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ISOLATION AND IDENTIFICATION OF P(3HB-*co*-4HB) PRODUCING BACTERIA FROM VARIOUS LOCATIONS IN KUALA TERENGGANU

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ABSTRACT

Polyhydroxyalkanoates (PHA) is a microbial bioplastic accumulated as a storage material under limited growth conditions in the presence of excess carbon sources. The ever increasing concern towards the depletion of petroleum resources and problems with utilization of a growing number of synthetic plastics, PHAs are being considered as a potential substitute for production of conventional non-degradable plastics. PHAs have been developed as biomaterials with unique properties for many years. Among all types of PHA, copolymer poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate) [P(3HB-*co*-4HB)] is widely sought after for biomedical application due to the biocompatibility, non-cytotoxicity and non-genotoxicity. Therefore, the aim of this study is to isolate and identify P(3HB-*co*-4HB) producers from water and soil sources in Kuala Terengganu. Samples of lake and soil were collected during November 2017 and then screened for P(3HB-*co*-4HB) producer. A total of 18 isolates were obtained, however only 5 isolates were identified as potential PHA producer. Interestingly, 3 isolates were confirmed as copolymer P(3HB-*co*-4HB) producer through gas chromatography analysis. These 3 isolated identified bacterial strain were *Cupriavidus* sp. TMT 11 with the highest 4HB molar fraction of 14.1%. The other 2 isolates were *Acinetobacter* sp. KPD 13 and *Cupriavidus* sp. PD 16. The properties of the copolymer P(3HB-*co*-4HB) produced by wide variety of bacteria isolated can be tailored for various biomedical applications.

Key words: Biomaterial, microbial bioplastics, PHA, P(3HB-*co*-4HB)

INTRODUCTION

Imagining a world without plastic is almost impossible. Application of plastic increases across the world in various sectors such as packing, construction, health care and more. Hence, to maintain world's sustainability, much preference is given to environmental-friendly and renewable resources. The usage of petrochemical plastic gripped world's attention because the disposal of this material gives a chronic impact towards the environment. This is because these plastics are not readily biodegradable and even have caused waste accumulation problems (Aziz *et al.,* 2017). Polymers of petrochemical origin have been substituting several conventional materials such as glass and metal. The growing use and incorrect way of disposal of these materials also can cause detrimental effect on the environment.

PHA is one of the polymers that had been drawing much attention. This is because these polymers are microbial biopolyster or bioplastics with vital environment capacity and high biotechnological properties (Rawte *et al.,* 2002). PHA has this characteristic because of its insolubility inside bacterial cytoplasm. This shows that PHA is a best replacement for the commercial plastics (Aziz *et al.,* 2017). In addition, *Alcaligenes eutrophus, Azotobacter vinelandii, Alcaligenes latus, Rhizobium* sp., *Bacillus* sp., *pseudomonads, methylotrophs,* and recombinant *Escherichia coli* are examples of PHA producing bacteria (Mikkili *et al.,* 2014). P(3HB-*co*-4HB) is one of the most widely recognized copolymer of PHA (John & Thomas, 2008). This copolymer had proven to play a role in medical and pharmaceutical field (Norhafini *et al.,* 2017). As an example P(3HB-*co*-4HB) is widely used in wound healing, cardiovascular, drug delivery and also tissue engineering applications (Chee *et al.,* 2008; Ali & Jamil, 2016). The monomer units of both 3HB

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and 4HB in P(3HB-*co*-4HB) copolymer are natural occurring metabolites in human body (Huong *et al.,* 2017).

Microorganisms that accumulate PHA survive in diverse environments which include soil (Wang & Bakken, 1998), sewage (Miliki *et al.,* 2014), marine sediments (Odham *et al.,* 1986), lake water (Mukai *et al.,* 1994) and ponds (Yellore & Desai, 1998). In a previous study, Amirul *et al.* (2008) reported that a novel bacterium which is capable of producing P(3HB-*co*-4HB) was first isolated and recognized as *Cupriavidus* sp. USMAA1020 from Lake Kulim, Malaysia. It became the origin of interest in this study since Kuala Terengganu ecosystem might be a promising ecosystem for the isolation of potential PHA producing bacteria. Hence, the objective of this study was to isolate microorganisms responsible for accumulation of P(3HB-*co*-4HB) from lake and soil spots in Kuala Terengganu for further productive use.

