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Optimum Condition for Polyhydroxyalkanoate Production from Crude Glycerol by *Bacillus* sp. Isolated from Lipid-Containing Wastewater

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Abstract

Polyhydroxyalkanoates (PHAs) are a group of biopolymers used as an alternative to petroleum-based synthetic plastics. Their industrial application is not widely available due to production cost constraints, mainly from raw materials used for carbon sources. This research focuses on selecting a *Bacillus* strain from lipid-containing wastewater that can produce PHAs from crude glycerol and investigating the optimum conditions for producing PHAs from crude glycerol. The *Bacillus* strain was isolated from the wastewater storage pond at a fermented pork sausage manufacturing plant in Thailand for PHAs production utilizing Mineral Salt Medium (MSM) with 1 % w/v glucose as a carbon source. PHAs synthesis was preliminarily investigated by staining cells with Sudan Black B. The isolated FMI3 produced the highest PHAs at 43 % of the dry cell weight (DCW). It was identified as *Bacillus pumilus* FMI3. Crude glycerol was obtained from a by-product of the transesterification of waste cooking oil catalysed by NaOH. It was partially purified by acidification and neutralization and exhibited about 80 % w/w glycerol, 1.90 ± 0.09 % ash content, 2.80 ± 0.14 % water content, light brown colour, and pH 7.11 ± 0.35. Isolated *B. pumilus* FMI3 grows best and produces the highest PHAs yields under optimum conditions, i.e., MSM containing crude glycerol and ammonium sulphate at 61:1 mole ratio as carbon and nitrogen sources respectively, 15 % inoculum, initial pH of neutral at 7, and shaking at 150 rpm at 40 °C for 72 h. These conditions achieved PHAs at 79 % of DCW, classified as medium-chain-length PHAs comprising 3-hydroxyoctanoate, 3-hydroxydecanoate, 3-hydroxydodecanoate, and 3-hydroxytetradecanoate at 9, 12, 67 and 12 %, respectively. This study reported the feasibility of utilizing crude glycerol as feedstock for PHAs production by the *B. pumilus* FMI3 strain, which was able to grow on and tolerate salts of crude glycerol.

Keywords: Crude glycerol, Polyhydroxyalkanoates, Production, Bacteria, Wastewater, Optimum condition, *Bacillus* sp.

Introduction

Polyhydroxyalkanoates (PHAs) are biodegradable polymers with properties similar to synthetic plastics. They can be synthesized from natural renewable carbon sources by numerous bacterial species. The accumulation of PHAs is a natural means for bacterial storage of carbon and energy when nutrient supplies are imbalanced, for example, in the presence of excessive carbon sources but nitrogen or phosphorous sources are restricted [1]. PHAs are biopolymers that possess numerous attractive properties, such as being thermoplastics similar to polyethene and polypropylene, bio-degradable, and compatible with living organisms. Coupled with a wide range of applications, PHAs thus have a strong potential to be used in place of synthetic plastics from petrochemicals [2]. PHAs accumulate inside the cells of bacteria in the form of inclusion bodies or granules, which are found in both gram-positive and gram-negative bacteria. PHA synthase in bacteria is the key enzyme involved in polymerizing PHAs, using *(R)*-3-hydroxyacyl-CoA as a substrate. Because various bacterial strains have different metabolic pathways for producing *(R)*-3-hydroxyacyl-CoA depending on the carbon sources, the resulting bacterially synthesized bio-polyesters vary immensely in their structures and molecular masses [3]. Generally, PHAs produced by microorganisms are distinguished into 2 main groups according to the side chains of their monomers: specifically, short-chain-length PHAs (SCL-PHAs) and medium-chain-length PHAs (MCL-PHAs) composed of (R)-3-hydroxy fatty acids with 3 - 5 carbon atoms and 6 - 14 carbon atoms, respectively [4]. The SCL-PHAs are structurally crystalline and are thus brittle; they also have high melting points but typically low resistance to compressive strength. In contrast, MCL-PHAs in general are naturally flexible with rubber-like material characteristics and a lower crystallinity compared to SCL-PHAs. These properties make MCL-PHAs and their co-polymers attractive for biomedical and industrial applications, given the need for flexible biomaterial-based devices [5]. However, a notable disadvantage of the industrial-scale production of PHAs is their high cost, which is around 5 - 10 times higher than producing plastic from petroleum sources [6]. Because roughly 50 % of the PHAs' production cost is the cost of their carbon sources [7], many studies have attempted to use industrial waste and by-products as alternative carbon sources to reduce the production cost of PHAs. These sources include molasses and vinasse, whey, crude glycerol, distillery wastewater, wastewater, used cooking oil, food waste, and wastewater from the vegetable oil refinery industry [8].

Crude glycerol is the principal by-product from the biodiesel manufacturing process, involving the transesterification of oils or fats using KOH or NaOH as a catalyst. In general, about 10 kg of crude glycerol will be obtained from every 100 kg of biodiesel produced. For example, in 2021, the world produced approximately 18.3 billion litres of biodiesel, which created around 1 - 2 billion litres of crude glycerol [9]. At present, there is a supply glut of crude glycerol due to increased biodiesel production for primarily vehicular use. Glycerol in pure form is highly-priced due to its use as a major ingredient of various high-value products in the cosmetic, pharmaceutical, food, cigarette tobacco, and chemical industries. However, crude glycerol as a by-product from the biodiesel manufacturing process has a low market value as a result of its low glycerol content, between 60 - 70 % w/w, and the presence of numerous impurities such as water, methanol, residual hydroxide, inorganic salts, fatty acid, fatty acid methyl esters, mono- and diacylglycerols, etc. [10] The low glycerol content and high level of impurities make the purification of crude glycerol for such high-value applications impractically expensive. This cost has resulted in the widespread availability of inexpensive crude glycerol, which can be an alternative carbon source for the biosynthesis of PHAs, which would help reduce the oversupply and increase the market value of crude glycerol. However, there are limitations in the use of impure crude glycerol as a carbon source for bacterial PHAs production. Specifically, inorganic salts, such as NaCl and K_2SO_4, which are present as impurities in crude glycerol will retard the bacterial growth and hence reduce the production of PHAs. Studies report that *Pandoraea* sp. MA03 isolated from an Atlantic rainforest accumulated PHB to a content of 63.6 % of dry cell weight (DCW) when supplemented with 2 % w/v crude glycerol. However, the PHB content was lower with the use of > 3 % w/v crude glycerol concentration [11]. Certain recombinants, such as *Burkholderia cepacian* ATCC 17759 have produced a PHB content of 31.35 % of DCW when supplemented with 3 - 9 % v/v crude glycerol [12], and *Escherichia coli*-ABC_{Ah} can accumulate PHB to a content of 14 % of DCW with the use of 1 % v/v crude glycerol [13].

Previous studies have demonstrated that most bacteria suitable for producing PHAs with crude glycerol supplementation belong to the gram-negative group. These bacteria can grow more easily and accumulate more PHAs in their cells compared to gram-positive bacteria. However, the production of PHAs from gram-negative bacteria often encounters contamination by lipopolysaccharides at the outer membrane of the bacterial cell wall. This contaminant has a high resistance to heat and is extremely difficult to remove unless solvents such as hydrogen peroxide or benzoyl peroxide are used for removal, which will cause the properties of the PHAs to change [14]. Consequently, the selection of gram-positive bacterial strains that can tolerate inorganic salts is an alternative way to utilize crude glycerol as the feedstock of PHAs production to help lower the overall cost of PHAs production and to directly exploit crude glycerol. Lipid-containing wastewater contains mixtures of various fats, oils, waxes, and other constituents that are related to fat. It is an effluent produced by many types of industries. The microorganisms that are capable of utilizing fatty acids in lipid-containing wastewater for energy sources consist of *Escherichia coli*, *Pseudomonas*, *Acinetobacter*, and various *Bacilli* [15]. Therefore, this study focuses on the isolation of PHAs producing a *Bacillus* strain from lipid-containing wastewater. Also, it concerns the optimization of PHAs production utilizing crude glycerol as feedstock to make the bio-polymers production process more competitive for industrial-scale manufacturing.

Materials and methods

Wastewater samples collection

Wastewater samples were collected from the wastewater storage pond at a fermented pork sausage manufacturing plant in Uttaradit Province, Thailand. These samples were collected at the mid-point of the pond at a depth of 30.0 cm below the water surface. They were then kept in 3 200 mL pre-sterilized glass bottles at 4 °C for further laboratory analysis [16].

