AJSTR ASEAN JOURNAL OF SCIENTIFIC AND TECHNOLOGICAL REPORTS
ONLINE ISSN: 2773-8752

*Research article*

# Inexpensive Production of Poly (3-hydroxybutyrate-*co*-3-hydroxyvalerate) from *Bacillus megaterium* PP-10 Using Pineapple Peel Waste

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**Citation:**
Chanasit, W.; Bunkaew, K. Inexpensive production of Poly(3-hydroxybutyrate-co-3-] hydroxyvalerate)from *Bacillus megaterium* PP-10 using pineapple peel waste. *ASEAN J. Sci. Tech. Report.* **2023**, *26*(3), 36-44. https://doi.org/10.55164.ajstr.v26i3.249473.

**Article history:**
Received: May 12, 2023
Revised: June 22, 2023
Accepted: June 23, 2023
Available online: June 28, 2023



**Abstract:** Pineapple peel waste has recently been interested in being utilized as a low-cost carbon source in PHA biosynthesis to reduce the production cost of PHA. The production of copolymer Poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) [PHBV] by the new *Bacillus megaterium* PP-10 was investigated. The bacteria were grown in a mixture of pineapple peel hydrolysate (PPH) and 3-hydroxyvalerate (3HV) precursor such as sodium propionate or sodium valerate at ratios of 1:1. Remarkably, the microbial growth and PHBV production in a mixture of PPH and sodium valerate exhibited higher biomass and higher PHA amount than that of sodium propionate, accounted about 2.40 ± 0.07 g/L of DCW and 0.71 ± 0.03 g/L of PHA concentration (PHA content of 29.6%DCW). Moreover, to control the 3HV molar fraction in PHBV, various sodium valerate concentration from 2 to 18 g/L was supplemented with PPH, and the result showed that the 3HV fraction increased linear trend with an increase in valerate concentration and was in the range between 6-35 mol%HV. In contrast, a maximum PHA concentration of 1.65 ± 0.04 g/L content (about 49%DCW) was obtained when *B. megaterium* PP-10 was cultivated in 18 g/L of total reducing sugar in PPH with 2 g/L of sodium valerate at 12 h of cultivation. Finally, the produced PHBV containing 20 mol%HV was further determined by some thermal properties and found that it possessed the melting and glass transition temperatures of 148°C and -10°C, respectively. Therefore, PHBV synthesized by *B. megaterium* PP-10 with various 3HV fractions was an excellent choice for biopolymer production.

**Keywords:** Poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) [PHBV], Pineapple Peel Waste, Pineapple Peel Hydrolysate (PPH), Sodium valerate, *Bacillus megaterium*

## 1. Introduction

Polyhydroxyalkanoates (PHAs) are polyesters synthesized and accumulated in bacteria as intracellular carbon and energy storage compounds under unbalanced growth conditions such as nitrogen limitation. [1-3]. Due to its physicochemical properties similar to synthetic plastics, i.e., polyethylene and polypropylene, PHAs are also completely biodegradable and show

biocompatibility properties. It has been extensively studied [3-5].Poly-3-hydroxybutyrate (PHB) is the first discovered and most commonly found in bacteria. However, PHB shows brittleness and high crystallinity [1,2,6]. An introduction of 3-hydroxyvalerate (3HV) to produce a copolymer of poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) or PHBV exhibited superior properties by enhancing the biodegradability and reducing the crystallinity and melting point of the polymer resulted in better plasticity and more toughness [1-3]. The properties of the copolymers PHBV change depending on their HV molar fraction [1, 4-6]. PHBV is generally synthesized when the acid's organic acids or sodium salt form, i.e., sodium propionate or sodium valerate, is added into the culture medium [4, 7]. Notably, both 3HV substrate acts as direct precursor that triggers the formation of 3HV through the condensation of acetyl-coenzyme A and propionyl-coenzyme A molecules. In contrast, PHB was synthesized by condensing two molecules of acetyl-coenzyme A from a 3-hydroxybutyrate (3HB) precursor such as glucose [7-10].

