasilkan enzim katalase kecuali A231, mampu mereduksi glukosa dan manitol, tidak dapat mereduksi laktosa, mampu memproduksi asam campuran kecuali A232, produksi 2,3-butanidiol, tidak dapat mereduksi sodium malonat, mampu mereduksi nitrat kecuali A232 dan C231, serta memproduksi hidrogen sulfida kecuali A221 dan A232. Keempat isolat bakteri memiliki kemiripan dengan genus *Mycobacterium*.

Kata kunci : bakteri pendegradasi polietilen *oxo-degradable*, isolasi, karakterisasi, TPA Benowo

During the past 25 years, plastic materials have gained widespread use in human life and substituted the role of metal and wood (Zusfahair *et al*. 2007; Raaman *et al*. 2012). Plastics characteristics are strong, lightweight, durable, easy to set up, and anti-rust (Kathiresan 2003; Kavitha *et al*. 2014). However, plastics disadvantageous characteristics are not heat-resistant, easy to damage at low temperatures, and hard to degrade (Kathiresan 2003; Akbar *et al*. 2013).

*Corresponding author; Phone: +62-85648219005, E-mail: mukamtomuhammad@gmail.com

Plastics are composed of petroleum based materials called resins (e.g polythene and polypropylene). This material is resistant to biodegradation, hence potentially cause pollution in the environment (Kumar *et al.* 2007; Kavitha *et al.* 2014).

One kind of the plastic waste that hard to degrade is polyethylene. Polyethylene (PE) is a linear hydrocarbon polymers consisting of long chains of the ethylene monomers (Tokiwa *et al*. 2009; Usha *et al*. 2011). Polyethylene is hard to degrade, which results of highly stable C-C and C-H covalent bonds, the addition of antioxidants and stabilizers, also high molecular weight and density (Arutchelvi *et al*. 2008; Leja and Lewandowicz 2009). Recently, biodegradable polyethylene called the oxo-degradable polyethylene was developed. The oxo-degradable polyethylene is a polyethylene with the addition of additives in the form of transition metals (Mn-stearate, Cu-stearate, Ni-stearate and Fi-stearate) and has exposed to UV irradiation, so it has a lower molecular weight than conventional polyethylene (Europeanbioplastics 2009). Transition metals are usually employed as pro-oxidant additives and they catalyzed the chain scission of PE through a free radical chain reaction (Suresh *et al*. 2011). According to Tokiwa *et al.* (2009), the presence of additives (such as starch) on the polymer PE made it easier to degrade by microorganisms in the environment than conventional polyethylene. Naturally, oxo-biodegradable polyethylene will be degraded in 2 years (Usha *et al*. 2011; Wahono 2010).

Continuous accumulation of plastic waste in environment can cause pollution and threat to humanity and environment (Deepika and Jaya 2015). In general, the plastic waste in the environment is processed by means of burn or bury. Burning or burying of the plastics releases harmful toxic gases, such as CO_2 and CO which is a major pollutant in environment (Zusfahair *et al*. 2007; Kavitha *et al*. 2014). Another method to reduce plastic waste is by landfilling technique, but this technique is less effective because it requires a wide area (Sihaloho 2011). Alternative solutions that can be offered is biodegradation.

Biodegradation is the chemical processes of material degradation caused by the activity of micro-organisms such as bacteria, fungi, algae and others (Leja and Lewandowicz 2009). The microbial species associated with the degrading capability were reported as bacteria *Pseudomonas* sp (Kathiresan 2003; Gupta *et al*. 2010; Usha *et al*. 2011), *Bacillus* sp (Gupta *et al*. 2010; Usha *et al*. 2011), *Staphylococcus* sp (Usha *et al*. 2011), *Streptomyces* sp, *Micrococus* sp, *Moraxella* sp (Kathiresan 2003), *Klebsiella* sp, *Nocardia* sp, *Mycobacterium* sp (Leja and Lewandowicz 2009), *Xanthomonas* sp, *Flavobacterium* sp, *Agrobacterium* sp (Gupta *et al*. 2010).