Isolation of PHAs producing *Bacillus* strains

The 3 wastewater samples were mixed thoroughly using a magnetic mixer. Next, 1 mL of the wastewater sample was pipetted into a test tube containing 9 mL of phosphate-buffered saline (PBS) then shaken well to produce a mixture (i.e., a sample solution at a concentration of 10^{-1}). This step was followed by pipetting 1 mL of the resulting solution (10^{-1}) into a sterile test tube containing 9 mL of PBS, which was then shaken to obtain a bacterial suspension at a 10^{-2} concentration. These serial dilutions continued until a bacterial suspension at a 10^{-10} concentration was obtained. Throughout the process, 1 mL of the bacterial suspension at each concentration level was pipetted into a sterile plate (2 replications), and 15 mL of Reasoner's 2A (R2A) medium was then added and mixed well for incubation at 35 °C for 48 h. Subsequently, bacillus-like colonies were picked and purified by streaking over the surface of nutrient agar. The R2A medium (1 L) consists of 0.50 g yeast extract, 0.50 g peptone, 0.50 g casamino acid, 0.50 g glucose, 0.50 g soluble starch, 0.30 g sodium pyruvate, 0.30 g K_2HPO_4, 0.050 g $MgSO_4.7H_2O$, and 15 g agar.

All the purified colonies were screened for PHAs production by incubation in 50 mL of growth medium (GM) supplemented with 0.2 % w/v glucose at 35 °C for 48 h in an incubator shaker at a speed of 150 rpm. Each litre of GM contains 10 g peptone, 10 g yeast extract, 5 g beef extract, and 5 g $(NH_4)_2SO_4$. In addition, 6 mL of bacterial suspension in GM was added to 114 mL of mineral salt medium (MSM) supplemented with 1 % w/v glucose for incubation at 35 °C for 72 h in an incubator shaker at a speed of 150 rpm. Each litre of MSM consists of 1 g $(NH_4)_2SO_4$, 6.7 g $Na_2HPO_4.7H_2O$, 0.1 g KH_2PO_4, 0.2 g $MgSO_4.7H_2O$, 60 mg $NH_3FeC_6H_8O_7$, 10 mg $CaCl_2.7H_2O$, and 1 mL Trace Element (TE), where the TE was prepared by dissolving 2.78 g $FeSO_4.7H_2O$, 1.98 g $MnCl_2.4H_2O$, 2.82 g $CoSO_4.7H_2O$, 1.67 g $CaCl_2.2H_2O$, 0.17 g $CuCl_2.2H_2O$, and 0.29 g $ZnSO_4.7H_2O$ in 1 L of 0.01 M HCl. The bacterial cell suspensions were taken for testing their PHAs production ability by the Sudan Black B dyeing method, DCW analysis, and measurement of the PHAs content. The cultures that could produce PHAs were then used in the Gram staining procedure and catalase enzyme production test [17].

Partial purification process of crude glycerol

The Chemistry Laboratory of Uttaradit Rajabhat University, Thailand, collected crude glycerol by the transesterification of waste cooking oil and methanol catalysed using NaOH. The method of acidification and neutralization of crude glycerol follows [18]. The first step is acidification. We started with a weight of 1 kg of crude glycerol and then added 1.19 M of sulfuric acid to achieve pH 2. This process produced 3 distinct layers, a free fatty acid top layer, a glycerol-rich middle layer, and an inorganic salt-rich bottom layer. The precipitated salt was removed from the mixture using a filter funnel with filter paper pore size 11 μm. The solution mixture of 2 layers was poured into the separating funnel to remove the glycerol-rich layer. After that, neutralization of crude glycerol was initiated by adding 12.5 M NaOH to achieve pH 7. We boiled the glycerol by-product at 105 °C for 2 h to evaporate the remaining water and then added 95 % ethanol at a ratio of 1:1. The mixture was stirred with a hotplate stirrer at 80 °C for 20 min. The glycerol by-product was analysed and kept for further experiment.

Study of the optimum conditions to produce PHAs

The study of the optimum conditions for PHAs accumulation, using crude glycerol as the carbon source of the wastewater-isolated *Bacillus* sp. FMI3, involves various factors that affect the DCW and PHAs yields. These factors include the concentration of crude glycerol, nitrogen source, a suitable C/N mole ratio, inoculum percentage, pH, and temperature. The 1-factor-at-a-time method was applied to examine the dynamics of 1 factor while the others were held constant (**Figure 1**). A starter culture (5 % v/v) of isolated *Bacillus* sp. FMI3 was added to 100 mL of MSM in 250 mL Erlenmeyer flasks. The first investigated factor was the concentration of crude glycerol. Various concentrations of partially purified crude glycerol (10, 20, 30, 40 and 50 g/L) were added into 100 mL of the MSM. The medium with different concentrations of crude glycerol was incubated at 35 °C with 150 rpm shaking for 72 h. The second examined factor was the nitrogen source, which can enhance cell growth and help produce PHAs of bacteria. This source was examined using the MSM, which contains the best concentration of crude glycerol as a carbon source. It was supplemented with nitrogen sources from other candidates (0.1 % w/v), such as ammonium sulphate, ammonium chloride, ammonium nitrate, ammonium acetate, monosodium glutamate (MSG), sodium nitrate, and urea. The medium with different nitrogen sources was incubated at 35 °C with 150 rpm shaking for 72 h. The third examined factor was the suitable C/N mole ratio. This ratio was determined using 40 g/L of crude glycerol and appropriate nitrogen sources identified in previous studies at various concentrations (0.1, 1, 5, 10, 15 and 20 g/L), which was equivalent to the C/N mole ratios of 565, 61, 11, 5.6, 3.9 and 2.9, respectively. The best nitrogen source and the best C/N mole ratio were chosen for further optimization. A study on how inoculum percentage and pH affect the growth and PHAs accumulation of the PHAs-producing bacteria was also performed using the starter culture at volumes of 5, 10, 15 and 20 mL, corresponding to 5, 10, 15 and 20 % v/v inoculum, respectively; initial medium pH values were 5 - 9, respectively. The media with different inoculum percentages and pH values were incubated at 35 °C with 150 rpm shaking for 72 h. Finally, the effects of incubation temperature were studied at various temperature (30, 35, 40, 50 and 60 °C).

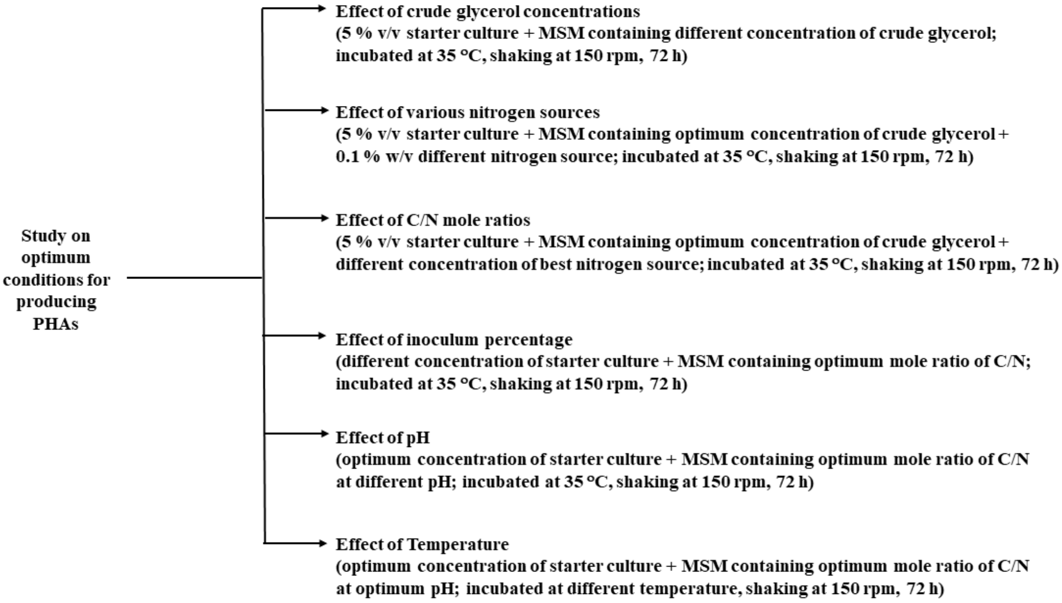

Figure 1 Experimental set-up for optimization of PHAs production.