Genus *Bacillus* has been reported previously. For example, *Bacillus cereus*, *Bacillus flexus*, *Bacillus aryabhattai* PHB10, and *Bacillus megaterium* were able to produce PHBV when fed with sugars or industrial wastes in the range of PHBV concentration of about 3.9-9.7 g/L with 2-84 mol% HV fraction [1]. However, the high production cost of PHA, which is more than 50% of the total cost is attributed to a carbon source, is a major obstacle for commercialized PHA; thus, the use of low-cost feedstock such as lignocellulosic waste is considered a good approach to reduce PHA production cost and at the same time can minimize waste disposal problem [7, 11]. Among these lignocellulosic waste, pineapple peel accounts for a significant part of waste accumulated in landfill which will further contribute to the release of greenhouse gases. However, with the rising pineapple demands worldwide such as Thailand that have been the largest exporter of pineapple products in the world with an estimation of the by-products from the canned pineapple industries based on the annual production year of 2020 account for more than 596,000 tons mainly, waste is pineapple peel waste (about 36%) followed by cores, stems, and crowns thus the abundance of pineapple peel waste and its disposal are a major concern currently[12, 13]. Other than the presence of protease in pineapple waste used for bromelain production and its high cellulose content, it is also a suitable substrate for the production of wine, vinegar, and organic acids, including use as a carbon source for supporting microbial growth due to its high sugar content, especially from the peel wastes [13, 14]. The highest reducing sugar concentration was obtained in pineapple peel waste compared with other feedstock, such as durian peel and sugarcane leaves, after being pretreated with 2.0%(w/v) sulfuric acid and autoclave [15]. The major components of pineapple peel consist of holocellulose, $\alpha$-cellulose, hemicellulose, and low lignin content. For sugar compositions in pineapple peel hydrolysate (PPH), there are various fermentable sugars, i.e., glucose, fructose, galactose, arabinose, and xylose, as previously reported by Sukruansuwan and Napathorn [14]. These sugars can be readily available carbon sources for microbial growth and PHBV production. Besides, PPH contains various nutrients such as vitamins, minerals, and trace elements. These nutrients can provide essential elements for microbial growth and metabolic processes, potentially enhancing PHBV production [13, 14, 16]. Therefore, the objective of this study is to investigate the biosynthesis of PHBV from newly isolated *Bacillus megaterium* PP-10 by using pineapple peel waste as an inexpensive carbon source and supplemented with 3HV-precursor to achieve high PHBV production with various 3HV molar fraction for broader applications.

## 2. Materials and Methods

### 2.1 Preparation of Pineapple Peel hydrolysate (PPH)

The pineapple peel waste (PPW) samples were collected from the pineapple plantation area, Pa Bon District, Phatthalung, Thailand [7°16′12″N 100°10′12″E] and were then dried in a hot air oven at 65°C followed by grinding and sieved to the particle sizes between 0.841-0.420 mm. Then, 10 g of fined PPW samples were acid-pretreated by using 1% (v/v) $H_2SO_4$ in an autoclave heating under 121°C at 15 psi for 30 min (method modified from Sukruansuwan and Napathorn 2018) for fermentable sugars production [14]. The obtained pineapple peel hydrolysate (PPH) was filtered through Whatman filter paper No. 1 and finally adjusted pH to 7.0. In addition, the PPH has further analyzed the total reducing sugar (TRS) amount by DNS [17] before use as a 3HB precursor.

**2.2 Bacterial strain and culture conditions**

The bacterial strain *Bacillus megaterium* PP-10 (accession no. OQ859945) used in this study was newly isolated from environmental soil and identified in our laboratory (16S rDNA sequence similarity, morphological and biochemical characteristics). For seed preparation, *B. megaterium* PP-10 was cultured in a basal culture medium (BCM) [18]. Then, 5%(v/v) seed was transferred to nitrogen-limiting mineral salt medium (MSM) consisting in g/L: $(NH_4)_2SO_4$, 1.0; $KH_2PO_4$, 2.0; $Na_2HPO_4$, 0.6; $MgSO_4 \cdot 7H_2O$, 1.0; 1 mL trace element [14, 18] supplemented with 1.0% (v/v) or 10 g/L of TRS in PPH and 10 g/L of 3HV precursor, i.e., sodium propionate (SP) or sodium valerate (SV). The pH of the medium was then adjusted to 7.0. The culture was incubated in a rotary shaker at 35°C and 200 rpm and the fermentation studies were conducted in 250 mL flasks with 50 mL culture medium for 48 h.

