Asia-Pacific Journal of Science and Technology: Volume: 30. Issue: 02. Article ID.: APST-30-02-03. Research Article

APST

Asia-Pacific Journal of Science and Technology

https://www.tci-thaijo.org/index.php/APST/index

Published by Research Department,
Khon Kaen University, Thailand

Effect of carbon, nitrogen and phosphorus on PHAs produced from *Novosphingobium* sp. THA_AIK7 and its structure

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Received 6 March 2024
Revised 30 May 2024
Accepted 30 May 2024

Abstract

The production of polyhydroxyalkanoates (PHAs) by microorganisms usually occurs in response to environmental stress and limited nutrient supply. Different types of carbon, nitrogen, and phosphorus sources were investigated to achieve optimal PHAs productivity. *Novosphingobium* sp. THA_AIK7 was cultured in a mineral salt medium (MSM) with various carbon, nitrogen, and phosphorus sources. Crude glycerol, monosodium glutamate (MSG), and $Na_2HPO_4 \cdot 7H_2O$ were the best sources for growth and PHA production. Biomass and PHAs derived from these nutrient sources were maximized at 5.23 g/L and 1.44 g/L, respectively with maximum PHA content 27.54%. Biomass and polymer productivity peaked in media containing $Na_2HPO_4 \cdot 7H_2O$ at Q_x 0.073 ± 0.003 and Q_p 0.015 ± 0.000 g/L h. Experimental and predicted values from the logistic and Gompertz models trended in the same direction and accurately predicted microbial growth and PHA production. The extracted polymer structure was verified as a poly(-3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) copolymer by ^{1}H nuclear magnetic resonance (NMR) spectrometry analysis. The hydroxyvalerate (HV) monomer contents in the polymers derived from crude glycerol, fructose, and glycerol were 10.85, 10.16, and 6.59 mol%, respectively. Results indicated that *Novosphingobium* sp. THA_AIK7 produced a PHBV copolymer from various carbon sources without precursor addition.

Keywords: Carbon, Nitrogen, Polyhydroxyalkanoates, Phosphorus

1. Introduction

Plastic materials have become an integral part of contemporary life and are increasingly used due to their durability, simplicity of molding, and resistance to biodegradation [1]. Common plastics made from fossil fuels such as polyethylene, polypropylene, and nylon are xenobiotic and non-biodegradable, consequently causing environmental pollution [2, 3]. Replacing fossil plastics with bioplastics can solve the problem of environmental pollution [4]. Bioplastics can be broken down by microorganisms such as bacteria and fungi. Polyhydroxyalkanoates (PHAs) are among the most well-known bioplastics; they are biosynthetic, produce zero toxic waste, create no hazardous waste, and are completely recyclable into organic waste [5].

PHA intracellular polyesters are naturally produced by a variety of bacterial species [6]. Under nutrient-limiting conditions with plenty of carbon sources, bacteria store PHAs as their carbon and energy reserves. PHAs have recently attracted interest due to their biodegradability and biocompatibility. They were first discovered by Lemoigne (1926) [7] and the production process has continuously improved. The first commercial PHA was

launched by Imperial Chemical Industries (ICI) under the tradename Biopol® in early 1980 [8]. However, PHAs cannot compete economically in the plastic market because of their higher cost compared with petroleum-based plastics. Two bottlenecks in PHA production are the carbon source price and the recovery process [9]. Providing optimal levels of carbon supply and limited nitrogen availability in the culture medium during fermentation are the most crucial fermentation conditions for improving Polyhydroxybutyrate (PHB) production [10] but high substrate concentrations may impede microbial growth and impact total biomass and PHA generation rates [11].

Novosphingobium sp. THA_AIK7, a gram-negative bacterium isolated from biodiesel-contaminated wastewater [12] can produce PHAs from a low-price carbon source as crude glycerol waste from the biodiesel process. PHA biosynthesis by *Novosphingobium* sp. THA_AIK7 was the first report of endotoxin-free PHAs produced from gram-negative bacteria that lack lipopolysaccharide (LPS) in their outer membrane [12]. Therefore, polymers derived from *Novosphingobium* sp. THA_AIK7 showed promise as an alternative choice for biomedical applications.

The most important factors for optimal PHA synthesis are temperature, time, pH, and nutrients as carbon sources, nitrogen sources, and phosphorus sources [13]. This research improved PHA production yield from *Novosphingobium* sp. THA_AIK7 by optimizing carbon, nitrogen, and phosphorus sources. Growth and product were modeled, and the derived polymers were analyzed by nuclear magnetic resonance (NMR).

2. Materials and methods

2.1 Effect of carbon, nitrogen, and phosphorus on PHA production

Inoculum of *Novosphingobium* sp. THA_AIK7 was cultured in nutrient broth (NB) and incubated at 30°C, 150 rpm for 24 h [12]. One liter of mineral salt medium (MSM) was used for PHA production, excluding carbon, nitrogen, and phosphorus, consisting of KH_2PO_4 1.50 g, $MgSO_4 \cdot 7H_2O$ 0.2 g, ferrous ammonium citrate 0.06 g, $CaCl_2 \cdot 2H_2O$ 0.01 g, and 1 mL of trace element solution. One liter of trace element solution contained H_3BO_3 0.3 g, $CoCl_2 \cdot 6H_2O$ 0.2 g, $ZnSO_4 \cdot 7H_2O$ 0.1 g, $MnCl_2 \cdot 4H_2O$ 0.03 g, $NaMoO_4 \cdot 2H_2O$ 0.03 g, $NiCl_2 \cdot 6H_2O$ 0.02 g, and $CuSO_4 \cdot 5H_2O$ 0.01 g [14]. The initial pH of the medium was set at 7.

2.1.1 Optimal carbon source for PHA production

Ten percent inoculum of *Novosphingobium* sp. THA_AIK7 was added to 150 mL of MSM broth containing 10 g/L of various carbon sources (glucose, glycerol, fructose, sucrose, lactose, maltose, xylose, and crude glycerol), 1 g/L of monosodium glutamate (MSG), and 6.7 g/L of $Na_2HPO_4 \cdot 7H_2O$. Non-refining crude glycerol used in this experiment contained 469 g/L of glycerol [15]. The culture was incubated at 30°C with an agitation rate of 150 rpm for 96 h. Samples were taken for biomass and PHA determination, while the polymer composition of films cast from the selected condition was analyzed by NMR. The volumetric production rates of biomass (Q_x; g/L h) and product (Q_p; g/L h) were calculated from the start of the experiment until maximum biomass and product concentration were reached.

2.1.2 Optimal nitrogen source for PHA production

Ten percent inoculum of *Novosphingobium* sp. THA_AIK7 was added to 150 mL of MSM broth containing 10 g/L of optimized carbon source from 2.1.1, 1 g/L of various nitrogen sources ($(NH_4)_2SO_4$, NH_4Cl, urea, KNO_3, $NaNO_3$, and MSG), and 6.7 g/L of $Na_2HPO_4 \cdot 7H_2O$. The culture condition and analysis were similar to 2.1.1.