Polyethylene degrading-bacteria capable to produce extracellular and intracellular depolimerase enzymes to catabolize the plastic polymers into monomers. Hence, the plastics is easier to accumulate in the cells of microorganisms and used as the source of carbon and energy (Prabhat *et al*. 2013). Plastics monomers metabolism by aerobic bacteria produce carbon dioxide and water, while anaerobic metabolism produces methane gas, hydrogen sulfide, carbon dioxide, and water (Usha *et al*. 2011).

This research was conducted to obtain indigenous isolates of oxo-degradable polyethylene degrading-bacteria of Benowo landfill Surabaya soil and characterize the bacterial isolates.

MATERIALS AND METHODS

Material. Oxo-degradable polyethylene plastic powder was obtained from Pusat Pengkajian dan Penerapan Bioteknologi Industri dan Pertanian, Puspiptek, Tangerang.

Sample Collection. Soil sample were collected from three sites (A, B, C) of Benowo landfill Surabaya. These sites were chosen based on the major waste type (which is plastics waste) and the degradation level of the palstics waste on the sites. Soil samples were taken from a depth of 5-10 cm and collected in sterile bottles, then the temperature and pH were measured and recorded.

Enrichment Media. This research use enrichment broth and enrichment agar media. Oxo-degradable polyethylene powder were added in both media as main carbon source for the bacteria. Enrichment broth media contain 0.1% $(NH_4)_2SO_4$, 0.1% $NaNO_3$, 0.1% KCl, 0.02% $MgSO_4$, 0.01% yeast extract, 2 g oxo-degradable PE powder and dissolved in 1 L steril H_2O (Burd 2008). Enrichment agar media contain 0.1% $(NH_4)_2SO_4$, 0.1% $NaNO_3$, 0.1% KCl, 0.02% $MgSO_4$, 0.01% yeast extract, 2 g oxo-degradable PE powder and added 12 g L^{-1} agar and 1 mL L^{-1} Tween 80. Tween 80 were added to optimize the biodegradation process. Both media were adjusted to pH 5 with 5N HCL.The growth media was sterilized at 121 °C for 20 min.

Isolation and Screening of Oxo-Degradable Polyethylene Degrading-Bacteria. Isolation and screening were done by culture technique (Burd 2008)

and clear zone method (Usha *et al*. 2011). 1 g of each soil sample was dissolved in erlenmeyer flasks contain 20 mL enrichment broth, then shaken at 32 °C for 4 weeks in a waterbath shaker (200 rpm). After 4 weeks incubation, each soil suspension was taken as 1 mL in test tubes containing 9 mL of enrichment broth media for the 10^{-1} dilution. Dilution series was done from 10^{-1} to 10^{-4} dilution. 0.1 mL of the each dilution was taken and planted in the enrichment agar media with pour plate method. For each sample, two replicas maintained and kept for incubated at 32 °C for 2-7 d. The isolates capable of forming clear zone were characterized based on colony morphology. Four of bacterial isolates that able to form the largest clear zone were further characterized for their cell morphology and physiology biochemistry characteristics.

Characterization of Oxo-Degradable Polyethylene Degrading-Bacteria. Degrading-bacteria were characterized by observing colony morphology, cell morphology and physiology biochemical properties. The cell morphology was observed based on cell shape, cell arrangement, Gram and size of the cell. Bacterial cell size was measured by using a compound microscope equipped with a camera (Olympus) and analyzed by software ScopeImage 9.0 (HIC). Physiological and biochemical test perform in this study were acid-fast staining, malonate test, and hydrogen sulfide production (Benson 2001); motility test (Pelczar and Chan 1986); catalase test, sugar reduction, nitrate reduction, and acids fermentation test (Hadioetomo 1993).

RESULTS

Total Abundance of Oxo-Degradable Polyethylene Degrading-Bacteria. The total abundance of oxo-degradable polyethylene-degrading bacteria of different sites varied from 1.24 x 10^4 CFU g^{-1} to 1.26 x 10^4 CFU g^{-1}. The average of total abundance of oxo-degradable polyethylene degrading-bacteria from Benowo landfill was 1.28 x 10^4 CFU g^{-1} (Table 1).