**2.3 Control of 3HV monomer composition**

Two different precursors of 3HV, such as sodium propionate (SP) or sodium valerate (SV), were compared in this study to achieve the high PHBV production with desired 3HV compositions by varying concentration of 3HB precursor, e.g., PPH and 3HV precursor from 2 to 18 g/L to reach a final concentration of carbon source at 20 g/L.

**2.4 Analytical procedures**

***2.4.1 Dry cell weight***

The cells were harvested by centrifugation (4,000x*g* for 10 min) followed by washing once with sterile distilled water and then lyophilized until constant cell weights were obtained. The dry cell weight (DCW) was calculated in g/L [5]

***2.4.2 PHA quantification***

Approximately 20 mg lyophilized cells were then subjected to methanolysis in the presence of 15% (v/v) sulphuric acid and 85%(v/v)methanol. The resulting methyl esters were then analyzed by gas chromatography (GC) analysis using poly(3-hydroxybutyric-*co*-3-hydroxyvaleric acid)(PHBV)(12% valerate, Sigma Aldrich) and benzoic acid as the external and internal standard, respectively to determine the PHA content and composition. The GC condition was performed according to the method described by Comeau et al. [18, 19]. The molar fraction of 3HV was calculated as this following equation; mol %3HV = [moles of 3HV/ total moles in PHBV] x 100 [1,5,10,19]

***2.4.3 Detection of PHA granule***

PHA accumulation was confirmed by transmission electron microscope (TEM) analysis. Briefly, the cell pellets were washed with a saline buffer (pH 7.2), and were then re-suspended in a 2.5% (v/v) glutaraldehyde solution overnight at 4°C before being fixed with 1.0% osmium tetroxide. The ultrathin sections were stained with uranyl acetate followed by lead citrate before viewing with JEOL JEM 2010F TEM (JEOL, Tokyo, Japan), with an accelerating voltage of 150-200 kV [20].

***2.4.4 Total reducing sugar***

DNS measured the total reducing sugar (TRS) concentration. Briefly, 500 µL of cell-free supernatant was added to 500 µL of the color reagent. These solutions were heated in boiling water for 10 min and immediately transferred to ice, and the absorbance was measured at 540 nm when the calibration curve was glucose [17].

**2.5 Thermal properties of PHA**

The thermal properties of the PHA sample were characterized by a differential scanning calorimeter (DSC) thermal analysis system (Perkin Elmer Pyris 1) in the range of -50 to 250°C at a heating rate of 20 °C/min. The glass transition temperature ($T_g$) and melting point temperature ($T_m$) were determined from the second scan of the DSC thermogram [21].

### 2.6 Statistical analysis

All the data represented the results of three independent experiments and were expressed as the mean values ± standard deviations (SD). The values were subjected to an independent t-test and values $p<0.05$ were taken as statistically significant [22]

## 3. Results and Discussion

### 3.1 Sugar analysis of Pineapple Peel hydrolysate (PPH)

The sugar compositions and concentrations in PPH were analyzed and it found that glucose was a significant component which reached about 1.51±0.02%(w/v) followed by fructose (1.30±0.01%w/v) and minor of sucrose and xylose (unpublished data). Moreover, the concentration of total reducing sugar (TRS) in PPH was determined and the result showed that the concentration of TRS in PPH was about 26.40±0.02 g/L. Similar to the previous study, the TRS concentration in PPH obtained in the range of 20 to 35 g/L after the pineapple peel residue was pretreated and showed that a major fermentable sugar in PPH was glucose followed by xylose, fructose, galactose, and arabinose, respectively [14]. However, the sugar compositions in PPH vary depending on the plant's age, growth conditions, soil conditions, geographic location, climate, and other environmental factors, such as temperature, stress, and humidity [13, 15].

### 3.2 The biosynthesis of PHBV from *Bacillus megaterium* PP-10

Incorporating 3HV monomer units into the PHBV polymer typically requires the addition of 3HV precursors, such as propionate or valerate, during the fermentation process. The only reported wild-type bacteria which can naturally synthesize PHBV from unrelated carbon sources like glucose are various species belonging to the Gram-positive genera *Nocardia* or *Rhodococcus* [4]. Thus in this study, sodium propionate or sodium valerate has to be supplemented in the production medium to enhance the availability of 3HV building blocks [1, 3-5]. The bacterial strain PP-10 investigated the PHBV copolymer production in the MSM culture medium containing 1.0% (v/v) of TRS in PPH and sodium propionate or sodium valerate at 10 g/L as mixed carbon sources. *B. megaterium* PP-10 produced the highest DCW and PHBV concentration of 2.40 ± 0.07 g/L (**Figure 1)** and 0.71 ± 0.03 g/L (about 29.6%DCW) after 12 h of cultivation (**Figure 2).** After 12 h, PHA content was likely to decrease along the cultivation time to maintain microbial growth.

