Journal of Tropical Resources and Sustainable Science

journal homepage: jtrss.org

Isolation and Characterization of Polyhydroxyalkanoates (PHAs) Producers from Kg Batu Melintang hotspring

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Received 25 August 2018
Accepted 16 January 2019
Online 30 June 2020

Keywords:
Biodegradable, mineral salt media, fluorescence, Nile Blue A

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Abstract

The increasing awareness on the negative environmental impact of petroleum-based plastics has driven industries to explore more efficient biodegradable polymers for production of bioplastic. Polyhydroxyalkanoates (PHAs) is one of the potential biodegradable polymers to replace petroleum-based plastic. It is synthesized and accumulated as intracellular granules in microorganism. In this study, polyhydroxyalkanoates (PHAs) producing bacteria were successfully isolated from sediment collected from Kg. Batu Melintang hotspring. Isolation process was carried on Minimal Salt Medium (MSM) agar supplemented with excess glucose as a carbon source. Potential PHA producers were screened by using Nile Blue staining plate assay. Out of 144 bacterial isolates, 12 bacterial isolates which showed strong orange fluorescence under ultraviolet (UV) light (365nm) were selected for further identification by morphological characterization and biochemical analysis. Based on the result obtained, possible species for Gram positive rod shape bacteria B75 and B87 is *Corynebacterium kutsceri* meanwhile Gram negative rod shape bacteria A4, A12, A50, A68, B2, B13, B22, B31, B73 and C3 showed affiliation to *Citrobacter sp., Enterobacter sp., Erwinia sp., Klebsiella sp., Proteus sp*., *Salmonella sp*., *Serratia sp*., *Shigella sp*., and *Yersinia sp.*

1. INTRODUCTION

Plastics are one of the most used materials in the modern world. The widespread use of plastics is due to their favourable thermal and mechanical properties (Liu et al., 2014). Extensive use of plastics has committed to a serious environmental pollution as plastics are not biodegradable or easily recycled. Hazardous gases emitted during the production of plastic also bring negative effect to the global climate (Andrady, 2003). Polyhydroxyalkanoates (PHAs) are types of polymer which can be a good source for the production of biodegradable plastic. PHAs had attracted a great attention in biotechnological applications due to their low toxicity, biodegradability, and their thermoplastic or elastomeric properties. This natural polymer is synthesized and accumulated as intracellular granules in some bacteria (Khanna & Srivastava, 2005).

PHAs can be produced by prokaryotes under limited nutrients source such as phosphorous or oxygen and with excess of carbon source (Pan et al., 2012). The polymers are intracellularly accumulated in the form of inclusion bodies and might consist up to 90 % of cellular dry weight. PHAs can be classified into two major groups based on the number of carbon atoms in the polymer chain. PHAs can be grouped into short-chain-length (SCL-PHA) and medium-chain-length (MCL-PHA). Tan et al., (2014) reported that more than 300 different bacterial species belonging to Gram positive and Gram negative bacteria are found to deposit PHA intracellularly. Unlike synthetic plastics that take very long time to degrade, PHAs can be fully degraded within a short period of time by enzymatic activities of microbes into carbon dioxide and water. In aerobic conditions, carbon dioxide and water are generated during biodegradation of PHAs while methane and carbon dioxide are produced during degradation anaerobic condition (Kolstad et al., 2012).

2. MATERIALS AND METHODS

2.1 Soil Sampling

Soil sediment was collected from a hotspring which is located at Kg. Batu Melintang, Jeli, Kelantan. (N 05°39'57.3", E 101°42'51.7"). The sediment samples were kept in zipper bag prior to isolation.

2.2 Minimal Salt Medium (MSM)

All the composition of the medium as shown in Table 1 was mixed with 1 mL trace elements solution (Table 2) and topped up to 1 L with distilled water. 20

eISSN Number: 2462-2389

gL^{-1} of glucose was used as sole carbon source and 1 gL^{-1} of ammonium sulphate, $(NH4)_2SO_4$ was added to the medium as a nitrogen source. Bacteriological agar was added at the concentration of 1.5% (w/v) and the pH of the medium was adjusted to pH 7 (Maytham & Nawres, 2016).

2.3 Isolation of Polyhydroxyalkanoates (PHAs) Producers

Serially diluted soil sediment was spread on MSM supplemented with 2% (w/v) glucose as carbon source and ammonium sulphate as nitrogen source. The plates were incubated at 30 ℃ for 3 days. Colonies grew on the plates were further transferred to new MSM agar supplemented with Nile Blue A solution and observed under UV (365 nm).

