Production of Biopolymer Polyhydroxyalkanoates (PHA) by Extreme Halophilic Marine Archaea *Haloferax mediterranei* in Medium with Varying Phosphorus Concentration

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Abstract. The development of plastics production from biodegradable resources such as polyhydroxyalkanoates (PHAs) is important due to the increasing demand for plastics. PHAs occur as intracellular solid materials produced by microorganisms as a result of an excess of carbon source and a limitation of nutrients. Poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) or PHBV, as one of the PHAs, is a combination of two homopolymers, namely poly-3-hydroxybutyrates (PHB) and poly-3-hydroxyvalerates (PHV). This study aimed to investigate the effect of the phosphorus concentration on the production of PHAs by *Haloferax mediterranei*. It is possible that phosphorus deficiency may affect the length of the HV chains in the copolymer structure and thus influence the copolymer properties. The experiment was done in triplicate at laboratory scale by culturing *H. mediterranei* in medium with phosphorus limitation using various phosphorus concentrations. During cultivation, the optical density, phosphorus concentration, pH, and dry cell weight were observed. The PHBV product was collected and analyzed using gas chromatography. The result shows that medium with a phosphorus concentration of 0.5 g/L produced higher PHAs than the other phosphorus concentrations. The accumulated PHA was 0.95 g/L with 15.6% of dry biomass and yield $Y_{PHA/S}$ of 0.1 g/g.

Keywords: *biopolymer; bioplastic; Haloferax mediterranei; PHA; PHBV; phosphorus limitation; polyhydroxyalkanoate.*

1   Introduction

Today, the major raw material source of plastics manufacturing is the fossil fuel industry. Despite their favorable properties, such as being easily moldable, cheap and robust, oil-derived plastics also have disadvantages. The main problem is that they produce non-biodegradable waste that will become

recalcitrant. Around 22-43% of plastics worldwide go to the landfill as non-biodegradable waste [1,2]. In the future, oil-derived plastics will compete with the energy-generation industry because of petroleum depletion. Non-degradable plastics have contributed to environmental damage at a rate of 25 million tonnes per year [3-7]. Therefore, it is important to discover, develop and exploit alternative methods of plastics production from renewable resources to minimize the environmental impact. Biopolymers are green alternatives to oil-based plastics and are ideal for many applications, such as product packaging, medical, construction, biofuels and many more [8-12]. Polyhydroxyalkanoates (PHAs) are an example of bio-based biodegradable polymers (biopolymers) [13-15].

Poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) or PHBV, as one of the PHAs, is a combination of two homopolymers, namely poly-3-hydroxybutyrates (PHBs) and poly-3-hydroxyvalerates (PHV), that can be arranged as block copolymer (PHB-b-PHV), higher-order copolymer (O-PHBV) or random copolymer (R-PHBV) [16]. PHBV has been studied for biomedical applications that need specific mechanical features. The main challenges and limitations of PHA production are the complexity of the biosynthesis process, high production costs, and variation in product properties. The monomer ratio in PHAs affect the mechanical and physical properties of the biopolymer and thus will make it easier to meet the desired properties.

Koller, *et al.* [17], Bosco & Chiampo [18], and Bhattacharyya, *et al.* [19] have carried out research on *Haloferax mediterranei* to produce PHBV with various carbon sources (whey sugars, rice-based ethanol stillage, and dairy residues) and nitrogen limitation. Their results show that nutrient limitation does affect PHBV production. It is known that under phosphate limitation, *H. mediterranei* will produce PHA [20]. However, Lilo & Rodriguez-Valera [20] did not discuss the material properties of PHA. Their research has limitations since they did not vary the amount of PHB and PHV content in the PHA. Therefore, the present study attempted to propose PHBV biosynthesis under phosphorus limitation to find the optimal fermentation process with optimum yield, productivity and desired material properties. It is possible that a phosphorus deficiency alters the metabolic flux towards the HV precursors, thereby affecting the length of the HV chains in the copolymer structure and thus influencing the copolymer properties. In biomedical applications, PHBV should be in a homogeneous form to provide specific properties. Consequently, a method of tailor-formed PHBV biosynthesis should be observed.