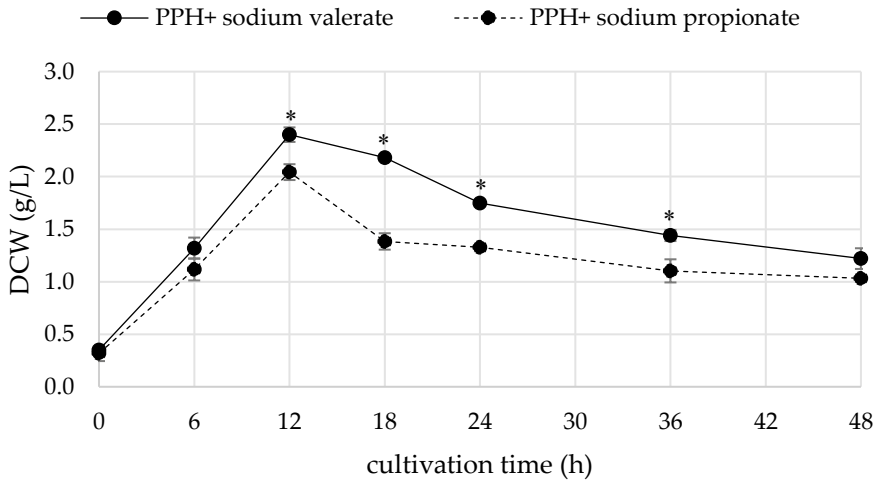


**Figure 1.** Cell growth (dry cell weight-DCW) produced by *B. megaterium* PP-10 when grown in the cells in PPH supplemented with sodium propionate or sodium valerate as 3HV-precursor for 48 h (* represents two independent groups were statistically significant, $p < 0.05$)

Interestingly, when growing, the cells in SV showed higher biomass than in SP. These results can be explained that the effect of propionic acid is more potent than valeric acid since carboxylic acids with shorter n-alkyl chains exhibit higher toxicity. In response to proton accumulation, free energy is released to expel protons out of cells to maintain the proton gradient [5, 10]. This excessive energy demand resulted in the decline of microbial activity, thus lowering the PHA yield. Therefore, valerate salt was identified as the better carbon substrate for synthesizing PHA compared to sodium propionate.

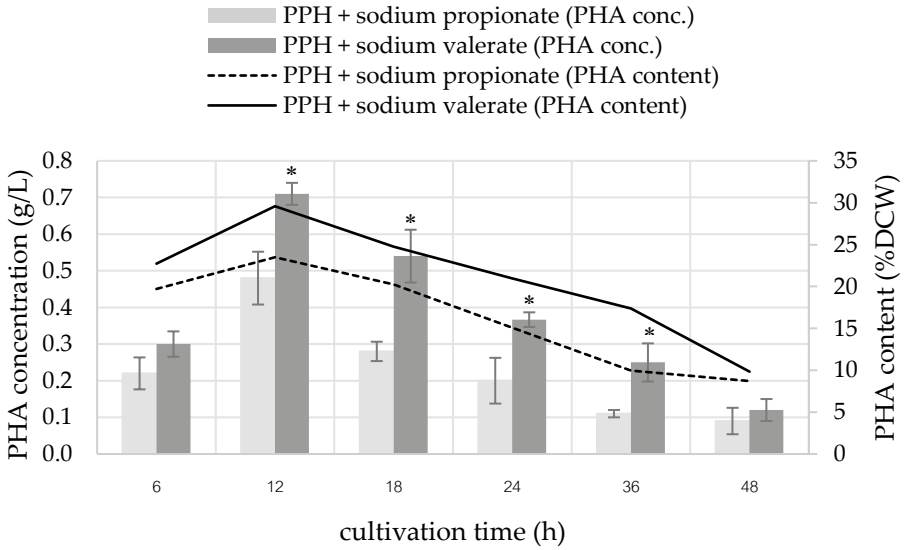


**Figure 2.** PHBV concentration (g/L) and the PHBV content (% DCW) produced by *B. megaterium* PP-10 when grown the cells in PPH supplemented with sodium propionate or sodium valerate as 3HV-precursor for 48 h (*represents two independent groups were statistically significant, $p < 0.05$)

