THE POTENCY OF BIOPLASTIC POLYHYDROXYALKANOATE (PHA) PRODUCING BACTERIA ISOLATED FROM PALM OIL MILL WASTE

Nur Haedar[*], Donny Suherman[*], Zasarwati Dwyana[*], Heriadi[*] Mashuri Masri[**]

[*]*Department of Biology, Mathematic and Natural Science Faculty, Hasanuddin University, Makassar, Indonesia, nurhaedar@unhas.ac.id, donny30798@gmail.com, zaraswatidwyana@unhas.ac.id, heriadi033@gmail.com*

[**]*Department of Biology, Science and Technology Faculty, State Islamic University Alauddin, Makassar, Indonesia, mashuri.masri@uin-alauiddin.ac.id*

Email Correspondence : nurhaedar@unhas.ac.id

Received : October 28, 2021 Accepted : April 10, 2022 Published : June 30, 2022

Abstract: Polyhydroxyalkanoate (PHA) is a polymer made up of biodegradable plastics that can be synthesized by microorganisms from various substrates that contain lots of carbon sources including fatty acids and sugars. One of the substrates that contain a lot of carbon sources is waste from palm oil processing plants. PHA-producing bacteria can take advantage of excess carbon sources in palm oil waste in the form of fatty acids to be converted into PHA. This study aims to determine the potential of bacteria isolated from palm oil mill waste to produce polyhydroxyalkanoates (PHA) and to determine the optimum time required for bacteria to produce PHA. Optimization of the fermentation time was carried out at 24, 48, and 72 hours using minimum Ramsay media added with 1% palm oil and 1% glucose. The results of the study obtained 20 isolates of bacteria isolated from palm oil mill waste, and 9 of them were able to produce polyhydroxyalkanoate (PHA) qualitatively. The results of quantitative selection obtained 2 bacterial isolates capable of producing PHA, namely isolates CPS 3 and LPS2 CPS 3, with crotonic acid absorbance values of 41.6 and 5.01, respectively with a fermentation time of 72 hours. Based on the results of genotypic identification using 16S rRNA DNA sequences, isolates of CPS 3, including Bacillus sp. strain CL 33 and isolate LPS 2, belonged to Bacillus flexus strain S5a. This shows that isolates from palm oil mill waste can be used as a source of isolate for PHA production.

Keywords: Palm oil mill waste; Biodegradable plastic; *Polyhydroxyalkanoate* (PHA)

Abstrak: *Polyhydroxyalkanoate* (PHA) merupakan polimer penyusun plastik *biodegredable* yang dapat di sintesis oleh mikroorganisme dari bermacam-macam substrat yang banyak mengandung sumber karbon asam lemak dan gula. Salah satu subtrat yang banyak mengandung sumber karbon adalah limbah yang berasal dari pabrik pengolahan kelapa sawit. Bakteri penghasil PHA dapat memanfaatkan sumber karbon berlebih dalam limbah kelapa sawit berupa asam-asam lemak untuk diubah menjadi PHA. Penelitian ini bertujuan untuk mengetahui potensi bakteri yang diisolasi dari limbah pabrik kelapa sawit dalam menghasilkan *Polyhydroxyalkanoate* (PHA) serta mengetahui waktu optimum yang dibutuhkan oleh bakteri dalam menghasilkan PHA. Optimasi waktu fermentasi dilakukan pada 24, 48 dan 72 jam menggunakan media minimal Ramsay yang ditambahkan minyak sawit 1% dan glukosa 1%. Hasil penelitian memperoleh 20 isolat bakteri yang diisolasi dari limbah pabrik kelapa sawit

dan 9 isolat diantaranya mampu menghasilkan *Polyhydroxyalkanoate* (PHA) secara kualitatif. Hasil seleksi secara kuantitatif diperoleh 2 isolat yang mampu menghasilkan PHA yaitu isolat CPS 3 dengan nilai absorbansi asam krotonoat sebesar 41,6 sedangkan LPS 2 memiliki nilai absorbansi asam krotonoat sebesar 5,01 dengan waktu fermentasi selama 72 jam. Berdasarkan hasil identifikasi secara genotipik dengan menggunakan sekuens DNA 16S rRNA isolat CPS 3 termasuk jenis *Bacillus sp. strain* CL33 dan isolat LPS 2 termasuk jenis *Bacillus flexus* strain S5a. Hal ini menunjukkan bahwa isolat dari limbah pabrik kelapa sawit dapat digunakan sebagai sumber isolat untuk produksi PHA.

Kata kunci: Limbah pabrik kelapa sawit; Plastik biodegradable; Polyhydroxyalkanoate (PHA)

Recommended APA Citation :

Hedar, N., Suherman, D., Dwyana, Z., Heriadi, & Masri, M. (2022). The Potency of Bioplastic Polyhydroxyalkanoate (PHA) Producing Bacteria Isolated From Palm Oil Mill Waste. *Elkawnie*, *8*(1), 93-107. https://doi.org/10.22373/ekw.v8i1.11145

Introduction

In today's modern era, human life cannot be separated from the use of plastic in daily activities. Non-degradable plastics that are widely used by humans can cause problems for the environment. These plastics accumulate a lot in nature and take years to decompose and become the biggest source of pollution in the environment. (Haedar et al., 2014; Możejko-Ciesielska and Kiewisz, 2016; Hopewell et al., 2008; Liu et al., 2019). One of the causes of the difficulty of non-degradable plastics to decomposing in nature is because it consists of polymers with relatively high molecular weights and is produced through a chemical synthesis process (Coppola et al., 2021).