Isolation of Oxo-Degradable Polyethylene Degrading-Bacteria. The four isolates with the largest diameter of clear zone indicatedthe highest degradation of oxo-degradable polyethylene capability, they were isolate A221, A231, A232, and C23 (Fig 1). Those four isolates were characterized for colony morphology, cell morphology, cell size,

Table 1 The total abundance of oxo-degradable polyethylene degrading-bacteria from soil sample of Benowo landfill Surabaya

Soil Sample	CFU/g
A	1,24 x 10^4
B	1,32 x 10^4
C	1,26 x 10^4
Average	1,28 x 10^4

A, B, and C were taken from the IC site of Benowo landfill Surabaya

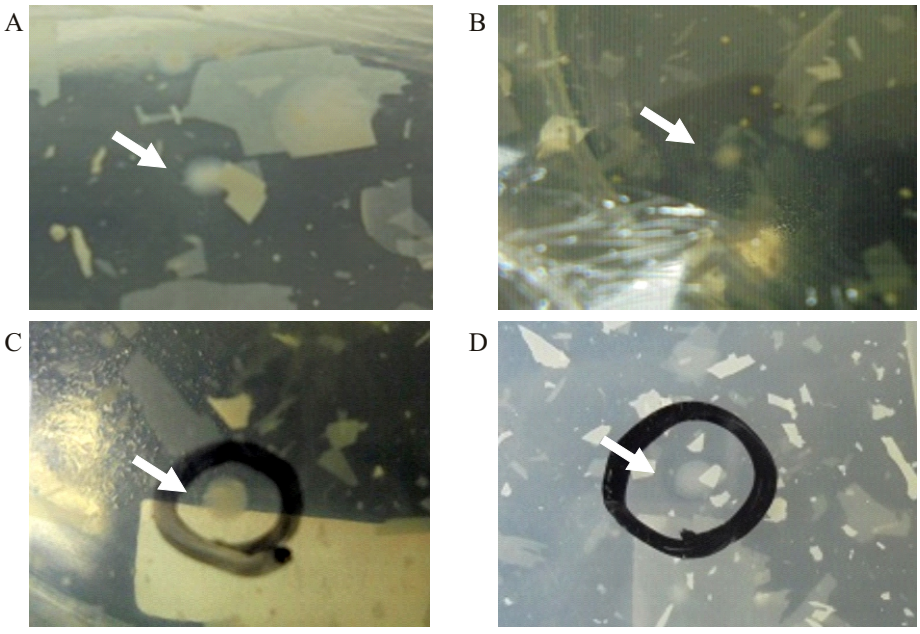

Fig 1 Formation of clear zone on bacterial isolates colony were A221 (A), A231 (B), A232 (C), and C231 (D).

Table 2 Characterization of four oxo-degradable polyethylene degrading-bacteria isolates with largest diameter of clear zone from soil sample of Benowo landfill Surabaya

Characteristics	Bacterial Isolates			
	A221	A231	A232	C231
Colony Morphology				
Shape	Irregular	Circular	Circular	Circular
Margin	Lobate	Entire	Lobate	Threed-like
Elevation	Flat	Flat	Flat	Flat
Optic	Opaque	Opaque	Opaque	Opaque
Pigmentation	Cream	Greenish	Yellowish	Dim yellow
Clear Zone diameter (cm)	0,7	1	0,6	1,3
Cell Morphology				
Shape	Bacilli	Bacilli	Bacilli	Bacilli
Arrangement	Monobacilli	Monobacilli	Diplobacilli	Streptobacilli
Gram	-	-	-	-
Size	3.077	3.094	3.610	4.375
Biochemical Physiology test				
Acids-tolerant	+	+	+	+
Hydrolysis H_2O_2	+	-	+	+
Motility Test	Non motile	Non motile	Non motile	Non motile
Glucose Reduction	+	+	+	+
Lactose Reduction	-	-	-	-
Mannitol Reduction	+	+	+	+
Methyl Red Test	+	+	-	+
Voges Proskauer Test	+	+	+	+
Malonate Utilization	-	-	-	-
Nitrate Reduction	+	+	-	-
H_2S Production	-	-	+	+
Location of bacterial isolates				
Soil Sample	A,B,C	A,B,C	A,B,C	C
Probable genus*	*Mycobacterium*	*Mycobacterium*	*Mycobacterium*	*Mycobacterium*

