Electronic Journal of Biotechnology 56 (2022) 1–11

Contents lists available at ScienceDirect

Electronic Journal of Biotechnology

journal homepage:

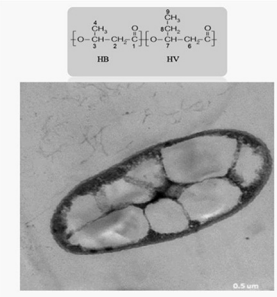
Research Article

Production and optimization of polyhydroxyalkanoates (PHAs) from *Paraburkholderia* sp. PFN 29 under submerged fermentation

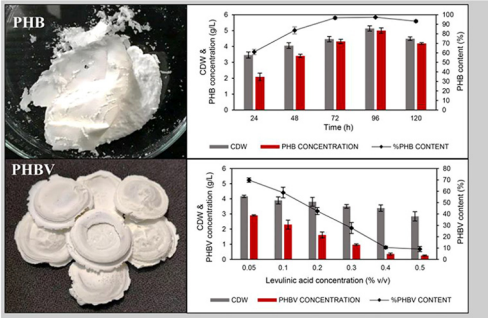
Thayat Sriyapai [a,b], Thitima Chuarung [c], Kazuhide Kimbara [d], Siritron Samosorn [e], Pichapak Sriyapai [b,f,*]

[a] *Faculty of Environmental Culture and Ecotourism, Srinakharinwirot University, Watthana, Bangkok 10110, Thailand*
[b] *Center of Excellence in Biosensors, Srinakharinwirot University, Watthana, Bangkok 10110, Thailand*
[c] *Department of Biology, Faculty of Sciences, Srinakharinwirot University, Watthana, Bangkok 10110, Thailand*
[d] *Department of Engineering, Graduate School of Integrated Science and Technology, Shizuoka University, Hamamatsu 432-8561, Japan*
[e] *Department of Chemistry, Faculty of Sciences, Srinakharinwirot University, Watthana, Bangkok 10110, Thailand*
[f] *Department of Microbiology, Faculty of Sciences, Srinakharinwirot University, Watthana, Bangkok 10110, Thailand*

GRAPHICAL ABSTRACT



Accumulation of intracellular-like PHAs granules in *Paraburkholderia* sp. PFN29



Optimization of PHAs production under submerged fermentation

ARTICLE INFO

Article history:
Received 15 April 2020
Accepted 21 December 2021
Available online 28 December 2021

Keywords:
Biodegradable plastics
Bioplastics
Optimization
Paraburkholderia sp.
Polyhydroxyalkanoates
Polyhydroxybutyrate
Polyhydroxybutyrate-co-3-polyhydroxyvalerate
Soil samples
Submerged fermentation

ABSTRACT

Background: Polyhydroxybutyrate (PHB) and polyhydroxybutyrate-*co*-hydroxyvalerate (PHBV) are the most common polyhydroxyalkanoates (PHAs). They are important candidates for biodegradable plastics that accumulate in the cytoplasm in the form of intracellular granules under unbalanced growth conditions. The present study focused on the production and optimization of PHAs from *Paraburkholderia* sp. PFN29 for the first time under submerged fermentation.

Results: Two hundred bacterial isolates were collected from soil samples in Thailand, six of which were found to accumulate PHB using the Nile red staining method. Six isolates were identified by 16S rDNA gene sequencing and were shown to be closely related to the genera *Azotobacter*, *Sphingobium* and *Paraburkholderia*. The best strain, *Paraburkholderia* sp. PFN29, showed the highest PHB concentration (5 ± 0.17 g/L). Optimization of PHB accumulation was achieved in modified medium containing 3% (w/v) glucose, and 0.1% (w/v) $(NH_4)_2Fe(SO_4)$ with incubation at pH 7.0 and 35°C for 96 h with shaking at 200 rpm. In preliminary PHBV synthesis, PFN29 was utilized to synthesize PHBV from glucose and organic acids (levulinic acid and propionic acid). ^{1}H nuclear magnetic resonance (^{1}H NMR) spectroscopy and gas chromatography-mass spectrometry (GC/MS) analyses confirmed the extracted PHAs compared to standard PHB and PHBV.

Conclusions: PFN29 can be used for feasible PHB production with a PHB content of up to 97.3% of 5.14 ± 0.17 g/L CDW. The PHBV concentration can be further improved by optimizing the production parameters

Peer review under responsibility of Pontificia Universidad Católica de Valparaíso

* Corresponding author.
E-mail address: peechapack@g.swu.ac.th (P. Sriyapai).

https://doi.org/10.1016/j.ejbt.2021.12.003
0717-3458/© 2022 Published by Elsevier B.V. on behalf of Pontificia Universidad Católica de Valparaíso.
This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

as cosubstrates. The present study provides useful data on PHA production by *Paraburkholderia*, which may be used as a candidate species for commercial PHA production.

How to cite: Sriyapai T, Chuarung T, Kimbara K, et al. Production and optimization of polyhydroxyalkanoates (PHAs) from *Paraburkholderia* sp. PFN 29 under submerged fermentation. Electron J Biotechnol 2022;56. https://doi.org/10.1016/j.ejbt.2021.12.003

© 2022 Published by Elsevier B.V. on behalf of Pontificia Universidad Católica de Valparaíso. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

1. Introduction

Polyhydroxyalkanoates (PHAs) are intracellular biodegradable polyesters that act as energy reserves under limiting nutritional conditions in the presence of excess carbon [1]. PHAs can be divided into two major classes based on the number of carbon atoms in the monomeric units. First, there is short-chain-length PHA (scl-PHA, C3-C5), which includes polyhydroxybutyrate (PHB) and polyhydroxybutyrate-*co*-hydroxyvalerate (PHBV). PHBV is a copolymer of hydroxybutyrate (HB) and hydroxyvalerate (HV) and is synthesized in medium with even- and odd-numbered carbon substrates (e.g., acetate, propionate and verlerate) to incorporate HV units into PHBV [2]. PHBV with a higher HV content shows greater flexibility, augmented plasticity, lower crystallinity and a lower crystallization rate [3] and has been employed in several important research fields, such as tissue engineering, biomaterial science, and biodegradable packaging [4]. The second class consists of medium-chain-length PHA (mcl-PHA, C6-C14), including homopolymers (such as polyhydroxyhexanoate, PHHx, and polyhydroxyoctanoate, PHO) and heteropolymers (such as polyhydroxyhexanoate-*co*-hydroxyoctanoate, PHHxHO) [5]. Industrially produced PHAs include PHB, PHBV, PHO, polyhydroxydecanoate (PHD) polyhydroxypentenoate (PHPE), polyhydroxy butyrate-*co*-hydroxyhexanoate (PHBHHx) and polyhydroxy butyrate-*co*-hydroxybutyrate (PHBHB) [6].

The genus *Burkholderia* (previously part of *Pseudomonas*) belongs to the class Betaproteobacteria and includes more than 100 species of gram-negative, aerobic, rod-shaped bacteria with validly published names (www.bacterio.net/burkholderia.html) [7,8]. Recently, this genus has been proposed to be divided into two genera according to phylogenetic clustering: one is a genus retaining this name and consisting mainly of animal and plant pathogens (i.e., *Burkholderia cepacia* complex (BCC) and *B. pseudomallei* and close relatives) and a new genus, *Paraburkhoderia* and *Caballeronia,* including environmental bacteria isolated from soil, water and endophytes [7,8,9]. *Paraburkhoderia* resembles the genus *Burkholderia*, and the morphological and metabolic characteristics are generally similar to those of the genus *Burkholderia* [7]. Studies on PHA production in the *Burkholderia* genus were initiated in the 1990s, and several *Burkholderia* spp. have been reported previously [10]. For example, *B. cepacia* ATCC 17759 produced maximal PHA production after 96 h (8.72 g PHA/L broth and 51.4% dry cell weight) using a detoxified sugar maple hemicellulosic hydrolytsate as a potentially inexpensive carbon source [1]. PHB may be produced by a few *Paraburkholderia* species. For example, *P. sacchari* IPT101 produced PHB using sugars and inhibitors of softwood hemicellulose hydrolysates as carbon sources, and the maximum cell dry weight reached 6.7 ± 0.1 g/L with 71 ± 5% PHB [6]. *P. xenovorans* LB400 was grown in M9 minimal medium with xylose (PHB_x), mannitol (PHB_m) or glucose (PHB_g) as the sole carbon sources, indicating that modifying the sugar used as the carbon source may be useful in tuning the structural properties of PHB and its performance as a component of electrospun scaffolds to better fit specific biomedical applications [11]. Furthermore, PHBV production was one of the few published findings on this genus and proved to be interesting for future industrial applications. This research focused on production and optimization of PHB and included a preliminary study of PHBV production by PHA-producing bacteria isolated from fifteen soil samples in Thailand. The polyesters produced were characterized using ^{1}H nuclear magnetic resonance (^{1}H NMR) spectroscopy and gas chromatography-mass spectrometry (GC/MS) analyses to determine their functional groups and chemical structure.

2. Material and methods

2.1. Sample collection and isolation of bacterial strains

Fifteen soil samples were collected from mangrove forests, palm tree gardens, paddy field soils, compost soils and forest soils in Thailand, kept in plastic bags, marked with collection details and protected from light and refrigerated at 4°C to screen for the best PHB production. One gram of soil sample was serially diluted with sterilized distilled water and plated on minimal agar with $Na_2HPO_4.7H_2O$ 30 g/L, KH_2PO_4 15 g/L, NaCl 2.5 g/L, NH_4Cl 5 g/L, $MgSO_4$ 2 g/L, $CaCl_2$ 0.1 g/L, glucose 0.4 g/L and agar 20 g/L and the pH was adjusted to 7. The plates were incubated at 30°C for 3–5 d. Several hundred colonies were selected and subsequently restreaked on nutrient agar plates. Cultures were maintained on nutrient agar plates and minimal agar plates at 4°C and subcultured monthly. The glycerol stock culture was maintained on nutrient agar slants overlaid with 20% (v/v) glycerol and maintained at −20°C.