However, incorporating 3HV precursors into the culture medium for PHBV biosynthesis is observed to reduce cell growth and PHBV content due to several factors, such as the toxicity and the induction of stress responses of 3HV precursors on microbial cells. In addition, 3HV precursors can disrupt the metabolic balance of the microbial culture through absent or insufficient enzymes responsible for the incorporation of 3HV monomers, resulting in inefficient utilization of the precursor [1, 5, 8]. This can divert cellular resources and energy toward stress mitigation. Moreover, adding 3HV precursors can introduce competition for carbon sources within the culture. For example, the 3HV precursor is metabolized preferentially over the primary carbon source, i.e., glucose, which may reduce cell growth and polymer content [4, 5, 10].

### 3.3 Control of 3HV monomer composition

The PHBV biosynthesis with different molar fractions of 3HV was produced when a mixture of 3HB-precursor (PPH) and 3HV-precursor (sodium valerate) was fed in MSM culture medium [1, 4, 5, 10]. To obtain the various 3HV molar fraction, the ratios between PPH and sodium valerate were varied from 0-20 g/L. **Table 1** demonstrated the biomass and PHBV production with different %mol of 3HV. A maximum DCW and PHA content of 3.38±0.01 g/L and 48.82%DCW were obtained when 18 g/L of PPH and 2 g/L of SV were used as mixed carbon sources. In contrast, the highest 3HV molar fraction of 35 mol% was produced when the cells were grown in 2 g/L of PPH and 18 g/L of SV, with the lowest cell growth and PHBV accumulation detected. This finding can indicate that *B. megaterium* PP-10 efficiently uptake and conversion of valerate into PHA [7, 8]. Therefore, a mixture of sodium valerate and PPH-containing glucose is a practical regulation of the 3HV molar fraction for tailor-made PHBV composition and properties. In many previous works, have been reported that genus *Bacillus*, for example, *Bacillus cereus*, *Bacillus flexus*, *Bacillus aryabhattai* PHB10, and *Bacillus megaterium*, were able to produce PHBV when fed with sugars or industrial wastes in the range of PHBV

concentration about 3.9-9.7 g/L with 2-84 mol% 3HV [1] as showed in **Table 2**. The PHBV amount in this study was the average of the PHBV content among the previous studies [7, 8, 23, 24].

**Table 1.** Biosynthesis of PHBV containing various 3HV compositions from *B. megaterium* PP-10 using a mixture of PPH and sodium valerate

| Precursor (g/L) | | DCW (g/L) | PHA concentration (g/L) | PHA content (%DCW) | Molar fraction | |
|---|---|---|---|---|---|---|
| PPH 3HB-precursor) | SV (3HV-precursor) | | | | 3HB | 3HV |
| 2 | 18 | 0.24 ± 0.01 | 0.03 ± 0.01 | 12.50 | 65 | 35 |
| 4 | 16 | 0.31 ± 0.02 | 0.05 ± 0.01 | 17.74 | 70 | 30 |
| 6 | 14 | 0.88 ± 0.02 | 0.18 ± 0.02 | 20.45 | 72 | 28 |
| 8 | 12 | 1.75 ± 0.02 | 0.37 ± 0.04 | 21.14 | 75 | 25 |
| 10 | 10 | 2.40 ± 0.07 | 0.71 ± 0.03 | 29.58 | 80 | 20 |
| 12 | 8 | 2.88 ± 0.05 | 0.88 ± 0.03 | 30.56 | 82 | 18 |
| 14 | 6 | 3.05 ± 0.02 | 1.12 ± 0.02 | 36.72 | 68 | 12 |
| 16 | 4 | 3.22 ± 0.01 | 1.44 ± 0.01 | 44.72 | 90 | 10 |
| 18 | 2 | 3.38 ± 0.01 | 1.65 ± 0.04 | 48.82 | 94 | 6 |