2.1.3 Optimal phosphorus source for PHA production

Ten percent inoculum of *Novosphingobium* sp. THA_AIK7 was added to 150 mL of MSM broth containing 10 g/L of optimized carbon source from 2.1.1, 1 g/L of optimized nitrogen source from 2.1.2, and 6.7 g/L of various phosphorus sources ($Na_2HPO_4 \cdot 7H_2O$, K_2HPO_4, and $(NH_4)_2HPO_4$. The culture condition and analysis were similar to 2.1.1.

2.2 Production kinetics

2.2.1 Biomass production kinetics

The logistic model was used to describe the growth of microorganisms during culture, and the relationship between growth and stable growth. The model presented the relationship between biomass (X) and initial cell concentration (X_0), maximum cell concentration (X_{max}), and maximum specific growth rate (μ_{max}) at a specific time (t), as shown in equation (1) [16].

$$x = \frac{X_0 \exp(\mu_{max} t)}{1-\left[\left(\frac{X_0}{X_{max}}\right)\left(1-\exp(\mu_{max} t)\right)\right]} \quad (1)$$

2.2.2 PHA production kinetics

The Gompertz model was used to describe the change in product concentration during culture for maximum product concentration (P_{max}), maximum product production rate (r_{max}), and lag time (t_L), as shown in equation (2) [16].

$$P = P_{max} \cdot \exp\left[-\exp\left(\frac{r_{max} \cdot \exp(1)}{P_{max}}\right)(t_L - t)+1\right] \quad (2)$$

The logistic and Gompertz models were used to fit the experimental data using Microsoft Excel and SigmaPlot 14.0 for non-linear regression analysis. Values of the fermentation parameters, as well as the statistical indicator for the coefficient of determination (R^2), were obtained directly from the software [17].

2.3 Analysis

2.3.1 Biomass yield

Two milliliters of cell suspension were centrifuged at 4°C and 6797 × g for 10 min. The supernatant was discarded and the cells were washed twice with distilled water, mixed well, and centrifuged again to reseparate the cell pellet before drying at 80°C until constant weight. The dried cell pellet was weighed and expressed as grams of biomass per liter of medium.

2.3.2 PHA yield

PHA-accumulating cells in a 10 mL sample were harvested following the above method. Five milliliters of 5% sodium hypochlorite were added to the centrifuge tube and incubated at 37°C for 1 h. The cell debris was cleaned twice with distilled water and then transferred to a glass bottle. Each glass bottle was added with 10 mL chloroform and 5 mL distilled water and incubated at 40°C for 6 h. The chloroform phase was transferred onto a glass plate, evaporated, and dried at 60°C for 24 h. The cast film was weighed and expressed as grams of PHA per liter of medium.

2.3.3 Sudan Black B staining

Cell suspension from 96 h cultivation was used for Sudan Black B staining to visualize PHA accumulation inside the bacterial cells under a compound microscope. Heat fixation of the bacterial cells was performed before Sudan Black B solution (0.3% (w/v) Sudan Black B in 70% ethanol) was dropped on the slide. After 10 min, the slide was washed with xylene, and safranin was dropped for another 45 sec. Dark blue spots scattered on the slide indicated that PHAs were stored inside the bacterial cells.

2.3.4 PHA characterization by NMR

The spectrum of the polymer synthesized by *Novosphingobium* sp. THA_AIK7 was analyzed by NMR. A 10 mg aliquot of the sample was dissolved in 1 mL deuterochloroform ($CDCl_3$) in a glass tube by heating at 45°C, with spectra recorded at 500 MHz [18] determined that characteristic peaks in the NMR spectrum at 0.9 ppm (HV unit) and 1.25 ppm (HB unit) could be utilized to calculate the HV composition in the poly(-3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) sample, as shown in equation (3).

$$\text{HV composition (\%)} = \frac{\text{Area}CH_3(\text{HV})}{\text{Area}CH_3\,(\text{HV})+\text{Area}CH_3(\text{HB})} \times 100\% \quad (3)$$

2.4 Statistical analysis

Significant differences were determined by one-way analysis of variance (ANOVA) at p-value < 0.05. The effect of each sample was defined by Duncan's multiple comparison test at p-value < 0.05 using SPSS version 15.

3. Results and Discussion

3.1 PHA granule staining

Preliminary identification of PHA lipid inclusions was carried out using Sudan Black B dye, a lipophilic dye that attaches to the ester bond in the polymeric chain of the PHB granule [19]. Dispersed dark blue spots of stained PHAs from cell cultivation in a medium containing crude glycerol, MSG and $Na_2HPO_4 \cdot 7H_2O$ for 96 h observed under a microscope for oil immersion at 1000x magnification indicated that PHAs occupied almost all the cell area, as shown in Figure 1. Results revealed that *Novosphingobium* sp. THA_AIK7 accumulated PHAs inside their cells under this condition. Yadav et al. (2021) found that under the appropriate condition, PHA accumulation as granules inside the cells varied from 1 to 90% of cell dry weight (CDW) [20].

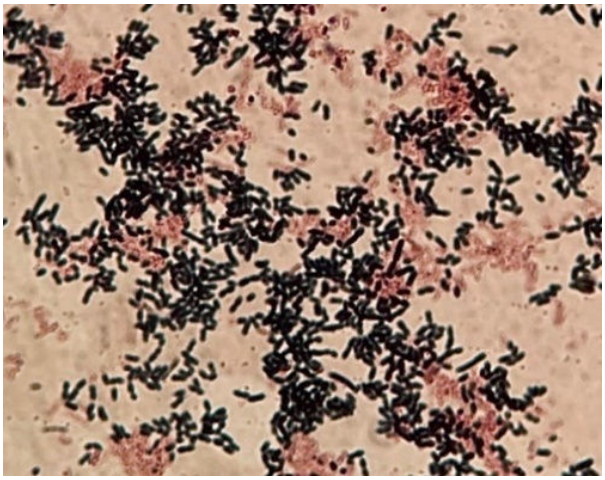

Figure 1 Sudan Black B staining of *Novosphingobium* sp. THA_AIK7 cultured in MSM for 96 h (magnification, 1000x).