2.2. Preliminary screening on Nile red agar plates for PHB production

All bacterial isolates were grown on modified minimal agar containing $Na_2HPO_4.7H_2O$ 30 g/L, KH_2PO_4 15 g/L, NaCl 2.5 g/L, NH_4Cl 5 g/L, $MgSO_4$ 2 g/L, $CaCl_2$ 0.1 g/L, glucose 10 g/L, agar 20 g/L, and Nile red dye (Sigma-Aldrich, USA) dissolved in dimethylsulfoxide (DMSO) at a final concentration of 0.5 μg dye per ml of medium [12]. The plates were incubated at 30°C for 3 d. The agar plates were exposed to ultraviolet light (312 nm) to detect the accumulation of intracellular PHB granules in bacteria. Colonies with bright orange fluorescence were selected for further studies. The DH5α strain of *Escherichia coli* was used as a negative control.

2.3. PHB production and extraction in submerged fermentation conditions

PHB-producing bacterial isolates were grown in 100 ml of nutrient broth with 1% (w/v) glucose in 250-ml Erlenmeyer flasks. The medium was inoculated with 10% (v/v) 24 h-old culture ($O.D._{600}$ = 0.8) as a starter and incubated with rotary shaking at 200 rpm at 30°C for 72 h. After 72 h, the cells were harvested by centrifugation at 9,100 × *g* for 10 min at 4°C. The cell dry weight (CDW) in grams per litre was determined. Measurements were performed in triplicate.

The PHB extraction method was modified by Hahn et al. [13]. Briefly, the cell pellet was washed with 0.85% NaCl solution. Chloroform and 5% (v/v) sodium hypochlorite were added to the cell pellets according to the following ratio: 10 ml chloroform to 10 ml of 5% (v/v) sodium hypochlorite per 1 g of pellet weight.

The mixture was placed at 30°C with shaking at 150 rpm for 12 h and then centrifuged at 9,100× *g* for 10 min at room temperature, resulting in a three-phase solution with the lower phase of chloroform containing PHB. The chloroform phase solution was carefully transferred to a fresh tube, and 2 volumes of 70% (v/v) methanol were added to the chloroform solution. The remaining solution was concentrated by a rotary evaporator. The white precipitate was washed twice with 95% (v/v) ethanol and then air-dried at room temperature for 24 h. The PHB concentration (g/L) and % PHB content were calculated. Measurements were performed in triplicate.

2.4. Phylogenetic and phenotypic characterization of the selected PHB-producing bacteria

The morphological and physiological properties of the strain analysed for PHA production were investigated according to Bergey's Manual of Determinative Bacteriology 9th ed [14], Gram staining and biochemical characterization using API 20 NE (bioMérieux, France). Molecular identification was performed by 16S rDNA sequencing analysis. Genomic DNA from this isolate was extracted using a modified method by Kieser et al. [15]. The 16S rDNA sequence was amplified using the universal oligonucleotide primers 16SmetaF (5′-AGAGTTTGATCCTGGCTCAG-3′) and 16SmetaR (5′-GGTTACCTTGTTACGACTT-3′) described by Lane [16], and the sequence was compared with 16S rDNA sequences from GenBank using the BLAST database on the NCBI website (http://www.ncbi.nlm.nih.gov/BLAST/). Multiple alignments of the sequences obtained were carried out with the program CLUSTAL X version 1.81 [17]. A phylogenetic tree was constructed according to the neighbour-joining method of Saitou and Nei [18] using the MEGA 5.1 program [19].

2.5. Optimization of culture medium constituents and conditions for PHB production

The strain yielding the highest amount of PHB was selected for studies on the effects of nutrient and environmental conditions. Starter culture (10%) was added to 100 ml of modified minimal medium pH 7.0 in 250-ml Erlenmeyer flasks. The effects of various carbon sources were studied by the addition of glucose, fructose, mannitol, galactose, xylose and sucrose individually at a final concentration of 1% (w/v). The medium with different carbon sources was incubated at 30°C with shaking at 200 rpm for 72 h. The carbon source concentration was prepared at 1%, 2%, 3%, 4% and 5% (w/v) of the final concentration of the best carbon sources as described above. The effects of different nitrogen sources were studied by adding different nitrogen sources, including NH_4Cl, $(NH_4)_2SO_4$, NH_4NO_3, $(NH_4)_2Fe(SO_4)$, KNO_3, $NaNO_3$, $NH_4H_2PO_4$, $(NH_4)_2HPO_4$, yeast extract, peptone and casein, with the best carbon source. The medium with different nitrogen sources was incubated at 30°C with shaking at 200 rpm for 72 h. The concentration of the nitrogen source was varied from 0-0.3% (w/v). To determine the effect of pH, the strain was grown in medium containing the best carbon and nitrogen sources at the optimum concentrations. Media were prepared at pH 5.0, 6.0, 7.0, 8.0, 9.0 and 10.0 and incubated at 30°C with shaking at 200 rpm for 72 h. To determine the effect of temperature, media were prepared by using the carbon source, nitrogen source and pH producing the highest yield and incubated at different temperatures, including 25°C, 30°C, 35°C, 40° and 45°C, with shaking at 200 rpm for 72 h. The effect of incubation time was studied for 5 d (24, 48, 72, 96 and 120 h) with the best parameters for PHB production. The PHB concentration (g/L), % PHB content and CDW (g/L) were determined for all the samples in different experiments, which were conducted in triplicate.

2.6. Effects of co-carbon sources and concentrations on preliminary PHBV production

To produce PHBV, the best bacterial strain was cultivated in 250-ml Erlenmeyer flasks containing 100 ml of modified minimal medium containing co-carbon sources of 3% (w/v) glucose and different concentrations (0.05–0.5% (w/v)) of levulinic acid (Sigma-Aldrich, USA) and propionic acid (Sigma-Aldrich, USA). All flasks were prepared with a nitrogen source, nitrogen concentration and pH that provided the highest yield for PHB production and were incubated at 35°C on a rotary shaker (200 rpm) for 96 h. The PHBV concentration (g/L), % PHBV content and CDW (g/L) were determined for all the flasks in triplicate.

2.7. Transmission electron microscopy (TEM) analysis

For TEM analysis of PHB accumulation in cells, the bacterium was cultured in optimum modified minimal medium under moderate shaking at 200 rpm and incubated at 35°C for 120 h. Cell pellets were collected by centrifugation at 9,000× *g* and washed with distilled water. Cells were dissolved in 2% (w/v) glyceraldehyde and refrigerated at 4°C for fixation on grids and cross-sectional observation [20]. The cells were observed with a TEM-Hitachi HT7700 at 5000X magnification (Scientific Equipment and Research Division, Kasetsart University Research and Development Institute).

2.8. Characterization of extracted PHB and PHBV

The bacterial strain was cultivated in a suitable medium for PHB and PHBV production. The chemical structures of PHB and PHBV in cell extracts were investigated using ^1H NMR spectroscopy. The extraction samples were dissolved in chloroform in glass tubes, and NMR spectra were obtained on a Bruker AVANCE 300 FT-NMR spectrometer (Bruker, Germany), where proton (^1H) spectra were obtained at 300 MHz. [21]. A spectrum was recorded in deuterochloroform ($CDCl_3$, Sigma-Aldrich, USA) and was referenced to the residual nondeuterated solvent signal. PHB and PHBV standards (Sigma-Aldrich, USA) were examined using ^1H NMR to compare the chemical structure of PHA samples as a standard reference.

PHBV samples were transferred to the Office of Scientific Instrument and Testing, Prince of Songkla University, Thailand for monomer composition and PHBV content analyses using the modified method of Van-Thuoc et al [22] with GC/MS (7890 B GC-5977A MSD, Agilent, USA).

3. Results and discussion

3.1. Primary screening and isolation of PHB-producing bacteria

Among the two hundred isolates collected from soil samples in Thailand, sixty-four isolates emitted bright orange fluorescence upon exposure to ultraviolet light after the Nile red dye staining process, suggesting that they had the ability to accumulate PHB in the cells, while the isolates that did not produce PHB displayed a dark smear. Nile red dye is a lipophilic fluorescent dye that can quickly quantify intracellular lipid-like inclusions, most commonly triacylglycerols and PHB [23].

3.2. Quantitative screening of PHB production in submerged fermentation conditions

Sixty-four isolates were cultured in 250-ml Erlenmeyer flasks in 100 ml nutrient broth with 1% (w/v) glucose as the carbon source

for 72 h with rotary shaking at 200 rpm at 30°C for PHB extraction. The results revealed that twelve isolates could synthesize PHB granules. These six isolates-RFN04, WJN12, WJN18, PFN29, RFN32 and LSN52 had PHB concentrations of 1.76 ± 0.14 g/L, 0.19 ± 0.03 g/L, 0.14 ± 0.02 g/L, 2.96 ± 0.18 g/L, 1.02 ± 0.15 g/L and 1.97 ± 0.13 g/L, respectively, and the PHB content percentage in these cells was between 3.35% and 87.24% (Table 1). The remaining six isolates, WJN11, WJ13, WJ19, KDN38, KDN40 and KDN48, produced PHB concentrations less than 0.01 g/L. PFN29, which had the highest PHB concentration, was selected as a high-potential strain for further optimization studies aimed at maximizing PHB production in a submerged fermentation process.