[a] Incubated in MSM for 12 h at 35°C, pH 7.0, 200 rpm

**Table 2.** PHBV production in various *Bacillus* sp.

| Bacterial strain | PHBV conc. (g/L) | PHBV content (%DCW) | 3HV (mol%) | References |
|---|---|---|---|---|
| *Bacillus megaterium* | 3.64 | 86.6 | 16.6 | [7] |
| *Bacillus aryabhattai* PHB10 | 2.8 | 71.15 | - | [8] |
| *Bacillus cereus* FA11 | 3.9 | 48.43 | 15 | [23] |
| *Bacillus flexus* | 4-9.7 | 32 | 2 | [24] |
| *Bacillus megaterium* PP-10 | 0.03-1.65 | 12.50-48.82 | 6-35 | This study |

### 3.4 Detection of PHA granule and Monomer characterization

To confirm the accumulation of PHA in *B. megaterium* PP-10, transmission electron microscopy was then examined for PHA granules in the bacterial cell, and the TEM micrograph was presented in **Figure 3**. Moreover, the monomer composition of the PHBV from *B. megaterium* PP-10 was identified by GC analysis. The GC-chromatogram of the tested PHBV consisted of 20 mol%HV, and 80mol%HB showed three significant peaks with retention times of 3.78, 4.58, and 5.61 min that referred to 3HB, 3HV, and benzoic acid (internal standard), respectively. This result corresponded with the PHBV standard presented in **Figure 4**. The PHA granule demonstrated about 30%DCW of its polymer content.

### 3.5 Thermal properties of PHBV

After the produced PHBV with 20 mol%HV was determined with DSC analysis, the result revealed that the melting temperature ($T_m$) and glass transition temperature ($T_g$)were 148°C and -10°C, respectively. This result was approximately equal to the PHBV reported by Policastro et al. (2021) [1] (**Table 3)**. The presence of $T_m$ and $T_g$ proves that the PHBV has both amorphous and crystalline regions [1, 2, 6, 7, 10]. Sudesh et al. [6] suggested that incorporating HV units in a homopolymer (PHB) reduced melting point, thereby increasing polymer flexibility and decreasing its brittleness. This property is advantageous for diverse applications such as cohesive and packaging [2, 3, 9]. PHBV with valerate fractions ranging from 8 mol% to 10 mol% is produced industrially using the bacterium *Ralstonia eutropha* [4].