3.2 Effect of medium composition on biomass and PHA production

To increase PHA yield, various carbon sources (10 g/L) namely glucose, glycerol, fructose, sucrose, lactose, maltose, xylose, and crude glycerol were added to the MSM with a 10% (v/v) inoculum and cultivated for 96 h at 30°C with shaking at 150 rpm. The carbon sources tested in this study impacted PHA production by *Novosphingobium* sp. THA_AIK7. Maximum and minimum biomass were obtained from crude glycerol and xylose at 4.41 and 1.20 g/L, while PHA concentrations were 1.01 and 0.01 g/L, respectively (Figure 2(A)). Glycerol obtained in soy-based biodiesel production (CSBP) was utilized as a substrate for bacterial growth, while free fatty acid (FFA) and fatty acid methyl esters (FAME) were good sources for PHA synthesis [21]. Poomipuk et al. [22] stated that lactose and sucrose, a disaccharide, were less preferable than monosaccharides such as glucose and fructose for PHA production [22], while *Cupriavidus* sp. KKU38 effectively utilized maltose, a disaccharide, and xylose, a monosaccharide for PHA production [22].

Novosphingobium sp. THA_AIK7 effectively exploited $(NH_4)_2SO_4$ and MSG for growth at 3.48 and 3.46 g/L, respectively whereas urea was less suitable because $(NH_4)_2SO_4$ served as a precursor for vitamins, amino acids, and growth factors [23]. The most favorable nitrogen source for PHA production was MSG at PHA concentration of 0.73 g/L, while urea was not appropriate for both biomass and PHA production (Figure 2(B)). These findings showed that PHA yield was not related to growth. PHA synthesis was enhanced by environmental stresses such as nitrogen, phosphate, or oxygen limitation [24]. Optimal biomass and polymer production in *Bacillus megaterium* was recorded using NH_4Cl in 2% sugarcane molasses at 1.27 g/L and 40.10% CDW, respectively [24]. Beaulieu et al. (1995) [25] investigated PHB production by *Alcaligenes eutrophus* in a synthetic 3% glucose medium supplemented with various ammonium substrates. They discovered that $(NH_4)_2SO_4$ provided optimal growth at 25.25 g/L and PHB yield of 43% CDW after 72 h of fermentation. Quillaguamán et al. (2008) [26] concluded that using MSG instead of glutamine in the medium resulted in a significant change in the final cell density of *Halomonas boliviensis*, with CDW of 44 g/L and PHB content of 81 wt% in fed-batch culture. Similar to carbon sources, each species preferred a different nitrogen source.

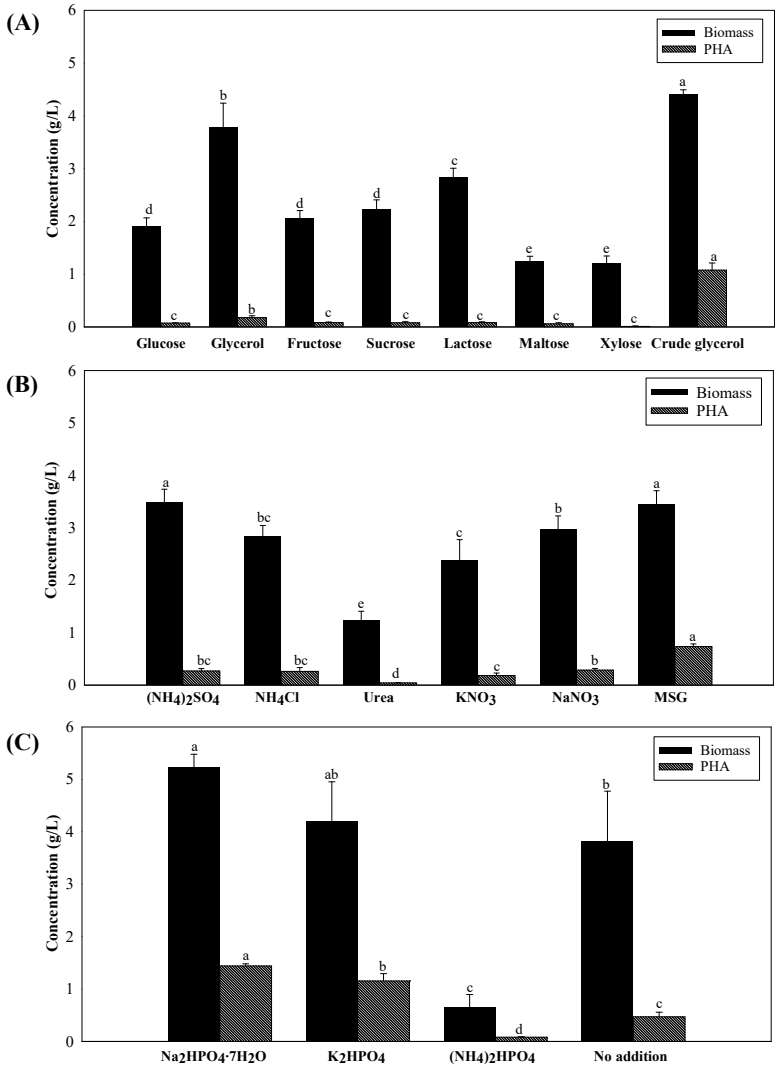

Figure 2 Influence of (A) carbon, (B) nitrogen, and (C) phosphorus sources on biomass production and PHA accumulation of *Novosphingobium* sp. THA_AIK7 at 96 h of cultivation. Data are represented as mean ± SD bar. Different superscripts indicate significant differences at p-value ≤ 0.05.

Three sources of phosphorus salts impacted biomass and PHA production that was maximized in media containing $Na_2HPO_4{\cdot}7H_2O$ at 5.23 and 1.44 g/L, respectively (Figure 2(C)), while $(NH_4)_2HPO_4$ was not suitable for biomass and polymer biosynthesis at 0.65 and 0.08 g/L, respectively. In the "No addition" treatment, the MSM contained KH_2PO_4 at 1.50 g/L as the phosphorus source. Therefore, phosphorus deficiency in the "No addition" treatment gave higher PHA accumulation than addition of $(NH_4)_2HPO_4$. Phosphorus deficiency yielded intracellular concentration of NADPH and stimulated PHA synthesis, while citrate synthase was inhibited in the TCA cycle resulting in higher PHA accumulation in cells [27]. Bustamantr et al. (2019) [28] found that higher concentrations of $(NH_4)_2HPO_4$ together with KH_2PO_4 reduced growth and PHB yield from *Caulobacter segnis* DSM 29236 due to the cytotoxic effect of ammonium on the cells. PHA accumulation in mixed microbial cultures was evaluated by Gao et al. (2021) [29] under various phosphorus sources. They determined Na_2HPO_4 as the best phosphorus source that increased 3HB and 3HO contents by 41% and 49%, respectively. Phosphorus supplements as $Na_2HPO_4.7H_2O$ and K_2HPO_4 improved both biomass and PHA content of the AIK7 strain as appropriate forms of nutrients for the cells.