Production of PHAs under the optimum conditions

The isolated *Bacillus* sp. FMI3 was incubated in a 250 mL Erlenmeyer flask containing 50 mL of GM at 35 °C for 48 h in a shaking incubator at an agitation speed of 150 rpm for use as a starting culture. A concentration of 15 % v/v from the above preculture was used to inoculate 250 mL of MSM at pH 7, supplemented with crude glycerol and ammonium sulphate at the C/N mole ratio of 61 as the carbon and nitrogen sources, respectively. The cultures were incubated at 40 °C for 72 h. The liquid nutrient (20 mL) was taken every 12 h for DCW analysis and quantity determination of PHAs and residual crude glycerol. Meanwhile, the compositions of the PHAs obtained from cultivation for 72 h were analysed using gas chromatography-mass spectrometry (GC-MS).

Identification of isolate *Bacillus* sp. FMI3

The isolate *Bacillus* sp. FMI3 was identified for its biochemistry characteristics using a VITEX 2 Compact automated bacteria identification and detection instrument. 16S rRNA gene sequencing was also used to identify this strain.

Analytical method

Cell growth was monitored by DCW. The PHAs in cell was extracted and quantified by UV-visible spectroscopy. The compositions of partially crude glycerol (e.g., ash content, water content, pH and free glycerol content) were investigated. The composition of monomeric PHAs was analysed by GC-MS. The structure of the monomers, the major function groups and glass transition temperature (Tg) were investigated and all analytical methods are presented below.

Analysis of DCW

A volume of 5 mL of each bacterial suspension was centrifuged at 7,000 rpm at 5 °C for 15 min. The obtained cell pellet was then washed twice with 5 mL of sterile distilled water and heated at 100 °C until dry to measure a constant weight [19].

PHAs extraction and quantification

Following the method of Getachew and Woldesenbet [20], 5 mL of each bacterial suspension was centrifuged at 7,000 rpm at 5 °C for 15 min. After the removal of the supernatant, the pellet was re-suspended with an equal volume of 4 % sodium hypochlorite and incubated at 37 °C in a shaker at 150 rpm for 2 h. The whole mixture was then centrifuged again to collect PHAs granules, and the supernatant was discarded. The obtained cell pellet was washed twice with 5 mL sterile distilled water, then re-washed with a mixture of 5 mL of acetone, ethanol, and diethyl ether at a 1:1:1 proportion and centrifuged once more at 7,000 rpm at 5 °C for 15 min. Finally, the obtained polymer granules were dissolved in 5 mL of hot chloroform and boiled until the chloroform evaporated entirely; subsequently, 5 mL of concentrated sulfuric acid was added for further boiling for 10 min in a water bath. The final mixture was taken for light absorption measurements under a UV spectrum at 235 nm. The concentration of PHAs was determined from an established standard crotonic acid graph where the light absorption (235 nm) was plotted against the concentration of crotonic acid as standard. The equation below was used for the quantification of PHAs.

$$\text{PHAs content (\%)} = [\text{PHAs concentration (g/L)} / \text{DCW (g/L)}] \times 100$$

Analysis of crude glycerol

The ash content and water content of crude glycerol were analysed according to standard method ISO2098-1972 [21,22]. For pH determination, crude glycerol (1.00 ± 0.1 g) was dissolved in 50 mL of deionized water and was measured by a digital pH meter at room temperature. The free glycerol content of crude glycerol was determined by an iodometric-periodic acid method [21,22].

Analysis of PHAs compositions by GC-MS

At the beginning, a sample of PHAs was prepared by centrifugation of bacterial suspension at 8,000 rpm for 15 min. The pellet was washed twice with sterile distilled water and then dried at 80 °C for 24 h. About 200 mg of the dried pellet was then placed into a 50 mL glass round bottom flask, to which 2 mL each of methanol and chloroform and 0.5 mL of sulfuric acid were added to perform the reflux process in a silicone oil bath at 100 °C for 2 h. The mixture was left to cool to room temperature, then 2 mL each of distilled water and saturated sodium hydrogen carbonate solution were added. The organic solvent in the bottom layer of the mixture (i.e., the layer of chloroform) was collected, and residual water was removed with a small amount of sodium sulphate. The solution was then filtered through a nylon membrane filter (pore size = 0.45 μm) to analyse the PHAs monomer compositions [23].

The analysis of PHAs monomer compositions using the GC-MS technique started by injecting 0.1 μL of the sample described above into a GC-MS device with a 30 m long HP-5ms capillary column (0.25 mm ID×0.25 μm film thickness). The initial column temperature was set at 65 °C for 5 min. Then, the temperature increased at a rate of 10 °C/min to a temperature of 150 °C then increased at a rate of 5 °C/min until it reached 200 °C, where it stayed for 6.5 min. The injector temperature was 250 °C, and the temperatures of the MS Quadrupole and MS Source were 150 and 230 °C, respectively. Helium was used as a carrier gas at a constant flow of 1 mL/min.

PHAs characterization

Tg was performed using differential scanning colorimetry (DSC) (STAR^{e} system). The temperature scanning was ramped from −100 - 100 °C at a heating rate of 10 °C/min under nitrogen. Analysis of the major functional group was conducted by using Fourier transform infrared spectroscopy (FTIR). An extracted sample of PHAs (1 mg) was dissolved in 5 mL of chloroform and then KBr was added to form a pellet. The spectra were recorded ranging 4,000 - 400 cm^{-1} by Spectrum GX (Perkin Elmer). The structure of the monomer's unit was characterized by proton nuclear magnetic resonance (^{1}H-NMR) spectroscopy. A PHAs sample (20 mg) was dissolved in deuterated chloroform on a Bruker Avance 400 spectrometer (Bruker, Helios, Singapore) at 400 MHz.

Statistical analysis

All experiments (i.e., the effect of crude glycerol concentration, nitrogen source, C/N mole ratio, inoculum percentage, pH, and temperature) were performed in triplicate. Experimental results were processed using simple linear regression. This method was used to test independent factors (i.e., concentration of crude glycerol, nitrogen source, C/N mole ratio, inoculum percentage, pH and incubation temperature) significantly predicted PHAs concentration and DCW. Independent factors were chosen base on regression coefficient (β) at significance ($p < 0.05$) and 95 % CI. All statistical analyses were performed using SPSS 21 for Windows.

Results and discussion

Selection of *Bacillus* sp. strain capable of producing PHAs

A total of 30 bacillus-like isolates were obtained from wastewater of fermented pork manufacturing plant. In the primary screening, 4 isolates exhibited black dots inside the cells, suggesting that these 4 bacterial strains could produce and accumulate PHAs [24]. These were named FMI1, FMI2, FMI3 and FMI4. All the isolates were rod-shaped and capable of producing catalase enzyme, as shown in **Table 1**. All isolates except FMI1 were gram-positive bacteria. The isolate FMI3 was found to have the highest PHAs accumulation (43 % of DCW) and was thus chosen for further investigation. This finding is consistent with the study of Penkhrue *et al.* [25], who found that *Bacillus* BP17 isolated from various soil sites exhibited the maximum PHAs content (19.9 % of DCW). *Bacillus* is a well-known source of the production and accumulation of PHAs between about 2 and 50 % [26]. In particular, *Bacillus megaterium* S29 and *Bacillus* sp. IPCB-403 accumulated PHB over 70 % of DCW in optimal conditions [27,28]. Consequently, the genus *Bacillus* is of interest for use in the production and accumulation of PHAs. From the tests of biochemical characteristics, the isolate FMI3 was found to resemble *Bacillus pumilus* (94 %), as shown in **Table 2**. The partial 16S rRNA gene sequence of isolate FMI3 (1,513 bp) was deposited into the GeneBank database under the accession number JX680133.1. The sequence was aligned and compared with those available in the public database. The result revealed that isolate FMI3 should be classified under the genus *Bacillus* with a high similarity level to *Bacillus Zhangzhouessis, Bacillus australimaris, Bacillus pumilus, Bacillus safensis, Bacillus altitudinis, Bacillus stratosphericus and Bacillus xiamenensis* (**Figure 2)**. The molecular evolutionary analyses also indicated that these strains belonged to the *Bacillus pumilus* clade [29]. Therefore, strain FMI3 isolated from this study is referred to as *Bacillus pumilus* FMI3.

The *B. pumilus* FMI3 could grow in 6.5 % w/v NaCl and utilize D-mannitol, D-mannose, D-tagatose, D-trehalose, and D-glucose as a carbon source and energy. This result is consistent with the work of Nicholson *et al.* [30], who found that *B. pumilus* is highly resistant to extreme environments, including those containing very limited or no availability of nutrients, drought, chemicals, and various types of radiation. These properties enable *B. pumilus* FMI3 to synthesize and accumulate PHAs in its cells more effectively than other isolates from the present study.