The raw material for making plastic comes from petroleum derivatives and natural gas. Petroleum and natural gas derivatives are non-renewable natural resources, so the process of making non-degradable plastics requires large natural resources (Hopewell et al., 2008; Yustinah et al., 2019). Polyethylene, polypropylene, acrylonitrile butadiene styrene, polycarbonate, polyamides, polystyrene, polytetrafluoroethylene, and acrylic polyurethane (PU, PUR) are compounds that make up non-degradable plastics. These two compounds are derived from the polymerization of ethylene and propane compounds which are derivatives of petrochemically. Polyethylene and polypropylene are long-chain polymers that are difficult to decompose naturally in nature (Coppola et al., 2021). Based on this, the use of non-degradable plastics cannot be maintained so plastic raw materials that can be degraded in nature are needed (Liu et al., 2019; Albuquerque and Malafaia, 2017; Haedar et al., 2014).

Biodegradable plastics continue to be developed to replace the use of non-degradable plastics. Biodegradable plastics are generally made from natural and environmentally friendly materials, for example, sago starch and cassava starch. Biodegradable plastics derived from starch are easier to manufacture and the raw

materials are easier to obtain because of their abundance in nature (Kasmiati et al., 2017). In addition to the use of natural ingredients such as starch and vegetable oils which are non-toxic and easily decomposed with hot water, the manufacture of biodegradable plastics can also be produced biologically specifically by bacteria (Hamzah et al., 2021). One of the bioplastic materials that can be used is polyhydroxyalkanoate (PHA). PHA is a plastic polymer that can be degraded by microorganisms and is environmentally friendly (Kresnawaty et al., 2014; Castilho et al., 2009).

Polyhydroxyalkanoate (PHA) is a polymer made up of biodegradable plastics that can decompose in nature and can be synthesized by microorganisms from various substrates such as fatty acids and sugars (Urtuvia et al., 2014). Polyhydroxyalkanoate (PHA) is a food reserve that is used by bacteria when there is a shortage of carbon sources (Możejko-Ciesielska and Kiewisz, 2016). Based on the results of the study, Tufail et al., (2017) found six types of bacteria, namely *Bacillus cereus*, *Bacillus subtilis*, *Brevibacterium halotolerance*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Stenotrophomonas rhizoposide*, which were isolated from used cooking oil and were able to produce PHA. The ability of these bacteria to produce Polyhydroxyalkanoate (PHA) is caused by the number of excess carbon sources in the form of triacylglycerol fatty acids contained in used cooking oil (Jiang et al., 2016). Tufail et al., (2017) stated that *P. aeruginosa* isolate was able to produce 52.3% PHA and 38.4% *B. subtilis* during its growth on a used cooking oil substrate.

The presence of Polyhydroxyalkanoate (PHA)-producing bacteria in nature is influenced by the substrate in which they live. Substrates containing excess carbon sources mainly contain fatty acids, which are indicators of the presence of these bacteria. Waste from palm oil processing is reported to contain about 0.5-1% emulsified oil (Nursanti, 2013).

Based on the description above, a study was conducted to know the potential of bacteria isolated from palm oil mill waste in producing Polyhydroxyalkanoate (PHA).

Material and methods

Material

The materials used in this study were a liquid waste, solid waste and Crude Palm Oil (CPO) from a palm oil mill in North Morowali Regency, Central Sulawesi Province, Nutrient Agar (Merck) medium, minimum Ramsay ($(NH_4)_2SO_4$, $Na_2HPO_4.7H_2O$, K_2HPO_4, $MgSO_4.7H_2O$, ferrous ammonium citrate, $CaCl_2.2H_2O$, trace element), glucose, palm oil, sodium hypochlorite, acetone (pa), diethyl ether (pa), chloroform (pa), H_2SO_4 (pa), alcohol 96% (pa), sudan black dye, safranin dye, and distilled water.

The equipment used in this study were petri dishes (Pyrex), Erlenmeyer (Pyrex), test tubes (Pyrex), beakers (Pyrex), measuring cups (Pyrex), round loops,

straight loops, and cuvette tubes, laminar airflow (Faster Bio60), incubator (Heraeus), shaker (HEALTH), hot plate (Thermo Scientific CIMARE), autoclave (American), microscope (Nikon), oven (Heraeus), centrifuge (Hettich), analytical balance (OHAUS), spectrophotometer UV-VIS (Thermo Scientific), PCR (Applied Biosystem Verity 96 Well Thermal Cycler), and electrophoresis (Thermos Scientific EC 200 XL)

Isolation of Polyhydroxyalkanoate (PHA) Producing Bacteria

A total of 1 g of the sample was diluted and graded to 10^{-6}. Then the sample was taken as much as 1 mL was grown on nutrient agar medium to which 1% palm oil and 1% glucose were added using the pour method. After that, it was incubated in an incubator at 37^{o}C for 1x24 hours. After that, each colony showing a different morphology was regrown on the same medium to obtain pure isolates.

Selection of Polyhydroxyalkanoate (PHA) Producing Bacteria

The selection stage was carried out to determine the best bacterial isolates capable of producing polyhydroxyalkanoates (PHA) characterized by purplish-blue colonies and dark granules. This selection stage included staining of the colony and cell morphology using sudan black dye.