* = Holt JG, Krieg NR, Sneath PHA, Staley JT, Williams ST. 1994. Bergey's Manual of Determinative Bacteriology. 8th edition.

biochemical and physiological characteristics. The characteristics of four isolates has similar properties with the genus *Mycobacterium* (Table 2).

DISCUSSION

The average of total abundance of oxo-degradable polyethylene degrading-bacteria from Benowo landfill was 1.28 x 10^4 CFU g^{-1} (Table 1). This result was nearly similar to the prior study of Kumar et al. (2007) who reported 1.07 x 10^4 CFU g^{-1} to 1.35 x 10^4 CFU g^{-1} bacteria was found in the mangrove soil sample at Suva, Fiji Islands. However, total abundance of oxo-degradable polyethylene degrading-bacteria from Benowo landfill was lower than those reported by Kathiresan (2003) and Usha *et al*. (2011). Kathiresan (2003) reported that total abundance of polyethylene degrading-bacteria from mangrove *Avicennia* rhizosfer was 24.50 x 10^4 CFU g^{-1} and *Rhizophora* rhizosfer was 41.33 x 10^4 CFU g^{-1}. Usha *et al*. (2011) reported that total abundance of polyethylene degrading-bacteria from garbage soil in India was 62.71 x 10^4 CFU g^{-1}. However, such variation of total abundance of bacteria can occur between different geographical locations owing to differences in the environmental parameters (Kumar *et al*. 2007).

Isolation and screening of oxo-degradable polyethylene degrading-bacteria were found 12 isolates. Those four isolates able to form clear zone, they were isolate A221, A231, A232 and C231 (Fig 1). Colony morphology characteristics of those four isolates were generally shape of circular and irregular; margin entire and lobate; elevation of flat; optics of opaque; and pigmentation of yellow, greenish and cream. Those four isolates had the characteristics of cell were bacilli, with cell arragement monobacill for A221 and A231, diplobacill for A232 and streptobacill for C231; Gram negative; and cell size range of 3-4 µm (Table 2). Thakur (2012) and Kathiresan (2003), reported that polyethylene-degrading bacteria were included as Gram positive and Gram negative bacteria.