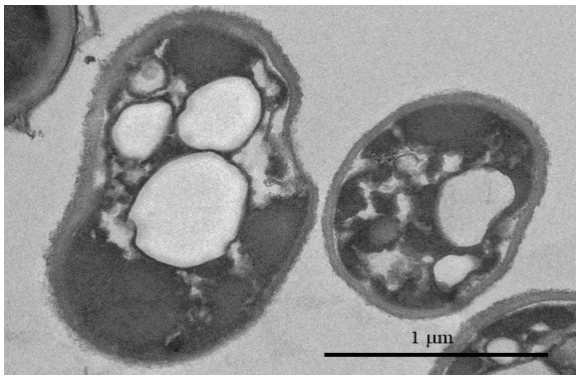


**Figure 3.** TEM images of *B. megaterium* PP-10 when grown in a mixture of PPH and sodium valerate for 12 h.

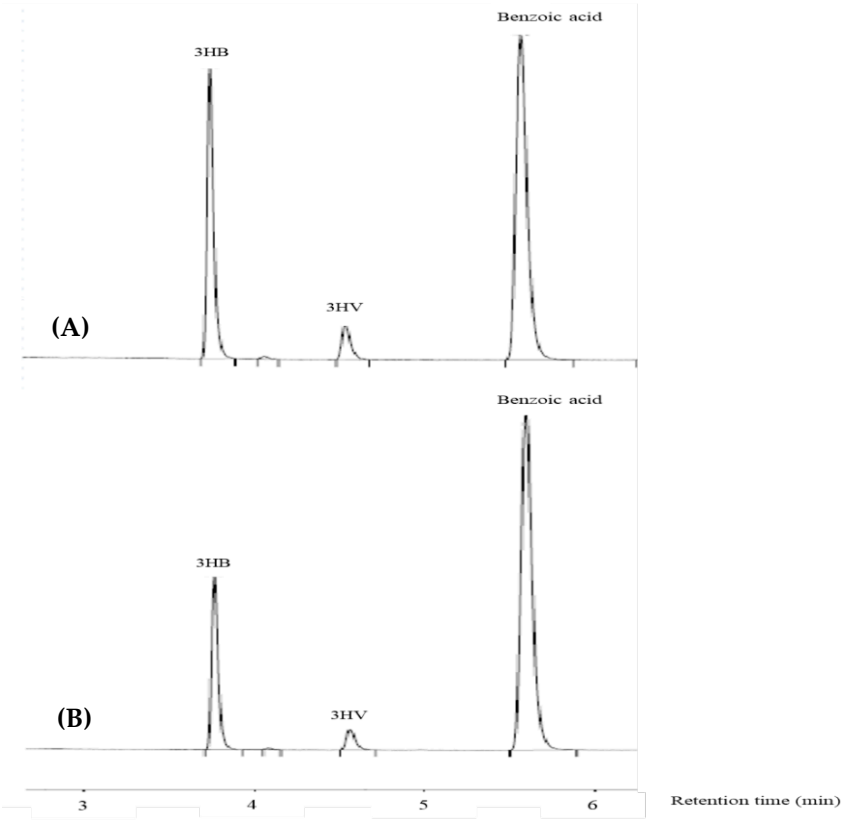


**Figure 4.** GC chromatogram of the poly (3-hydroxybutyrate-*co*-12 mol% 3-hydroxyvalerate) (A) PHBV with 20 mol%HV produced from *B. megaterium* PP-10 when cultivated the cells in PPH supplemented with sodium valerate at ratios of 1:1 for 12 h (B)

**Table 3.** Thermal properties of polyhydroxybutyrate (PHB) and poly(3-hydroxybutyrate-*co*-3-hydroxy valerate) (PHBV) and low-density polypropylene (LDPE)

| Polymer | melting temperature (°C) | glass transition temperature (°C) | reference |
|---|---|---|---|
| PHB | 180 | 4 | [1, 6, 9] |
| P(3HB-*co*-20mol%3HV) | 145 | -1 | [1, 3] |
| P(3HB-*co*-20mol%3HV) | 148 | -10 | This study |
| Low density polypropylene(LDPE) | 130 | -30 | [9] |

In this regard, *Bacillus megaterium* PP-10 is another attractive choice for the biosynthesis of copolymer PHBV from the low-cost feedstock. In addition, the tailored-made PHBV with various 3HV fractions was obtained in this study by varying the ratio of carbon substrate resulting in desired variations in the PHBV composition and properties. Moreover, the mechanical properties of the produced PHBV will be carried out.

## 4. Conclusions

In conclusion, the findings of this study demonstrate the successful accumulation of a copolymer of PHBV by *Bacillus megaterium* PP-10, reaching a significant concentration of 1.65 g/L. This copolymer accounted for 48.82% of the dry cell weight (DCW) and exhibited a 3HV molar fraction ranging from 6 to 35 mol%. The manipulation of 3HV-precursors enabled the regulation of different 3HV molar fractions, providing versatility in tailoring the polymer properties. Furthermore, the thermal properties of the produced copolymer, P3HB-*co*-20mol%3HV, were investigated. Notably, this copolymer exhibited a relatively low melting temperature (Tm) of 148°C and a glass transition temperature (Tg) of -10°C. These lower Tm and Tg values enhance polymer elasticity, making it more desirable for applications than PHB. Although the microbial growth and overall PHBV content achieved in this study are still relatively low, it is crucial to recognize the potential for further optimization of fermentation conditions. By fine-tuning various parameters such as the concentration of 3HV precursors, carbon source availability, carbon-to-nitrogen ratios, and the expression of relevant enzymes, as well as considering culture conditions and employing fermentation strategies like fed-batch cultivation, it is possible to overcome the current challenges and achieve higher cell growth and PHBV content. The pursuit of large-scale production of PHBV presents exciting opportunities for future research. By tackling the challenges mentioned above and exploring genetic engineering approaches, the potential for scaling up the production process becomes even more promising. These advancements would pave the way for the broader utilization of PHBV and its copolymers in various industrial applications, contributing to a more sustainable and eco-friendly future.

## 5. Acknowledgements

I would like to thank the Microbial Technology for Agriculture, Food and Environment Research Center, Faculty of Science, Thaksin University for supporting the laboratory facilities and equipment.

**Author Contributions**: Conceptualization, investigation, validation, and writing—original draft preparation, W.C.; methodology, investigation, and formal analysis, K.B.

**Funding**: National Higher Education, Science, Research and Innovation Policy Council, Thaksin University (Research project grant no. TSU-65A105000021) Fiscal Year 2022.

**Conflicts of Interest**: The authors declare no conflict of interest.

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