The volumetric production rates of biomass (Q_x) and PHAs (Q_p) for the above treatments were calculated (Table 1). Crude glycerol gave the highest PHA content at 24.45% with xylose at only 0.90%. Highest Q_p was obtained from crude glycerol at 0.011 ± 0.001 g/L h, while highest Q_x was recorded from glycerol at 0.053 ± 0.006 g/L h, proving that the bacteria used glycerol for their growth. Maximum PHA content of the nitrogen source was achieved from MSG at 21.32%, with the minimum from urea at 3.49%. MSG and $(NH_4)_2SO_4$ recorded the same level of Q_x at 0.036 ± 0.003 g/L h, showing that the AIK7 strain used these two nitrogen sources equally for growth. Highest productivity of the polymer Q_p was gained from MSG at 0.008 ± 0.001 g/L h, while

$Na_2HPO_4 \cdot 7H_2O$ and $(NH_4)_2HPO_4$ gave maximum and minimum PHA contents at 27.54% and 12.31%, respectively. Biomass and polymer productivity peaked in media containing $Na_2HPO_4 \cdot 7H_2O$ at Q_x 0.073 ± 0.003 and Q_p 0.015 ± 0.000 g/L h. PHA production is a complex process, with the final quality and quantity of product yield depending on the strain, metabolic pathway involved, fermentation parameters, PHA production phase, carbon source, nitrogen source, phosphorus source, and nutrient depletion condition required for PHA synthesis [30]. Results suggested that the appropriate medium for growth and PHA production of *Novosphingobium* sp. THA_AIK7 contained crude glycerol, MSG, and $Na_2HPO_4 \cdot 7H_2O$ as carbon, nitrogen, and phosphorus sources, respectively. The variation in PHA content derived from each condition was low (0.90-27.54%) compared to the observed dark blue spots of stained PHAs in Figure 1 because the extraction process was unable to recover all the stored polymer inside the bacterial cells. Large-scale extraction achieved enhanced PHA recovery at up to 56.67% CDW by physical methods coupled with chloroform extraction (data not shown).

Table 1 Biomass and PHA production of *Novosphingobium* sp. THA_AIK7 under different conditions at 96 h of cultivation.

Condition	PHA content (%)	Q_x (g/L h)	Q_p (g/L h)
Carbon sources			
Glucose	3.87^{b}	0.020±0.002^{de}	0.001±0.000^{bc}
Glycerol	4.66^{b}	0.053±0.006^{a}	0.002±0.000^{b}
Fructose	4.04^{b}	0.021±0.002^{de}	0.001±0.000^{bc}
Sucrose	3.55^{b}	0.023±0.002^{d}	0.001±0.000^{bc}
Lactose	2.92^{bc}	0.030±0.002^{c}	0.001±0.000^{bc}
Maltose	4.77^{b}	0.017±0.001^{e}	0.001±0.000^{bc}
Xylose	0.90^{c}	0.017±0.002^{e}	0.000±0.000^{c}
Crude glycerol	24.45^{a}	0.046±0.001^{b}	0.011±0.001^{a}
Nitrogen sources			
$(NH_4)_2SO_4$	7.76^{b}	0.036±0.003^{a}	0.003±0.000^{b}
NH_4Cl	9.25^{b}	0.030±0.002^{bc}	0.003±0.001^{b}
Urea	3.49^{c}	0.017±0.002^{d}	0.000±0.000^{d}
KNO_3	7.66^{b}	0.025±0.004^{c}	0.002±0.000^{c}
$NaNO_3$	9.64^{b}	0.031±0.003^{ab}	0.003±0.000^{b}
MSG	21.32^{a}	0.036±0.003^{a}	0.008±0.001^{a}
Phosphorus sources			
$Na_2HPO_4 \cdot 7H_2O$	27.54^{a}	0.073±0.003^{a}	0.015±0.000^{a}
K_2HPO_4	27.41^{a}	0.058±0.010^{ab}	0.012±0.001^{b}
$(NH_4)_2HPO_4$	12.31^{b}	0.007±0.003^{c}	0.001±0.000^{d}
No addition	12.31^{b}	0.053±0.013^{b}	0.005±0.001^{c}

Different lowercase superscripts in the same column indicate significant differences at p-value ≤ 0.05.

3.3 Kinetic modeling of microbial growth and PHA production

The logistic and Gompertz models were used to simulate microbial growth and PHA production under various conditions to calculate the growth kinetics and polymer production rate [16, 17]. Non-linear regression analysis was performed using Microsoft Excel to determine the values of the cultivated parameters and the statistical indicator for the coefficient of determination (R^2). The microbial growth kinetics and PHA production from *Novosphingobium* sp. THA_AIK7 cultivated under different carbon, nitrogen, and phosphorus sources at 0-96 h, with estimated kinetics of biomass and PHA production obtained from both models are summarized in Table 2.

The simulation of microbial growth by the logistic model showed that crude glycerol, glycerol, and lactose had progressively increasing prediction at maximum predicted biomass (X_{max}) of 4.841, 3.939, and 2.969 g/L, respectively (Table 2). The predicted specific growth rate (μ_{max}) was highest at 0.104 h^{-1} from fructose, showing that the AIK7 strain used fructose for growth faster than the other carbon sources. The AIK7 strain also utilized glucose, the most common carbon source for bacterial growth, faster than crude glycerol, glycerol, and lactose with predicted μ_{max} of 0.094 h^{-1}, while μ_{max} values from these three carbon sources were 0.044, 0.070, and 0.062 h^{-1}, respectively. The predicted μ_{max} was maximized from fructose and glucose but the maximum predicted X_{max} reached only 1.597 and 1.781 g/L (Table 2) with Q_x only 0.021 and 0.020 g/L h, respectively (Table 1), indicating that these two monosaccharides were easily assimilated into the cell but might not be used directly for cell growth. Statistical analysis revealed that X_{max} and μ_{max} obtained from glucose and fructose were not significantly different. The simulated values of crude glycerol, glycerol, and lactose were close to the experimental data with R^2 0.984, 0.990, and 0.984, respectively (Table 2) indicating that this model could be used to explain the growth kinetics of *Novosphingobium* sp. THA_AIK7 under these conditions.

The Gompertz model was chosen to simulate PHA production. Crude glycerol had a progressively increasing prediction, with maximum predicted PHAs (P_{max}) of 1.200 g/L, while the simulation curve of xylose showed the least amount of PHAs with P_{max} at 0.014 g/L. Maximum production rate (r_{max}) was obtained from crude glycerol at 0.030 g/L h, with minimum r_{max} given by xylose, glucose, fructose, and maltose at 0.002 g/L h (Table 2). The start time for PHA production from crude glycerol was 44.5 h, with R^2 of 0.995 (Table 2). Xylose was not

appropriate for supporting growth or PHA production, with P_{max}, r_{max}, and Q_p of 0.014 g/L, 0.002 g/L h, and 0.000 g/L h, respectively.