Sudan black staining on bacterial colonies

Bacterial isolates were grown on a Nutrient Agar medium enriched with 1% palm oil and 1% glucose and incubated for 3x24 hours. On the surface of the growing bacterial colonies, excess Sudan black dye was added and allowed to stand for 30 minutes. The dye was removed and then rinsed with 96% ethanol. Colonies that show a purplish-blue color was observed (Haedar et al., 2014).

Polyhydroxyalkanoate (PHA) granule staining on bacterial cells

Bacterial isolates were grown on NA media enriched with 1% palm oil and 1% glucose and incubated for 3x24 hours. A smear of bacteria was made on a glass slide, then it was fixed over a spirit fire for a few seconds to attach the bacteria to the slide. This staining uses 2 types of reagents, such as sudan black and safranin reagents. After that, Sudan black dye was dripped on top of the preparation, left for 5-10 minutes then decolorized using 96% ethanol, and then dried. After that, it was dripped with safranin and left for 10 seconds then washed with running distilled water. Then observed under a light microscope (Nikon) with 1000 times magnification using oil immersion. PHA-producing bacteria can be seen by marked red bacterial cells while PHA granules are dark in color (Jinda and Paniticharoenwong., 2016).

Ability Test of Polyhydroxyalkanoate (PHA) Producing Bacteria

A total of 5 mL of isolate inoculum which had equalized OD value (25% T) was grown in 100 mL of minimal medium Ramsay with 1% (w/v) carbon sources in the form of palm oil and 1% glucose (w/v). Incubated in an incubator shaker (HEALTH) at a speed of 150 rpm with variations in incubation time of 24 hours,

48 hours, and 72 hours at room temperature. Bacterial cultures that have been incubated with incubation time intervals of 24, 48, and 72 hours, were centrifuged at 4000 rpm for 15 minutes. The separated cell mass was then washed with distilled water which was then centrifuged again for 15 minutes. Then, the separated and washed cell mass was suspended with 5 mL of distilled water. Then 1 mL of cell suspension was taken for analysis of Polyhydroxyalkanoate (PHA) levels with a UV-Vis spectrophotometer and 1 mL of cell suspension was taken to measure the dry weight of the cell mass (Haedar et al., 2014).

Measurement of dry weight cell mass

A total of 1 mL of cell suspension was put into a container whose dry weight was known. The cell suspension was dried using an oven (Heraeus Instruments) at 70 ^{o}C and weighed until a constant weight was obtained (Haedar et al., 2014).

Polyhydroxyalkanoate (PHA) analysis using UV-Vis spectrophotometer

A total of 1 mL of cell suspension, 3 mL of phosphate buffer pH 7.0 and 1 mL of sodium hypochlorite or 5% NaOCl were added. Then incubated at room temperature at 180 rpm for 24 hours. The remaining pellets were then collected by centrifugation at 4000 rpm for 15 min. The supernatant was discarded and the cell pellet was added with 5 mL of distilled water, then centrifuged at 4000 rpm for 15 minutes. The supernatant was discarded and the cell pellet was added with 3 mL of acetone, then centrifuged at 4000 rpm for 15 minutes. The supernatant was discarded and the pellet was slowly washed with 3 mL of diethyl ether, allowed to stand for 5 minutes, then the ether was discarded. After the dry pellets were added 3 mL of concentrated H_2SO_4, then heated in a Water Bath at 100 ^{o}C for 10 minutes. The value of crotonic acid formed was measured using a UV-Vis spectrophotometer (Genesys 10S, Thermo Scientific) at a wavelength of 235 nm and H_2SO_4 as a blank (Haedar et al., 2014).

Molecular Identification of Selected Bacterial

Identification of the selected bacteria was carried out using the 16S rRNA gene method using universal primers, namely Forward Primer 63F (5'-CAGGCCTAACACATGCAAGTC-3') and reverse primer 1387R (5'-GGGCGGTGTGTACAAGGC-3') (Marchesi et al., 1998). PCR (Applied Biosystem Verity 96 Well Thermal Cycler) products from samples that showed electrophoresis (Thermos Scientific EC 200 XL) results that matched the primary target band of the 16S rRNA gene were sent to PT. Indonesian genetics for DNA sequencing.

Data Analysis

The data from the isolation and selection of Polyhydroxyalkanoate (PHA) producing bacteria are presented in the form of tables and figures. Data from dry weight measurement and analysis of Polyhydroxyalkanoate (PHA) are presented

in the form of a histogram, and data from sequencing results were BLAST for nucleotide sequences using the database available on the website www.ncbi.nlm.nih.gov.

Results and Discussion

Isolation of Polyhydroxyalkanoate (PHA) Producing Bacteria

The results of bacterial isolation from several types of palm oil mill waste in the North Morowali district obtained 20 bacterial isolates, namely 8 isolates from liquid waste, 7 isolates from Crude Palm Oil (CPO), and 5 isolates from solid waste. The determination of the number of isolates was based on differences in the morphological characteristics of the colonies which included the shape, color, elevation and margins of each of these bacterial isolates. The results of observations of bacterial colony morphology can be seen in Table 1.