MATERIALS AND METHODS

Isolation and screening of P(3HB-*co*-4HB) producing bacteria from various sampling sites

A total of 18 samples were collected from various environments such as water and soil in Kuala Terengganu areas. Universiti Malaysia Terengganu, Pulau Duyong, Tok Jembal and Kampong Gong Kijang are few sites where samples were collected. Water sample was collected 0.5 m below the water surface while soil sample was also collected by digging 0.5 m below the surface. The samples were collected in universal bottles. The collected samples was enriched with mineral salts medium (MSM) consisting of 1.80 g/L KH_2PO_4, 2.90 g/L K_2HPO_4, 0.55 g/L $(NH_4)_2SO_4$, 0.10 g/L $MgSO_4.7H_2O$ and 0.50 mL/L trace elements (1.390 g/L $FeSO_4.7H_2O$, 0.990 g/L $MnCl_2.4H_2O$, 0.145 g/L $ZnSO_4.7H_2O$, 0.085 g/L $CuCl_2.2H_2O$, 0.840 g/L $CaCl_2.2H_2O$ and 1.410 g/L $CoSO_4.7H_2O$). Medium was added with 8 g/L filter-sterilized γ-butyrolactone as the sole carbon source but with limited nitrogen source to maintain the C/N ratio at 20. The PHA producers were identified using Nile red (1 mg/mL) which was then exposed to ultraviolet (320 nm) illumination (Spiekermann *et al.,* 1999). Intracellular PHA granules in the microbial cells were stained by Nile red and observed under fluorescence microscopy (Amirul *et al.,* 2008).

Identification of P(3HB-*co*-4HB) producer

The 16S rRNA sequencing was performed to determine the identity of the isolates. The bacteria were grown on nutrient rich plates for 24-48 hours and then inoculated into the test reagent. The 16S rRNA gene sequence was determined by direct sequencing of PCR-amplified 16S rDNA. The sequences of purified PCR products were determined using the CEQ^{TM} DTCS-Quick Start Kit (Beckmann Coluter) as directed in the manufacturer's protocol. Sequence reactions were electrophoresed using the CEQ^{TM} 8000 Genetic Analysis System (Amirul *et al.,* 2009a).

Analytical procedures

The culture media was collected after incubation and then centrifuged at 10000 rpm for 15 min. The cell pellet was dried to estimate the dry cell weight. P(3HB-*co*-4HB) content and composition was determined using gas chromatography (GC) analysis (Braunegg *et al.,* 1978). PHA yield was calculated as the multiplication of PHA content with the dry cell weight. Residual biomass was estimated as the difference between dry cell weight and dry weight of PHA (Mikkili *et al.,* 2014). Approximately 10-15 mg of freeze-dried cells were subjected to methanolysis and further analysed using GC as previously described (Amirul *et al.,* 2009a).

RESULTS AND DISCUSSION

Isolation and screening of potential P(3HB-*co*-4HB) producing strain

A total of 18 colonies were isolated from water and soil environment around selected Kuala Terengganu area, after being cultured and streaked on the mineral salt medium (MSM) agar plate containing γ-butyrolactone. γ-butyrolactone is generally used as feeding carbon precursor since it triggers the formation of 4HB via the biosynthesis pathway of P(3HB-*co*-4HB) (Amirul *et al.,* 2009b). Next, Nile Red was filtered over the MSM plate to look for the intense fluorescence. According to this method, about 5 out of 18 isolates were recognised as possible PHA producer strains. Table 1 demonstrates the sample sites, sources and fluorescence intensity according to the isolates. All the 5 potential producers emitted orange/yellow fluorescence when exposed under UV illuminator as shown in Figure 1.