The predicted logistic model of MSG, NH_4Cl, and $(NH_4)_2SO_4$ sharply increased and reached X_{max} peak at 13.500, 13.333, and 13.267 g/L, respectively (Table 2). Trends of the predicted lines and experimental lines of these nitrogen sources were in the same direction, with R^2 0.991, 0.986, and 0.979, respectively showing that the logistic model could be used to predict the growth of this bacteria. The $(NH_4)_2SO_4$ gave the maximum predicted μ_{max} at 0.026 h^{-1}, while MSG reached the second highest μ_{max} at 0.022 h^{-1}, exhibiting that the AIK7 strain quickly utilized these two sources of nitrogen for biomass generation. The predicted X_{max} and μ_{max} values of urea were lowest among all sources of nitrogen at 6.667 g/L and 0.011 h^{-1} (Table 2), corresponding with the lowest Q_x of 0.017 g/L h (Table 1) and suggesting that urea was not suitable for their growth.

The predicted PHAs from MSG steadily increased with P_{max} of 2.083 g/L, whereas urea produced the least amount with P_{max} of 0.052 g/L. Maximum and minimum production rates (r_{max}) were also obtained from MSG and urea at 0.016 and 0.001 g/L h, respectively (Table 2). The start time for PHA production of MSG was 50 h with R^2 of 0.995 (Table 2). The NH_4Cl and $(NH_4)_2SO_4$ showed support for growth, with high predicted X_{max}, while the P_{max} values were 0.283 g/L and 0.343 g/L, with r_{max} 0.004 and 0.004 g/L h (Table 2.), respectively. This result indicated that these two nitrogen sources were used for biomass generation in preference to product formation.

The predicted biomass from $Na_2HPO_4.7H_2O$, K_2HPO_4, and No addition increased with time. The retrieved biomass values from the model were 5.146, 4.833, and 3.900 g/L, respectively (Table 2). The R^2 values of 0.991, 0.940, and 0.977 from $Na_2HPO_4.7H_2O$, K_2HPO_4, and No addition revealed that the model results were similar to the experimental data (Table 2). The μ_{max} of $Na_2HPO_4.7H_2O$ peaked at 0.116 h^{-1}, while the predicted X_{max} and μ_{max} of $(NH_4)_2HPO_4$ were lowest at 0.770 g/L and 0.004 h^{-1}, concurring with the lowest Q_x of 0.007 g/L h (Table 1) and confirming that this phosphorus source was not preferred by this bacterium. The estimated kinetic results showed that the logistic model accurately predicted microbial growth under these conditions.

The predicted PHA values for $Na_2HPO_4{\cdot}7H_2O$ and K_2HPO_4 sharply increased and reached P_{max} of 3.367 g/L and 2.767 g/L, respectively. No addition and $(NH_4)_2HPO_4$ produced the lowest amount of PHAs with P_{max} 0.883 and 0.387 g/L, respectively. Maximum and minimum production rates (r_{max}) were obtained from $Na_2HPO_4{\cdot}7H_2O$ and $(NH_4)_2HPO_4$ at 0.035 and 0.001 g/L h, respectively (Table 2). The start time for PHA production from $Na_2HPO_4{\cdot}7H_2O$ was 50 h with R^2 of 0.965 (Table 2). Results indicated that $(NH_4)_2HPO_4$ was not suitable for both growth and product formation by this bacterium.

Table 2 Estimated kinetics of biomass and PHA production of *Novosphingobium* sp. THA_AIK7 under different conditions.

Condition	X_{max} (g/L)	P_{max} (g/L)	μ_{max}(h^{-1})	r_{max} (g/L h)	R^2 (Biomass)	R^2 (PHAs)
Carbon sources						
Glucose	1.781±0.030^{d}	0.079±0.008^{c}	0.094±0.009^{a}	0.002±0.001^{b}	0.950	0.812
Glycerol	3.939±0.467^{b}	0.400±0.100^{b}	0.070±0.013^{b}	0.003±0.001^{b}	0.990	0.891
Fructose	1.597±0.074^{de}	0.103±0.015^{c}	0.104±0.010^{a}	0.002±0.000^{b}	0.804	0.974
Sucrose	2.773±0.492^{c}	0.078±0.013^{c}	0.029±0.009^{d}	0.005±0.001^{b}	0.948	0.952
Lactose	2.969±0.119^{c}	0.083±0.015^{c}	0.062±0.009^{bc}	0.003±0.000^{b}	0.984	0.942
Maltose	1.416±0.210^{de}	0.090±0.056^{c}	0.040±0.013^{d}	0.002±0.001^{b}	0.917	0.776
Xylose	1.067±0.115^{e}	0.014±0.006^{c}	0.043±0.015^{cd}	0.002±0.002^{b}	0.872	0.525
Crude glycerol	4.841±0.439^{a}	1.200±0.200^{a}	0.044±0.006^{cd}	0.030±0.005^{a}	0.984	0.995
Nitrogen sources						
$(NH_4)_2SO_4$	13.267±1.079^{a}	0.343±0.093^{bc}	0.026±0.002^{a}	0.004±0.001^{b}	0.979	0.975
NH_4Cl	13.333±3.055^{a}	0.283±0.029^{bc}	0.019±0.002^{bc}	0.004±0.000^{bc}	0.986	0.971
Urea	6.667±0.289^{b}	0.052±0.003^{c}	0.011±0.001^{d}	0.001±0.000^{c}	0.611	0.995
KNO_3	13.200±0.346^{a}	0.450±0.278^{b}	0.017±0.001^{c}	0.004±0.001^{bc}	0.912	0.980
$NaNO_3$	11.333±1.041^{a}	0.400±0.087^{bc}	0.020±0.001^{b}	0.003±0.001^{bc}	0.941	0.909
MSG	13.500±1.732^{a}	2.083±0.333^{a}	0.022±0.002^{b}	0.016±0.004^{a}	0.991	0.995
Phosphorus sources						
$Na_2HPO_4{\cdot}7H_2O$	5.146±0.067^{a}	3.367±0.321^{a}	0.116±0.021^{a}	0.035±0.004^{a}	0.991	0.965
K_2HPO_4	4.833±0.577^{a}	2.767±1.168^{a}	0.045±0.000^{b}	0.034±0.003^{a}	0.940	0.981
$(NH_4)_2HPO_4$	0.770±0.139^{c}	0.387±0.032^{b}	0.004±0.002^{c}	0.001±0.000^{b}	0.318	0.953
No addition	3.900±0.656^{b}	0.883±0.362^{b}	0.103±0.023^{a}	0.007±0.002^{b}	0.977	0.841

Different lowercase superscripts in the same column indicate significant differences at p-value ≤ 0.05.

