Malays. Appl. Biol. (2020) 49(4): 79–84

EFFECTS OF VARIOUS CARBON PRECURSORS COMBINATION IN REGULATING THE MOLAR FRACTION OF P(3HB-*co*-4HB) USING LOCALLY ISOLATED *Cupriavidus* sp. TMT11

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Accepted 10 November 2020, Published online 25 December 2020

ABSTRACT

The P(3HB-*co*-4HB) is commonly used for biomedical applications. This is due to the desired mechanical properties as well as biocompatibility, non-genotoxicity, and non-cytotoxicity of this copolymer. However, the production of P(3HB-*co*-4HB) with a specific 4HB molar fraction is still limited. In this study, carbon precursors at different combinations and concentration ratios have been used in the production of P(3HB-*co*-4HB) bioplastics using locally isolated *Cupriavidus* sp. TMT11. The carbon precursors 1,6-hexandiol, 1,4-butanediol, and γ-butyrolactone were used to screen for high cell biomass, PHA content, and 4HB molar fraction through Gas Chromatography (GC) analysis. Generally, the combination of various carbon precursors showed an increase in cell biomass. The carbon combination of 1,4-butanediol and γ-butyrolactone at the ratio of 4:2 and carbon combination of 1,6-hexanediol and γ-butyrolactone at a ratio of 1:5 showed high amount of cell biomass above 0.35 g/L on day 3. Nevertheless, the 4HB molar fraction of both the combination was recorded as 9±0.27 mol% and 14±1.1 mol% respectively. The lowest amount of cell biomass and PHA yield were recorded with the carbon combination of 1,6-hexanediol and 1,4-butanediol with the ratio 5:1 at 0.55±0.13 g/L. However, the highest 4HB molar fraction of 89.37±3.6 mol% 4HB was recorded with this combination. The 4HB molar fraction above 80 mol% was reported with a carbon combination of 1,6-hexanediol +1,4-butanediol at 5:1, 1:1, and 2:4 ratio. The varying combination of carbon precursors biosynthesized a wide range of 4HB molar fractions ranging from 9.07 to 89.37 mol% 4HB.

Key words: Polyhydroxyalkanoate (PHA), bioplastics, P(3HB-*co*-4HB), carbon precursor

INTRODUCTION

Polyhydroxyalkanoate (PHA) is a family of microbial polymers produced intracellularly as carbon and energy storage material by a wide variety of bacteria under stress conditions. One of the unique characteristics of PHA is that it can be completely degraded in various environments and this garnered attention worldwide (Vigneswari *et al*., 2019).

Poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate) [P(3HB-*co*-4HB)] copolymer is a member of the bacterial PHAs. P(3HB-*co*-4HB) has been found to have desirable mechanical properties for applications in the medical and pharmaceutical fields. The biosynthesis of P(3HB-*co*-4HB) involved the utilization of specific carbon precursors such as 1,4 butanediol, 1,6-hexanediol, γ-butyrolactone, 1,6-hexanediol ω-alkanediols with even number carbon atom numbers (Huong *et al*., 2018). P(3HB-*co*-4HB) was first produced by *Cupriavidus necator*, which is previously known as *Ralstonia eutropha.* There are many types of bacterial strains that accumulate P(3HB-*co*-4HB) such as *Hydrogenophaga pseudoflava* (Choi & Lee, 1999), *Comamonas acidovorans* (Lee *et al*., 2004), and *Alcaligenes latus* (Lefebvre *et al*., 1997). The bacterial strain that has been used in this study is *Cupriavidus* sp. TMT11, a locally isolated from Teguh Megah Timur pond in Kuala Terengganu, Malaysia (Chai *et al*., 2017).

The production of P(3HB-*co*-4HB) with a wide range of 4HB molar faction is vital to tailor the various application of this copolymer. Effective approaches in biosynthesizing P(3HB-*co*-4HB) with

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varying 4HB molar fraction is necessary due to the increasing applications in medical and pharmaceutical sectors such as cardiovascular, orthopedic, drug delivery, and tissue engineering applications (Chee *et al*., 2008; Rahayu *et al*., 2008; Faezah *et al*., 2011). In this study, the three main carbon precursors namely 1,6-hexandiol, 1,4-butanediol, and γ-butyrolactone which are commonly used in the biosynthesis of P(3HB-*co*-4HB) are used to determine the effect the biomass of P(3HB-*co*-4HB) and the varying 4HB molar fraction using the locally isolated *Cupriavidus* sp. TMT11.