Table 1: Composition of Minimal Salt Medium

Composition	Concentration (g/L)
Disodium hydrogen phosphate, Na_2HPO_4	3.5
Potassium dihydrogen phosphate, KH_2PO_4	1.5
Ammonium sulfate, $(NH_4)_2SO_4$	1.0
Magnesium sulphate heptahydrate, $MgSO_4.7H2O$	0.2
Calcium chloride 2-hydrate, $CaCl_2.2H_2O$	0.01
Iron (III) chloride hexahydrate, $FeCl_3.6H_2O$	0.06

Table 2: Composition of trace element solution

Composition	Concentration (g/L)
Boric acid, H_3BO_3	0.30
Cobalt (II) chloride hexahydrate, $COCl_2.6H_2O$	0.20
Zinc sulfate heptahydrate, $ZnSO_4.7H_2O$	0.10
Manganese (II) chloride tetrahydrate, $MnCl_2.4H_2O$	0.03
Sodium molybdate dehydrate, $NaMoO_4.2H_2O$	0.03
Nickel (II) chloride hexahydrate, $NiCl_2.6H_2O$	0.02
Copper (II) sulphate pentahydrate, $CuSO_4.5H_2O$	0.01

2.4 Screening of Polyhydroxyalkanoates (PHAs) Producers

Nile Blue A dissolved in dimethyl sulfoxide (DMSO; 0.25 mg/mL) was added to MSM agar with the final concentration of 0.5 µg/mL (Hassan et al., 2016). Bacterial isolates were streaked on this plate and incubated at 30 ℃ for 3 days. A 0.05 % (w/v) Nile Blue sulfate staining solution was prepared by dissolving 0.05g of Nile Blue sulfate in 100 mL of absolute ethanol. Colonies on the agar plates were stained with 5 mL of staining solution and shaken gently at room temperature. After 20 minutes, staining solution was removed from the agar plates and the plates were stood to dry the surface. The agar plates were exposed to UV light at 365 nm after staining to detect potential PHAs producing bacteria. Potential PHA producer will fluoresce when observed under UV light at 365 nm (Kitamura & Doi, 1994).

2.5 Identification of Polyhydroxyalkanoates (PHAs) Producers

Identification of potential PHA producers were carried out by morphological and biochemical analysis. Bacteria isolates were streaked on nutrient agar and the size, form, margin, elevation and colour of the colonies were observed and recorded. Gram staining and endospore staining were conducted to analyse the cell wall of the bacterial isolates and also their ability to form spores. The isolates were also observed under microscope to identify their cellular morphology. Standard biochemical tests namely acid fast test, catalase test, starch hydrolysis, oxidase test, lactose fermentation and indole test were conducted to identify the possible genus and species of the isolates based on Bergey's Manual of Determinative Bacteriology (1974).

3. RESULTS AND DISCUSSION

After a preliminary screening, a total of 144 potential PHA producers were successfully isolated. These isolates demonstrated fluorescence colonies under UV (365 nm) after staining with Nile Blue A solution. PHAs accumulating colonies will show bright orange fluorescence under UV light which deduced that the presence of PHAs in the cells (Bhuwal et al., 2013). Out of 144 bacterial isolates, 12 bacterial isolates showing relatively strong fluorescence intensity were chosen for further characterization and identification. Figure 1 shows the fluorescence colonies of isolate B87 after staining with Nile Blue A and observed under UV light. The content of polyhydroxyalkanoates (PHAs) in bacterial cell increase as the fluorescence intensity increased (Reddy et al., 2013). Nile Blue stain showed a strong positive orange fluorescence when it attached to the hydrophobic material such as PHA granules and lipids (Desouky et al., 2007). The selected bacterial isolates were then labelled as A4, A12, A50, A68, B2, B13, B22, B31, B73, B75, B87 and C3.

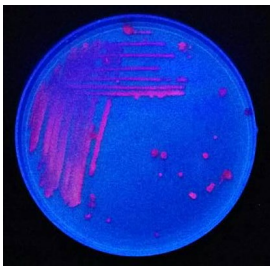


Figure 1: Isolate B87 stained with Nile Blue A under UV light (365 nm).

Potential PHA producers were further characterized by morphological and biochemical analysis. Bacterial isolates were examined for their colony morphologies which are size, form, margin, elevation and colour. The results are tabulated in Table 3.

eISSN Number: 2462-2389

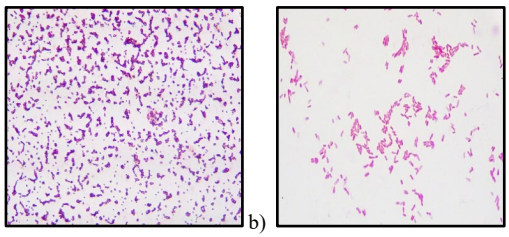
Several biochemical tests were carried out to further characterize the potential PHA producers. Results from Gram staining showed that majority of the isolates (n=10) are Gram negative while only two isolates are Gram positive. All of the bacteria isolates are rod shape (Table 4). Figure 2 shows the cellular morphology for two of the isolates, Gram positive isolate B87 and Gram negative isolate C3. Gram positive isolates, B75 and B87 were preceded for endospore staining and both isolates showed negative results. Table 5 shows the results for the rest of biochemical test namely acid fast test, catalase test, starch hydrolysis, oxidase test, lactose fermentation and indole test.