3.3. Identification and phylogenetic studies based on 16S rDNA sequences

Based on the partial sequence of the 16S rDNA gene, the six isolates were assigned to four genera: *Azotobacter, Sphingobium*, *Candidatus Burkholderia* and *Paraburkholderia*. The GenBank accession numbers for the 16S rDNA sequences are shown in Table 1, and the phylogenetic tree of the six bacterial strains was studied using their 16S rDNA sequences, which revealed the closest resemblance for each genus (Fig. 1). The RFN04 and RFN32 sequences had 99% similarity with the sequence of *Azotobacter vinelandii* strain ISSDS-436 (EF620452). WJN12 and WJN18 sequences had 100% similarity with the sequence of *Sphingobium barthaii* strain KK22 (NR137223). PFN29 and LSN52 sequences were 99% identical to those of *Candidatus Burkholderia schumannianae* strain SD1099 (HQ849259) and *Paraburkholderia phenoliruptrix* (CP003864), respectively. *Candidatus Burkholderia schumannianae* is also known as *Candidatus Paraburkholderia schumannianae* or *Paraburkholderia schumannianae* [9,24]. The new genus *Paraburkholderia* includes plant growth-promoting species isolated from natural environments, which are used in agricultural crops to reduce the use of agrochemicals and encourage sustainable agricultural management strategies [25]. A few *Paraburkholderia* species, including *P. sacchari* DSM 17165 [26] and *P. xenovorans* LB400 [11] have previously been shown to be capable of producing PHB from a wide variety of carbon sources, but *P. schumannianae* has not previously been reported. As a result, this is a preliminary report for PHB production by the strain PFN29.

3.4. Optimization of PHB production by Paraburkholderia sp. strain PFN29

According to the experimental findings, a one-factor-at-a time technique under shaking flask conditions was used to study the effects of various parameters in modified minimal medium on PHB production by the strain PFN29, and the enhancement parameters revealed that optimizing cultivation parameters had a considerable impact on the rate of PHB production.

3.4.1. Effects of carbon sources and carbon concentrations

In this work, the PHB-producing bacterium PFN29 was cultured in modified minimal medium pH 7.0 for 72 h at 30°C with shaking at 200 rpm with various carbon sources. The results showed that the PHB concentration (2.63 ± 0.06 g/L) and PHB content (82.67% of 3.27 ± 0.05 g/L CDW) were maximal when glucose was used for PHB production and bacterial cell growth (Fig. 2A). Comparable results have been reported for *Vibrio azureus* BTKB33 [27] and *Erythrobacter aquimaris* [28]. Glucose is an easily assimilable substrate and promotes bacteria to produce more PHB [29]. In addition, mannitol and fructose were inferior, yielding PHB concentrations of 2.27 ± 0.1 g/L (73.13% PHB content of 3.04 ± 0.08 g/L CDW) and 2.12 ± 0.12 g/L (68.61% PHB content of 3.07 ± 0.07 g/L CDW), respectively, but sucrose yielded the lowest PHB concentration of 0.18 ± 0.02 g/L (9.48% PHB content of 2.13 ± 0.15 g/L CDW). In the present study, all substrates were used as the sole carbon source to enhance PHB production, although glucose supported maximal PHB synthesis. Jiang et al. [30] reported that substrates derived from various carbon sources for PHA production can be classified into three categories: carbohydrates (simple sugars), triacylglycerol (oils from plants and fats from animals) and hydrocarbons (waste plastics). The substrates for PHA biosynthesis in bacterial cells are usually restricted to small molecules since bacteria have thick, rigid cell walls surrounding membranes. Large polymeric molecules cannot be transported into the cell, and extracellular transformation by either microorganisms or chemical processes is required for the use of polymeric molecules [30]. The types and compositions of PHAs produced by a biological system depend on the PHA synthase substrate specificity, the carbon sources supplied and the metabolic pathways functioning in the cells [31]. Since increasing the carbon source concentration may be directly related to cell growth and PHB synthesis, different glucose concentrations (1–5% (w/v), 10–50 g/L) were also investigated for this experiment, and 3% (w/v) glucose provided the highest PHB concentration (3.52 ± 0.04 g/L) and PHB content (92.3% of 3.80 ± 0.26 g/L CDW) and was selected for further experiments (Fig. 2B). When glucose concentration was increased, the growth and PHB concentration of PFN29 were decreased. According to Lasemi et al. [32], PHB production from *A. beijerinckii* was maximized at the desired concentration of glucose (50 g/L) as the carbon source; however, when the substrate concentration was increased to 60 g/L, the substrate consumption rate and cell growth were reduced, which was attributable to the presence of extra substrate above the optimum carbohydrate concentration. Excess glucose may inhibit cell growth.

Table 1
Identification of bacterial isolates by 16S rDNA sequencing and PHB accumulation.

Isolates (accession number)	16S rDNA gene (accession number) (%similarity)	CDW (g/L)	PHB concentration (g/L)	PHB content (%)
RFN04 (KY701515)	*Azotobacter vinilandii* (EF620452) (99%)	10.84 ± 1.24	1.76 ± 0.14	16.2
RFN32 (KY701518)	*Azotobacter vinilandii* (EF620452) (99%)	10.52 ± 0.77	1.02 ± 0.15	9.66
WJN12 (KY701516)	*Sphingobium barthaii* (NR137223) (100%)	4.09 ± 0.05	0.19 ± 0.03	4.18
WJN18 (KY701517)	*Sphingobium barthaii* (NR137223) (100%)	4.27 ± 1.10	0.14 ± 0.02	3.35
PFN29 (KY425763)	*Candidatus Burkholderia schumannianae* (HQ849259) (99%)	3.39 ± 0.32	2.96 ± 0.18	87.24
LSN52 (KY701521)	*Paraburkholderia phenoliruptrix* (CP003864) (99%)	2.38 ± 0.13	1.97 ± 0.13	82.58

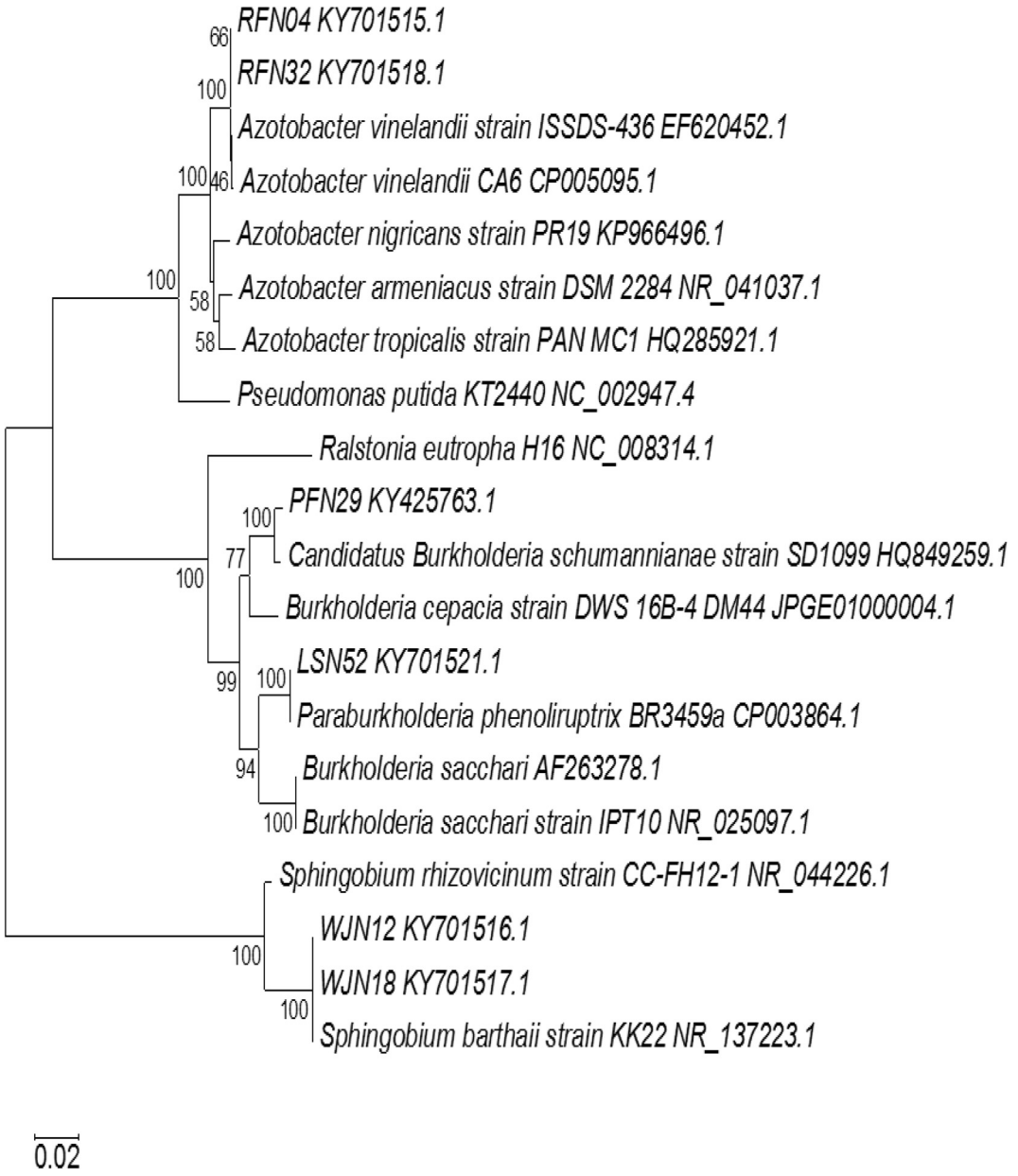


Fig. 1. Phylogenetic relationship of PHAs-producing strains based on partial 16S rDNA sequences. Accession numbers are given in back of taxonomic assignment. The numbers at the nodes indicate the level of bootstrap support based on 1,000 replicates. Bar = 20% sequence divergence.