The experiment was performed to understand the synthesis of PHA by *H. mediterranei* under phosphorus limitation. The first experiment was conducted with various phosphorus concentrations. It was expected that PHA accumulation increased with phosphorus deficiency in a fed-batch process.

2   Experiment

The experiment of PHA production consisted of the preparation of culture medium, PHA biosynthesis, PHA extraction, and PHA characterization. The analytical procedures were done in triplicate.

2.1   Medium preparation

All the glassware used in the experiment were sterilized in an autoclave at 121 °C for 20 minutes at 15 psig to reduce contamination. *H. mediterranei* DSM 1411 was obtained from ATCC and maintained in agar plates using the following media: NaCl 156 g/L, $MgCl_2 \cdot 6H_2O$ 13 g/L, $MgSO_4 \cdot 7H_2O$ 20 g/L, $CaCl_2 \cdot 6H_2O$ 1 g/L, KCl 4 g/L, $NaHCO_3$ 0.2 g/L, NaBr 0.5 g/L, yeast extract 5 g/L, glucose 10 g/L, and agar if required. The medium for cell culture used the same components but without agar.

The yeast extract in this medium acts as phosphorus and nitrogen source, which can be substituted with 0.5 g/L KH_2PO_4, 0.005 g/L $FeCl_3$, 2 g/L NH_4Cl and 1.25 mL/L trace element solution SL-6.

2.2   Variation of Phosphorus Concentrations

The second experiment used a variation of phosphorus concentrations. The phosphorus source in the second experiment came from potassium phosphate monobasic (KH_2PO_4). The second experiment was performed in 500 mL shake flasks containing 100 mL cultures. Fermentation shake flasks were inoculated with 10% (v/v) inoculum from the inoculum step. The fermentation process used the same medium as before, but a certain amount of phosphate salts (see Table 1), 0.005 g/L $FeCl_3$, 2 g/L NH_4Cl and 1.25 mL/L trace element solution SL-6, and 15 g/L buffer PIPES were added instead of yeast extract.

Table 1   Various concentrations of phosphorus sources in the experiment.

Flask code	$[KH_2PO_4]$ (g/L)
C	0.5
D	0.25
E	0.00375
L	Without phosphorus source

The cultures were incubated at 37 °C and 200 rpm for a week and observed. Every 6 hours samples were taken to measure optical density, while pH, phosphorus concentration, and glucose concentration sampling were done every 24 h.

2.3   PHA Extraction

The extraction of PHA produced by *H. mediterranei* is easier and faster when compared to other PHA producing organisms. *H. mediterranei* cells have high salinity content due to its natural growth environment and lyse in contact with distilled water.

After biomass was obtained from the fermentation process it was rinsed with distilled water for cell disruption and centrifuged at 13000 rpm for 5 min. The extraction process can be enhanced by adding sodium dodecyl sulphate (SDS) to digest the lipid and protein content of the disrupted cells [21]. The solution was then centrifuged at 13000 rpm for 5 min and washed with distilled water to remove the salts. The pellet of PHA was dried at 60 °C until it reached a constant weight.

2.4   Sample Analysis

Optical density was used to measure cell growth, using a UV-visible spectrophotometer. 1 mL of culture broth was placed in an Eppendorf tube and centrifuged at 13000 rpm for 5 min. The supernatant was discarded and the solid pellet was resuspended with 1 mL of washing solution (10% w/v of NaCl). Optical density was measured with a blank of washing solution as a comparison at $\lambda = 600$ nm [22-24]. Each sample was done in triplicate at a 6-hour interval.

Dry cell weight (DCW) estimation is a method to determine the growth rate of biomass in g/L. DCW was measured gravimetrically at a 24-hour interval in triplicate. The dry weight of the pellet was obtained by spinning 3 mL of culture broth at 13000 rpm for 5 min and the pellet was dried at 60 °C until it reached a constant weight.

The supernatant from the DCW measurement was used for measuring the pH of the culture broth. The pH of the culture broth was measured with a pH meter

every 24 hour. *H. mediterranei*, like most halophile archaea, grows on pH optima 7 [25].