These simulation results of PHA production by the Gompertz model suggested that both the experimental and predicted data trended to go in the same direction, confirming that this model explained the product formation profile of *Novosphingobium* sp. THA_AIK7. He et al. (2022) [31] isolated *Novosphingobium percolationis* sp. nov. and *Novosphingobium huizhouense* sp. nov., from the leachate of a domestic waste treatment plant. They concluded that these two strains had broad carbon metabolic abilities and harbored the complete Embden-Meyerhof-Parnas pathway, citrate cycle, Entner-Doudoroff pathway, pentose phosphate pathway, and glyoxylate cycle for central carbon metabolism. Koller and Obruča (2022) [32] summarized PHA production from glycerol by various bacterial strain. They drew a metabolic pathway generating a pyruvate intermediate from glycerol uptake into the cell via glycolysis and Entner-Doudoroff pathway. This pyruvate intermediate was then converted

to central metabolite acetyl-CoA, a common precursor of PHA biosynthesis pathway. Free fatty acids (FFAs) and fatty acid methyl esters (FAMEs) contained in crude glycerol were other sources of PHA production via β-oxidation and fatty acid *de novo* biosynthesis [21, 33], explaining why *Novosphingobium* sp. THA_AIK7 produced more PHAs from crude glycerol than from other carbon sources.

3.4 NMR spectra of PHAs derived from various conditions

The extracted polymers from *Novosphingobium* sp. THA_AIK7 cultivated with different sources of carbon, nitrogen, and phosphorus were subjected to ^{1}H NMR spectrometry analysis. ^{1}H NMR peaks of each condition were detected at 0.9, 1.26, 2.6, and 5.2 ppm, as shown in Figure 3, corresponding to the absorption resonance of methyl (CH_3), methyl (CH_3), methylene (CH_2), and methane (CH) groups, respectively [34, 35]. The identical peaks of PHBV were designated as methyl group protons in HB and HV at 0.9 and 1.27 ppm, respectively. The detected signals resembled those from an earlier study of pure P(HB-co-HV) polymers [36, 37], proving that the extracted polymer from every condition was PHBV. The NMR spectra at 3.5-4.0 ppm revealed the presence of glycerol end-capping resonance [12, 38, 39], as shown in Figure 3(A) in crude glycerol, Figure 3(B) in KNO_3 and NH_4Cl, and Figure 3(C) in No addition and K_2HPO_4 used as medium composition.

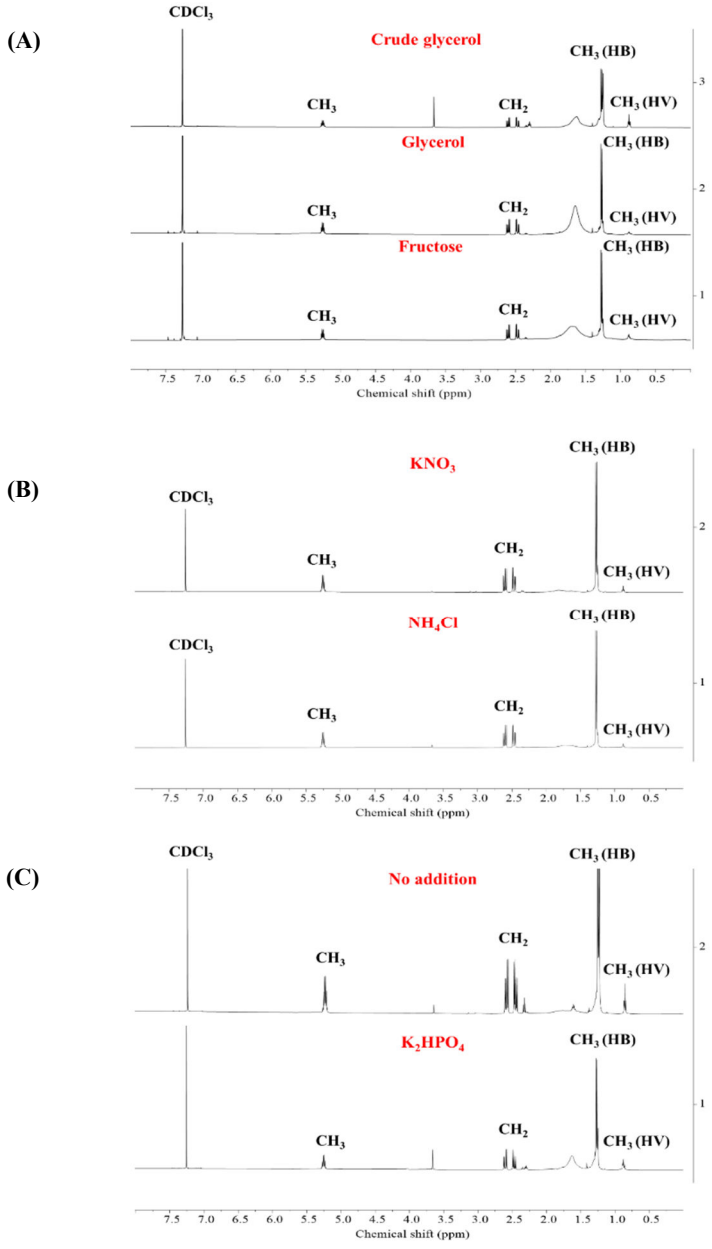

Figure 3 NMR spectra of PHAs produced under various conditions (A) carbon, (B) nitrogen, and (C) phosphorus sources.

Table 3 Peak area and mol% of PHBV extracted polymer under various conditions.

Carbon source	Nitrogen source	Phosphorus source	Peak area at 0.9 ppm	Peak area at 1.25 ppm	HV monomer (mol%)
Fructose	MSG	$Na_2HPO_4{\cdot}7H_2O$	1.59	14.06	10.16
Glycerol	MSG	$Na_2HPO_4{\cdot}7H_2O$	0.39	5.53	6.59
Crude glycerol	MSG	$Na_2HPO_4{\cdot}7H_2O$	1.61	13.23	10.85
Crude glycerol	NH_4Cl	$Na_2HPO_4{\cdot}7H_2O$	0.19	4.18	4.35
Crude glycerol	KNO_3	$Na_2HPO_4{\cdot}7H_2O$	0.27	4.81	5.32
Crude glycerol	MSG	K_2HPO_4	0.46	7.49	5.79
Crude glycerol	MSG	No addition	0.38	5.65	6.30