3.4.2. Effects of nitrogen sources and nitrogen concentrations

The influence of the nitrogen source was investigated by inoculating PFN29 in various nitrogen sources at 0.1% (w/v), including 3% (w/v) glucose as the carbon source. The PHB concentration, PHB content, and cell dry weight were increased by inorganic nitrogen sources compared to the control and organic nitrogen sources (Fig. 3A). The PHB concentration and PHB content with ammonium iron(II) sulfate and ammonium chloride were 3.9 ± 0.05 g/L (93.1% PHB content of 4.19 ± 0.12 g/L CDW) and 3. 50 ± 0.29 g/L (91.5% PHB content of 3.81 ± 0.05 g/L CDW), respectively, and ammonium iron(II) sulfate containing two different cations (Fe^{2+} and NH^{4+}) was the best for PHB accumulation. PHB synthesis related to autotrophy using ferrous iron, Fe(II), as an electron donor might be leveraged for long-term PHB production such as production by *Gallionella ferruginea* [33]. Ammonium is an important parameter to be controlled in feast and famine processes for PHB production [34]. This study is the first to show that PHB can be produced by *Paraburkholderia* sp. using ammonium iron(II) sulfate as a nitrogen source. Ammonium chloride was the second-most useful nitrogen source and was most suitable for PHB accumulation in *Alcaligenes eutrophus* PTCC 1615 [35] and *V. azureus* BTKB33 [27], most likely due to its simple structure that these strains can easily use. The nitrogen concentration in culture medium strongly influences the production of intracellular PHB [29]. Therefore, the concentration of a suitable nitrogen source must also be optimized. Different nitrogen concentrations (0, 0.01, 0.05, 0.1, 0.2 and 0.3% (w/v)) were examined (Fig. 3B). For ammonium iron(II) sulfate at 0.1% (w/v), 1 g/L was the optimum concentration for PHB accumulation, and the PHB concentration was 4.10 ± 0.05 g/L (95.1% PHB content of 4.35 ± 0.17 g/L CDW), which is consistent with a previous report showing that ammonium sulfate at 1 g/L is the optimal nitrogen concentration for maximal PHB production by bacterial strains isolated from rhizospheric soil [36]. When bacteria were cultivated in medium

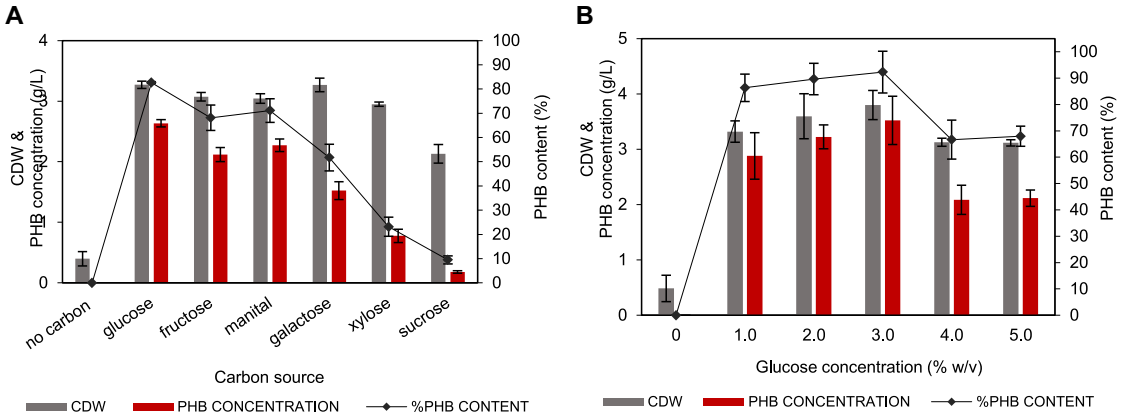


Fig. 2. Optimization of PHB production by *Paraburkholderia* sp. strain PFN29 under submerged fermentation. PFN29 was cultured in modified minimal medium pH 7.0 for 72 h at 30°C with shaking at 200 rpm. (A) Effect of carbon sources and (B) Effect of carbon concentrations.

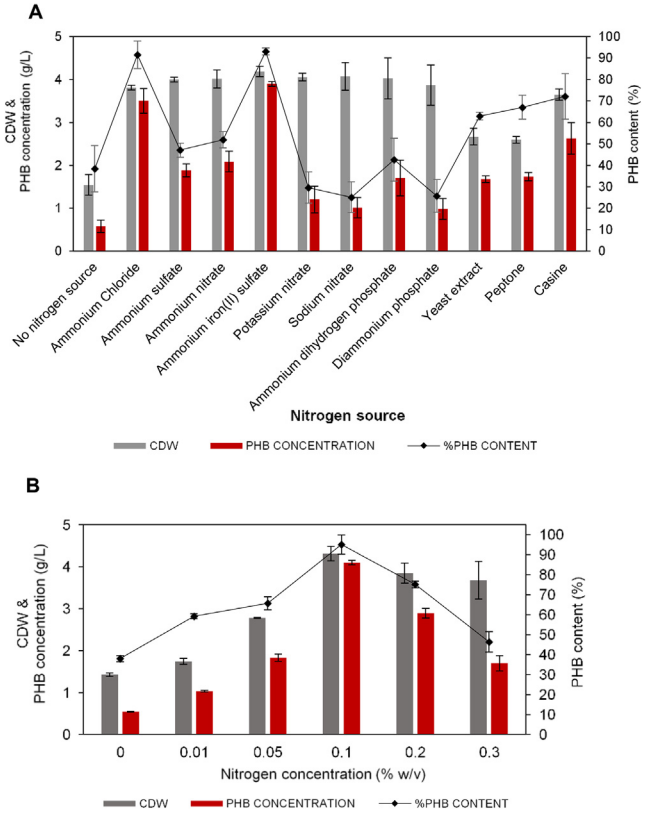


Fig. 3. Optimization of PHB production by *Paraburkholderia* sp. strain PFN29 under submerged fermentation. PFN29 was cultured in modified minimal medium containing 3% (w/v) glucose as the carbon source pH 7.0 for 72 h at 30°C with shaking at 200 rpm. (A) Effect of nitrogen sources and (B) Effect of nitrogen concentrations.

containing more than 0.1% (w/v) nitrogen, their cell growth and PHB concentrations were both reduced. Similar results have been reported by Lathwal et al. [36], where high nitrogen concentrations (above 1 g/L) in medium were shown to inhibit PHB accumulation. PFN29 produced PHB during growth under nitrogen-deficient conditions as a control (0.54 ± 0.01 g/L PHB concentration and 38% PHB content of 1.43 ± 0.04 g/L CDW); however, this strain required nitrogen-limited conditions for PHB synthesis. This finding is consistent with previous reports for *P. xenovorans* LB400 [37] and *P. sacchari* LMG 19450 [38], which have been shown to produce PHB under nitrogen-limited conditions.

3.4.3. Effect of pH

The effect of the pH of the medium on PHB production was investigated. PFN29 was cultured in the optimum medium containing 3% (w/v) glucose as the carbon source and 0.1% (w/v) ammonium iron(II) sulfate as the nitrogen source with different pH levels ranging between 5.0 and 10.0. The results revealed that after 72 h of incubation using the optimal medium at pH 7.0, a maximum PHB concentration of 4.04 ± 0.11 g/L (95.3% PHB content of 4.24 ± 0.22 g/L CDW) was obtained (Fig. 4A), and pH 6.0 was the second-most useful pH for PHB production (3.05 ± 0.06 g/L PHB concentration), which is consistent with previous reports for *V. azureus* BTKB33 [27] and *P. sacchari* IPT 101 LMG 19450 [39] where the optimal pH was 7.0. Little PHB production was observed at pH levels < 6.0 and >8.0, possibly because pH values above and below the optimal level affect the degenerative enzymes of biopolymer-producing bacteria. pH is an important parameter in the synthesis of metabolites and the growth of bacteria because it influences metabolic processes [40].

3.4.4. Effect of temperature

The effects of several temperatures, including 25, 30, 35, 40 and 45°C, on PHB production were studied. PFN29 was cultivated in optimum medium containing 3% (w/v) glucose as the carbon source and 0.1% (w/v) ammonium iron(II) sulfate as the nitrogen source at pH 7.0 for 72 h. Incubation temperatures in the range of 30–35°C were appropriate for PHB production, with a maximum PHB concentration of 4.51 ± 0.26 g/L (96.9% PHB content of 4.66 ± 0.32 g/L of CDW) achieved at 35°C (Fig. 4B). Similar findings have also been reported for *V. azureus* BTKB33 [27] and *E. aquimaris* [28]. The PHB concentration and cell dry weight decreased when the temperature dropped below 30°C and increased beyond 35°C, suggesting that very low or very high temperatures reduced the activity of the enzymes responsible for PHB synthesis.