MATERIALS AND METHODS

Bacterial strain and maintenance

Cupriavidus sp. TMT 11 used in this study was isolated from Teguh Megah Timur pond in Kuala Terengganu, Malaysia as previously described (Meng *et al*., 2019). Briefly, it is maintained in 20% (v/v) glycerol solution and stored at -20°C for long term storage. The bacteria were revived in nutrient-rich (NR) broth for 24 hr at 200 rpm at 30°C (Huong *et al*., 2018).

Biosynthesis of P(3HB-*co*-4HB) copolymer

The production of the P(3HB-*co*-4HB) copolymer was carried out in a shake flask system. The inoculum was prepared with bacterial strain *Cupriavidus* sp. TMT11 was grown in nutrient broth containing 10 g/L peptone, 10 g/L meat extract, and 2 g/L yeast extract at 30°C and placed in an orbital shaker for 15-18 hr at 200 rpm. Later, 10% (v/v) inoculum was transferred into the mineral medium to produce P(3HB-*co*-4HB) copolymer when the bacterial growth based on optical density measured using a spectrophotometer (540 nm) reaches 0.4-0.5. The mineral salt media (MSM) was prepared with 5.8g/L K_2HPO_4, 3.7 g/L KH_2PO_4 and 1.1 g/L $(NH_4)_2SO_4$, trace elements (2.78 g/L $FeSO_4$.$7H_2O$, 1.98 g/L $MnCl_2.4H_2O$, and 2.81 g/L $CoSO_4.7H_2O$, 1.67 g/l $CaCl_2.2H_2O$, 0.17 g/L $CuCl_2.2H_2O$ and 0.29 g/L $ZnSO_4.7H_2.O$ and 0.29 g $ZnSO_4.7H_2O$) and $MgSO_4.7H_2O$ (0.1M) which were sterilised at 121°C for 15 min. The MSM was prepared in 50 mL in a 250 mL conical flask was added with a specific combination and concentration ratio of carbon precursor (Table 1). The MSM was then inoculated with inoculum grown in NR and incubated at 30°C in an orbital shaker at 150 rpm for 72 hr. Bacterial growth will be monitored at intervals of 24 hr. The carbon sources used were 1,6-hexanediol, 1,4-butanediol, and γ-butyrolactone with a total carbon concentration of 0.6 wt% C but with a varying ratio as listed in Table 2.1. The cells were harvested by centrifugation (Centrifuge 5804 R) at 10000 rpm for 15 min. The cell pellet was subjected to lyophilization before further analysis (Norhafini *et al*., 2017).

Analytical procedure

The contents of P(3HB-*co*-4HB) and its composition which includes the 4HB molar fraction was obtained using gas chromatography (GC) analysis using Shimadzu GC-17A (Shimadzu, Japan) analysis (Braunegg *et al*., 1978) with Supelco SPBTM -1(L × I.D. 30 M × 0.25 mm) [Sigma, USA] column (Amirul *et al*., 2009).

Statistical analysis

All experimental values are presented as means and standard deviation. The data were analyzed using ANOVA and Turkey's Test using SPSS 25 software. The $p \leq 0.05$ were considered significant.

RESULTS AND DISCUSSION

Effect of different ratio of carbon precursors on bacterial growth

Cupriavidus sp. TMT11 obtained from Teguh Megah Timur pond in Kuala Terengganu, Malaysia was cultivated using 1,6-hexanediol, 1,4-butanediol, and γ-butyrolactone with varying combinations and concentration ratio of carbon precursors. The effect of different ratios of carbon source in the biomass of P(3HB-*co*-4HB) production was observed at intervals of 24 hr for a total of 72 hr. Figure 1 highlights the biomass of the cells at different time intervals. Based on the results obtained in Figure 1, the carbon combination of 1,4-butanediol and γ-butyrolactone at the ratio of 4:2 and carbon combination of 1,6-hexanediol and γ-butyrolactone at a ratio of 1:5 showed the highest amount of cell biomass above 0.35 g/L on day 3. The carbon combination of 1,6-hexanediol and 1,4-butanediol at a ratio of 5:1 recorded the lowest amount of cell biomass of 0.045 ± 0.004 g/L at day 3 of cultivation. The varying combination of carbon precursors using the mixed-substrate strategy of 1,4-butanediol and 1,6-hexanediol to regulate the molar fraction of