GC analysis to identify P(3HB-*co*-4HB) producing bacteria strain

GC analysis further confirmed that 3 out of the 5 isolates were P(3HB-*co*-4HB) producing strains. TMT 11, KPD 13 and PD 16 were the 3 isolates from water bodies that produced copolymer P(3HB-*co*-4HB). There was no isolate from soil sources identified as P(3HB-*co*-4HB) producing strain. As seen in Table 1, there was only one potential PHA producer isolated from soil environment but it was not P(3HB-*co*-4HB) producer. This was probably

Table 1. Isolation and screening of PHA producer using Nile Red staining method

Isolates code	Place	Coordinate[a] (Lat/Long)	Source	Fluorescent intensity[b]
TMT 11	Teguh megah timur (pond)	N 5°20'27" E 103°6'19"	water	+++
KPD 13	Kampung Pulau Duyong (pond)	N 5°20'23" E 103°6'29"	water	+++
KPD 14	Kampung Pulau Duyong (pond)	N 5°20'23" E 103°6'29"	water	+++
PD 16	Pulau Duyong (river)	N 5°20'21" E 103°6'18"	water	++
PTJ 17	Pantai Tok Jembal (sand)	N 5°23'13" E 103°6'18"	soil	+

[a] Latitude and longitude of samples that had being isolated.
[b] Concentration of Nile Red: 1mg/mL; Fluorescence intensity: strong (+++); moderate (++); weak (+); non-fluorescence (–).

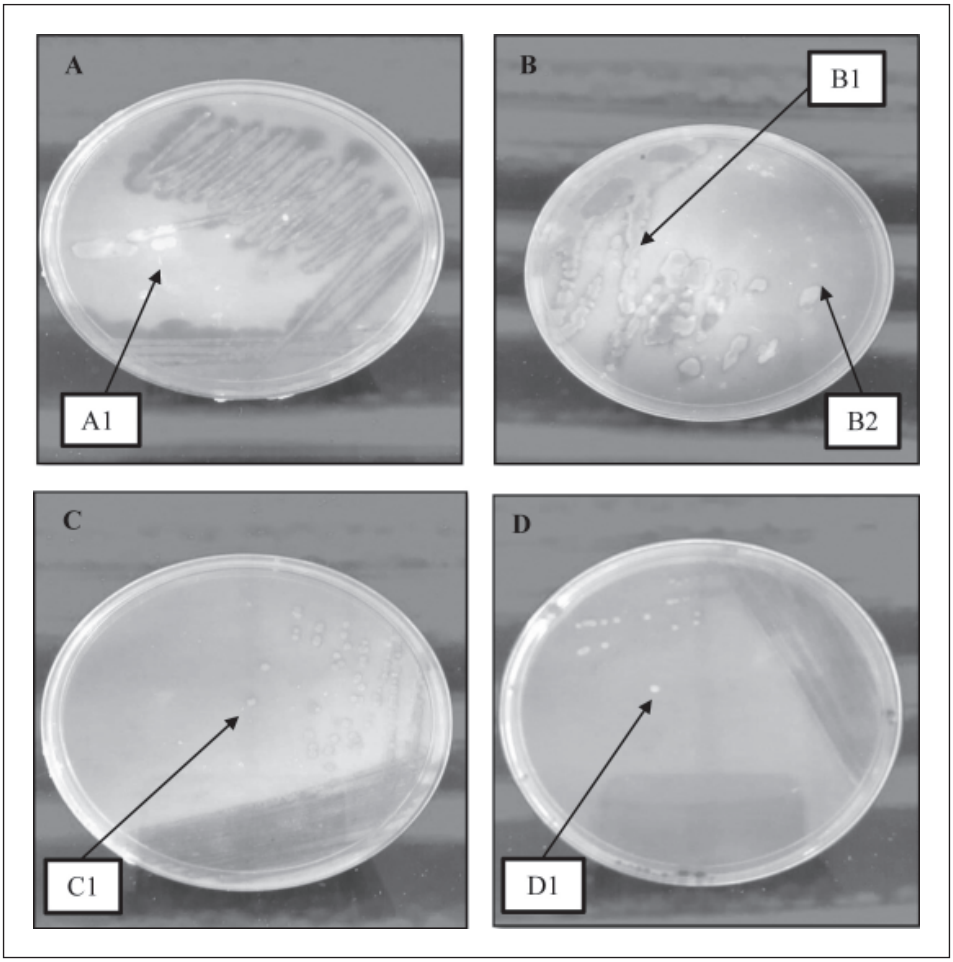

Fig. 1. Colonies of PHA accumulating cell emitted orange fluorescence under UV illuminator; (A1, B1 and B2) shows strong intense of fluorescence; (C1) moderate fluorescence; (D1) weak fluorescence.