Table1. Morphological characteristics of bacterial colonies isolated from palm oil mill waste

Isolate Source	Isolate Code	Bacterial colony morphological characteristics			
		Shape	**Color**	**Elevation**	**Margin**
Liquid waste	LCS 1	*Circular*	Milky White	*Flat*	*Rhizoid*
	LCS 2	*Circular*	Yellowish White	*Convex*	*Entire*
	LCS 3	*Circular*	Milky White	*Flat*	*Filamentous*
	LCS 4	*Circular*	Beige	*Convex*	*Entire*
	LCS 5	*Circular*	Milky White	*Convex*	*Entire*
	LCS 6	*Circular*	Milky White	*Convex*	*Entire*
	LCS 7	*Circular*	Milky White	*Convex*	*Entire*
	LCS 8	*Circular*	Milky White	*Convex*	*Entire*
CPO	CPS 1	*Circular*	Yellowish White	*Raised*	*Lobate*
	CPS 2	*Circular*	Milky White	*Raised*	*Lobate*
	CPS 3	*Circular*	Milky White	*Raised*	*Lobate*
	CPS 4	*Circular*	Milky White	*Raised*	*Rhizoid*
	CPS 5	*Circular*	Yellowish White	*Raised*	*Rhizoid*
	CPS 6	*Circular*	Milky White	*Raised*	*Rhizoid*
	CPS 7	*Circular*	Milky White	*Flat*	*Rhizoid*
Solid waste	LPS 1	*Circular*	Yellowish White	*Raised*	*Entire*
	LPS 2	*Circular*	Milky White	*Raised*	*Entire*
	LPS 3	*Circular*	Milky White	*Convex*	*Entire*
	LPS 4	*Circular*	Milky White	*Raised*	*Entire*
	LPS 5	*Circular*	Milky White	*Convex*	*Entire*

Selection of Polyhydroxyalkanoate (PHA) Producing Bacteria

The qualitative selection stage was carried out to determine the bacterial isolates that are capable of producing Polyhydroxyalkanoate (PHA). This stage

includes colony morphology staining and cell morphology staining using Sudan black dye.

Table 2. The results of the selection of polyhydroxyalkanoate (PHA) producing bacterial isolates with Sudan Black dye

Isolate Source	Isolate Code	Absorbing Ability Sudan Black	
		Colony	Cell
Liquid waste	LCS 1	-	-
	LCS 2	-	-
	LCS 3	-	-
	LCS 4	-	-
	LCS 5	-	-
	LCS 6	-	-
	LCS 7	-	-
	LCS 8	-	-
CPO	CPS 1	+	+
	CPS 2	+	+
	CPS 3	+	+
	CPS 4	+	+
	CPS 5	+	+
	CPS 6	+	+
	CPS 7	+	+
Solid waste	LPS 1	+	+
	LPS 2	+	+
	LPS 3	-	-
	LPS 4	-	-
	LPS 5	-	-

Description :
(+) Able to absorb Sudan black dye
(-) Unable to absorb Sudan black dye

Based on the results of the qualitative selection presented in Table 2, 9 isolates were able to absorb the Sudan black dye which indicated that the isolates were able to accumulate PHA in their cells after being grown on Nutrient Agar medium enriched with 1% palm oil and 1% glucose. The addition of 1% palm oil and 1% glucose was intended to provide an excess of carbon sources in the growth medium. According to Urtuvia et al., (2014) stated that the substrates in the form of fatty acids and excess sugar are used by bacteria to synthesize Polyhydroxyalkanoate (PHA) in cells.

Macroscopic observations on the staining of bacterial colonies using 0.5% sudan black dye can be seen in Figure 1. The dark blue color of the bacterial colonies is due to the presence of PHA granules formed in the bacterial cells

which can absorb the sudan black dye after washing using 96% ethanol in excess. According to Haedar et al., (2014) Polyhydroxyalkanoate (PHA) granules are known to be sudanophillic or can be stained by lipid staining, namely sudan black.

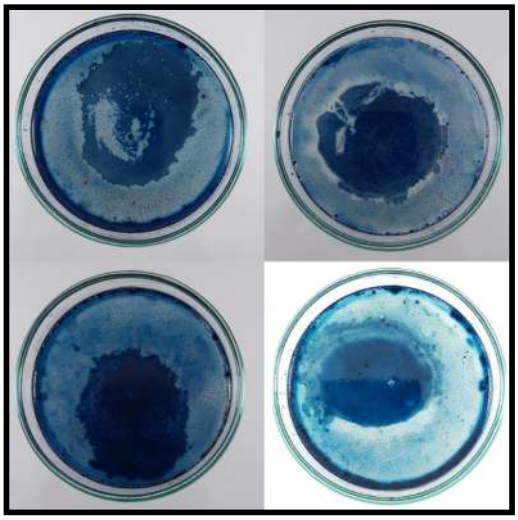

Figure 1. Staining bacterial colonies using Sudan black dye

Microscopic observation of the staining of polyhydroxyalkanoate (PHA)-producing bacterial cells is shown in Figure 2. Bacterial isolates were declared positive for being able to absorb Sudan black dye which was indicated by the presence of PHA granules in the bacterial cells which were dark blue or dark purple.