Table 1. Carbon concentration of carbon sources

Carbon sources	Carbon concentration ratio
1,6-Hexanediol + 1,4-Butanediol	5:1
1,6-Hexanediol + γ-Butyrolactone	1:5
1,6-Hexanediol + γ-Butyrolactone	4:2
1,4-Butanediol + γ-Butyrolactone	1:5
1,4-Butanediol + γ-Butyrolactone	4:2
1,6-Hexanediol + 1,4-Butanediol	4:2
1,6-Hexanediol + 1,4-Butanediol	1:5

Table 2. Regulating the molar fraction of P(3HB-*co*-4HB) through combined carbon precursors using *Cupriavidus* sp. TMT11

Carbon combination	Carbon concentration ratio	PHA content* (wt%)	PHA composition (mol%)*		PHA yield (g/L)*
			4HB	3HB	
1,4-butanediol + γ-butyrolactone	1:5	24.48 ± 10.98^{a}	33.87 ± 6.37^{a}	66.13 ± 26.37^{a}	0.36 ± 0.14^{a}
1,4-butanediol + γ-butyrolactone	4:2	51.09 ± 12.69^{bc}	9.07 ± 0.27^{b}	90.93 ± 0.27^{b}	4.70 ± 1.70^{b}
1,6-hexandiol + γ-butyrolactone	1:5	54.82 ± 10.33^{c}	14.06 ± 1.10^{c}	85.94 ± 14.10^{a}	4.89 ± 0.74^{b}
1,6-hexandiol + γ-butyrolactone	4:2	82.38 ± 93.31^{c}	20.10 ± 9.78^{a}	79.90 ± 19.78^{a}	3.54 ± 4.05^{b}
1,6-hexanediol +1,4-butanediol	1:5	44.62 ± 14.25^{ab}	30.12 ± 18.77^{a}	69.88 ± 18.77^{a}	2.96 ± 0.80^{c}
1,6-hexanediol +1,4-butanediol	4:2	24.15 ± 5.36^{ab}	23.77 ± 3.07^{a}	76.23 ± 3.07^{a}	1.55 ± 0.37^{ac}
1,6-hexanediol +1,4-butanediol	5:1	1.68 ± 1.08^{d}	89.37 ± 3.60^{d}	10.63 ± 3.60^{c}	0.55 ± 0.13^{a}

Data show the mean ± standard deviation of three replicates. Means with different alphabets (a–d) within the same column are significantly different at $p \leq 0.05$ level (Tukey test).
* Data determined from GC analysis.