Table 1 Primary test results of bacterial strains isolated from lipid-containing wastewater.

Isolate No.	Sudan black B staining	Gram-staining	Shape	Catalase enzyme production	DCW (g/L)	PHAs concentration (g/L)	PHAs content (%)
FMI1	black	gram-negative	rod	positive	1.25 ± 0.06	0.25 ± 0.01	20
FMI2	black	gram-positive	rod	positive	1.20 ± 0.06	0.13 ± 0.06	11
FMI3	black	gram-positive	rod	positive	1.36 ± 0.06	0.58 ± 0.03	43
FMI4	black	gram-positive	rod	positive	1.08 ± 0.05	0.36 ± 0.02	33

Table 2 Biochemical characteristics of the FMI3 strain.

Characteristics	Reaction	Characteristics	Reaction
Enzyme production:		Substrate utilization	
β-xylosidase	+	Cyclodextrin	−
L-lysine arylamidase	−	D-galactose	−
Leucine arylamidase	−	Glycogen	−
Phenylalanine arylamidase	+	Myo-inositol	−
L-proline arylamidase	−	Methyl-α-D-glucopyranoside acidification	−
β-galactosidase	+	Methyl-D- xyloside	−
L-pyrrolidonyl arylamidase	−	Maltotriose	−

Characteristics	Reaction	Characteristics	Reaction
α-galactosidase	+	D-mannitol	+
Alanine arylamidase	−	D-mannose	+
Tyrosine arylamidase	+	D-melezitose	−
β-N-acetyl-glucosaminidase	+	N-acetyl-D-glucosamine	−
Ala-Phe-Pro arylamidase	+	Palatinose	−
Glycine arylamidase	(−)	L-rhamnose	−
β-glucosidase	+	Phosphoryl choline	−
α-glucosidase	−	Pyruvate	(+)
β-mannosidase	−	D-tagatose	+
α-mannosidase	+	D-trehalose	+
Gram reaction	+ve	Inulin	−
Putrescine assimilation	−	D-glucose	+
Growth in 6.5% NaCl	+	D-ribose	(−)
Kanamycin resistance	−	Tetrazolium red	+
Esculin hydrolyse	+	Polymixin B resistance	−

Note: + = positive reaction; − = negative reaction; (+) = weak-positive reaction; (−) = weak-negative reaction; +ve = gram positive bacteria.

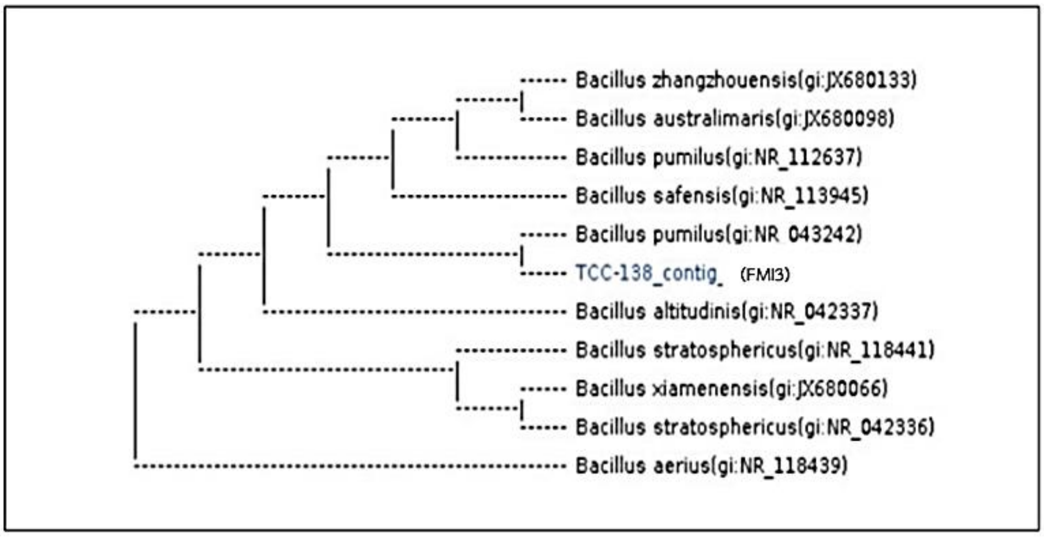

Figure 2 Neighbor joining phylogenetic tree showing the interrelationship between isolate FMI3 and top 10 Blast hits from database.

Composition of partially purified crude glycerol

Crude glycerol from biodiesel production was acidified with concentrated H_2SO_4 and left for phase separation. After complete separation, the upper phase consisted of free fatty acid, methanol, water, and acids, while the middle phase was a glycerol-rich layer with some methanol, water, and salt. The lower phase also had a small fraction of an inorganic salt-rich layer [18]. The chemical composition of partially purified crude glycerol indicated the glycerol content at 80.04 ± 4.00 % w/w, light brown colour with pH 7.11 ± 0.35, 1.90 ± 0.09 % ash content, and 2.80 ± 0.14 % water content.

Optimal conditions for producing PHAs

B. pumilus FMI3 was cultured in MSM supplemented with 5 different concentrations of partially purified crude glycerol to investigate the utilization of crude glycerol on its growth and PHAs production. Increasing concentration of crude glycerol from 10 - 40 g/L affected DCW and PHAs accumulation of *B. pumilus* FMI3, where a slight increase in DCW and PHAs content was obtained with higher glycerol concentration (**Table 3**). The isolate *B. pumilus* FMI3 provides the highest DCW and PHAs yield at 2.10 ± 0.10 g/L and 44 % of DCW, respectively, when using 40 g/L and decreased to 1.92 ± 0.09 g/L and 40 % of DCW at 50 g/L crude glycerol. A potential explanation for this finding is that the isolate *B. pumilus* FMI3 can thrive on crude glycerol that contains some salt. However, its growth performance will become poorer with higher crude glycerol concentrations in MSM because some contaminants in crude glycerol might make unsuitable environments such as high pH, high salts, or the effect of higher osmotic stress on the cell [31]. This observation is consistent with the finding of Phithakrotchanakoon *et al.* [13] on the production and accumulation of PHB by *E. coli-ABC$_{Ah}$*, which indicated that higher concentrations of crude glycerol in the M9 medium can be harmful to the cells, leading to a reduced PHB yield.

Table 3 Effect of crude glycerol concentration on *Bacillus pumilus* FMI3's cell growth and PHAs production.

Concentration of crude glycerol (g/L)	DCW (g/L)	PHAs Concentration (g/L)	PHAs content (% DCW)
10	1.46 ± 0.07^{a**}	0.43 ± 0.02^{b**}	29
20	1.72 ± 0.09	0.65 ± 0.03	38
30	1.80 ± 0.09	0.69 ± 0.03	38
40	2.10 ± 0.10^{c*}	0.92 ± 0.05^{d*}	44
50	1.92 ± 0.09	0.77 ± 0.04	40

The mean and standard deviation per treatment were calculated from triplicates experiment. Lower case letter indicates a significant difference ($^{*}p < 0.05$; $^{**}p < 0.001$).
^{a**} represent β = −0.423, 95 % CI = −0.644 - −0.202;
^{b**} represent β = −0.329, 95 % CI = −0.475 - −0.183;
^{c*} represent β = 0.377, 95 % CI = 0.127 - 0.626;
^{d*} represent β = 0.288, 95 % CI = 0.113 - 0.462.

In general, the bacterial synthesis of PHAs occurs in 2 phases. The first is the growth phase when bacteria require nutrients for cell growth and multiplication. The second is the growth-limiting phase to enhance the accumulation of intercellular PHAs granules by limitation of nutrients [32]. A nitrogen source is an important requirement during the growth phase to maximize the concentration of biomass responsible for the accumulation of PHAs. The effect of various nitrogen sources on cell growth and PHAs production of *B. pumilus* FMI3 was tested by growing in MSM containing crude glycerol as a carbon source and a variety of nitrogen sources from inorganic salt and organic compound using a C/N mole ratio of 11. The results presented in **Table 4** reveal the best nitrogen source to be ammonium sulphate among the various nitrogen sources belonging to the inorganic salts group under testing. Ammonium sulphate helps to provide the highest PHAs concentration at 0.94 ± 0.05 g/L (45 % of DCW); the next best nitrogen sources are ammonium nitrate, ammonium acetate, ammonium chloride, and sodium nitrate, in descending order. Meanwhile, the use of organic nitrogen sources, including MSG and urea, resulted in limited cell growth and PHAs synthesis in *B. pumilus* FMI3. It may be explained that ammonium sulphate is an inorganic salt as a source of nitrogen that served as a precursor for vitamins, amino acids, and growth factors [33]. Our findings are consistent with the study results of Gomaa [34], which showed that ammonium sulphate was the best supporter for growth and PHAs production by *Bacillus subtilis* as it increased the cell dry mass and PHAs production up to 36.98 and 22.98 g/L, respectively. Therefore, ammonium sulphate was the nitrogen source in the subsequent experiments.