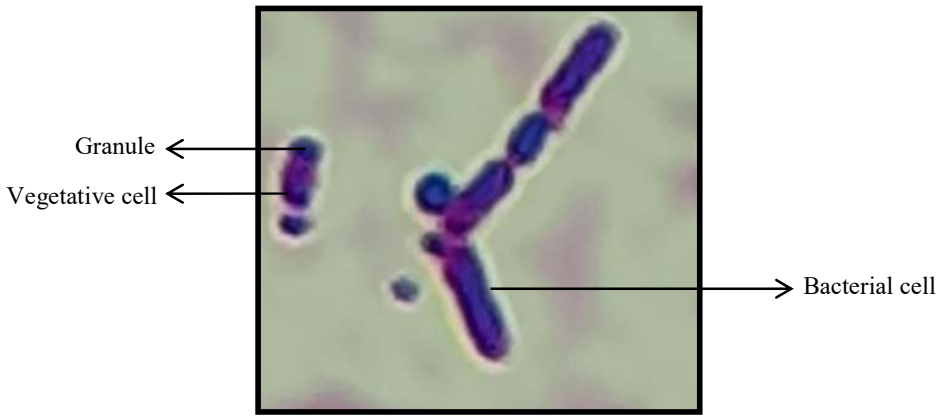

Figure 2. Staining results of bacterial cells using a light microscope (1000 times magnification)

The dark blue color of the granules was maintained after washing with 96% ethanol because the granules in bacterial cells producing Polyhydroxyalkanoate (PHA) could bind strongly to the Sudan black dye. While the bacterial cells

without granules during washing using 96% ethanol, the Sudan black dye will fade or decolorize and the bacterial cells will be stained with safranin so that they show a red color in vegetative cells when observed using a light microscope (Haedar et al., 2014; Sadasivam et al., 2018).

The Ability of PHA Producing Bacteria

The bacterial isolates that had been selected qualitatively were then grown on minimal Ramsay media which was added to an excess of carbon sources in the form of 1% palm oil and 1% glucose. The minimum Ramsay medium was enriched with excess carbon sources in the form of palm oil and glucose so that the nine isolates were able to form carbon stocks in the form of PHA granules stored in their cells. The growth of PHA-producing bacteria will be optimum if the nitrogen and phosphate content is limited. But the carbon sources are present in the overgrowth media (Jiang et al., 2016).

Variations in incubation time of 24 hours, 48 hours and 72 hours were used to obtain the optimum incubation time required for each bacterial isolate to produce Polyhydroxyalkanoate (PHA). Analysis of the ability of the nine bacterial isolates to produce PHA was carried out quantitatively by calculating the dry weight of cells and analyzing the crotonoic acid formed at each observation time interval.

Cell Dry Weight

The results of the dry weight calculation of bacterial cells can be seen in Figure 3 which shows at 24-hour intervals, CPS 4 isolates had the highest cell dry weight of 0.004 g/mL. The lowest cell dry weight was obtained by CPS 1 and LPS 1 isolates, which were 0.001 g/mL. At the time interval of 48 hours, the highest cell dry weight value was obtained by isolates of CPS 5, which was 0.024 g/mL, while the lowest cell dry weight was obtained by CPS 6 and LPS 1 isolate were about 0.004 g/mL. At intervals of 72 hours, the highest cell dry weight value was obtained by an isolate of CPS 3, which was 0.045 g/mL. while the lowest cell dry weight was obtained by CPS 4, CPS 5, CPS 6, and LPS 1 isolates of 0.009 g/mL.

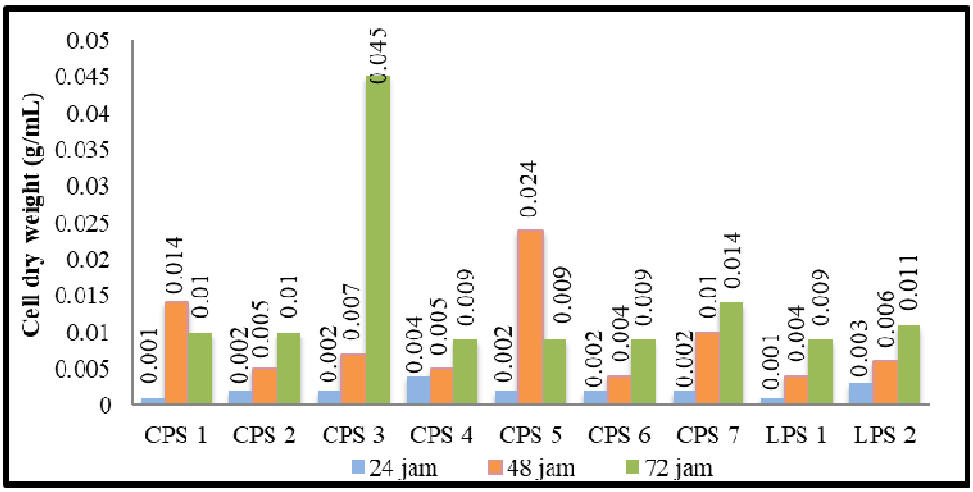

Figure 3. Histogram of dry weight comparison of cells (g/mL) at each incubation time interval (24, 48, and 72 hours)

Based on the data results of cell dry weight measurements, it can be seen that the highest cell dry weight values were obtained at intervals of 72 hours for isolates of CPS 2, CPS 3, CPS 4, CPS 6, CPS 7, LPS 1 and LPS 2. As for isolates CPS 1 and CPS 5, the highest cell dry weight value was obtained at 48-hour intervals. The same thing was stated by Haedar et al., (2014) who stated that the highest dry weight value of bacterial cells was obtained at 72 hours of incubation. Chee et al., (2010) state that the concentration of vegetable oil in growth media in the form of lauric acid and myristic acid used by bacteria was able to increase cell biomass. This is also supported by the research of Pena et al., (2014) that the carbon source in the form of palm oil can produce relatively high cell biomass at a concentration of 0.5% (w/v).