The glucose concentration was measured every 24 hours using a glucometer by taking a sample of 1 mL culture in an Eppendorf tube and centrifuging it at 13000 rpm for 5 min. The supernatant was diluted with distilled water.

The phosphorus concentration of the culture was measured every 24 hours using the Vanadate-Molybdate method [26] by measuring the supernatant of the culture broth after centrifugation. The absorbance of the samples was measured at 470 nm using a UV-visible spectrophotometer.

The PHBV product in the dry cells and 3HV molar fraction of PHBV were analyzed using gas chromatography mass spectrometry (GC-MS) as described in Han, *et al.* [16]. Benzoic acid was utilized as quantitative estimation standard.

Scanning electron microscopy (SEM) was used to observe the uniformity of the PHBV particle size with a Hitachi SU 3500 (Hitachi) device. The PHBV samples were covered with gold dust prior to observation.

The thermal characterization of PHBV product was measured with thermogravimetry (TGA) and differential scanning calorimeter (DSC) using an STA PT1600 TG-DSC/DTA (Linseis) device. The temperature scanning was done from ambient temperature up to 600 °C at a rate of 5 °C/min in air atmosphere. The crystallization temperature (Tc), melting temperature (Tm) were measured successively at the top point of the crystal peak and the top point of the melting peak.

3 Results and Discussions

The experiment was aimed at investigating the effect of different phosphate concentrations on PHBV production and properties. *H. mediterranei* needs a carbon source and the presence of a nutrient source such as nitrogen and phosphorus to grow. There is a strong possibility that the *H. mediterranei* has slow growth at low concentrations of phosphorus salt.

Optical density measurement of the *H. mediterranei* culture was used to calculate the specific growth rate. The graphical determination of the growth rate can be plotted in a diagram, as shown in Figure 1. The specific growth rate was obtained from the slope of the linear regression of $Ln(X/X_0)$ against time. The maximum specific growth rate was obtained between 24 to 52.5 hours after inoculation. The growth rate started to decrease after 52.5 hours and reached the stationary phase after 70.5 hours. The maximum specific growth rate and

doubling time of *H. mediterranei* fermentation can be seen in Table 2. The best specific growth rate was obtained from the flask with a concentration of phosphorus source of 0.25 g/L. It had a specific growth of 0.061 h^{-1} and a doubling time of 11.3 hr. The doubling time is a valuable parameter for estimating the ability of a cell population to double. This parameter can also be used in a scaled-up process.
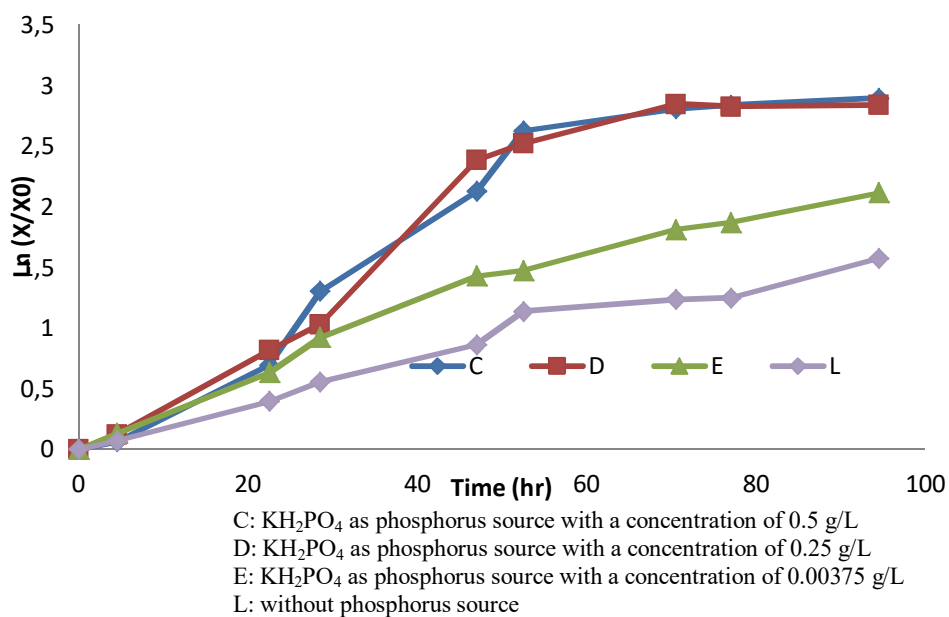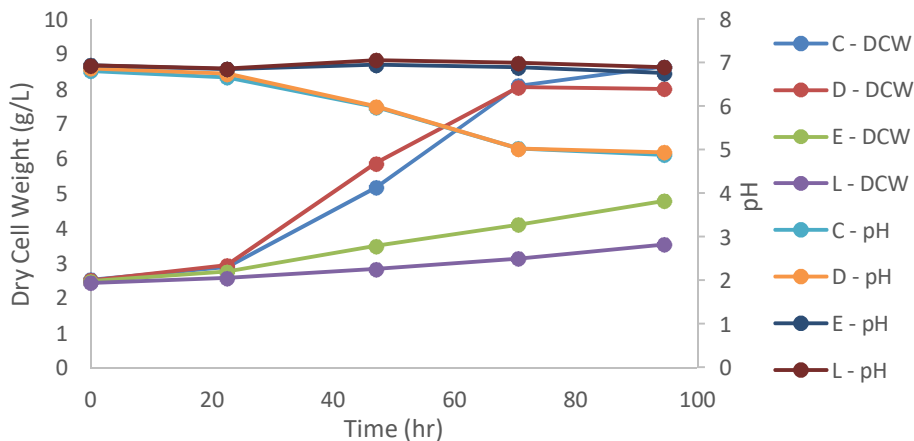