Isolation and screening of oxo-degradable

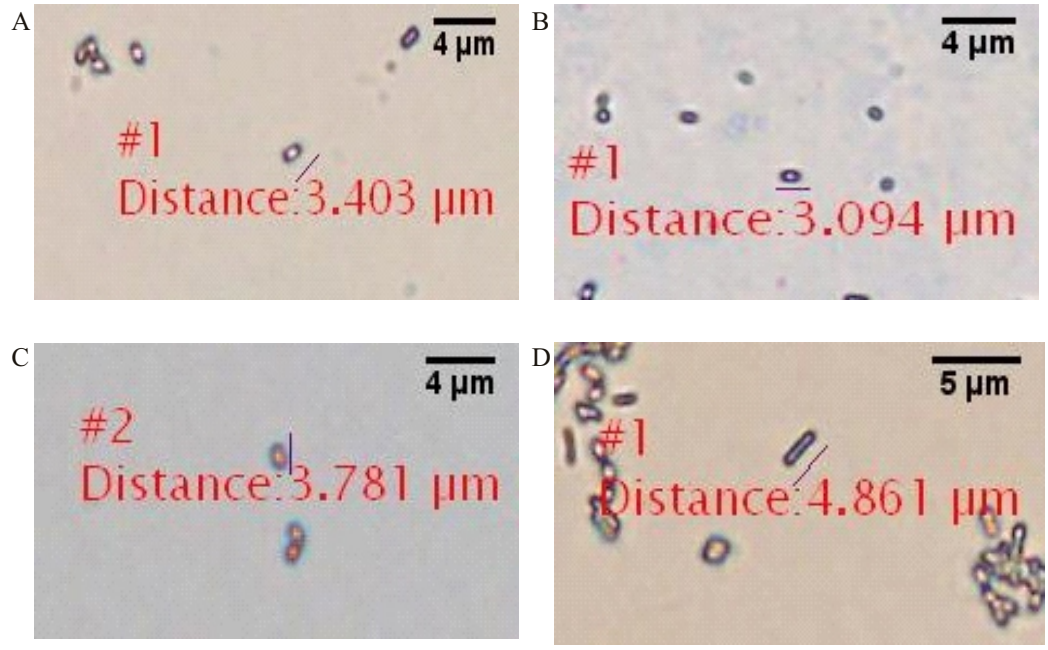

Fig 2 Oxo-degradable polyethylene degrading-bacteria isolates from Benowo landfill soil Surabaya were A221 (A), A231(B), A232 (C), and C231 (D).

polyethylene degrading-bacteria from Benowo landfill Surabaya use two method, they are culture technique (Burd, 2008) and clear zone (Usha *et al*. 2011). Tokiwa *et al*. (2009), reported that clear zone method is a widely and effective to be used for screening polymer degrading-bacteria and assessment of the degradation potency of different microorganisms towards a polymer. The clear zone around the colonies showed polymer degrade as carbon source by bacteria. In the enrichment media was added oxo-degradable polyethylene powder as primary carbon source for bacteria, so bacteria grew in this media was bacteria with oxo-degradable polyethylene degrading capability. In addition, bacteria isolates with low degrade capability was not formed clear zone around colonies. The size of clear zone around the colonies depands on bacteria capability to degrade of oxo-degradable polyethylene, availability of functional groups that increases hydrophilicity, size molecular weight and density of the polymers, structural complexity (linearity or presence of branching) in the polymers, nature and physical form of the polymers (films or powder), and presence of ester or amida bonds (Arutchelvi *et al*. 2008).

The enrichment agar media was added emulsifier of Tween 80. Tween 80 is non-ionic surfactants. The function of Tween 80 is to enhance biodegradation of polyethylene by increasing the adhesion of the bacteria towards the polyethylene surface (Albertsson *et al*. 1993).

Oxo-degradable polyethylene is biodegradable plastics. It is easier to degrade than conventional polyethylene (Wahono 2010). The presence of additives on oxo-degradable polyethylene polymers such as transition metals will result in easier to oxidize process at thermal degradation and photodegradation (Kalus 2007). Jeon and Kim (2014) reported that the transition metals of Fe-stereate additives is more suitable as the photodegradation catalyst of PE than Mn-stereate and Co-stereate. Polymers oxidation will cause lower molecular weight of PE, hence its easier to degrade by microorganisms (Europeanbioplastics 2009; Tokiwa *et al*. 2009).

Luckachan (2006) states that the biodegradation is divided into two stages, they are abiotic degradation and biotic degradation. Abiotic degradation can occur through thermal degradation, photooxidative, hydrolytic, chemical, mechanical-chemical, and addition-induction, whereas biotic degradation is performed by bacterial degradation.

At the abiotic degradation, oxidation of the carbon chains (e.g photodegradation) will cease the change of the backbone structure of oxo-degradable polyethylene polymers (Leja and Lewandowicz 2009). Oxidation of the carbon chains polymers results in the formation of functional groups such as carboxylic acids, aldehydes, ketones, lactones, and low molecular weight hydrocarbons (Chiellini *et al*. 2006). The formation of functional groups in the polymers properties cause the change of hydrocarbon polymers

characteristics from hydrophobic to hydrophilic. The change of the hydocarbon polymers characteristics made it able to absorb water and facilitate to degrade of polymers by microorganisms (Ammala *et al*. 2011).