because the soil conditions in the sampling sites was not suitable for bacteria to accumulate P(3HB-*co*-4HB) but stored other types of PHA. GC chromatogram of these 3 isolates showed three peaks, the first peak represents 3-hydroxybutyrate methyl ester, second peak indicates the γ-butyrolactone whereas the third peak represents 4-hydroxybutyrate methyl ester. Interestingly, the peaks in chromatogram from GC analysis for TMT 11, KPD 13 and PD 16 shows that these environmental isolates are capable of producing P(3HB-co-4HB) from carbon precursor γ-butyrolactone as shown in Figure 2, 3 and 4 respectively. The data of P(3HB-*co*-4HB) copolymer production of these isolates from γ-butyrolactone is shown in Table 2. Overall the PHA accumulation for the three isolates ranged from 21-77 wt% of the dry cell weight, 3HB composition ranged from 85-92 mol% and 4HB

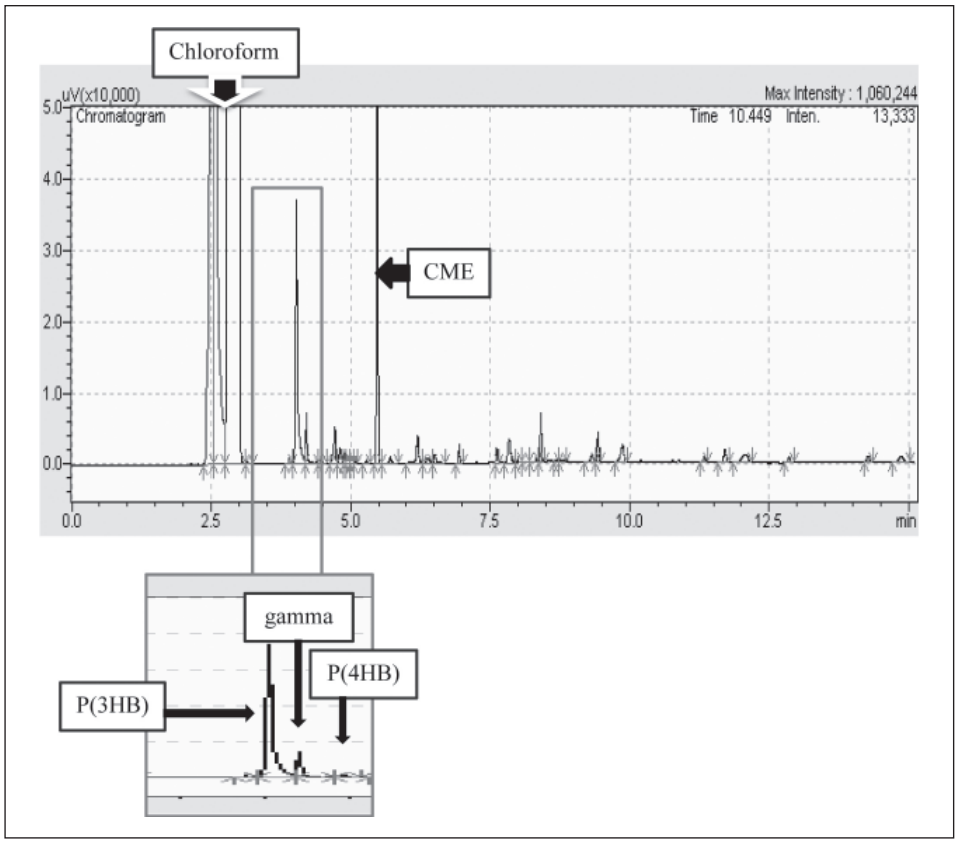

Fig. 2. GC analysis results showing the presence of copolymer P(3HB-*co*-4HB) in the bacteria isolate TMT 11.