C: KH_2PO_4 as phosphorus source with a concentration of 0.5 g/L
D: KH_2PO_4 as phosphorus source with a concentration of 0.25 g/L
E: KH_2PO_4 as phosphorus source with a concentration of 0.00375 g/L
L: without phosphorus source

Figure 1 Graphical determination of the specific growth rate of *H. mediterranei* using optical density measurement for *H. mediterranei* culture at various phosphorus salt concentrations.

Table 2 Specific growth rate (μ) and doubling time (Td) of *H. mediterranei* culture with different phosphate concentrations.

| P Source	P Salt Concentration (g/L)	μ (h^{-1})	Td (hr)
KH_2PO_4	0.5	0.059	11.7
	0.25	0.061	11.3
	0.00375	0.029	23.7
No P source	-	0.021	33.6

C: KH$_2$PO$_4$ as phosphorus source with a concentration of 0.5 g/L
D: KH$_2$PO$_4$ as phosphorus source with a concentration of 0.25 g/L
E: KH$_2$PO$_4$ as phosphorus source with a concentration of 0.00375 g/L
L: without phosphorus source

Figure 2   Dry cell weight (DCW) of *H. mediterranei* fermentation and pH of cultures with various phosphorus salt concentrations.

Figure 2 shows the DCW during the fermentation process against time. In this experiment, the DCW during fermentation followed the optical density increment. In their experiment, Lillo & Rodriguez-Valera found that PHB started to accumulate with the increase of the biomass at logarithmic phase and reached its maximum at the start of the stationary phase [20]. After a lag phase at 24 hours after incubation, the amount of biomass proportional to the optical density increased, until it reached its maximum at 70.5 hours at the beginning of the stationary phase. PHA accumulation began at the log phase, increased with the dry cell weight but the cell growth was delayed.

The result of the pH measurement is shown in Figure 2. The culture medium started at adjusted pH 7, but after 22.5 hours the pH of the fermentation process of *H. mediterranei* dropped to 5.1 at 70.5 hours and remained stable for a certain time for flasks C and D. The phenomenon of pH decrease was captured by Rodriguez-Valera *et al.* [22], where the pH of a non-buffered liquid medium with glucose as a carbon source decreased from 7 to 5.8 after 24 hours of incubation. A possible explanation for this phenomenon is that the fermentation becomes slightly acidic, which is related to the metabolism of the cells during the fermentation process. The pH decrease could occur when acidic compounds are produced during fermentation. However, the culture with a low concentration of phosphorus (flasks E and L) did not experience this phenomenon, since the pH stabilized at approximately pH 7. It is almost certain