The next stage is biodegradation by micro-organisms (bacteria). Oxo-degradable polyethylene degrading-bacteria consume polyethylene as the primary carbon source. It able to reduce of partial plastic. Firstly, the bacteria will colonize the oxo-degradable polyethylene surfaces by forming biofilms (Usha *et al*. 2011). The bacteria also produce extracellular enzyme to degrade the polymer backbone into smaller fragments and lower molecular weight (oligomers, dimers, monomers). The polymer backbone are degraded through some steps, involve several extracellular enzymes. The functional groups (carbonyl groups) are converted to alcohol by the monooxygenase enzyme. After that, alcohol is oxidized to aldehyde by the alcohol dehydrogenase enzyme. Next, aldehyde is converted to the fatty acid/carboxylic acid, ketone or ester by dehydrogenase enzyme. The fragments are then transferred into bacteria cells (mineralization) (Premraj and Doble 2004; Ammala *et al*. 2011; Roy *et al*. 2011; Usha *et al*. 2011). Inside the cells, it is metabolized via β-oxidation cycle with the aid of intracellular enzymes (Leja and Lewandowicz 2009). In the β-oxidation cycle, the carbon polymer molecules are broken down to produce 2-carbon acetyl CoA. Then acetyl-CoA are moved to Citric Acid cycle and produce CO_2 and H_2O in aerobic conditions or CO_2, H_2O, Ch_4, and H_2S in anaerobic conditions (Premraj and Doble 2004; Leja and Lewandowicz 2009).

The results of physiological biochemical characterization of four isolates showed isolates were acid-resistant condition, and capable to produce the catalase enzyme except isolate A231. Motility test of bacteria used semisolid medium NA, showed isolates was non-motile. Non-motile of bacteria isolates was characterized by bacteria growth does not spread (Pelczar and Chan 1986). Reduction test of glucose and mannitol showed positive results for 4 isolates, but it can not reduce lactose. This suggests that the bacterial isolates uncapable to produce the lactase enzyme to breakdown lactose and utilize glucose as a carbon source. These results are consistant with prior study that polyethylene degrading-bacteria isolated from soil samples do not produce the lactase enzyme. Marista *et al*. (2013) stated that most of the soil bacteria uncapable to reduce lactose.

MR-VP test is used to determine of acids fermentation presence. The bacteria with acids fermentation ability can produce CO_2 and H_2, because formic hydrogenylase enzyme presence (Hadioetomo 1993; Benson 2001). Three of four isolates showed positive results and one isolate showed a negative result, that is isolate A232. This indicated that the isolate A232 unable to reduce sugar in anaerobic conditions, hence acidic compounds is not form. The fermentation test of 2,3-butanediol in the four isolates showed positive results, showed the presence of acetoin as the precursor for 2,3-butanediol formation (Hadioetomo 1993).

Nitrate reduction test showed two isolates were able to utilize nitrate as an electron acceptor, which are isolates A221 and A231. Two other isolates unable to utilize nitrate, which are isolates A232 and C231. In the malonate utility test, showed all bacteria isolates were not utilize the sodium malonat as carbon source. It is indicated by the absence of color change in the malonate broth media.

Hydrogen sulfide production test done using TSIA media. In sulfite, plastic polymer will be bioassimilitated to produce CO_2, H_2O, H_2S, and biomassa by bacteria (hydrogen sulfide) (Roy *et al.* 2011). Certain bacteria can produce hydrogen sulfide from the amino acid cysteine. The presence of cysteine desuffurase enzyme will transform cysteine into an alfa-amino cacrylic acid, then alfa-amino cacrylic acid is transformed to amino acid. Next, amino acid is formed pyruvaic acid (Benson 2011). The result of hydrogen sulfide production test were two isolates able to produce hydrogen sulfide, they are A232 and C231. This indicated that both isolates capable to degrade the polyethylene polymers. In aerobic condition, degradation process of plastics polymer will generate residues such as, CO_2, H_2O, CH_4 and H_2S (Leja and Lewandowicz 2007).

This present study only performed isolation, characterization, and initial screening of biodegradation ability of the isolates. Thus, further characterization were needed to identify the isolates. Analysis of the degradation potency of the isolates as microbial consortia were also needed.

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