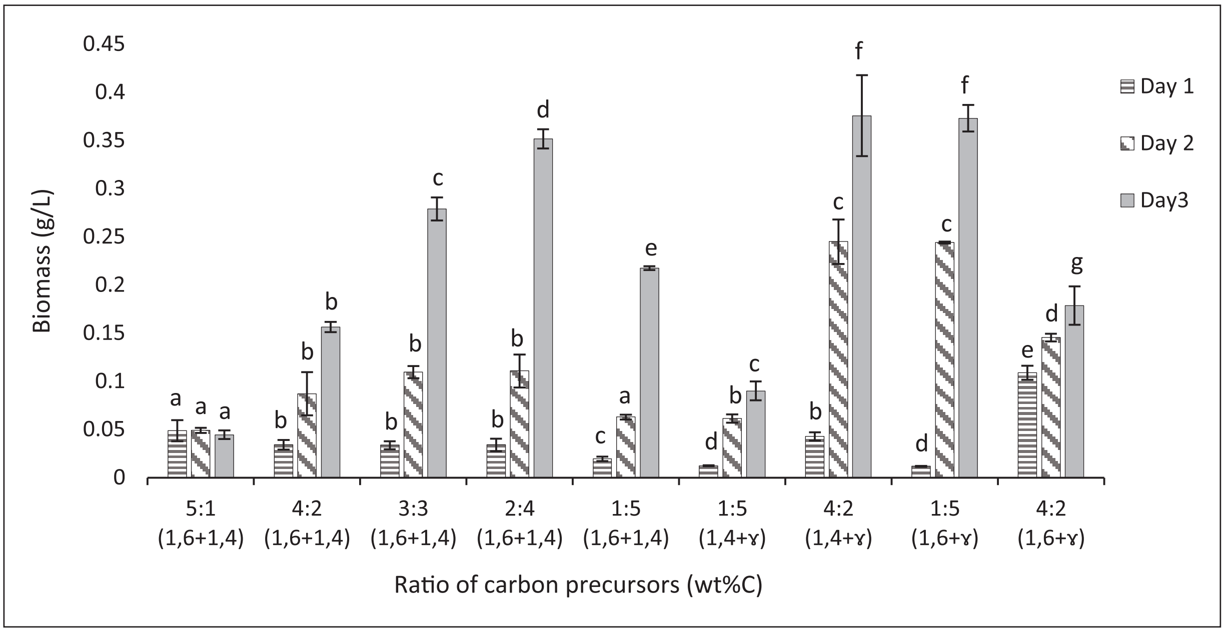

Fig. 1. Biomass of cells obtained at every 24 hr time interval using different combinations and concentration ratio of carbon precursors. Means with different alphabets (a–g) are significantly different at $p \leq 0.05$ level (Tukey test).

P(3HB-*co*-4HB) had been demonstrated by Huong *et al*. (2015) through their work. It was reported that the combination of carbon precursors showed a better increment of the bacteria biomass as compared to using sole carbon precursor. It was reported that the combination of more than one physiologically similar substrate provides increasing cell biomass, which can overcome the limitation faced by single-stage cultivation in biosynthesis (Syafiq *et al*., 2017). Furthermore, it was observed that as the concentration of 1,6-hexanediol decreases while 1,4-butanediol increases, the biomass of bacteria showed an increment. A similar trend was observed across different 1,6-hexanediol and 1,4-butanediol combination. A similar observation was also reported by Chanprateep *et al*. (2008), which stated that 1,4-butanediol is one of the effective carbon precursors for the increase in the 4HB molar fraction as compared to γ-butyrolactone. Biosynthesis of P(3HB-*co*-4HB) involves the conversion of 1,4-butanediol to 4-hydroxybutyryl-CoA, before being converted to 4-hydroxybutyrate, where only a portion of 4-hydroxybutyrate will undergo a complex pathway to form 3-hydroxybutyrate (Steinbüchel & Lütke-Eversloh, 2003; Iqbal & Amirul, 2014; Norhafini *et al*., 2017). Hence, this further confirmed that the utilization of the optimum amount of 1,4-butanediol could greatly promote the production of 4HB monomer in copolymer P(3HB-*co*-4HB).

The percentage of increase in cell biomass between 24 hr to 72 hr was shown in Figure 2. It was reported that the carbon combination of 1,6-hexanediol and 1,4-butanediol at a ratio of 4:2

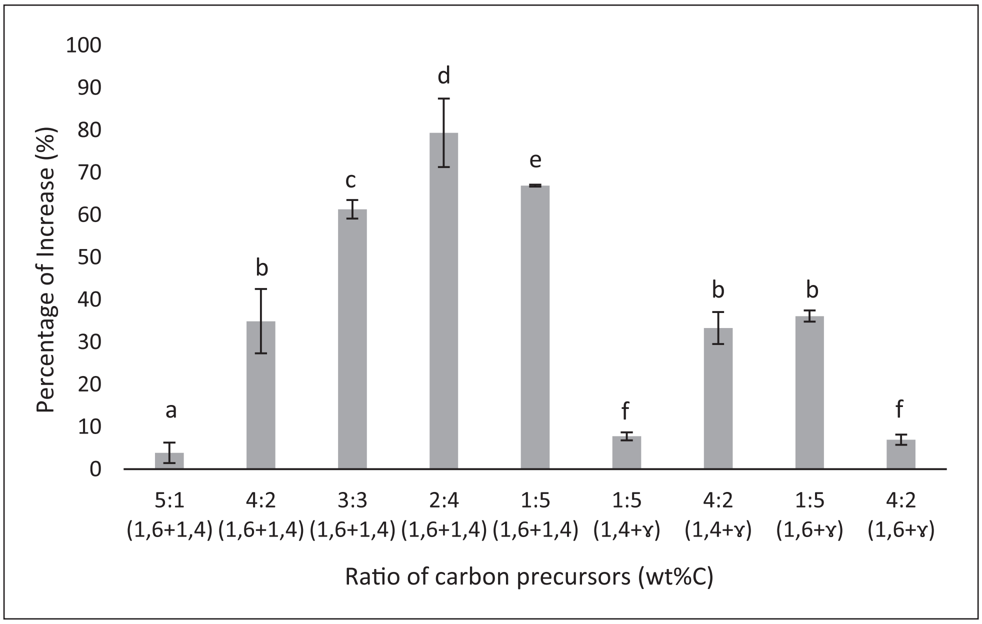

Fig. 2. Percentage of increase in cell biomass between time intervals. Means with different alphabets (a–f) are significantly different at $p \leq 0.05$ level (Tukey test).

reported the highest percentage of increment at 99.14%. Whereas the carbon combination of 1,6-hexanediol and α-butyrolactone at 4:2 ratio has the lowest percentage of increment at 39.08%. This could probably be attributed to the presence of 1,4-butanediol which further supports the fact that this carbon precursor 1,4-butanediol is one of the effective carbon precursors to not only increase the 4HB molar fraction but also cell biomass.