3.4.5. Effect of incubation time

Incubation time is an important factor associated with bacterial growth and PHB accumulation. The effects of various incubation times for PFN29 on PHB production was evaluated by growing this strain in medium prepared using 3% (w/v) glucose and 0.1% (w/v) ammonium iron(II) sulfate at pH 7.0 and 35°C for different durations (24, 48, 72, 96 and 120 h) with shaking at 200 rpm (Fig. 4C). The results revealed that after 96 h of incubation in the medium under the stationary phase of growth, the highest PHB concentration and cell dry weight were 5 ± 0.17 g/L and 5.14 ± 0.17 g/L (97.3% PHB content), respectively, which gradually diminished with increasing incubation time. These results are

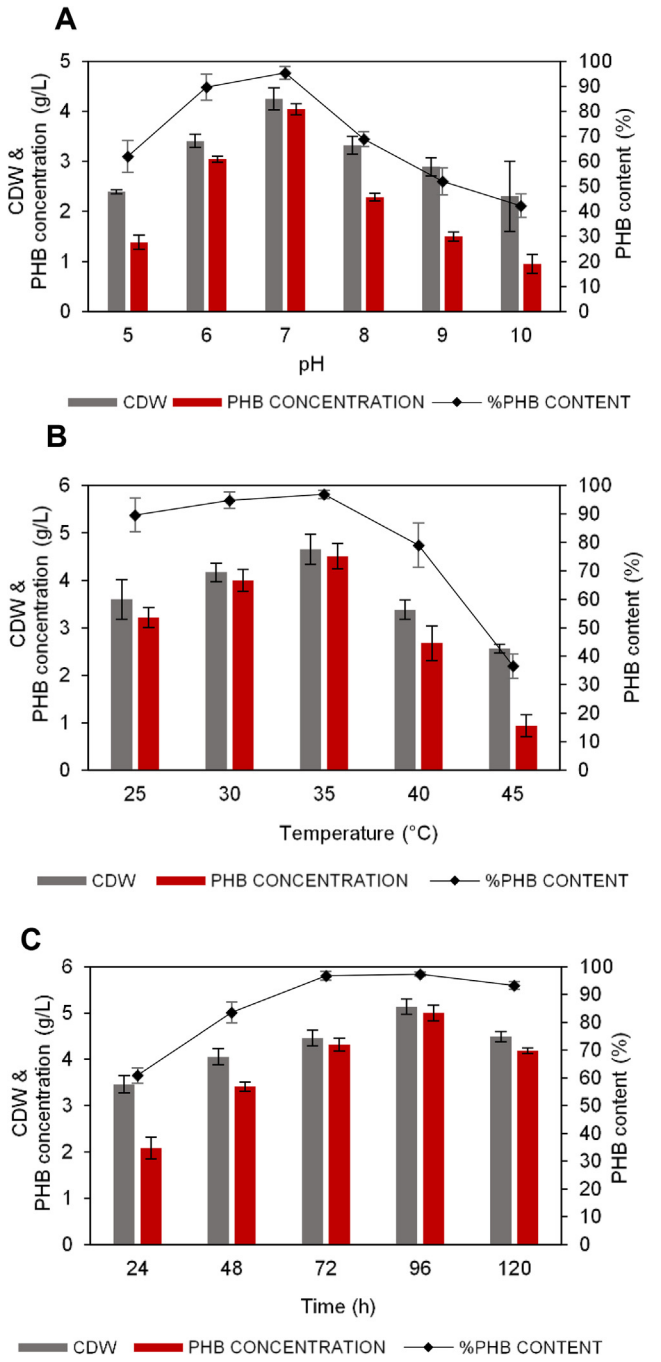


Fig. 4. Optimization of PHB production by *Paraburkholderia* sp. strain PFN29 under submerged fermentation. PFN29 was cultured in modified minimal medium containing 3% (w/v) glucose as the carbon source and 0.1% (w/v) ammonium iron (II) sulfate as the nitrogen source with shaking at 200 rpm. (A) Effect of pH, (B) Effect of temperature, and (C) Effect of incubation time.

consistent with a report on the cultivation of *Rhodobacter sphaeroides* N20 in GM medium at pH 7.0 with incubation at 37°C for 96 h [41]. The PHB concentration at 24 h was minimal (2.09 ± 0. 24 g/L) because the culture was in the acclimatization period. The reduction in PHB production after 96 h may be due to a lack of nutrients and an increase in metabolites, toxins and inhibitors that might have a negative effect on PHB synthesis [36].

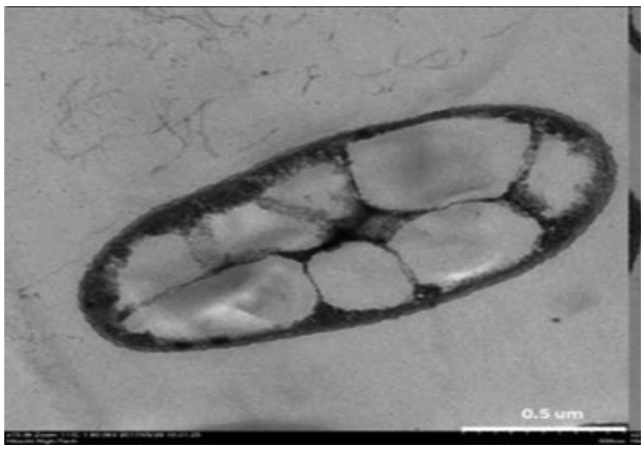


Fig. 5. Accumulation of intracellular-like PHB granules in *Paraburkholderia* sp. PFN29 under transmission electron microscope. PFN 29 was grown in optimum medium containing 3% (w/v) glucose and 0.1% (w/v) ammonium iron(II) sulfate at pH 7.0 and 35°C for 96 h with shaking at 200 rpm.

3.4.6. Accumulation of intracellular-like PHB granules in Paraburkholderia sp. PFN29

The TEM images revealed PHB granules with a diameter approximately 0.2–0.5 μm (Fig. 5) accumulating inside *Paraburkholderia* sp. PFN29, which is consistent with Kunasundari and Sudesh [42] who reported that the average size of intracellular PHA granules is approximately 0.2–0.5 μm in diameter.

3.5. Effects of the co-carbon sources and concentrations on preliminary PHBV production

A wide range of both gram-positive and gram-negative bacteria have been found to produce PHBV copolymers instead of PHB homopolymers if appropriate precursors are present. The presence of precursors is also essential for adjusting the 3-hydroxyvalerate (3HV) monomer fraction in PHBV and thus changing the polymer characteristics [43]. As precursors, levulinic acid and propionic acid were employed as cosubstrates or auxiliary carbon sources for cell growth and PHBV biosynthesis in *Paraburkholderia* sp. PFN29 cultivated in modified minimal medium with 3% (w/v) glucose as the main carbon source (Fig. 6). The results revealed that using 3% (w/v) glucose and 0.05% (w/v) propionic acid as co-carbon sources resulted in the highest PHBV concentration (3.08 ± 0.06 g/L, 62.8% PHBV content of 4.89 ± 0.20 g/L CDW) (Fig. 6A). When bacteria were cultivated in medium containing more than 0.05% (w/v) propionic acid, their cell growth, PHBV concentration and PHBV content were reduced. Similar results were achieved when these bacteria were grown in medium containing 3% (w/v) glucose and 0.05% (v/v) levulinic acid, which yielded a maximum PHBV concentration of 2.91 ± 0.04 g/L (69.8% PHBV content of 4.17 ± 0.08 g/L CDW) (Fig. 6B). PHBV production was decreased when the medium contained more than 0.05% (w/v) levulinic acid. The growth rate of bacteria was significantly influenced by increasing levulinic acid and propionic acid concentrations, and high concentrations are known to be harmful to microorganisms [44]. In particular, propionic acid was found to be considerably more hazardous than valeric acid [43]. The results are consistent with a previous report for *Alcaligenes* sp. SH-69 in which the use of levulinic acid as a cosubstrate was found to enhance growth and PHA accumulation at concentrations ranging from 0.05 to 0.1% (w/v) with glucose as a

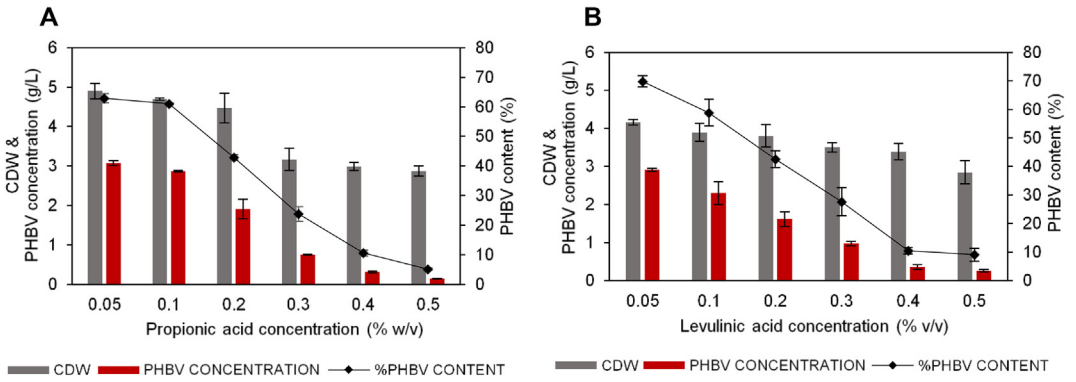


Fig. 6. Effects of the co-carbon sources and concentrations on preliminary PHBV production. *Paraburkholderia* sp. PFN29 was grown in optimum medium containing 3% (w/v) glucose with (A) propionic acid and (B) Levulinic acid as co-carbon sources and 0.1% (w/v) ammonium iron(II) sulfate at pH 7.0 and 35°C for 96 h with shaking at 200 rpm.