Table 4 Effect of different nitrogen sources on the growth and PHAs synthesis of *Bacillus pumilus* FMI3 incubated in MSM supplemented with crude glycerol as the carbon source.

Nitrogen source	DCW (g/L)	PHAs concentration (g/L)	PHAs content (% DCW)
Ammonium acetate	2.04 ± 0.10	0.77 ± 0.04	38
Ammonium chloride	2.01 ± 0.10	0.71 ± 0.03	35
Ammonium nitrate	2.02 ± 0.10	0.87 ± 0.04	43
Ammonium sulphate	2.10 ± 0.10^{a*}	0.94 ± 0.05^{b*}	45
MSG	1.23 ± 0.06	0.28 ± 0.01	23
Sodium nitrate	1.14 ± 0.05	0.12 ± 0.01^{c*}	10
Urea	0.56 ± 0.02^{d**}	0.016 ± 0.01^{e*}	2.8

The mean and standard deviation per treatment were calculated from triplicates experiment. Lower case letter indicates a significant difference ($^{*}p < 0.05$; $^{**}p < 0.001$).
^{a*} represent $\beta = 0.727$, 95 % CI = 1.230 - 1.776;
^{b*} represent $\beta = 0.477$, 95 % CI = 0.051 - 0.903;
^{c*} represent $\beta = -0.476$, 95 % CI = −0.902 - −0.049;
^{d**} represent $\beta = -1.218$, 95 % CI = −1.767 - 0.669;
^{e*} represent $\beta = -0.600$, 95 % CI = −0.989 - −0.212.

To investigate the effect of C/N mole ratios, *B. pumilus* FMI3 was incubated in MSM supplemented with crude glycerol as the carbon source and ammonium sulphate as the nitrogen source at C/N mole ratios from 2.9 to 565 by fixing the crude glycerol concentration at 40 g/L while varying the ammonium sulphate concentration (0.1 - 20 g/L). It revealed that higher C/N mole ratios (low concentration of ammonium sulphate) result in lower cell growth while enhancing PHAs accumulation (**Figure 3**). The optimum C/N mole ratio was 61 (1 g/L of ammonium sulphate) - this value enabled *B. pumilus* FMI3 to produce the highest PHAs accumulation at 1.40 ± 0.07 g/L (58 % of DCW). Our finding is consistent with the study results of Hungund *et al.* [19], which identified a positive relationship between C/N ratio and PHAs accumulation in the form of PHB. Consequently, the nutrient limitation is necessary to trigger the PHAs accumulation. However, in this study, C/N mole ratios greater than 61 reduced PHAs output to 33 % of DCW. This outcome may indicate that an overly high concentration of carbon source stops the growth of *B. pumilus* FMI3 and its PHAs synthesis. Meanwhile, C/N mole ratios lower than 61 result in reduced PHAs yields due to the comparatively large amount of nitrogen, which promotes bacterial growth but reduces PHAs production. This phenomenon may be explained by the bacteria using nitrogen for cell growth, so the synthesis and accumulation of PHAs in bacterial cells do not occur. Therefore, the concentration of crude glycerol and ammonium sulphate at a C/N mole ratio of 61 were used as the carbon and nitrogen sources, respectively, in the further experiments.

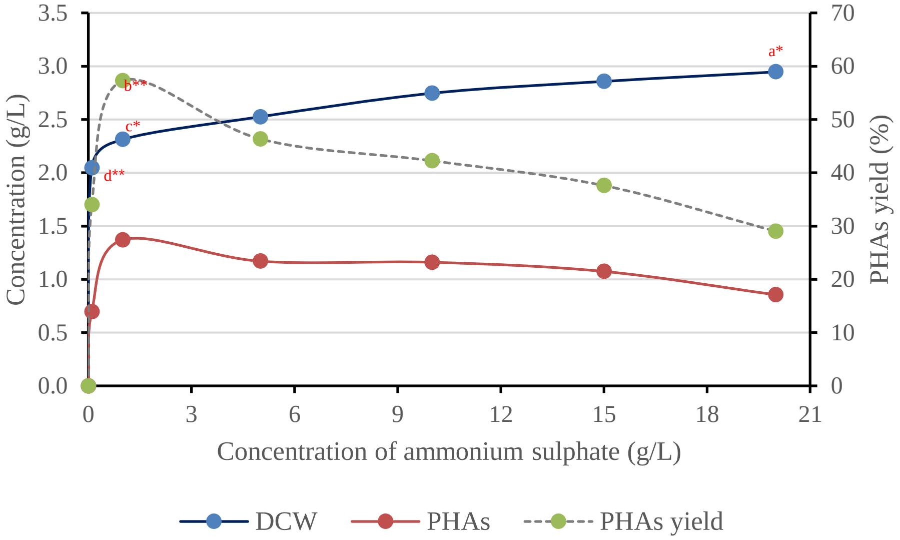

Figure 3 PHAs yield, Dry cell weight (DCW) and polyhydroxyalkanoate (PHAs) concentration of the *Bacillus pumilus* FMI3 strain cultured in MSM containing various concentration of ammonium sulphate. The mean and standard deviation per treatment were calculated from triplicates experiment. Lower case letter indicates a significant difference ($^{*}p < 0.05$; $^{**}p < 0.001$).
^{a*} represent β = 0.433, 95 % CI = 0.030 - 0.836;
^{b**} represent β = −0.647, 95 % CI = −0.959 - −0.335;
^{c*} represent β = 0.377, 95 % CI = 0.121 - 0.632;
^{d**} represent β = −0.430, 95 % CI = −0.660 - −0.199.

The effects of inoculum percentage on the growth and PHAs accumulation by *B. pumilus* FMI3 appear in **Figure 4**. It revealed that an increase in the inoculum percentage will increase the DCW and PHAs concentration. The optimum PHAs production of 1.87 ± 0.09 g/L (67 % of DCW) was obtained using 15 % v/v inoculum. However, the inoculum concentration higher than 15 % v/v results in a lower PHAs concentration. At a higher inoculum concentration, the nutrients are no longer sufficient for bacterial cell growth, thus it must use nutrients and energy from stored PHAs instead [35]. Therefore, the following experiment used inoculum at a 15 % v/v concentration.

The studies on the effect of pH of the MSM on PHAs production by *B. pumilus* FMI3 showed that the medium with an initial pH of 7.0 gave the highest production of PHAs at 1.90 ± 0.09 g/L (67 % of DCW) (**Figure 5**). The pH values other than 7.0 tend to reduce PHAs synthesis and cell growth. This effect can be explained by the fact that pH 7.0 is neutral and the most favourable for bacterial growth. The growth media with an initial pH of less or more than 7 causes hydrogen ion and hydroxide ion concentrations to be imbalanced, resulting in the inhibition of bacterial nutrient uptake for intercellular storage. Thus, adjusting the initial pH of the culture medium to a value of 7 has the effect of reducing the concentration of free hydrogen ions (H^{+}) using hydroxide ions (OH^{-}), such that the hydrogen ions do not have a direct inhibitory effect on bacterial cell growth [34].