The peak areas of HB and HV were calculated to obtain HV mol% in each polymer extract, as shown in Table 3. Commercial PHBV polymer from Sigma-Aldrich contained HV units at 8% and 10% [40, 41], while PHBV produced from *Novosphingobium* sp. THA_AIK7 varied from 4.35-10.85 mol%. The highest PHBV percentage of 10.85 mol% was obtained from crude glycerol with MSG and $Na_2HPO_4.7H_2O$ in the medium formula, while the lowest percentage of 4.35 mol% was achieved from crude glycerol with NH_4Cl and $Na_2HPO_4.7H_2O$ in the medium. Many researchers have attempted to utilize the crude glycerol by-product from biodiesel for PHA production. *Halomonas taeanenisis* YLGW01 was selected for PHB production because of its high rate of glycerol consumption and tolerance to salt. This strain produced poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (P(3HB-co-3 HV)) with 17% 3 HV mol fraction when a precursor was added [42]. The biosynthesis of P(3HB-co-3HV) copolymer from waste glycerol and sodium valerate was tested by Kaddo et al. [43]. The 3HV incorporation was only 8 mol% after 24 h of precursor addition together with NH_4Cl [43]. PHB-producing halophile *Vibrio proteolyticus* isolated from Korean seas created PHBV with 15.8 mol% 3HV using co-substrates of propionic acid and fructose [44]. Contrasting with other research, the data in Table 3 indicated that *Novosphingobium* sp. THA_AIK7 utilized only crude glycerol, glycerol, or fructose to polymerize a PHBV copolymer without the addition of any precursor, suggesting that *Novosphingobium* sp. THA_AIK7 could produce a PHBV copolymer with high 3HV content from a low-priced carbon source.

4. Conclusions

This study optimized carbon, nitrogen, and phosphorus sources to increase PHA accumulation in *Novosphingobium* sp. THA_AIK7 by modeling the growth and production and analyzing the structure of the derived polymer by NMR. The appropriate medium for growth and PHA production of *Novosphingobium* sp. THA_AIK7 contained crude glycerol, MSG, and $Na_2HPO_4{\cdot}7H_2O$ as carbon, nitrogen, and phosphorus sources, respectively. The structure of the extracted polymer verified by ^{1}H NMR spectrometry revealed that *Novosphingobium* sp. THA_AIK7 produced a PHBV copolymer from different carbon sources without the addition of the HV monomer precursor.

5. Acknowledgments

We would like to express our grateful to Division of Biology, Faculty of Science and Technology, Rajamangala University of Technology Thanyaburi, and the Innovation and Medical Biotechnology Center (iMBC), Medical Life Sciences Institute, Department of Medical Sciences, Ministry of Public Health, Thailand, for technical support and providing scientific instruments in our research. This research was supported by the Thailand Science Research and Innovation (TSRI), Promotion Funding [Grant no. FRB660012/0168] managed under Rajamangala University of Technology Thanyaburi [FRB66E0621].

6. Reference

[1] Ali SS, Abdelkarim EA, Elsamahy T, Al-Tohamy R, Li F, Kornaros M, Zuorro A, Zhu D, Sun J. Bioplastic production in terms of life cycle assessment: A state-of-the-art review. Environ Sci Ecotechnol. 2023;15:100254.

[2] Mondal S, Subramaniam C. Xenobiotic Contamination of water by plastics and pesticides revealed through real-time, ultrasensitive, and reliable surface-enhanced raman scattering. ACS Sustain Chem Eng. 2020;8:639–648.

[3] Raza ZA, Abid S, Banat IM. Polyhydroxyalkanoates: Characteristics, production, recent developments and applications. Int Biodeterior Biodegradation. 2018;126:45–56.

[4] Dalton B, Bhagabati P, De Micco J, Padamati RB, O'Connor K. A review on biological synthesis of the biodegradable polymers polyhydroxyalkanoates and the development of multiple applications. Catalysts. 2022;12:319.

[5] Sirohi R, Prakash Pandey J, Kumar Gaur V, Gnansounou E, Sindhu R. Critical overview of biomass feedstocks as sustainable substrates for the production of polyhydroxybutyrate (PHB). Bioresour Technol. 2020;311:123536.

[6] Wang Y, Dao J, Chen GQ. Polyhydroxyalkanoate/Polyhydroxybutyrate. Comprehensive Biotechnology. Elsevier; 2019. p. 244–257.

[7] Lemoigne M. Produits de deshydration et de polymerisation de L'acide β=Oxybutyrique. Bull Soc Chim Biol. 1926;8:770–782.

[8] Palmeiro-Sánchez T, O'Flaherty V, Lens PNL. Polyhydroxyalkanoate bio-production and its rise as biomaterial of the future. J Biotechnol. 2022;348:10–25.

[9] Gonzalez K, Navia R, Liu S, Cea M. Biological approaches in polyhydroxyalkanoates recovery. Curr Microbiol. 2021;78(1):1–10.

[10] Zhang L, Jiang Z, Tsui TH, Loh KC, Dai Y, Tong YW. A Review on Enhancing *Cupriavidus necator* Fermentation for Poly(3-hydroxybutyrate) (PHB) Production From Low-Cost Carbon Sources. Front Bioeng Biotechnol. 2022;10:946085.

[11] Kaur G, Roy I. Strategies for large-scale production of polyhydroxyalkanoates. Chem Biochem Eng Q. 2015;29:157–172.

[12] Teeka J, Imai T, Reungsang A, Cheng X, Yuliani E, Thiantanankul J, Poomipuk N, Yamaguchi J, Jeenanong A, Higuchi T, Yamamoto K, Sekine M. Characterization of polyhydroxyalkanoates (PHAs) biosynthesis by isolated *Novosphingobium* sp. THA_AIK7 using crude glycerol. J Ind Microbiol Biotechnol. 2012;39(5):749–758.

[13] Hameed K, Gatea I, Hussein A. Biosynthesis and optimization of polyhydroxyalkanoate (PHA) production by *Acinetobacter lwoffii* isolate. J Appl Sci Nanotechnol. 2023;3(1):90–103.

[14] Ramsay BA, Lomaliza K, Chavarie C, Dubé B, Bataille P, Ramsay JA. Production of poly-(beta-hydroxybutyric-co-beta-hydroxyvaleric) acids. Appl Environ Microbiol. 1990;56(7):2093–2098.

[15] Rakkitkanphun C, Teeka J, Kaewpa D, Areesirisuk A. Purification of biodiesel-derived crude glycerol by acidification to be used as a carbon source for microbial oil production by oleaginous yeast. Biomass Convers Biorefin. 2023;13:15381-15391.

[16] Zajšek K, Goršek A. Modelling of batch kefir fermentation kinetics for ethanol production by mixed natural microflora. Food Bioprod Process. 2010;88(1):55–60.

[17] Dodić JM, Vučurović DG, Dodić SN, Grahovac JA, Popov SD, Nedeljković NM. Kinetic modelling of batch ethanol production from sugar beet raw juice. Appl Energy. 2012;99:192–197.

[18] Bloembergen S, Holden DA, Hamer GK, Bluhm TL, Marchessault RH. Studies of composition and crystallinity of bacterial poly(β-hydroxybutyrate-co-β-hydroxyvalerate). Macromolecules. 1986;19(11):2865–2871.

[19] Rathore Dr P. Screening of PHA producing bacteria from diverse sources. Int J Biosci. 2011;1:27–32.