Table 3: Colony morphology of bacterial isolates

Isolate	Size	Form	Margin	Elevation	Colour
A4	Small	Circular	Entire	Raised	Creamy
A12	Small	Circular	Entire	Raised	Creamy
A50	Small	Circular	Entire	Raised	Creamy
A68	Small	Circular	Entire	Raised	Creamy
B2	Small	Circular	Entire	Raised	Creamy
B13	Small	Circular	Entire	Raised	Creamy
B22	Small	Circular	Entire	Raised	Creamy
B31	Small	Circular	Entire	Raised	Creamy
B73	Small	Circular	Entire	Raised	Creamy
B75	Large	Irregular	Undulate	Craterifom	Orange
B87	Large	Irregular	Undulate	Craterifom	Orange
C3	Large	Irregular	Undulate	Craterifom	Orange

Table 4: Gram staining reactions and cellular morphology of bacterial isolates

Isolates	Gram's reaction	Cell morphology
A4	Negative	Short rod
A12	Negative	Short rod
A50	Negative	Short rod
A68	Negative	Short rod
B2	Negative	Short rod
B13	Negative	Short rod
B22	Negative	Short rod
B31	Negative	Short rod
B73	Negative	Short rod
B75	Positive	Short rod
B87	Positive	Short rod
C3	Negative	Short rod



a) b)

Figure 2: Gram staining of bacteria isolates seen under microscope with 100x magnification. a) Gram positive isolate B87 b) Gram negative isolate C3

Table 5: Biochemical characteristics of the bacterial isolates

Isolates	T1	T2	T3	T4	T5	T6	T7	T8
A4	-	Nt	Nt	Nt	Nt	-	+	-
A12	-	Nt	Nt	Nt	Nt	-	-	-
A50	-	Nt	Nt	Nt	Nt	-	+	-
A68	-	Nt	Nt	Nt	Nt	-	+	-
B2	-	Nt	Nt	Nt	Nt	-	+	-
B13	-	Nt	Nt	Nt	Nt	-	-	-
B22	-	Nt	Nt	Nt	Nt	-	-	-
B31	-	Nt	Nt	Nt	Nt	-	-	-
B73	-	Nt	Nt	Nt	Nt	-	+	-
B75	+	-	-	+	+	Nt	Nt	Nt
B87	+	-	-	+	+	Nt	Nt	Nt
C3	-	Nt	Nt	Nt	Nt	-	-	-

Key: (+): positive reaction; (-): negative reaction; Nt: Not tested

Legend:
T1: Gram staining
T2: Spore forming test
T3: Acid fast test
T4: Catalase test
T5: Starch hydrolysis test
T6: Oxidase test
T7: Lactose fermentation test
T8: Indole test

Identifications of the potential PHA producers were carried out based on comparative analysis using Bergey's Manual of Determinative Bacteriology (1974). Based on the biochemical analysis, the possible genus and species for Gram positive isolate B75 and B87 is *Corynebacterium kutsceri.* As for Gram negative isolates A4, A12, A50, A68, B2, B13, B22, B31, B73 and C3, negative result in oxidase test indicates that they are from the family of *Enterobacteriaceae*. Further test showed that the potential species for the isolates A4, A50, A68, B2 and B73 are *Citrobacter sp., Enterobacter sp.*, *Erwinia sp*., *Klebsiella sp*., or *Serratia sp*. While the potential species for isolates A12, B13, B22, B31 and C3 are *Erwinia sp*., *Proteus sp*., *Salmonella sp*., *Serratia sp*., *Shigella sp*., or *Yersinia sp.*

Several studies reported on the production of PHA from *Citrobacter sp.* (Rehman et al., 2007); *Enterobacter aerogenes* (Arumugam et al., 2014); *Erwinia sp. USMI-20* (Majid et al., 1999); *Klebsiella pneumonia (*Ivanov et al., 2013*)*; *Serratia rubidaea* (Bologun et al., 2014) and *Yersinia frederiksenii (*Lam et al., 2017*)*. However, there was no report on *Proteus sp., Salmonella sp*., and *Shigella sp.,* as PHAs producing bacteria.

eISSN Number: 2462-2389

4. CONCLUSION

As a conclusion, a total of 144 potential PHA producers were isolated from soil sediment of Kg. Batu Melintang hotspring. 12 bacterial isolates with relatively strong fluorescence intensity under UV (365 nm) were selected for further characterization and identification by morphological and biochemical analysis. Based on Bergey's Manual of Determinative Bacteriology (1974) the possible genus and species of the bacteria isolates were identified. Gram positive rod shape bacteria B75 and B87 were identified as *Corynebacterium kutsceri* meanwhile Gram negative rod shape bacteria A4, A12, A50, A68, B2, B13, B22, B31, B73 and C3 showed affiliation to *Citrobacter sp., Enterobacter sp., Erwinia sp., Klebsiella sp., Proteus sp*., *Salmonella sp*., *Serratia sp*., *Shigella sp*., and *Yersinia sp.*

ACKNOWLEDGEMENT

Authors would like to thank Faculty of Bioengineering and Technology and Universiti Malaysia Kelantan for the support to this research project.

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eISSN Number: 2462-2389