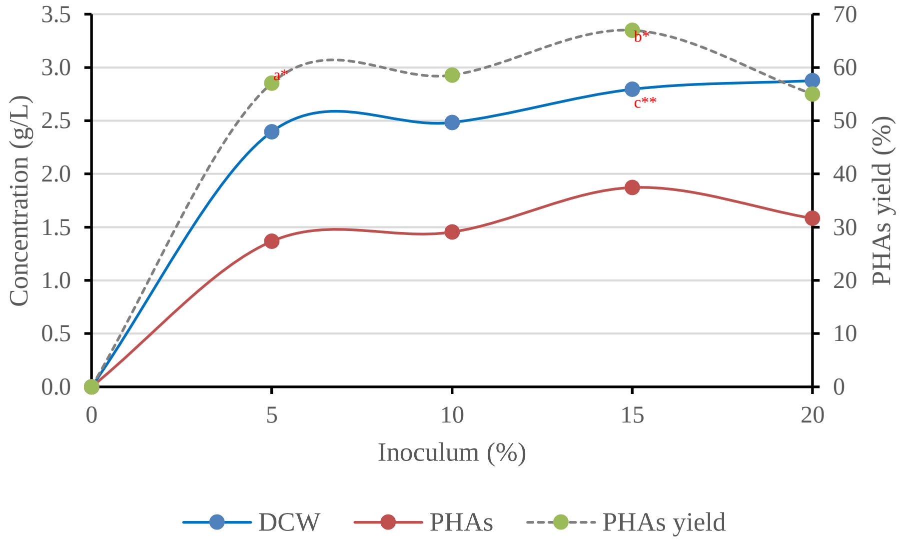

Figure 4 The isolated *Bacillus pumilus* FMI3 growth and its PHAs production using various inoculum percentages.

The mean and standard deviation per treatment were calculated from triplicates experiment. Lower case letter indicates a significant difference ($^{*}p < 0.05$; $^{**}p < 0.001$).

^{a*} represent β = −0.322, 95 % CI = −0.614 - −0.029;
^{b*} represent β = 0.318, 95 % CI = 0.023 - 0.613;
^{c**} represent β = 0.405, 95 % CI = 0.208 - 0.602.

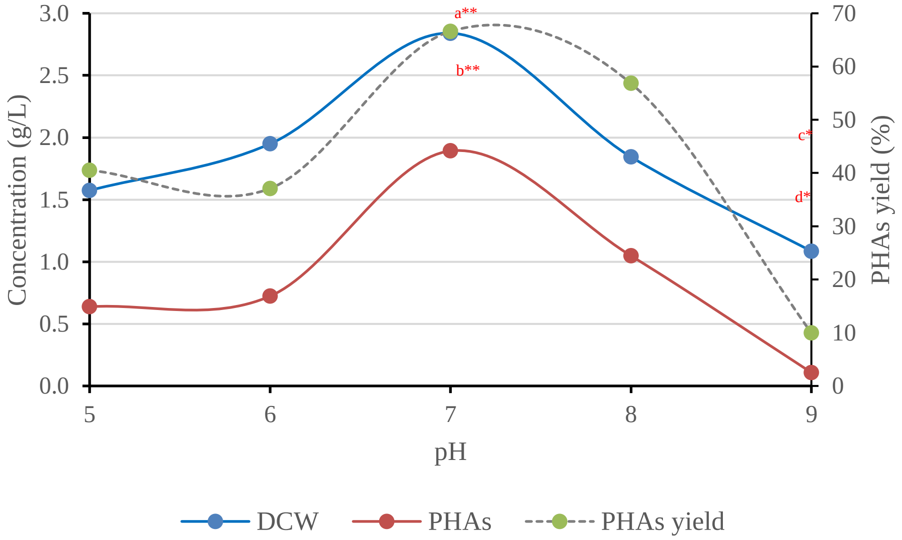

Figure 5 The isolated *Bacillus pumilus* FMI3 growth and its PHAs production at various initial pH of the medium.

The mean and standard deviation per treatment were calculated from triplicates experiment. Lower case letter indicates a significant difference ($^{*}p < 0.05$; $^{**}p < 0.001$).

^{a**} represent β = 1.227, 95 % CI = 1.404 - 1.822;
^{b**} represent β = 1.263, 95 % CI = 0.423 - 0.836;
^{c*} represent β = −0.967, 95 % CI = −1.617 - −0.316;
^{d*} represent β = −0.967, 95 % CI = −1.638 - −0.296.

The incubation temperature is another important factor for attaining high bacterial growth and PHAs content. It plays a crucial role in cell metabolic processes, with the optimum temperature varying across different types of micro-organisms. The effects of various temperatures on the growth and PHAs production by the isolate *B. pumilus* FMI3 showed 40 °C as the optimum temperature for this strain. The maximum PHAs synthesis and accumulation of 2.0 ± 0.1 g/L (71 % of DCW) was obtained. Temperatures at lower and higher than 40 °C will result in lowered cell growth and PHAs production (**Figure 6**). Previous studies indicate that the optimum temperature for the growth of *B. pumilus* lies within a wide range between 5 and 50 °C [36]. They report that a higher temperature will induce a higher reaction rate because a reduced substrate viscosity will increase the coefficient value of substrate diffusion, and high temperatures can generate an equilibrium displacement in endothermic reactions [37]. However, overly high temperatures will reduce bacterial growth and PHAs production.

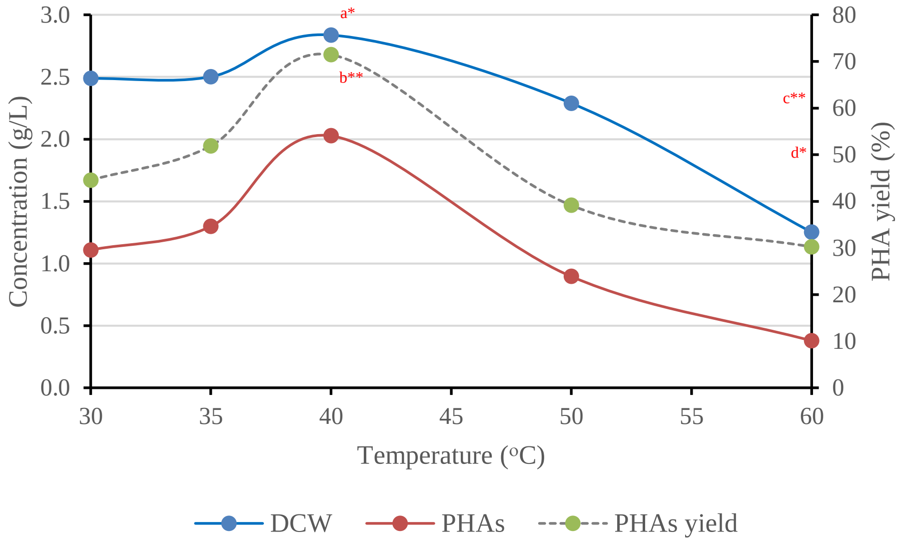

Figure 6 The isolated *Bacillus pumilus* FMI3 growth and its PHAs production at different temperatures. The mean and standard deviation per treatment were calculated from triplicates experiment. Lower case letter indicates a significant difference ($^*p < 0.05$; $^{**}p < 0.001$).
^{a*} represent β = 0.705, 95 % CI = 1.817 - 2.448;
^{b**} represent β = 1.106, 95 % CI = 1.579 - 1.708;
^{c**} represent β = −1.267, 95 % CI = −1.556 - −0.977;
^{d*} represent β = −0.954, 95 % CI = −1.533 - −1.374.

PHAs production from crude glycerol under optimum conditions

The optimized PHAs production from crude glycerol by the isolate *B. pumilus* FMI3 in MSM was achieved with crude glycerol and ammonium sulphate at a C/N mole ratio of 61, 15 % v/v inoculum concentration, a pH value of 7, and incubation at 40 °C in a shaker at 150 rpm. The experimental results of this configuration appear in **Figure 7**. Bacterial growth was rapid in the first 24 h of incubation and became stable after 48 h; in addition, the PHAs production of *B. pumilus* FMI3 tends to correspond with its growth. The maximum PHAs content was 2.30 ± 0.12 g/L (79 % of DCW), produced at 72 h of cultivation with about 9.0 ± 0.4 g/L of crude glycerol residual unused. This result indicated that the isolate *B. pumilus* FMI3 can use crude glycerol for its cell growth and PHAs production. The optimization of medium composition improved PHAs yield about 1.8-fold as compared to an un-optimized medium. The present experimental results confirm that the indigenous *B. pumilus* FMI3 strain isolated from lipid-containing wastewater can use crude glycerol for PHAs synthesis and accumulation more efficiently than the recombinant or genetically modified bacteria. For example, recombinant *E. coli* cultivated in M9 medium supplemented with 1 g/L yeast extract and 1 % v/v crude glycerol as the carbon source yielded an optimal PHAs production of 14 % of DCW [13], in contrast to the maximum of 79 % of DCW achieved in this study. Furthermore, *B. pumilus* FMI3 can outperform a new bacterial strain, *Pandoraea* sp. MA03, isolated from Atlantic rainforest soil in Brazil. In terms of PHAs production, by cultivation in MSM with 2 % w/v crude glycerol and a pH of 7 at 30 °C incubation in a flask shaken at 150 rpm for 120 h, this species produced a maximum PHAs of 64 % of DCW [11]. This finding demonstrated that the isolate *B. pumilus* FMI3 is highly attractive for producing PHAs from crude glycerol at an industrial scale. It is ideal because it is a gram-positive bacterium with an absence of lipopolysaccharides such that the properties of the extracted PHAs do not change, and it has good tolerance to salts in crude glycerol. Therefore, the use of a by-product of the biodiesel manufacturing process as a raw material for producing PHAs can reduce the cost of biopolymer production at an industrial scale while also adding market value to crude glycerol. Comparing the isolate *B. pumilus* FMI3 with *B. megaterium* LVN01, which is also a gram-positive bacterial strain, in producing PHAs from crude glycerol reveals similar performance. *B. megaterium* LVN01 achieved a maximum PHAs value of 75 % of DCW from cultivation in glycerol synthetic medium (WGM) containing crude glycerol and ammonium sulphate as the carbon and nitrogen sources, respectively, with a C/N mole ratio of 45 and fermentation at 31 °C for 40 h [38].