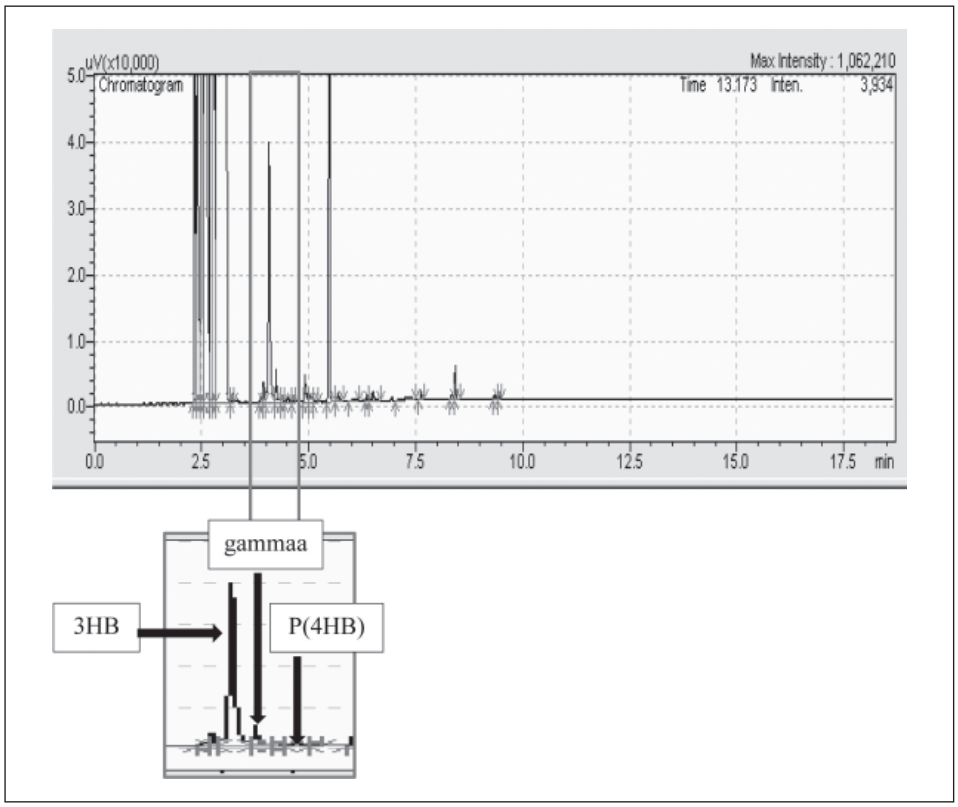

Fig. 3. GC analysis results showing the presence of copolymer P(3HB-*co*-4HB) in the bacteria isolate KPD 13.