that at a very low growth rate, the acidic compounds produced during the fermentation process are very low and thus the pH of the culture is not affected or only slightly.
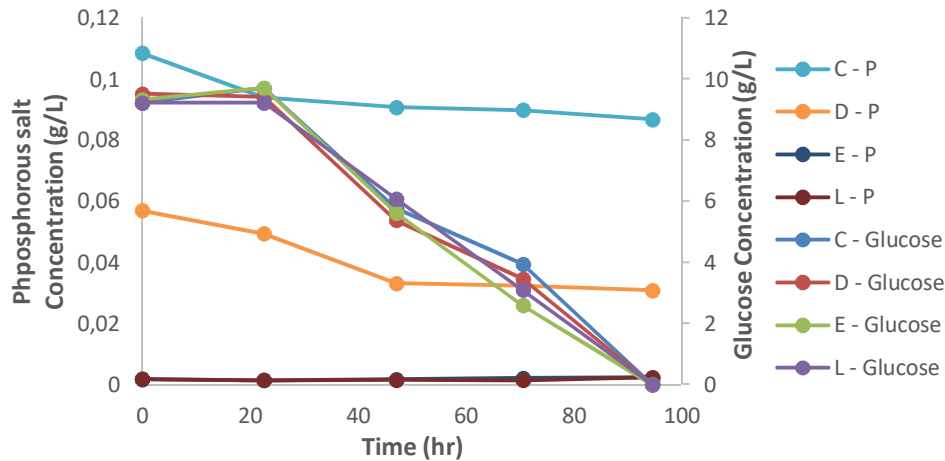
C: KH$_2$PO$_4$ as phosphorus source with a concentration of 0.5 g/L
D: KH$_2$PO$_4$ as phosphorus source with a concentration of 0.25 g/L
E: KH$_2$PO$_4$ as phosphorus source with a concentration of 0.00375 g/L
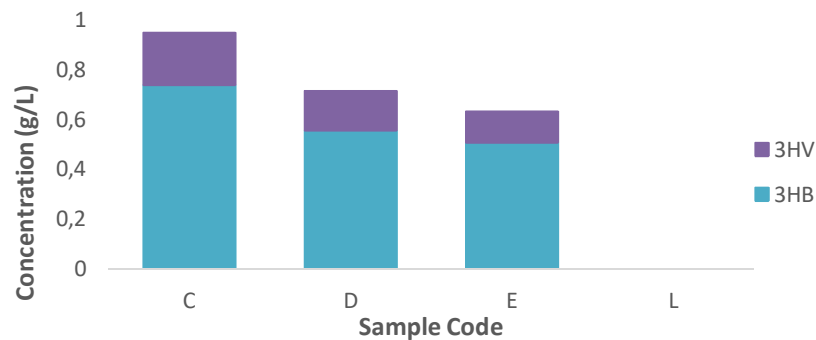L: without phosphorus source.

Figure 3 Glucose and phosphorous salt concentrations of *H. mediterranei* cultures.

The glucose concentration during fermentation in the second experiment is shown in Figure 3. The initial concentration was 10 g/L for every flask, however, the readings on the glucometer were not 10 g/L. It is likely that this was due to the limitations of the glucometer, which can only read a glucose concentration between 1.1 mmol/L to 33.3 mmol/L and is not entirely accurate for glucose measurement. However, the glucose concentration pattern in this experiment was similar to that in the previous research by Lillo & Rodriguez-Valera [20], where the glucose concentration decreased after 23 hours of incubation. However, in the experiment carried out by Lillo & Rodriguez-Valera [20], the glucose was completely consumed more rapidly, after 65 hours of incubation. This is probably because of the difference in initial glucose concentration. The *H. mediterranei* cell requires a sufficient amount of phosphorus to grow. However, even though the glucose requirement was

fulfilled, insufficient phosphorus will result in slow growth of *H. mediterranei* during the log phase [20]. Thus, it can lead to lower PHBV production, in accordance with the dry cell weight of the *H. mediterranei* culture.

The phosphorus concentrations in the culture of *H. mediterranei* for all flasks are shown in Figure 3. The phosphorus content in flask C decreased and remained stable after 23 hours. The phosphorus concentration of flask D also decreased from the start of the fermentation process until 47 hours and had a stable concentration of 0.031 g/L. The flasks with a low concentration of phosphorus or without a phosphorus source (E and L) had almost zero concentration of phosphorus.