PHAs monomeric compositions from the GC-MS analysis

The GC-MS technique was applied to analyse methyl esters of the polymer extracted from bacterial cells of the *B. pumilus* FMI3 strain cultivated in MSM under optimum conditions. The PHAs obtained were determined to belong to 4 types of monomers, including 3-hydroxyoctanoate (C8-3HO), 3-hydroxydecanoate (C10-3HD), 3-hydroxydodecanoate (C12-3HDD), and 3-hydroxytetradecanoate (C14-3HTD). The peak retention times of 3HO, 3HD, 3HDD, and 3HTD were 16.25, 19.70, 23.36 and 27.47 min, respectively, according to the mass spectra of methyl esters of 3-hydroxyalkanoates (**Table 5**). The fragment m/z 103 is characteristic of methyl esters formed by α-cleavage of the hydroxyl functional group. The loss of the CH_3OH group from the fragment at m/z = 103 could instead explain the presence of the intense signal at m/z = 71 [39]. Thus, the PHAs derived from *B. pumilus* FMI3 can be classified as MCL-PHAs [4] containing 3HDD as the most prevalent component, followed by 3HTD, 3HD, and 3HO in descending order. The 3HDD unit can only be synthesized from the fermentation of crude glycerol by *B. pumilus* FMI3, and no known previous reports on the use of crude glycerol as a carbon source in bacterial fermentation indicate 3HDD availability. In contrast, only the 3-hydroxybutyrate (3HB) type was obtained from *Bacillus licheniformis* M2-12 (mutant) grown in MSM [40] and *E. coli*-ABC$_{Ah}$ grown in M9 medium [13] supplemented with 3 % w/v and 1 % v/v crude glycerol as a carbon source, respectively. The MCL-PHAs were first discovered in *Pseudomonas oleovorans* [41], which had not been previously reported produced from the *Bacillus* sp. It implies that *B. pumilus* FMI3 uses crude glycerol as a carbon source for producing PHAs through a *de novo* fatty acid biosynthesis pathway. This pathway has been reported in *Pseudomonas putida and Pseudomonas aeruginosa* [42], which produced (*R*)-3-hydroxyacyl-CoA precursors from a non-related carbon source, such as glucose and gluconate. The MCL-PHAs are an amorphous substance or are semi-crystalline and elastic while being less brittle than SCL-PHAs. Moreover, the MCL-PHAs containing a high amount of 3HDD will be more crystalline and have greater tensile strength than MCL-PHAs with a lower amount of 3HDD [43]. These properties make it a particularly attractive polymer for industrial applications in areas of biomedicine that require products and equipment made of flexible biomaterials [44].

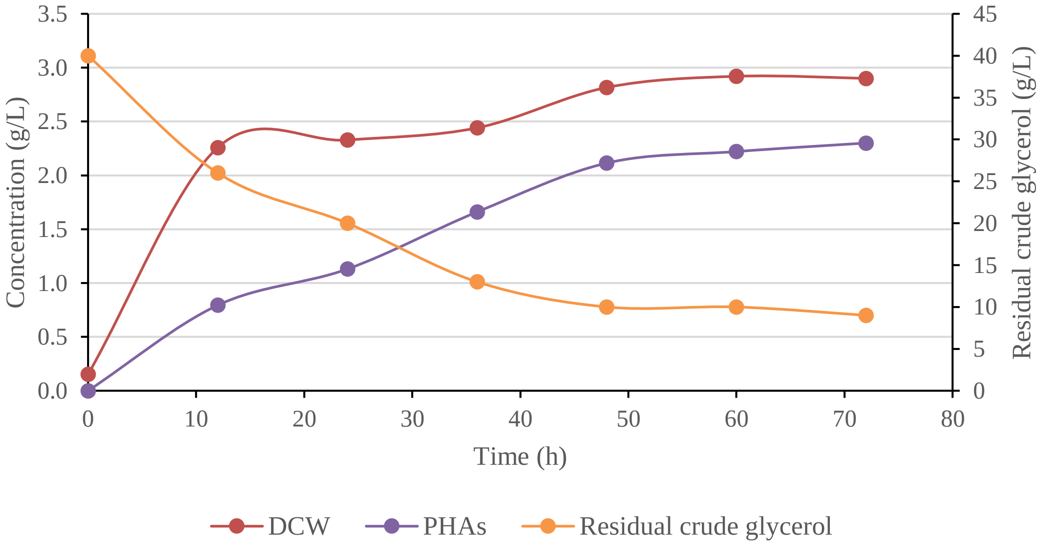

Figure 7 Profile of cell growth, PHAs production of the isolated *Bacillus pumilus* FMI3, and residual crude glycerol under optimum conditions.

Table 5 Monomer compositions of PHAs analysed by Gas chromatography and mass spectrometry.

Monomer composition	Mol%	Retention time (min)	M^+	m/z
C8-3HO	8.94	16.25	141	31, 39, 41, 43, 55, 61, 71, 74, 83, 103
C10-3HD	12.23	19.70	201	45, 61, 71, 74, 84, 103, 127, 153, 184
C12-3HDD	66.56	23.36	229	31, 33, 41, 43, 55, 57, 59, 61, 69, 71, 74, 83, 87, 96, 101, 103
C14-3HTD	12.27	27.47	260	29, 31, 43, 55, 61, 71, 74, 83, 97, 103, 111, 123

PHAs characterization

The thermogram for DSC analysis of the PHAs obtained from *B. pumilus* FMI3 by using crude glycerol as the carbon source are shown in **Figure 8**. The Tg of PHAs was 20.89 °C. Generally, the Tg of PHAs ranges 5 - 20 °C, depending on the composition and thermal history of the polymer [45,46]. MCL-PHAs that contain phenoxy group are completely amorphous with a Tg of 20 °C [42]. The temperature of Tg in this study is higher than the Tg of copolymer P(3HB-*co*-3HHx) that are produced by *E. coli*-$ABC_{Ah}J_{Ah}$ cultured in crude glycerol (Tg = 7 °C) [13]. This phenomenon may be explained by the fact that the PHAs produced from *B. pumilus* FMI3 with the use of crude glycerol as the carbon source were in amorphous form.