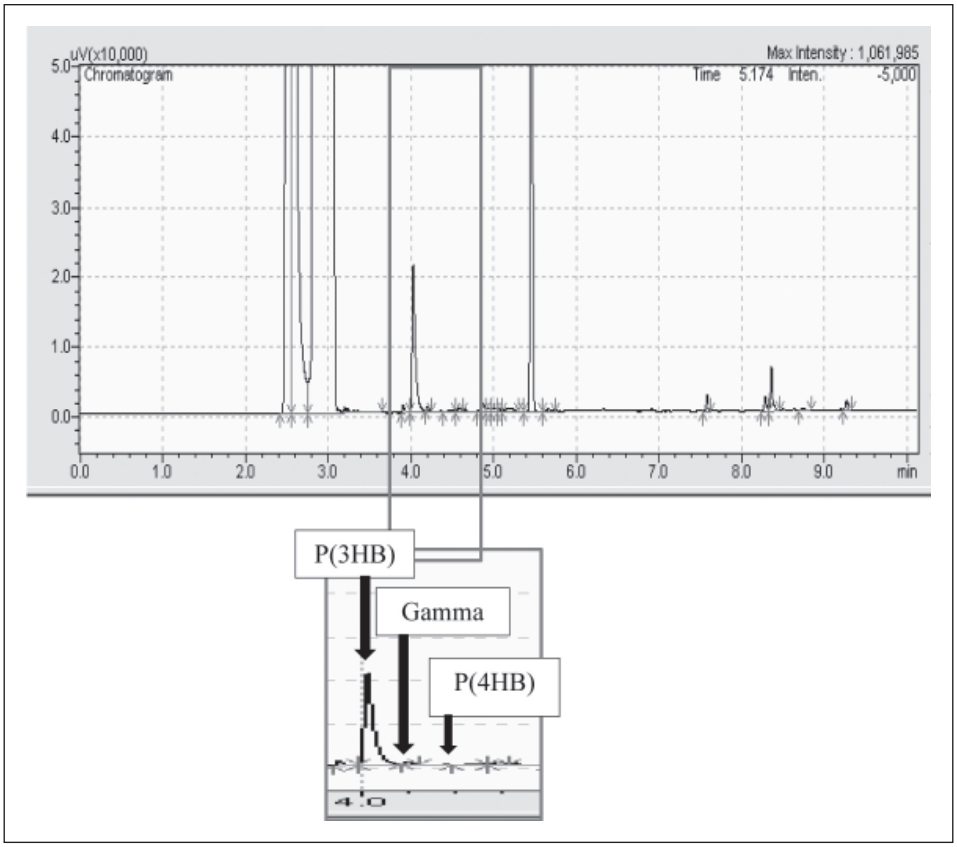

Fig. 4. GC analysis results showing the presence of copolymer P(3HB-*co*-4HB) in the bacteria isolate PD 16.

Table 2. Biosynthesis of copolymer P(3HB-*co*-4HB) by the isolated colonies

Isolates	Dry cell weight (g/L)[a]	PHA content (wt%)[b]	PHA composition (mol%)[b]		PHA yield (g/L)[b]	Residual biomass[c]
			3HB	4HB		
TMT 11	0.726	77.23 ± 9.95	85.90 ± 2.61	14.10 ± 2.61	0.56	0.166
KPD 13	0.859	62.93 ± 2.07	93.93 ± 4.80	7.99 ± 3.78	0.54	0.319
PD 16	1.948	29.57 ± 1.69	92.27 ± 4.33	3.28 ± 0.76	0.58	1.368

[a] The cell pellets were harvested after 72 hours.
[b] Data determined from GC analysis. Values are mean ± SD of triplicates.
[c] Data determined by subtracting PHA yield from dry cell weight.

composition ranged from 3-14 mol%. It was revealed that isolate from TMT 11 resulted in higher accumulation of PHA 77.23 wt% of its dry cell weight with 85.9 mol% fraction of 3HB monomer and 14.1 mol% fraction of 4HB monomer. KPD 13 showed 62.93 wt% of its dry cell weight with 93.93 mol% of 3HB composition while 7.99 mol% of 4HB composition. Finally, isolate of PD 16 produced 29.57 wt% of PHA out of its dry cell weight whereas 92.27 mol% of 3HB composition and 3.28 mol% of 4HB composition. In addition, the PHA yield of TMT 11, KPD 13 and PD 16 was 0.56 g/L, 0.54 g/L and 0.58 g/L respectively. In general, the three isolates that produced copolymer P(3HB-*co*-4HB) were collected from water bodies. This analysis shows that water bodies in Kuala Terengganu are among potential sources of P(3HB-*co*-4HB) producing microorganisms. This probably is because the water bodies contain excess carbon and less nitrogen providing a suitable environment for potential bacteria to accumulate P(3HB-*co*-4HB) inside their intracellular granules as compared to soil environment in sampling sites.