Polyhydroxyalkanoate (PHA) Analysis Using UV-Vis Spectrophotometer

Polyhydroxyalkanoate (PHA) analysis was based on the formation of crotonoic acid with the addition of H_2SO_4 whose value was measured using a UV-Vis spectrophotometer at a wavelength of 235 nm. The result of bacterial cell extraction in the form of PHA will be dehydrated after the addition of sulfuric acid and form crotonoic acid which can absorb ultraviolet light with a wavelength of 235 nm (Hanson and Philips., 1981).

The results of the measurement of the absorbance value of crotonoic acid can be seen in Figure 4 which shows that the highest absorbance value at 24-hour intervals was found in LPS 2 isolate with an absorbance value of 0.423. The lowest absorbance value obtained by CPS 5 isolate was 0.021. At a time interval of 48 hours, the highest absorbance value of crotonoic acid was obtained by CPS 5 isolate, which was 1,520, while the lowest absorbance value was found in LPS 1 isolate, which was 0.129. At the interval of 72 hours, the highest absorbance value

of crotonoic acid was found in CPS 3 isolate, which was 41.6, while the lowest absorbance value was found in LPS 1 isolate, which was 0.416.

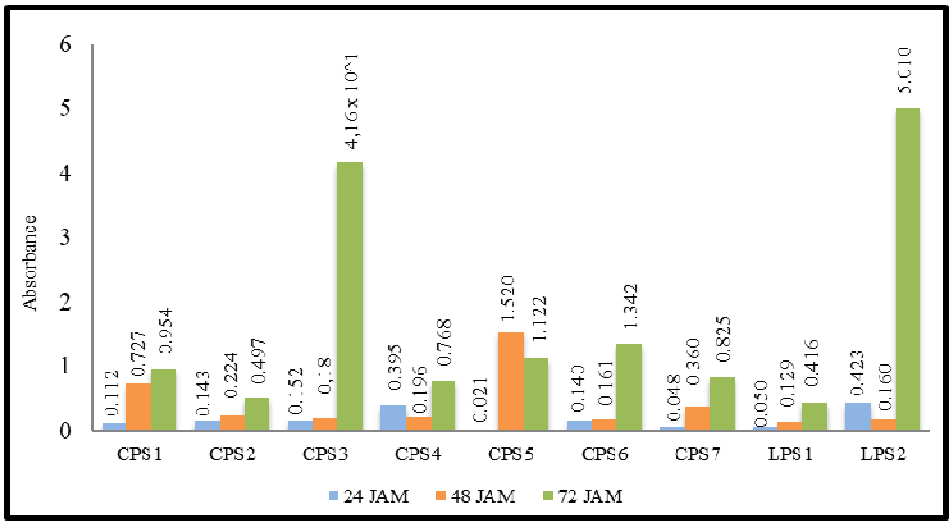

Figure 4. Comparative histogram of crotonoic acid absorbance values formed at each incubation time interval (24, 48, and 72 hours)

Based on the histogram presented, it is known that the highest absorbance value of crotonoic acid was obtained at 72 hours of incubation. CPS 3 and LPS 2 isolates had the highest absorbance value about 41.6 and 5.01 respectively at intervals of 72 hours. This is supported by the research of Kresnawaty et al., (2014), that the polyhydroxyalkanoate (PHA) producing bacteria isolated from the soil of landfills and palm oil mill effluent showed PHA accumulation values of 6.67% and 9.44% after 72 hours incubation, respectively. In previous research conducted by Ratnaningrum et al., (2019), bacteria of the type Bacillus sp B58 produced a PHA concentration of 35.6% isolated from soil in the Mekongga Forest Area. Bacillus flexus strain S5a bacteria isolated from palm oil waste produced a PHA concentration value of 2.61%.

The incubation time required to obtain the amount of Polyhydroxyalkanoate (PHA) can be influenced by several factors, such as the carbon content in the form of fatty acids in palm oil which takes a long time to be used by bacteria directly and produces PHA. Therefore, anaerobic treatment is needed so that hydrolysis and acidogenesis processes will occur to produce volatile short-chain fatty acids (volatile fatty acids) such as acetic acid, butyric acid, valeric acid and caproic acid which are more easily converted into PHA by PHA producing bacteria (Kresnawaty et al., 2014).

Identification of Bacterial Types Using Molecular Markers

The results of sequencing using the BLAST program with a database of 16S ribosomal RNA sequences on isolates of bacteria CPS 3 had the highest similarity with the type of bacteria *Bacillus sp*. strain CL33 with a DNA sequence similarity value in the Gen Data Bank of 97.86%. While the LPS 2 bacterial isolates had the highest similarity with the type of bacteria *Bacillus flexus* strain S5a with a DNA sequence similarity value in the Gen Data Bank of 99.06%.

Table 3. Results of BLAST Bacterial Isolates of CPS 3 and LPS 2

Isolat	Bacterial Species	E Value	Identities	Accession
CPS 3	*Bacillus sp.* (in: Bacteria) strain CL33	00.00	97,86%	MH605368.1
LPS 2	*Bacillus flexus strain* S5a	00.00	99,06%	MT585375.1

The results of the sequencing of CPS 3 and LPS 2 bacterial isolates using the NCBI BLAST program, then a phylogeny construction was made using the UPGMA using the MEGA X program can be seen in Figure 5.