[20] Yadav B, Talan A, Tyagi RD, Drogui P. Concomitant production of value-added products with polyhydroxyalkanoate (PHA) synthesis: A review. Bioresour Technol. 2021;337:125419.

[21] Ashby RD, Solaiman DKY, Foglia TA. Bacterial poly(hydroxyalkanoate) polymer production from the biodiesel co-product stream. J Polym Environ. 2004;12(3):105–112.

[22] Poomipuk N, Reungsang A, Plangklang P. Poly-β-hydroxyalkanoates production from cassava starch hydrolysate by *Cupriavidus* sp. KKU38. Int J Biol Macromol. 2014;65:51–64.

[23] Shenbagarathai R. Effect of nitrogen and calcium sources on growth and production of PHA of *Pseudomonas* sp. LDC-5 and its Mutant. Curr Res J Biol Sci. 2010;2(3):1664-167.

[24] Gouda MK, Swellam AE, Omar SH. Production of PHB by a *Bacillus megaterium* strain using sugarcane molasses and corn steep liquor as sole carbon and nitrogen sources. Microbiol Res. 2001;156(3):201–207.

[25] Beaulieu M, Beaulieu Y, Melinard J, Pandian S, Goulet J. Influence of ammonium salts and cane molasses on growth of *Alcaligenes eutrophus* and production of polyhydroxybutyrate. Appl Environ Microbiol. 1995;61(1):165–169.

[26] Quillaguamán J, Doan-Van T, Guzmán H, Guzmán D, Martín J, Everest A, Hatti-Kaul R. Poly(3-hydroxybutyrate) production by *Halomonas boliviensis* in fed-batch culture. Appl Microbiol Biotechnol. 2008;78(2):227–232.

[27] García G, Sosa-Hernández JE, Rodas-Zuluaga LI, Castillo-Zacarías C, Iqbal H, Parra-Saldívar R. Accumulation of PHA in the *Microalgae Scenedesmus* sp. under nutrient-deficient conditions. Polymers. 2021;13:131.

[28] Bustamante D, Segarra S, Tortajada M, Ramón D, Del Cerro C, Auxiliadora Prieto M, Iglesias JR, Rojas A. In silico prospection of microorganisms to produce polyhydroxyalkanoate from whey: *Caulobacter segnis* DSM 29236 as a suitable industrial strain. Microb Biotechnol. 2019;12(3):487–501.

[29] Gao M, Li Y, Ma X, Li D, Li J. Biotransformation of palm oil wastewater to scl- and mcl-polyhydroxyalkanoates by mixed microbial consortia using different nitrogen and phosphorus sources. Biomass Convers Biorefin. 2023;13:12961–12973.

[30] Divyashree MS, Rastogi NK, Shamala TR. A simple kinetic model for growth and biosynthesis of polyhydroxyalkanoate in *Bacillus flexus*. N Biotechnology. 2009;26(1):92–98.

[31] He X, Lu H, Hu W, Deng T, Gong X, Yang X, Song D, He M, Xu M. *Novosphingobium percolationis* sp. nov. and *Novosphingobium huizhouense* sp. nov., isolated from landfill leachate of a domestic waste treatment plant. Int J Syst Evol Microbiol. 2022;72(5):005394.

[32] Koller M, Obruča S. Biotechnological production of polyhydroxyalkanoates from glycerol: A review. Biocatal Agric Biotechnol. 2022;42:102333.

[33] Liu MH, Chen YJ, Lee CY. Characterization of medium-chain-length polyhydroxyalkanoate biosynthesis by *Pseudomonas mosselii* TO7 using crude glycerol. Biosci Biotechnol Biochem. 2018;82(3):532–539.
[34] Kumar M, Singhal A, Verma PK, Thakur IS. Production and characterization of polyhydroxyalkanoate from lignin derivatives by *Pandoraea* sp. ISTKB. ACS Omega. 2017;2(12):9156–9163.
[35] Sriyapai T, Chuarung T, Kimbara K, Samosorn S, Sriyapai P. Production and optimization of polyhydroxyalkanoates (PHAs) from *Paraburkholderia* sp. PFN 29 under submerged fermentation. Electron J Biotechnol. 2022;56:1–11.
[36] Lau N-S, Sam K-K, Amirul AA-A. Genome features of moderately halophilic polyhydroxyalkanoate-producing *Yangia* sp. CCB-MM3. Stand Genomic Sci. 2017;12:12.
[37] Tao J, Song C, Cao M, Hu D, Liu L, Liu N, Wang S. Thermal properties and degradability of poly(propylene carbonate)/poly(β-hydroxybutyrate-co-β-hydroxyvalerate) (PPC/PHBV) blends. Polym Degrad Stab. 2009;94(4): 575–583.
[38] Ashby RD, Solaiman DKY, Strahan GD. Efficient utilization of crude glycerol as fermentation substrate in the synthesis of poly(3-hydroxybutyrate) biopolymers. J Am Oil Chem Soc. 2011;88(7):949–959.
[39] Zhu C, Nomura CT, Perrotta JA, Stipanovic AJ, Nakas JP. Production and characterization of poly-3-hydroxybutyrate from biodiesel-glycerol by *Burkholderia cepacia* ATCC 17759. Biotechnol Prog. 2010;26(2):424–430.
[40] Adamus G, Sikorska W, Janeczek H, Kwiecień M, Sobota M, Kowalczuk M. Novel block copolymers of atactic PHB with natural PHA for cardiovascular engineering: Synthesis and characterization. Eur Polym J. 2012;48(3):621–631.
[41] Patel M, Gapes DJ, Newman RH, Dare PH. Physico-chemical properties of polyhydroxyalkanoate produced by mixed-culture nitrogen-fixing bacteria. Appl Microbiol Biotechnol. 2009;82(3):545–555.
[42] Kim B, Oh SJ, Hwang JH, Kim HJ, Shin N, Bhatia SK, Jeon JM, Yoon JJ, Yoo J, Ahn J, Park JH, Yang YH. Polyhydroxybutyrate production from crude glycerol using a highly robust bacterial strain *Halomonas* sp. YLGW01. Int J Biol Macromol. 2023;236:123997.
[43] Al-Kaddo KB, Sudesh K, Samian MR. Screening of bacteria for PHA production using waste glycerol as carbon source and the ability of new strain to produce P(3HB-co-3HV) copolymer. Malays J Microbiol. 2016;12(3):245-253
[44] Hong JW, Song HK, Moon YM, Hong YG, Bhatia SK, Jung HR, Choi TR, Yang SY, Park HY, Choi YK, Yang YH. Polyhydroxybutyrate production in halophilic marine bacteria *Vibrio proteolyticus* isolated from the Korean peninsula. Bioprocess Biosyst Eng. 2019;42(4):603–610.