The dried sample was analyzed using a GC-MS assay to measure the 3HB and 3HV fractions. The results of the GC-MS assay were calculated by comparing the peak area of the 3HB and 3HV fractions of the samples, with the peak area of 3HB and 3HV fractions of the standard sample. The product from the flask without phosphorus source (L) was undetectable by the GC-MS assay, maybe due to the very low amount of sample. The calculation results are shown in Figure 4.

C: KH_2PO_4 as phosphorus source with a concentration of 0.5 g/L
D: KH_2PO_4 as phosphorus source with a concentration of 0.25 g/L
E: KH_2PO_4 as phosphorus source with a concentration of 0.00375 g/L

Figure 4 PHBV distribution of *H. mediterranei* culture with various concentration of phosphorous salts.

From Figure 4, it can be seen that the cultures with a higher phosphorus concentration had a higher content of 3HB and 3HV fraction compared to the cultures with a lower concentration. The best results were obtained from flask C (KH_2PO_4 0.5 g/L) with 3HB, 3HV and total PHBV content at 0.74 g/L, 0.21 g/L, and 0.95 g/L respectively. According to Lillo & Rodriguez-Valera [20], a lower phosphate concentration results in lower PHB formation and a decrease in

growth. This is in accordance with this experiment's result, where the flasks with a low concentration of phosphate salts (E) gave poorer PHB accumulation. The GC-MS did not capture the PHBV from the culture without phosphorus source (flask L). This is likely due to the very low dry cell weight (0.29 g/L) compared to the other flasks, making it difficult to weigh.

Introducing hydroxyvaleric units to the PHB structure forms a copolymer poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) and therefore decreases its crystallinity, melting point and glass transition temperature [4]. As a result, PHBV is more flexible, with a higher elongation break. According to Jiang and Zhang [27], the crystallinity of the copolymer decreases with the increase of HV percentages, while the elongation to break increases along with the rise of HV percentages. Therefore, the product will be easier to process. The result from this experiment had higher HV percentages than the PHBV produced from *Escherichia coli* in the experiment done by Wang [28].

Table 3 Growth kinetics parameters and productivity of *H. mediterranei* fermentation for various phosphorus concentrations.

P source	Conc. (g/L)	Operation mode	μ (h^{-1})	Td (h)	ΔX (g/L)	ΔS (g/L)	PHA (g/L)	$Y_{X/S}$	$Y_{PHA/S}$	$Y_{PHA/X}$
KH$_2$PO$_4$	0.5	Batch	0.06	11.7	6.1	9.2	0.95	0.66	0.10	0.156
	0.25	Batch	0.06	11.3	5.5	9.5	0.72	0.58	0.08	0.130
	0.00375	Batch	0.03	23.7	2.3	9.3	0.64	0.25	0.07	0.276
No P	0	Batch	0.02	33.6	1.1	9.2	0.29	0.12	0.03	0.261

μ	: specific growth rate
Td	: doubling time
ΔX	: total biomass
ΔS	: total substrate
$Y_{X/S}$	: biomass yield with respect to substrate
$Y_{PHA/S}$	: PHA yield with respect to substrate
$Y_{PHA/X}$	: PHA yield with respect to biomass

The productivity for each phosphorous concentration in this experiment is shown in Table 3. The best specific growth rate (μ) was obtained from the concentration of phosphorus source 0.5 and 0.25 g/L, with μ of 0.06 h^{-1}. However, if the comparison is with the $Y_{PHA/S}$ yield, the best phosphorous source concentration was 0.5 g/L with the highest PHA accumulated at 0.95 g/L with 15.6% of dry biomass and a $Y_{PHA/S}$ yield of 0.10 g/g. Using KH$_2$PO$_4$ as phosphorus source, Lillo & Rodriguez-Valera [20] reported higher results, i.e. 3.09 g/L of PHA production and a yield of 0.309 g/g. A reasonable explanation