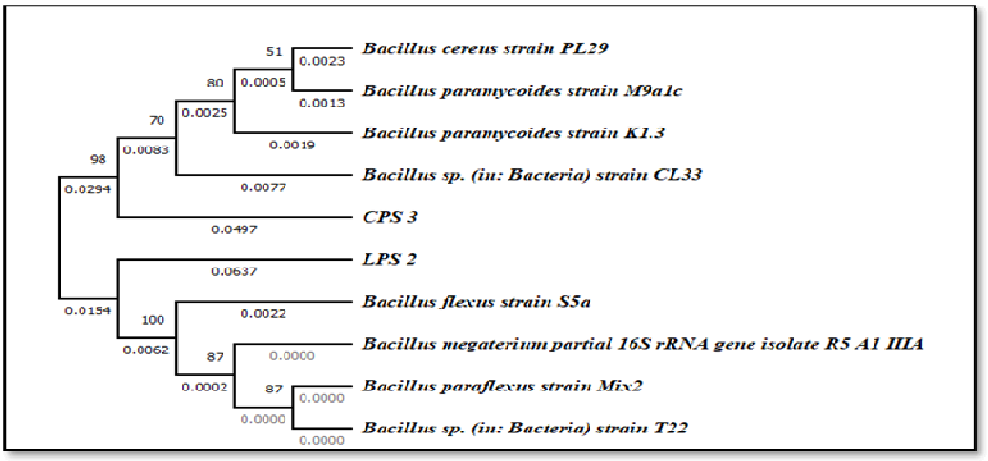

Figure 5. Phylogeny with UPGMA Method on CPS 3 and LPS 2 isolates

The results of the phylogeny analysis using the UPGMA method on isolates of bacteria CPS 3 and LPS 2 can be divided into 2 clades. The first and second clades both show a group of bacteria from the genus Bacillus. The bacterial isolate CPS 3 had the closest similarity to Bacillus sp. strain CL33 with a genetic distance of 0.0497 and a bootstrap value of 98. While the LPS 2 isolate had the closest similarity to Bacillus flexus strain S5a with a genetic distance of 0.0637 and a bootstrap value of 100.

According to Ohimain et al., (2012) stated that bacteria of the genus Bacillus are bacteria that can be found in palm oil mill waste. Bacillus bacteria can adapt to extreme environmental conditions by forming stress-resistant endospores obtained by synthesizing Polyhydroxyalkanoate (PHA).

Conclusions

A total of 20 isolates were successfully obtained from palm oil mill waste and 9 of them were able to produce polyhydroxyalkanoate (PHA) qualitatively. Quantitative selection results obtained 2 isolates capable of producing PHA, namely isolates CPS 3 with a crotonoic acid absorbance value of 41.6 while LPS 2 had a crotonoic acid absorbance value of 5.01 at 72 hours intervals. Results Based on genotypes using 16S rRNA DNA sequences of isolates of CPS 3 including Bacillus sp. strain CL33 and isolate LPS 2 belongs to Bacillus flexus strain S5a.

Acknowledgement

The authors would like to thank Hasanuddin University for facilitating this research and for its financial support for the 2021 academic advisory research grant, No: 915/UN4.22/PT.01.03/2021.

References

Albuquerque, P. B., & Malafaia, C. B. (2018). Perspectives on the production, structural characteristics and potential applications of bioplastics derived from polyhydroxyalkanoates. *International journal of biological macromolecules*. *107*: 615-625. http://dx.doi.org/10.1016/j.ijbiomac.2017.09.026

Castilho, L. R., Mitchell, D. A., & Freire, D. M. (2009). Production of polyhydroxyalkanoates (PHAs) from waste materials and by-products by submerged and solid-state fermentation. *Bioresource Technology*, *100*(**23**): 5996-6009. https://doi: 10.1016/j.biortech.2009.03.088.

Chee, J. Y., Tan, Y., Samian, M. R., & Sudesh, K. (2010). Isolation and characterization of a Burkholderia sp. USM (JCM15050) capable of producing polyhydroxyalkanoate (PHA) from triglycerides, fatty acids and glycerols. *Journal of Polymers and the Environment*, *18* (4), 584-592. https://doi: 10.1007/s10924-010-0204-1

Coppola, G., Gaudio, M. T., Lopresto, C. G., Calabro, V., Curcio, S., & Chakraborty, S. (2021). Bioplastic from renewable biomass: a facile solution for a greener environment. *Earth Systems and Environment, 5*(2), 231-251. https://doi.org/10.1007/s41748-021-00208-7

Djamaan, A., Fitra, F., Pusmegdewi., Dillasamola, D., Asiska, P.D., &Anthoni, A. (2016). Isolation and Identification of Polyhydroxyalkanoates Producing Bacteria from Soil Sample in Tropical Forest of Anai Valley, West

Sumatra, Indonesia. *Der Pharmacia Lettre*. *8* (**7**) : 169-174. http://scholarsresearchlibrary.com/archive.html

Haedar, N., Fahruddin, Firdaus, Z., & Nurlaela, N. (2014). ProduksiPoli-B-HidroksiButirat (PHB) Pada Isolat Bakteri Dari Molasses Dan Tanah Pabrik Gula. Publikasi Jurusan Biologi dan Kimia. *Fakultas Matematika dan Ilmu Pengetahuan Alam. Universitas Hasanuddin*.