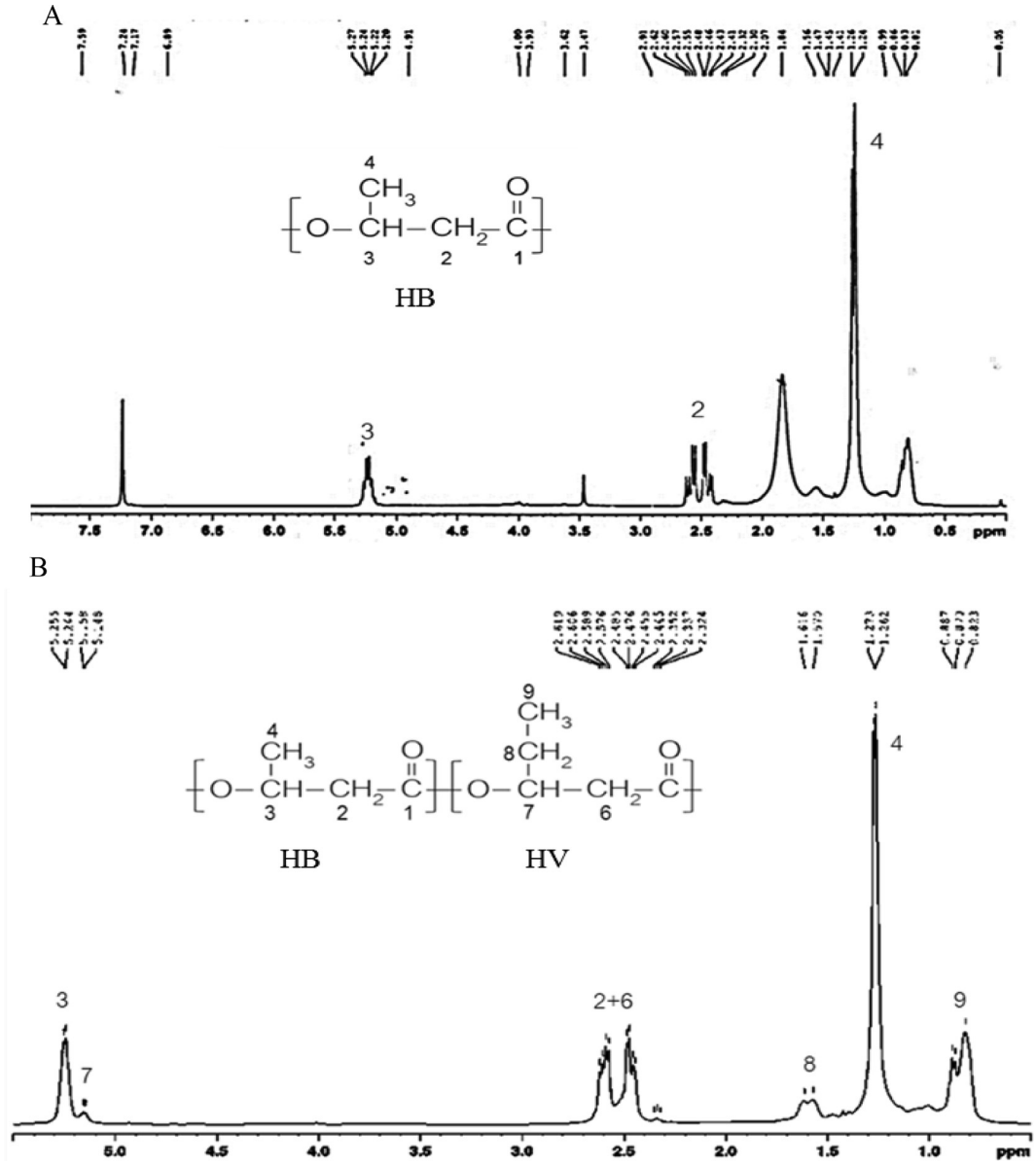


Fig. 7. [1]H NMR spectrum of PHA from *Paraburkholderia* sp. PFN 29. (A) extracted PHB; PFN29 was grown in optimum medium containing 3% (w/v) glucose and 0.1% (w/v) ammonium iron(II) sulfate at pH 7.0 and 35°C for 96 h with shaking at 200 rpm and (B) extracted PHBVp; PFN29 was cultivated in optimum medium containing 3% (w/v) glucose plus 0.05% (w/v) propionic acid with 0.1% (w/v) ammonium iron(II) sulfate at pH 7.0 and 35°C for 96 h with shaking at 200 rpm.

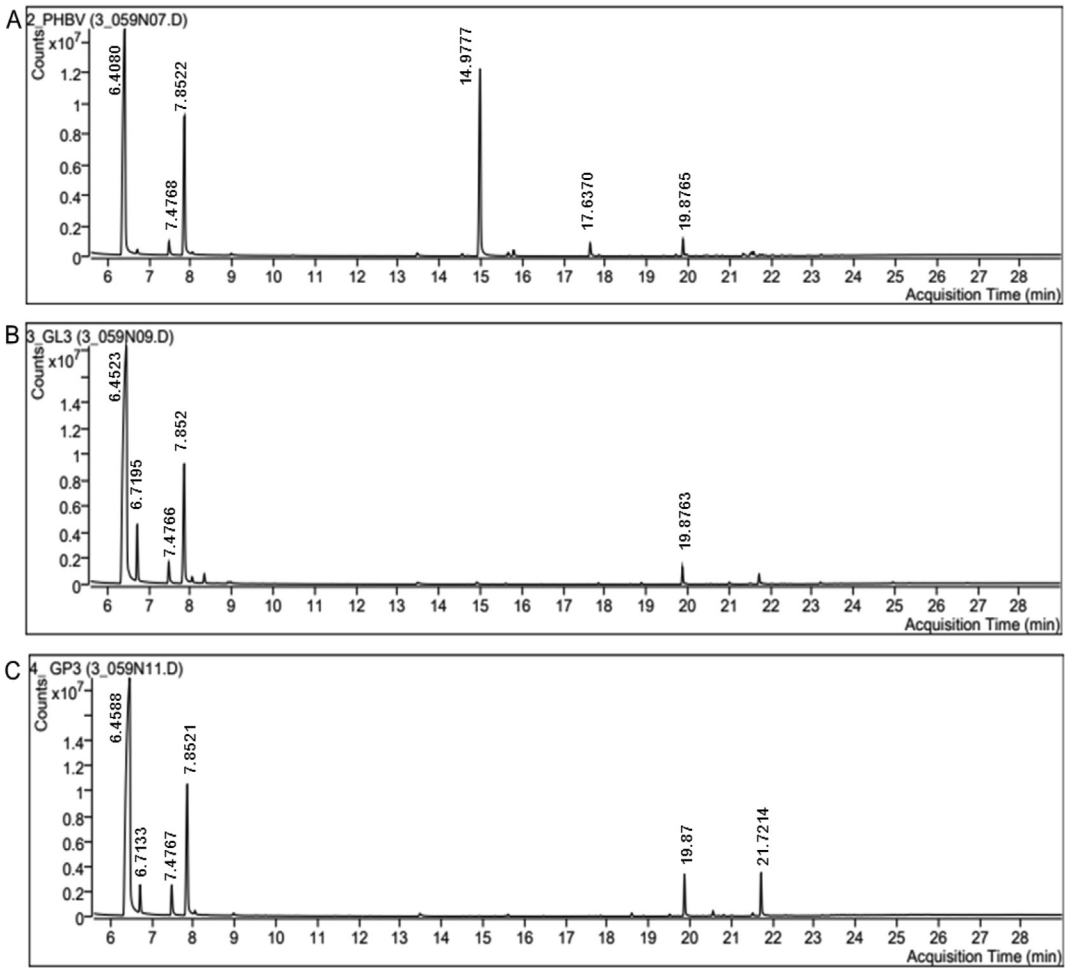


Fig. 8. GC/MS chromatogram of PHBV. (A) The chromatogram shows six monomeric compositions of standard PHBV; butanoic acid, 3-hydroxy-, methyl ester; ethyl 3 hydroxy butyrate; pentanoic acid, 3-hydroxy-, methyl ester; citric acid, trimethyl ester; 3-hydroxy-3-methoxycarbonyl-pentanedioic acid dimethyl ester; and hexadecanoic acid, methyl ester, (B) extracted $PHBV_l$, and (C) extracted $PHBV_p$.

principal carbon source, while concentrations greater than 0.15% (w/v) resulted in minimal cell growth and only trace quantities of recovered PHA [45]. In addition, PHBV synthesis from *Cupriavidus necator* was observed when low concentrations of levulinic acid were combined with other potentially less toxic substrates, such as glycerine and simple sugars [46]. Propionic acid was also shown to be an effective cosubstrate in the synthesis of PHBV from *Herbaspirillum seropedicae* Z69 [47]. PFN29 was utilized to synthesize PHBV from glucose, levulinic acid, and propionic acid as part of preliminary research to optimize the concentration of co-carbon substrates for PHBV synthesis. However, we should investigate additional conditions that yield the highest PHBV accumulation and have potential for industrial utilization.