of the lower productivity compared to the product obtained from Lillo & Rodriguez-Valera [20] may be due to the different operation process chosen. Lillo & Rodriguez-Valera [20] used fed-batch culture, while this experiment used batch culture with phosphorous limitation from the beginning of the process. It is almost certain that a phosphorous limitation will reduce the growth rate of the culture. This result is comparable to the experiment done by Koller *et al.* [29], who reported an accumulated PHA of 0.21 g/L and a yield of 0.23 g/g with fed-batch culture.
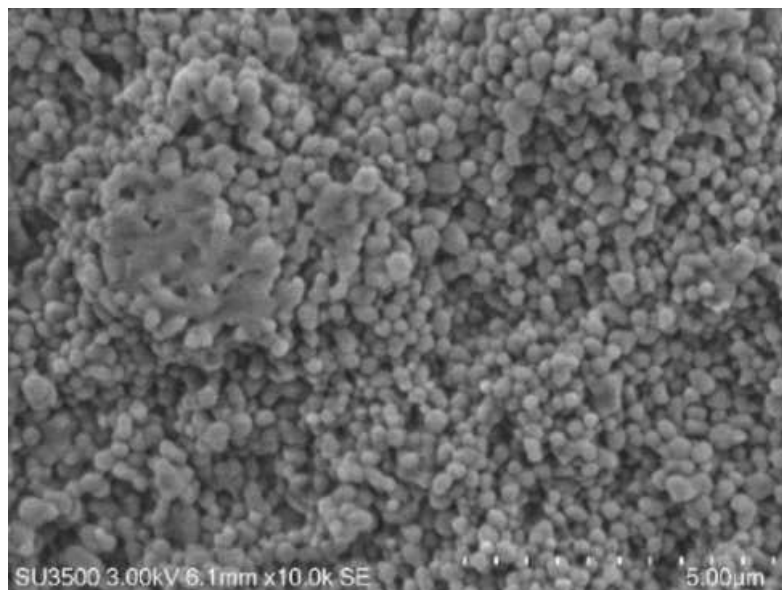

Figure 5 Figure 1 SEM images of PHBV with phosphorous salt concentration 0.5 g/L with 10,000x magnification.

The results from scanning electron microscopy (SEM) observation gained from the best concentration of phosphorous salt is shown in Figure 5. It shows that the PHBV particles produced from this experiment are quite uniform with an average particle size of 200-300 nm. This result is useful for application in biomedical applications where PHBV particles should be homogenous in order to provide specific properties. Leimann *et al.* [30] reported that the average diameters for HPHBV (PHBV with high molecular weight) and LPHBV (PHBV with low molecular weight) nanoparticles were 151 nm and 91 nm respectively, while for LPHBV and HPHBV the microparticle diameters were 33 μm and 58 μm, respectively. According to the study by Leimann *et al.* [30], based on the average size of the particles, the PHBV produced in this