Identification of P(3HB-*co*-4HB) producer

The identification of the P(3HB)-degrading bacteria was carried out by using molecular analysis, 16S rDNA. Then, the PCR products were sent for sequencing. Table 3 shows the results of bacterial identification based on 16S rDNA analysis. The 16S rRNA sequence of strain TMT11 exhibited 98% similarity to *Cupriavidus plantarum.* Therefore, the strain was denoted as *Cupriavidus* sp. TMT11. The phylogenetic comparison of strain *Cupriavidus* sp. TMT11 within its genus is shown in Figure 5a. Meanwhile, isolates KPD13 and PD16 exhibited 99% and 98% similarity to *Acinetobector bereziniae* and *Cupriavidus numazensis* respectively. Therefore, the strains were denoted *Acinetobector* sp. KPD13 and *Cupriavidus* sp. PD16 and the phylogenetic comparison of these strains within their genus are shown in Figure 5b and 5c.

Table 3. Preliminary genus identification of P(3HB-*co*-4HB) producer

P(3HB-*co*-4HB) producer strain[a]	Genus[b]	Similarity index[b]
TMT11	*Cupriavidus* sp.	98%
KPD13	*Acinetobacter* sp.	99%
PD16	*Cupriavidus* sp.	98%

[a] Strains that was isolated from water source.
[b] Interpreted identification data from 16S rDNA identification.

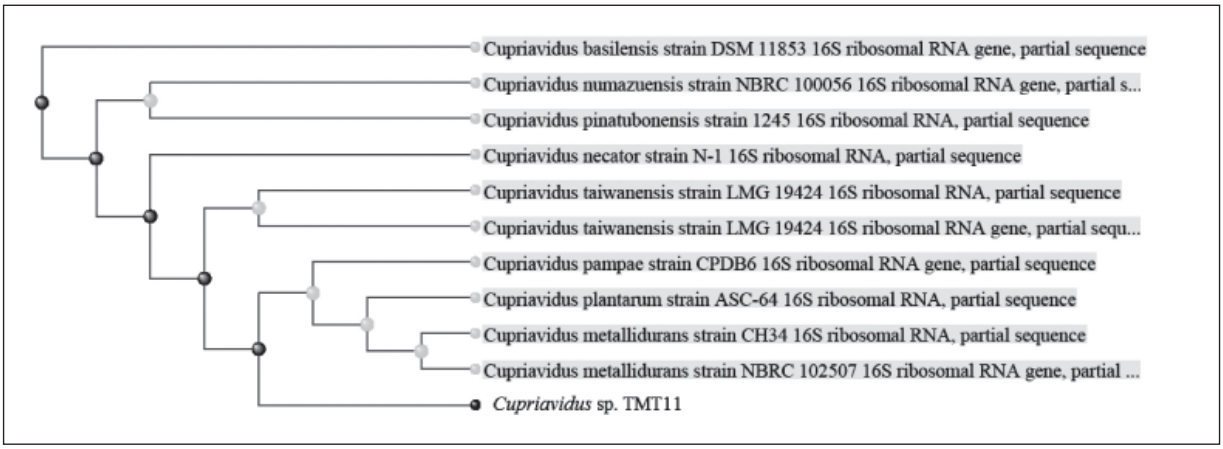

Fig. 5a. Neighbour-joining phylogenetic tree of *Cupriavidus* sp. TMT11 and related bacteria based on 16S rRNA sequence comparisons. Accession numbers are given.