3.6. Confirmatory analysis of PHB and PHBV products

The ^{1}H NMR spectrum of extracted PHB from PFN29 was obtained, and each peak corresponded to a certain carbon atom in monomers, similar to standard PHB. PHB peaks were detected at 1.26, 2.6 and 5.2 ppm, which correspond to the resonance absorption of methyl ($-CH_3$), methylene ($-CH_2$) and methane (CH) groups, respectively, in 3-hydroxybutyrate (3-HB), confirming its structure as a PHB homopolymer (Fig. 7A). The ^{1}H NMR spectra of extracted PHB from gram-negative and gram-positive bacteria were consistent with those reported in previous studies. Narayanan et al. [48] discovered that the first peak started at 1.67, reflecting to the $-CH_3$ proton, multiplet peaks from 2.37 to 2.71, indicating the existence of a $-CH_2$ proton, and peaks at 5.28 to 5.31, indicating the presence of a –CH proton in the PHB molecule formed by *Bacillus cereus* according to ^{1}H NMR analysis. For PHBV production, PFN29 was cultivated in medium containing co-carbon substrates and (i) 3% (w/v) glucose plus 0.05% (v/v) levulinic acid and (ii) 3% (w/v) glucose plus 0.05% (w/v) propionic acid with 0.1% (w/v) ammonium iron(II) sulfate at pH 7.0 and 35°C for 96 h with shaking at 200 rpm, and the extracted PHAs were designated $PHBV_l$ and $PHBV_p$, respectively. The ^{1}H NMR spectra of extracted PHAs implied that the polymers contained the two monomeric units, HB and HV (Fig. 7B). In the ^{1}H NMR spectrum resonances of $PHBV_l$, peaks at approximately 0.83–0.89 and 1.27 ppm were designated as methyl group protons in HB and HV, while multiple peaks at approximately 1.57–1.61 and 2.32–2.62 ppm were assigned to methylene protons. The existence of methane protons gave multiple peaks around 5.14–5.25 ppm. Similarly, the ^{1}H NMR spectrum resonances of PHBVp were noted at 0.83–0.9, 1.27, 1.55–1.63, 2.44–2.62 and 5.13–5.26 ppm for $-CH_3$ (HV side group), $-CH_3$ (HB side group), $-CH_2$ (HV side group), $-CH_2$ (HV and HB bulk structure) and CH (HV and HB bulk structure), respectively (Fig. 7B). These findings are in agreement with those of previous reports [49]. The chemical composition of extracted PHAs from PFN29 was characterized by GC/MS analysis. The results were compared with the standard PHBV (Fig. 8A). Four chromatogram peaks of $PHBV_l$ (Fig. 8B) and $PHBV_p$ (Fig. 8C) with

acquisition times of 6.452:6.4588, 7.4766:7.4767, 7.852:7.8521 and 19.8763:19.87 min were observed in the mass spectrum. The mass spectra from the MS library were identified as (i) butanoic acid, 3-hydroxy-, methyl ester, (ii) ethyl 3 hydroxy butyrate, (iii) pentanoic acid, 3-hydroxy-, methyl ester, and (iv) hexadecanoic acid, methyl ester, and were also observed in the chromatogram of the PHBV standard (18 mol% 3HV). These findings are consistent with the GC/MS analysis of the PHBV extracted from *Bacillus aryabhattai* PHB10, which exhibited a major peak resembling 3-hydroxybutyric acid methyl ester, pentadecanoic acid methyl ester and hexadecanoic acid methyl ester, confirming the PHBV structure [50]. Glucose and levulinic acid were used as co-carbon sources, resulting in 3HV contents of 15 mol% which was similar to glucose and propionic acid.

4. Conclusions

PFN 29 was cultured in optimum medium containing 3% (w/v) glucose and 0.1 % (w/v) ammonium iron(II) sulfate at pH 7.0 and 35°C for 96 h with shaking at 200 rpm. The highest PHB concentration was 5 ± 0.17 g/L (97.3 PHB content of 5.14 ± 0.17 g/L CDW). For preliminary PHBV production, PFN 29 was grown in medium containing 3% (w/v) glucose with 0.05% (v/v) levulinic acid and 3% (w/v) glucose with 0.05% (w/v) propionic acid as co-carbon sources. The extracted PHAs were characterized by ^{1}H NMR and GC/MS analyses to confirm their structure containing PHB and PHBV, with PHBV containing 15 mol% 3HV. This is the first report on PHB and PHBV production by *P. schumannianae* PFN 29. According to the findings of this study, PFN 29 is a promising candidate for PHA production in a variety of industrial applications as a substitute for petroleum-based plastics.

Financial support

This study was financially supported by research fund 2018 (gran no. 79/2562) of Srinakharinwirot University.

Conflict of interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

Acknowledgments

The authors acknowledge financial support from Srinakharinwirot University.

References

[1] Pan W, Perrotta JA, Stipanovic AJ, et al. Production of polyhydroxyalkanoates by Burkholderia cepacia ATCC 17759 using a detoxified sugar maple hemicellulosic hydrolysate. J Ind Microbiol Biotechnol 2012;39:459–69. https://doi.org/10.1007/s10295-011-1040-6. PMid: 21953365.

[2] Guho NM, Pokhrel D, Abbasi M, et al. Pilot-scale production of poly-3-hydroxybutyrate-co-3-hydroxyvalerate from fermented dairy manure: Process performance, polymer characterization, and scale-up implications. Bioresour Technol Rep 2020;12. https://doi.org/10.1016/j.biteb.2020.100588. PMid: 28952534.

[3] Ke Y, Liu C, Zhang X, et al. Surface modification of polyhydroxyalkanoates toward enhancing cell compatibility and antibacterial activity. Macromol Mater Eng 2017;302:1700258. https://doi.org/10.1002/mame.201700258.

[4] Rivera-Briso AL, Serrano-Aroca A. Poly(3-hydroxybutyrate-co-3-hydroxyvalerate): enhancement strategies for advanced applications. Polymers 2018;10:732. https://doi.org/10.3390/polym10070732. PMid: 30960657.

[5] Keshavarz T, Roy I. Polyhydroxyalkanoates: bioplastics with a green agenda. Curr Opin Microbiol 2010;13:321–6. https://doi.org/10.1016/j.mib.2010.02.006. PMid: 20227907.

[6] Dietrich K, Dumont M-J, Orsat V, et al. Consumption of sugars and inhibitors of softwood hemicellulose hydrolysates as carbon sources for polyhydroxybutyrate (PHB) production with *Paraburkholderia sacchari* IPT 101. Cellulose 2019;26:7939–52. https://doi.org/10.1007/s10570-019-02664-x.

[7] Dobritsa AP, Samadpour M. Transfer of eleven species of the genus *Burkholderia* to the genus *Paraburkholderia* and proposal of *Caballeronia* gen. nov. to accommodate twelve species of the genera *Burkholderia* and *Paraburkholderia*. Int J Syst Evol Micr 2016;66:2836–46. https://doi.org/10.1099/ijsem.0.001065. PMid: 27054671.

[8] Bournaud C, Moulin L, Cnockaert M, et al. *Paraburkholderia piptadeniae* sp. nov. and *Paraburkholderia ribeironis* sp. nov., two root-nodulating symbiotic species of *Piptadenia gonoacantha* in Brazil. Int J Syst Evol Micr 2017;67:432–40. https://doi.org/10.1099/ijsem.0.001648. PMid: 27902255.

[9] Sawana A, Adeolu M, Gupta RS. Molecular signatures and phylogenomic analysis of the genus Burkholderia: proposal for division of this genus into the emended genus *Burkholderia* containing pathogenic organisms and a new genus *Paraburkholderia* gen. nov. harboring environmental species. Front Genet 2014;5:429. https://doi.org/10.3389/fgene.2014.00429. PMid: 25566316.

[10] Hang X, Zhang G, Wang G, et al. PCR cloning of polyhydroxyalkanoate biosynthesis genes from *Burkholderia caryophylli* and their functional expression in recombinant *Escherichia coli*. FEMS Microbiol Lett 2002;210:49–54. https://doi.org/10.1111/j.1574-6968.2002.tb11158.x. PMid: 12023076.

[11] Sanhueza C, Diaz-Rodriguez P, Villegas P, et al. Influence of the carbon source on the properties of poly-(3)-hydroxybutyrate produced by *Paraburkholderia xenovorans* LB400 and its electrospun fibers. Int J Biol Macromol 2020;152:11–20. https://doi.org/10.1016/j.ijbiomac.2020.02.080. PMid: 32057856.

[12] Spiekermann P, Rehm BH, Kalscheuer R, et al. A sensitive, viable-colony staining method using nile red for direct screening of bacteria that accumulate polyhydroxyalkanoic acids and other lipid storage compounds. Arch Microbiol 1999;171:73–80. https://doi.org/10.1007/s002030050681. PMid: 9914303.

[13] Hahn SK, Chang YK, Kim BS, et al. Optimization of microbial poly(3-hydroxybutyrate) rec3 using dispersions of sodium hypochlorite solution and chloroform. Biotechnol Bioeng 1994;44:256–61. https://doi.org/10.1002/bit.260440215. PMid: 18618692.

[14] Holt JG, Krieg NR, Sneath PHA, et al. Bergey 's manual of determinative bacteriology. 9th ed. Baltimore, USA: Williams and Wilkins Press; 1994. p. 787. ISBN 9780683006032.

[15] Kieser T, Bibb MJ, Buttner MJ, et al. Practical Streptomyces genetics. Norwich, UK: The John Innes Foundation; 2000. ISBN 9780708406236.

[16] Lane DJ. 16S/23S rRNA sequencing. In: Stackebrandt E, Goodfellow M, editors. Nucleic acid techniques in bacterial systematic. John Wiley and Sons: New York; 1991. p. 115–75.

[17] Thompson JD, Gibson TJ, Plewniak F, et al. The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res 1997;25:4876–82. https://doi.org/10.1093/nar/25.24.4876. PMid: 9396791.