experiment can be classified as PHBV nanoparticles with high molecular weight.
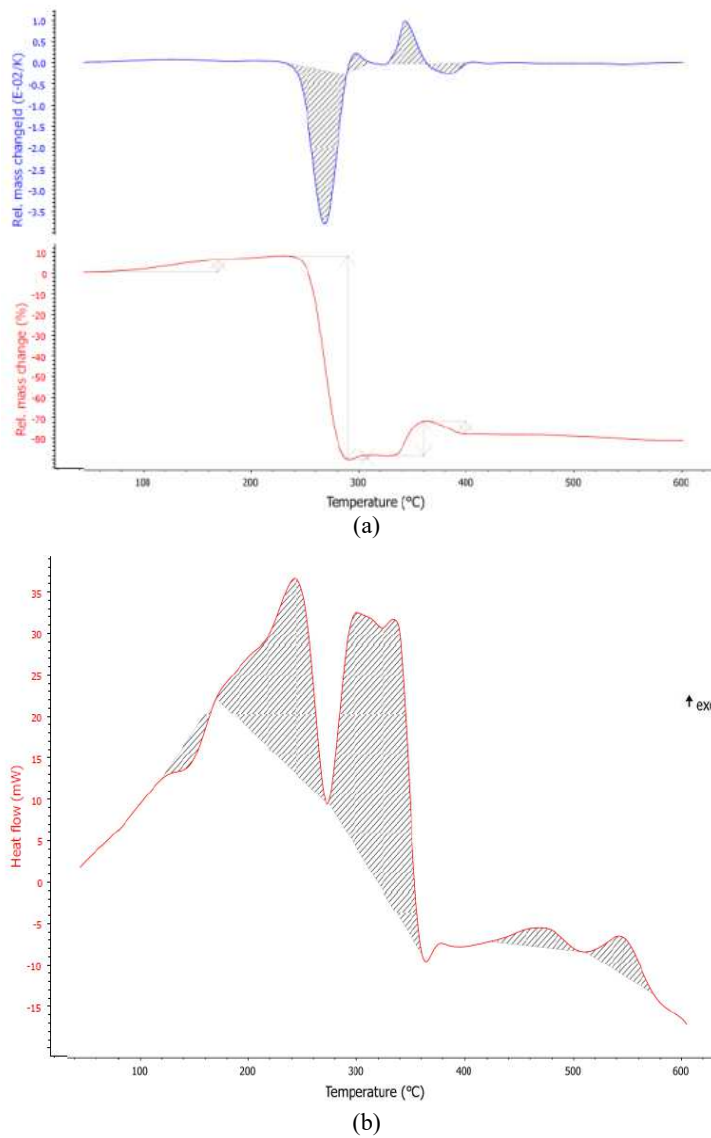

(a)

(b)

Figure 6 Thermal analysis of PHBV from *H. mediterranei*: (a) TGA/DTA and (b) DSC.

The thermal analysis of the PHBV particles in this experiment is shown in Figure 6. From the TGA/DTA graph it can be seen that the PHBV sample had its melting point at approximately 147 °C and the decomposition process started at 247 °C. This experiment gave a comparable result to previous studies, as can be seen from Table 4. From the DSC curve, the typical below initial thermal decomposition temperature can be considered negligible. The exothermic reaction after melting, followed by initial thermal decomposition and ended at the final thermal decomposition process indicated by the oxidation process of PHBV at relatively lower temperature compare to others, suggesting an easier biodegradation process. At higher temperature, the endothermic reaction was dominant since extra heat was required to break chemical bonds in the compound undergoing decomposition.

Table 4   Thermal properties of PHBV obtained in this experiment compared to previous studies.

Thermal properties	PHBV from *H. mediterranei* with P limitation	Wang, *et al.* [24]	Bouza, *et al.* [31]	Goncalves *et al.* [32]	Ojumu, *et al.* [33]
HV content (%)	22.36	16	12	6.2	25
T_m (°C)	147.4	150.18	143	167.7	137
T_{di} (°C)	247.0	266.6	n.r.	n.r.	n.r.
T_{do} (°C)	268.1	298.3	285	n.r.	n.r.
T_{df} (°C)	287.3	315.9	n.r.	n.r.	n.r.

T_m: melting point
T_{di}: temperature of initial thermal decomposition
T_{do}: onset temperature of thermal decomposition
T_{df}: temperature of final thermal decomposition
n.r.: not reported

4   Conclusions

The best result from this experiment was obtained from the culture with 0.5 g/L of KH_2PO_4 as phosphorus source. The accumulated PHA was 0.95 g/L with 15.6% of dry biomass and a $Y_{PHA/S}$ yield of 0.1. Medium with a higher concentration of phosphate salt gave higher PHA accumulation. This confirms the information from the literature that *H. mediterranei* needs phosphorus to grow. The PHA product yield obtained from this experiment was lower than in the literature. Fed-batch culture gives a higher product yield compared to batch culture, as can be found in the literature. With total phosphorus deficiency and excess of carbon source, *H. mediterranei* cells started to accumulate PHA.

With the slight difference in total PHBV production between the flask with the highest concentration of phosphorus source (flask C) and that with the second highest concentration (flask D), with half the concentration of phosphorus source, it can be concluded that it is more advantageous to use the second

highest concentration to produce PHBV. A lower concentration of phosphorus concentration makes less phosphorus required for the medium, thus incurring lower costs. However, the fermentation conditions need to be optimized to obtain maximum PHA accumulation.

Acknowledgments

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