Effect of mixed-substrates with a various concentration on copolymer P(3HB-co-4HB) production

The regulation of 4HB molar fraction of P(3HB-*co*-4HB) through combined carbon precursors using *Cupriavidus* sp. TMT11 was shown in Table 2. A high 4HB molar fraction of above 80 mol% was recorded with a carbon combination of 1,6-hexanediol +1,4-butanediol at the ratio of 5:1, 1:1, and 2:4. The highest PHA yield of the samples was observed with the carbon combination of 1,6-hexanediol +1,4-butanediol at the ratio of 1:1 and 2:4. However, the carbon combination of 1,6-hexanediol + γ-butyrolactone at 1:5 ratio recorded the lowest PHA yield at 0.36 g/L. A similar observation was reported by Syafiq *et al*. (2017). The carbon precursors used during biosynthesis affect the composition of polymer produced by the bacterial cells. Generally, the carbon combination of 1,4-butanediol and α-butyrolactoneat at the ratio of 1:5 has recorded relatively the lowest PHA yield. This could be because γ-butyrolactone could have been toxic to the bacterial cells (Steinbüchel & Lütke-Eversloh *et al*., 2003). Huong *et al*. (2015) reported that synergistic of 1,4-butanediol and 1,6-hexanediol shows a better increment of the bacteria biomass as well as 4HB molar fraction as compared to using sole carbon precursor. This study was carried out to evaluate the bacterial growth as well as regulating the 4HB molar fraction by varying the carbon precursor. This indicates that the varying combination of carbon precursors used gave a wide range of P(3HB-*co*-4HB) ranging from 9.07 to 89.37 mol% 4HB biosynthesized using *Cupriavidus* sp. TMT11.

CONCLUSION

The 4HB molar fractions of P(3HB-*co*-4HB) copolymer can be regulated using the combination of varying carbon precursor with a wide range of 4HB molar fraction exhibited. This work confirmed that the *Cupriavidus* sp. TMT11 has the highest bacterial biomass and highest 4HB mol% accumulated when the combination of 1,6-hexanediol and 1, 4-butanediol were used. This will enable the scale-up of P(3HB-*co*-4HB) copolymer production in a bioreactor to regulate the 4HB molar fractions. Carbon precursor concentration and combination based on ratio should be optimized to prevent the carbon precursors to become toxic to the bacteria, thus enhance the production of P(3HB-*co*-4HB). The biosynthesized P(3HB-*co*-4HB) copolymer can be potentially used for various biomedical applications in the future.

ACKNOWLEDGEMENTS

The authors acknowledge the Ministry of Education Malaysia for supporting this work under the FRGS grant (FRGS/1/2017/STG05/UMT/03/1).

REFERENCES

Amirul, A.A., Yahya, A.R.M., Sudesh, K., Azizan, M.N.M. & Majid, M.I.A. 2009. Isolation of poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate) producer from Malaysian environment using γ-butyrolactone as carbon source. *World Journal of Microbiology and Biotechnology*, **25(7)**: 1199-1206.

Braunegg, G., Sonnleiter, B. & Lafferty, R.M. 1978. A rapid gas chromatographic method for the determination of poly-3-hydroxybutyric acid in microbial biomass. *European Journal of Applied Microbiology and Biotechnology*, **6(1)**: 29-37.

Chanprateep, S., Katakura, Y., Visetkoop, S., Shimizu, H., Kulpreecha, S. & Shioya, S. 2008. Characterization of new isolated Ralstoniaeutropha strain A-04 and kinetic study of biodegradable copolyester poly (3-hydroxybutyrate-*co*-4-hydroxybutyrate) production. *Journal of Industrial Microbiology & Biotechnology*, **35(11)**: 1205-1215.

Choi, J. & Lee, S.Y.1999. Factors affecting the economics of polyhydroxyalkanoate production by bacterial fermentation. *Applied Microbiology and Biotechnology*, **51(1)**: 13-21.