Hamzah, F. H., Sitompul, F. F., Ayu, D. F., & Pramana, A. (2021). Effect of the Glycerol Addition on the Physical Characteristics of Biodegradable Plastic Made from Oil Palm Empty Fruit Bunch. Industria: *Jurnal Teknologi dan Manajemen Agroindustri, 10*(3). https://doi.org/10.21776/ub.industria.2021.010.03.5

Hanson, R. S., & Philips J. A. (1981). Chemical Composition In Manual of Method or general Bacteriology. *American Society for Microbiology*. USA. pp, 343-344.

Hopewell, J., Dvorak, R., & Kosior, E. (2009). Plastics recycling: challenges and opportunities. *Philosophical Transactions of the Royal Society B: Biological Sciences*. *364* (**1526**), 2115-2126. https://doi:10.1098/rstb.2008.0311

Jiang, G., Hill, D. J., Kowalczuk, M., Johnston, B., Adamus, G., Irorere, V., & Radecka, I. (2016).Carbon sources for polyhydroxyalkanoates and an integrated biorefinery. *International journal of molecular sciences*. *17* (**7**): 1157. DOI: 10.3390/ijms17071157

Jinda, N., & Paniticharoenwong, T. (2016). The isolation and screening of polyhydroxyalkanoates producing bacteria from wastewater and soil from palm oil manufacturing plant. *J. Biotechnol, 14*, 547-555.

Kamsiati, E., Herawati, H., & Purwani, E. Y. (2017). Potensi pengembangan plastik biodegradable berbasis pati sagu dan ubi kayu di indonesia. *Jurnal Penelitian dan Pengembangan Pertanian, 36* (**2**) : 67-76. http://dx.doi.org/10.21082/jp3.v36n2.2017.p67-76

Kresnawaty, I., Prakoso, H. T., Eris, D. D., & Mulyatni, A. S. (2016). Screening of bioplastics polyhydroxyalkanoic producing bacteria from landfill and palm oil mill effluents. *E-Journal Menara Perkebunan*, *82* (**1**) : 25-31. http://dx.doi.org/10.22302/iribb.jur.mp.v82i1.28

Liu, F., Li, J., & Zhang, X. L. (2019). Bioplastic production from wastewater sludge and application. In *IOP Conference Series: Earth and Environmental Science*. *344*(**1**). https://doi.org/10.1088/1755-1315/344/1/012071

Marchesi, J. R., Sato, T., Weightman, A. J., Martin, T. A., Fry, J. C., Hiom, S. J., & Wade, W. G. (1998). Design and evaluation of useful bacterium-specific PCR primers that amplify genes coding for bacterial 16S rRNA. *Applied and environmental microbiology*, *64*(2), 795-799. DOI: 10.1128/aem.64.2.795-799.1998

Możejko-Ciesielska, J. & R. Kiewisz. (2016). Bacterial polyhydroxyalkanoates : Still fabulous. *Microbiological Research 192*: 271–282. http://dx.doi.org/10.1016/j.micres.2016.07.010

Nursanti, I. (2013). Karakteristik limbah cair pabrik kelapa sawit pada proses pengolahan anaerob dan aerob. *Jurnal Ilmiah Universitas Batanghari Jambi*, *13*(**4**): 67-73. DOI: http://dx.doi.org/10.33087/jiubj.v13i4.324

Ohimain E. L., C Daokoru-Olukule., C Svlvester, Izah, A Rita AC Eke., dan J Okonkwo. (2012). Microbiology of palm oil mill effluents. *Microbiol Biotech Res*. *2*(**6**); 852-857.

Peña, C., Castillo, T., García, A., Millán, M., and Segura, D. (2014). Biotechnological strategies to improve production of microbial poly-(3-hydroxybutyrate): a review of recent research work. *Microbial biotechnology*, *7* (**4**), 278-293. DOI: 10.1111/1751-7915.12129.

Ratnaningrum, D., Saraswaty, V., Priatni, S., Lisdiyanti, P., Purnomo, A., and Pudjiraharti, S. (2019). Screening of polyhydroxyalkanoates (PHA)-producing bacteria from soil bacteria strains. In *IOP Conference Series: Earth and Environmental Science*. *277*(**1**); 012003. DOI:10.1088/1755-1315/277/1/012003

Sadasivam, S., Sigamani, S., Venkatachalam, H., and Ramamurthy, D. (2018). A New Method for the Production of Polyhydroxyalkanoates by Bacillus sp. and Detect the Presence of PHA Synthase. *Smart Science*, *6*(**2**), 105-116. https://doi.org/10.1080/23080477.2018.1437332

Tufail, S., Munir, S., & Jamil, N. (2017). Variation analysis of bacterial polyhydroxyalkanoates production using saturated and unsaturated hydrocarbons. *Brazilian journal of microbiology*. *48* (**4**) : 629-636. https://doi.org/10.1016/j.bjm.2017.02.008

Urtuvia, V., Villegas, P., González, M., & Seeger, M. (2014). Bacterial production of the biodegradable plastics polyhydroxyalkanoates. *International journal of biological macromolecules*. *70*: 208-213. Doi: 10.1016/j.ijbiomac.2014.06.001

Yustinah, N., Hidayat, R., Alamsyah, A. M., Roslan, H., Hermansyah, & Gozan, M. (2019). Production of polyhydroxybutyrate from oil palm empty fruit bunch (OPEFB) hydrolysates by Bacillus cereus suaeda B-001. *Biocatalysis and Agricultural Biotechnology*. *18* : 101-19. https://doi.org/10.1016/j.bcab.2019.01.057