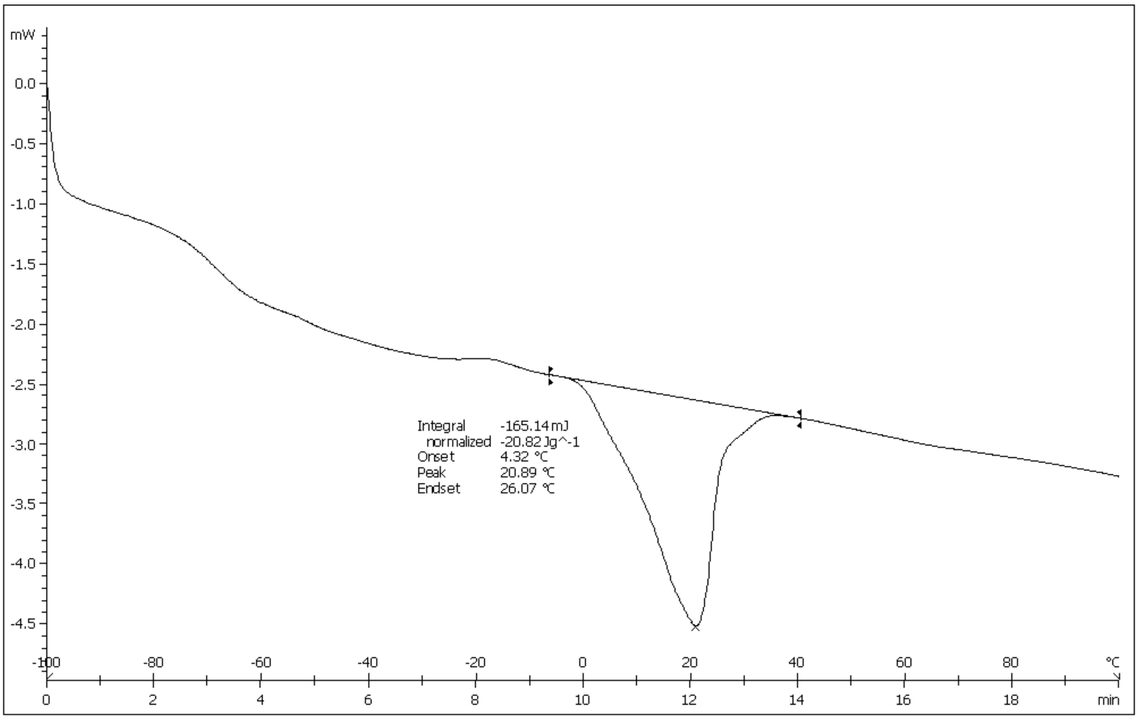

Figure 8 Differential scanning calorimetry (DSC) analysis of extracted MCL-PHAs produced from *Bacillus pumilus* FMI3.

In the FTIR analysis results, the peak at 3,419.25 cm^{-1} indicated the hydroxyl (OH) group of the polymer chain (**Figure 9**). Similar results have been reported in research by Getachew and Woldesenbet [20]. The peaks at 2,961.15, 2,925.14 and 2,852.14 cm^{-1} were assigned to asymmetric methyl (CH_3) group, the asymmetric methylene (CH_2) group of the lateral monomeric chains, and the symmetrical methyl group, respectively [34]. The peak at 1,655.57 cm^{-1} has been indicated a weak carbonyl (C=O) bond extended for conjugated carbonyl or the amide group [47]. The peak at 1,407.92 cm^{-1} was characteristic of the asymmetric stretching of the methyl and methylene groups [20]. The peak at 1,230.39 cm^{-1} represents –C–O– polymeric group and the absorption peaks from 1,169.04 - 468.92 cm^{-1} were assigned to C–O and C–C stretching vibration in the amorphous phase [34].

The ^1H NMR scan of PHAs obtained from *B. pumilus* FMI3 with the use of crude glycerol as a carbon source is represented in **Figure 10**. The spectrum appears at almost identical chemical shifts with those that were previously reported [48]. Chemical shift of the peak b at 1.27 ppm and peak a at 0.89 ppm can be assigned to the hydrogen of CH_2 groups in the saturated lateral chain and the terminal CH_3 group, respectively [49]. The chemical shifts of the peak c at 1.62 ppm and peak e at 5.25 ppm are assigned to CH_2 proton adjacent to the β-carbon in the side chain and methine protons of the β-carbon, respectively [48]. The peak d at 2.30 ppm can be assigned to diastereotopic methylene (CH_2) [25], but some literature assign it to unsaturation of PHAs monomer [50], which is related to hydrophobic oleate and palmitate from cellular membrane lipids [48].

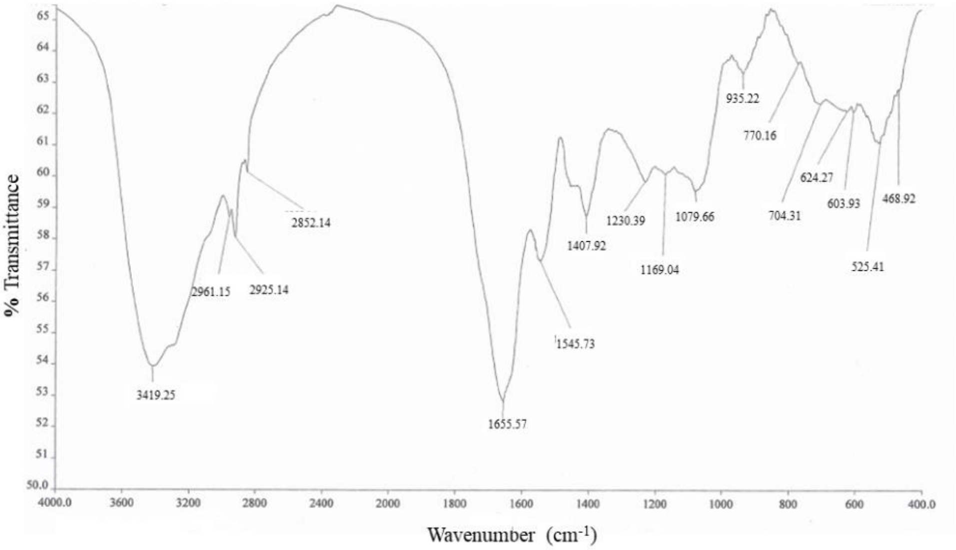

Figure 9 FTIR spectra of extracted PHAs produced by *Bacillus pumilus* FMI3 strain.

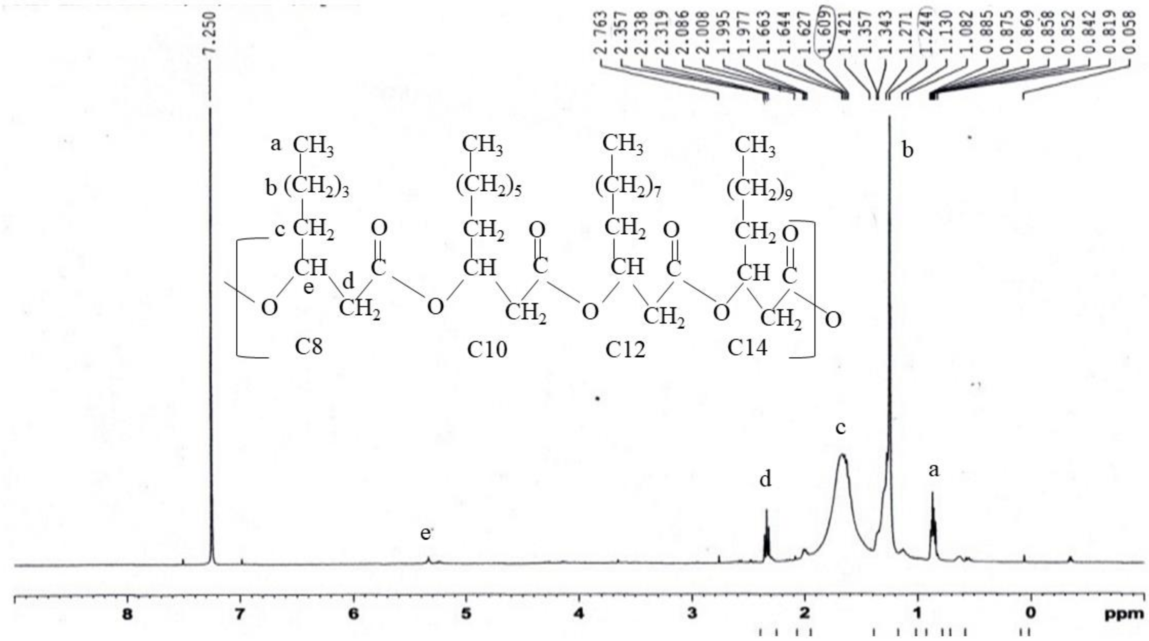

Figure 10 ^1H NMR spectra of extracted PHAs produced by *Bacillus pumilus* FMI3 strain.

Conclusions

The *B. pumilus* FMI3 strain isolated from lipid-containing wastewater showed a practicable potential to produce PHAs from crude glycerol as the sole carbon source. The production of PHAs from crude glycerol is a promising alternative to support biodiesel production from fats and oils. *B. pumilus* FMI3 is a new strain of *Bacillus* sp. used to convert crude glycerol to MCL-PHAs polyester with a high accumulation rate. The optimum condition of PHAs production from this study can be guidelines for reducing the cost of PHAs production at an industrial scale, which will improve the biopolymer's competitive efficiency in replacing petroleum-based synthetic plastics.

Acknowledgements

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