Chee, J.W., Amirul, A.A., Tengku Muhammad, T.S., Majid, M.I.A. & Mansor, S.M. 2008. The influence of copolymer ratio and drug loading level on the biocompatibility of P(3HB-co-4HB) synthesized by *Cupriavidus* sp. (USMAA2-4). *Biochemical Engineering Journal*, **38(3)**: 314-318.

Faezah, A.N., Rahayu, A., Vigneswari, S., Majid, M.I.A. & Amirul, A.A. 2011. Regulating the molar fraction of 4-hydroxybutyrate in poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate) by biological fermentation and enzymatic degradation. *World Journal of Microbiology and Biotechnology*, **27(10)**: 2455-2459.

Huong, K.H., Elina, K.A.R. & Amirul, A.A. 2018. Production of high molecular weight poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate) copolymer by *Cupriavidus malaysiensis* USMAA1020 utilising substrate with longer carbon chain. *International Journal of Biological Macromolecules*, **116**: 217-223.

Huong, K.H., Kannusamy, S., Lim, S.Y.H. & Amirul, A.A. 2015. Biosynthetic enhancement of single-stage poly (3-hydroxybutyrate-*co*-4-hydroxybutyrate) production by manipulating the substrate mixtures. *Journal of Industrial Microbiology & Biotechnology*, **42(9)**: 1291-1297.

Iszatty, I., Aidda, O.N., Hema, R. & Amirul, A.A. 2017. Combination of 4-Hydroxybutyrate carbon precursors as substrate for simultaneous production of P(3HB-*co*-4HB) and yellow pigment by *Cupriavidus* sp. USMAHM13. *Arabian Journal for Science and Engineering*, **42(6)**: 2303-2311.

Iqbal, N. & Amirul, A.A. 2014. Synthesis of P (3HB*co*4HB) copolymer with targetspecific 4HB molar fractions using combinations of carbon substrates. *Journal of Chemical Technology & Biotechnology*, **89(3)**: 407-418.

Lee, S.Y. & Choi, J. 1998. Effect of fermentation performance on the economics of poly(3-hydroxybutyrate) production by *Alcaligenes latus*. *Polymer Degradation and Stability*, **59(3)**: 387-393.

Lee, W.H., Azizan, M.N.M. & Sudesh, K. 2004. Effects of culture conditions on the composition poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate) synthesized by *Comamonas acidovorans*. *Polymer Degradation and Stability*, **84(1)**: 129-134.

Lefebvre, G., Rocher, M. & Braunegg, G. 1997. Effects of low dissolved-oxygen concentrations on poly-(3-hydroxybutyrate-*co*-3-hydroxybutyrate) production by *Alcaligenes eutrophus*. *Applied and Environmental Microbiology*, **63(3)**: 827-833.

Meng, C.J., Sadasivam, M. & Vigneswari, S. 2019. Isolation and identification of P(3HB-*co*-4HB) producing bacteria from various locations in Kuala Terengganu. *Malaysian Applied Biology Journal*, **48(1)**: 199-206.

Norhafini, H., Thinagaran, L., Shantini, K., Huong, K., Syafiq, I.M., Bhubalan, K. & Amirul, A.A. 2017. Synthesis of poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate) with high 4HB composition and PHA content using 1,4-butanediol and 1,6-hexanediol for medical application. *Journal of Polymer Research*, **24(11)**: 189.

Rahayu, A., Zaleha, Z., Yahya, A.R.M., Majid, M.I.A. & Amirul, A.A. 2008. Production of copolymer poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate-) through one-step cultivation process. *World Journal of Microbiology and Biotechnology*, **24(11)**: 2403-2409.

Steinbüchel, A. & Lütke-Eversloh, T. 2003. Metabolic engineering and pathway construction for biotechnological production of relevant polyhydroxyalkanoates in microorganisms. *Biochemical Engineering Journal*, **16(2)**: 81-96.

Syafiq, I.M., Huong, K.-H., Shantini, K., Vigneswari, S., Aziz, N.A., Amirul, A.A. & Bhubalan, K. 2017. Synthesis of high 4-hydroxybutyrate copolymer by *Cupriavidus* sp. transformants using one-stage cultivation and mixed precursor substrates strategy. *Enzyme and Microbial Technology*, **98**: 1-8.

Vigneswari, S., Rashid, N.S.B.T. & Amirul, A.A. 2019. Biodegradation of polyhydroxyalkanoates (PHA) films in soil and lake environment. *Malaysian Applied Biology*, **48(1)**: 193-198.