[18] Saitou N, Nei M. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 1987;4(4):406–25. https://doi.org/10.1093/oxfordjournals.molbev.a040454. PMid: 3447015.

[19] Tamura K, Peterson D, Peterson N, et al. MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol 2011;28:2731–9. https://doi.org/10.1093/molbev/msr121. PMid: 21546353.

[20] Berlanga M, Montero MT, Fernández-Borrell J, et al. Rapid spectrofluorometric screening of poly-hydroxyalkanoate-producing bacteria from microbial mats. Int Microbiol 2006;9(2):95-102. [cited 9 February 2021]. Available from: http://www.im.microbios.org/ PMid: 16835839..

[21] Han J, Hou J, Zhang F, et al. Multiple propionyl coenzyme A-supplying pathways for production of the bioplastic poly(3-hydroxybutyrate-co-3-hydroxyvalerate) in *Haloferax mediterranei*. App Environ Microbiol 2013;79(9):2922–31. https://doi.org/10.1128/AEM.03915-12. PMid: 23435886.

[22] Van-Thuoc D, Huu-Phong T, Thi-Binh N, et al. Polyester production by halophilic and halotolerant bacterial strains obtained from mangrove soil samples located in Northern Vietnam. Microbiologyopen 2012;1(4):395–406. https://doi.org/10.1002/mbo3.44. PMid: 23233461.

[23] Choi JE, Na HY, Yang TH, et al. A lipophilic fluorescent LipidGreen1-based quantification method for high-throughput screening analysis of intracellular poly-3-hydroxybutyrate. AMB Express 2015;5:48. https://doi.org/10.1186/s13568-015-0131-6. PMid: 26253390.

[24] Lemaire B, Oevelen SV, Block PD, et al. Identification of the bacterial endosymbionts in leaf nodules of *Pavetta* (Rubiaceae). Int J Syst Evol Microbiol 2012;62:202–9. https://doi.org/10.1099/ijs.0.028019-0. PMid: 21378132.

[25] Vio SA, García SS, Casajus V, et al. *Paraburkholderia*. In: Amaresan N, Kumar MS, Annapurna K, editors. Beneficial microbes in agro-ecology: bacteria and fungi. Academic Press; 2020. p. 271–311. https://doi.org/10.1016/B978-0-12-823414-3.00015-0.

[26] Li M, Wilkins MR. Fed-batch polyhydroxybutyrate production by *Paraburkholderia sacchari* from a ternary mixture of glucose, xylose and arabinose. Bioprocess Biosyst Eng 2021;44:185–95. https://doi.org/10.1007/s00449-020-02434-1. PMid: 32895870.

[27] Sasidharan RS, Bhat SG, Chandrasekaran M. Biocompatible polyhydroxybutyrate (PHB) production by marine Vibrio azureus BTKB33 under submerged fermentation. Ann Microbiol 2015;65:455–65. https://doi.org/10.1007/s13213-014-0878-z.

[28] Mostafa YS, Alrumman SA, Otaif KA, et al. Production and characterization of bioplastic by polyhydroxybutyrate accumulating *Erythrobacter aquimaris* isolated from mangrove rhizosphere. Molecules 2020;25:1–21. https://doi.org/10.3390/molecules25010179. PMid: 31906348.
[29] Getachew A, Woldesenbet F. Production of biodegradable plastic by polyhydroxybutyrate (PHB) accumulating bacteria using low cost agricultural waste material. BMC Res Notes 2016;9:509. https://doi.org/10.1186/s13104-016-2321-y. PMid: 27955705.
[30] Jiang G, Hill DJ, Kowalczuk M, et al. Carbon sources for polyhydroxyalkanoates and an integrated biorefinery. Int J Mol Sci 2016;17(7):1157. https://doi.org/10.3390/ijms17071157. PMid: 27447619.
[31] Lau N-S, Sudesh K. Revelation of the ability of *Burkholderia* sp. USM (JCM 15050) PHA synthase to polymerize 4-hydroxybutyrate monomer. AMB Express 2012;2:41. https://doi.org/10.1186/2191-0855-2-41. PMid: 22877240.
[32] Lasemi Z, Darzi GN, Baei MS. Media optimization for Poly(β-hydroxybutyrate) production using *Azotobacter Beijerinckii*. Int J Polym Mater 2013;62(5):265–9. https://doi.org/10.1080/00914037.2012.664205.
[33] Ranaivoarisoa TO, Singh R, Rengasamy K, et al. Towards sustainable bioplastic production using the photoautotrophic bacterium *Rhodopseudomonas palustris* TIE-1. J Ind Microbiol Biotechnol 2019;46(9–10):1401–17. https://doi.org/10.1007/s10295-019-02165-7. PMid: 30927110.
[34] Fang F, Liu X-W, Xu J, et al. Formation of aerobic granules and their PHB production at various substrate and ammonium concentrations. Bioresour Technol 2009;100(1):59–63. https://doi.org/10.1016/j.biortech.2008.06.016. PMid: 18674897.
[35] Aramvash A, Dashti Aghjeh S. One-factor-at-a-time optimization of polyhydroxybutyrate production and growth of Alcaligenes eutrophus by altering culture parameters and incubation time. J Sci Islam Repub Iran 2015;26:303-13. [cited 5 March 2021]. Available from: https://jsciences.ut.ac.ir/article_55997.html..
[36] Lathwal P, Nehra K, Singh M, et al. Optimization of culture parameters for maximum polyhydroxybutyrate production by selected bacterial strains isolated from rhizospheric soils. Pol J Microbiol 2015;64(3):227–39. PMid: 26638531.
[37] Urtuvia V, Villegas P, González M, et al. Bacterial production of the biodegradable plastics polyhydroxyalkanoates. Int J Biol Macromol 2014;70:208–13. https://doi.org/10.1016/j.ijbiomac.2014.06.001. PMid: 24974981.
[38] Oliveira-Filho ER, Silva JGP, De Macedo MA, et al. Investigating nutrient limitation role on improvement of growth and poly(3-hydroxybutyrate) accumulation by *Burkholderia sacchari* LMG 19450 from xylose as the sole carbon source. Front Bioeng Biotechnol 2020;7:416. https://doi.org/10.3389/fbioe.2019.00416. PMid: 31970153.
[39] Dietrich K, Oliveira-Filho ER, Dumont M-J, et al. Increasing PHB production with an industrially scalable hardwood hydrolysate as a carbon source. Ind Crops Prod 2020;154. https://doi.org/10.1016/j.indcrop.2020.112703.
[40] Bora L. Polyhydroxybutyrate accumulation in *Bacillus megaterium* and optimization of process parameters using response surface methodology. J Polym Environ 2013;21:415–20. https://doi.org/10.1007/s10924-012-0529-z.
[41] Sangkharak K, Prasertsan P. Nutrient optimization for production of polyhydroxybutyrate from halotolerant photosynthetic bacteria cultivated under aerobic-dark condition. Electron J Biotechnol 2008;11:1–12. https://doi.org/10.2225/vol11-issue3-fulltext-2.
[42] Kunasundari B, Sudesh K. Isolation and recovery of microbial polyhydroxyalkanoates. Express Polym Lett 2011;5:620–34. https://doi.org/10.3144/expresspolymlett.2011.60.
[43] Policastro G, Panico A, Fabbricino M. Improving biological production of poly (3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) co-polymer: a critical review. Rev Environ Sci Biotechnol 2021;20:479–513. https://doi.org/10.1007/s11157-021-09575-z.
[44] Wang Y, Chen R, Cai JY, et al. Biosynthesis and thermal properties of PHBV produced from levulinic acid by *Ralstonia eutropha*. PLoS One 2013;8(4). https://doi.org/10.1371/journal.pone.0060318. PMid: 23593190.
[45] Jang JH, Rogers PL. Effect of levulinic acid on cell growth and poly-β-hydroxyalkanoate production by *Alcaligenes* sp.SH-69. Biotechnol Lett 1996;18:219–24. https://doi.org/10.1007/BF00128683.
[46] Jaremko M, Yu J. The initial metabolic conversion of levulinic acid in *Cupriavidus necator*. J Biotechnol 2011;155(3):293–8. https://doi.org/10.1016/j.jbiotec.2011.07.027. PMid: 21821073.
[47] Catalán AI, Malan AK, Ferreira F, et al. Propionic acid metabolism and poly-3-hydroxybutyrate-co-3-hydroxyvalerate production by a *prpC* mutant of *Herbaspirillum seropedicae* Z69. J Biotechnol 2018;286:36–44. https://doi.org/10.1016/j.jbiotec.2018.09.008. PMid: 30240592.
[48] Narayanan M, Kandasamy S, Kumarasamy S, et al. Screening of polyhydroxybutyrate producing indigenous bacteria from polluted lake soil. Heliyon 2020;6. https://doi.org/10.1016/j.heliyon.2020.e0538. PMid: 33163664.
[49] Kumar M, Singhal A, Verma PK, et al. Production and characterization of polyhydroxyalkanoate from lignin derivatives by *Pandoraea* sp. ISTKB. ACS Omega 2017;2(12):9156–63. https://doi.org/10.1021/acsomega.7b01615. PMid: 30023602.
[50] Pillai AB, Kumar AJ, Kumarapillai H. Biosynthesis of poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) in *Bacillus aryabhattai* and cytotoxicity evaluation of PHBV/poly(ethylene glycol) blends. 3 Biotech 2020;10:32. https://doi.org/10.1007/s13205-019-2017-9. PMid: 31988826.