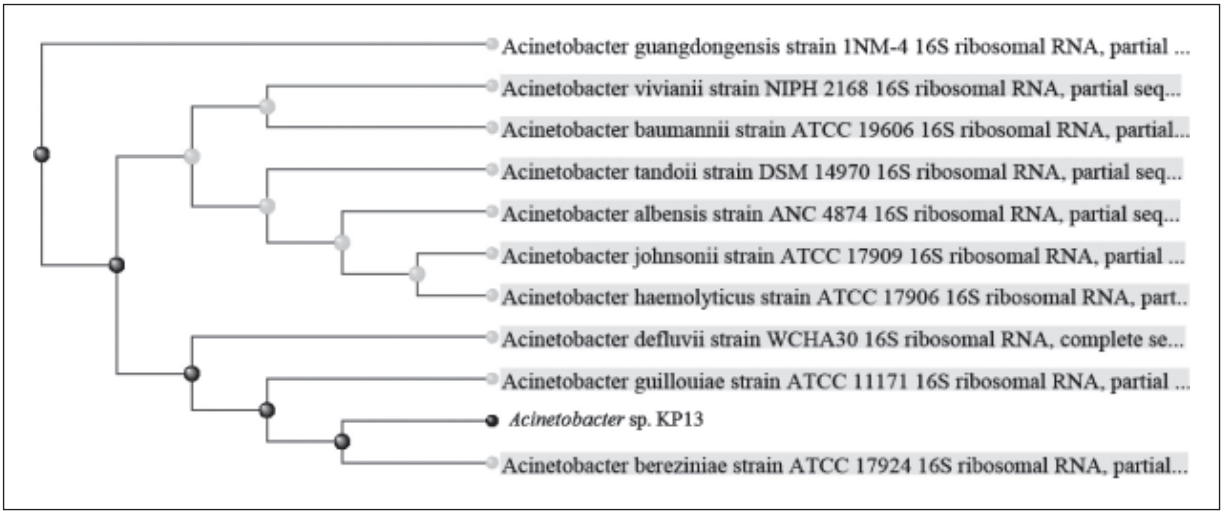

Fig. 5b. Neighbour-joining phylogenetic tree of *Acinetobacter* sp. KPO 13 and related bacteria based on 16S rRNA sequence comparisons. Accession numbers are given.

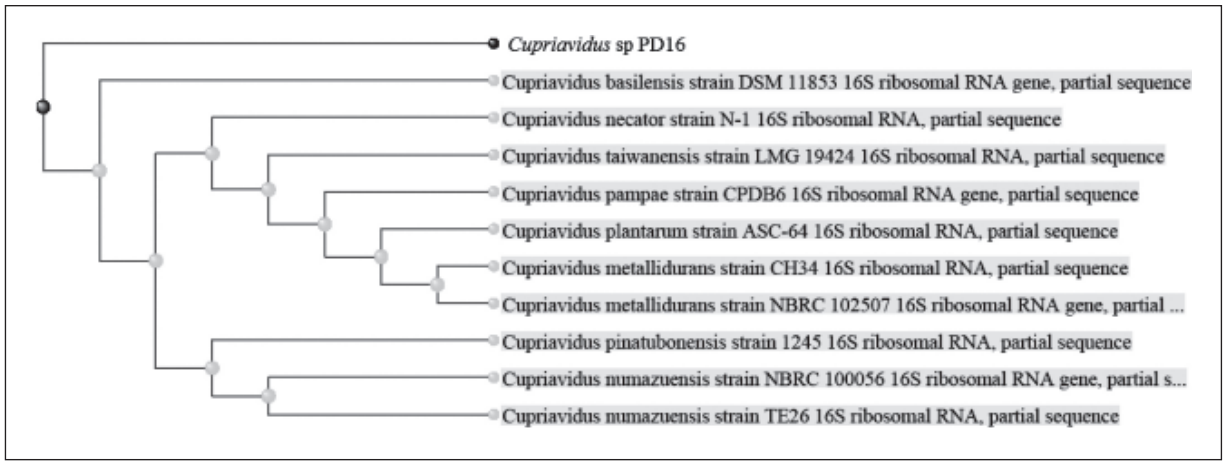

Fig. 5c. Neighbour-joining phylogenetic tree of *Cupriavidus* sp. PD16 and related bacteria based on 16S rRNA sequence comparisons. Accession numbers are given.

CONCLUSION

The isolated bacteria TMT 11 (*Cupriavidus* sp.), KPD 13 (*Acinetobacter* sp.) and PD 16 (*Cupriavidus* sp.) from water bodies in Kuala Terengganu were capable of producing P(3HB-*co*-4HB) using γ-butyrolactone as carbon precursor. The PHA production and 4HB fraction in the copolymer produced by these three locally isolated bacteria could be further regulated by implying various carbon sources and feeding techniques. Further studies are also required to efficiently scale-up P(3HB-*co*-4HB) copolymer production using these three bacterial strains in order to establish a high yield PHA production system. Manipulating the copolymer is vital in order to reach the desirable properties of copolymer to be fully utilised in developing biomaterial such as scaffolds, sutures, wound dressings, cardiovascular patches and medical grafts.

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