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Optimization of Production of Polyhydroxyalkanoates (PHAs) from Newly Isolated *Ensifer* sp. Strain HD34 by Response Surface Methodology

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Abstract: Petroleum-based plastics have become a big problem in many countries because of their non-degradability and that they become microplastics in the environment. This study focused on the optimization of production medium and conditions of polyhydroxyalkanoates (PHAs), which are biodegradable bioplastics and are accumulated in microbial cells. Among 341 isolates from 40 composted soil samples, the best isolate was the HD34 strain, which was identified using morphological, molecular, and biochemical methods. The results showed that the strain was most closely related to *Ensifer adhaerens* LMG20216^T, with 99.6% similarity. For optimization of production medium and conditions using response surface methodology, it exhibited an optimal medium containing 3.99% (*w*/*v*) of potato dextrose broth (PDB) and 1.54% (*w*/*v*) of D-glucose with an adjusted initial pH of 9.0. The optimum production was achieved under culture conditions of a temperature of 28 °C, inoculum size of 2.5% (*v*/*v*), and a shaking speed of 130 rpm for 5 days. The results showed the highest PHA content, total cell dry weight, and PHA yield as 72.96% (*w*/*w*) of cell dry weight, 9.30 g/L, and 6.78 g/L, respectively. The extracted PHA characterization was studied using gas chromatography, ^{1}H NMR, FTIR, and XRD. The results found that the polymer was a polyhydroxybutyrate (PHB) with a melting temperature (T_m) and degradation temperature (T_d) of 173.5 °C and 260.8 °C, respectively.

Keywords: polyhydroxyalkanoates; polyhydroxybutyrate; response surface methodology; central composite design

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1. Introduction

Petroleum-based plastics are being used in many fields, which caused global plastic production to reach almost 370 million tons in 2020 [1], and they have become a big problem in the world because they are non-degradable plastics. Polyhydroxyalkanoates (PHAs) are one family of biodegradable plastics and are compelling as environmentally friendly polymers instead of petroleum-based or non-degradable plastics. The interesting aspects of PHAs are their ability to be completely degraded by various microorganisms, their biocompatibility with humans, and their properties that are similar to the petroleum polymers polypropylene (PP) and polyethylene (PE) [2]. However, the production cost of PHAs is still higher than petroleum-based plastics. Therefore, it is necessary to develop bacterial genetic engineering, optimize the production medium and circumstances, and reduce production costs in order to produce PHAs.

There have been reports of numerous microorganisms with PHA production capabilities. The first PHA-producing bacterium was found in 1926 [3]. PHAs can be accumulated in bacterial cells as intracellular granules which are used as a carbon/energy source, or

a reducing power storage material. Gram-negative bacteria are typically used for the production of PHAs on an industrial scale [4]. Several studies show that *Rhizobium* can accumulate PHAs as their reserve energy in both free-living and legume-nodule-associated bacteria [5]. Rhizobial species that are PHAs producers include *Azorhizobium*, *Bradyrhizobium*, *Ensifer*, *Mesorhizobium*, and *Rhizobium*. *Rhizobium elti* was reported to accumulate 81.8% PHA content using mannitol as the sole carbon source, while *Pseudomonas stutzeri* accumulated 83% PHA content using sucrose as a carbon source [6]. Moreover, *Burkholderia cepacian* showed 49% PHA content as the result of cultivation with glucose provided by rice husks [7].

The monomer of the PHA bioplastic family is hydroxyalkanoic acid arranged in a chain with an R-group at the third carbon. The different amounts of carbon atoms in the R-group result in differential properties and polymer types in the PHA family. For example, the R-group of polyhydroxybutyrate (PHB) is one carbon atom, and polyhydroxyvalerate (PHV) is two carbon atoms [8]. PHB has the most promising properties for the plastic industry, such as being water insoluble and resistant, biocompatible, and non-toxic, with a high melting point and low flexibility [9]. Because of its biocompatibility and biodegradability, the PHA family has been applied to the nanotechnology, medical, tissue engineering, and packing industries [10]. In particular, PHA nanocomposites are safe for various emerging medical applications, for example cardiovascular and orthopedic uses, drug delivery, and tissue engineering [11]. The PHA family has been studied with the application of blending with chitosan for improving its compatibility, degradability, and strength properties. Furthermore, blending with boron nitride nanoparticles is also used to feasibly enhance nanocomposite properties such as antibacterial and cell viability efficacy [12].

Many campaigns are applied to reduce plastic waste while demand for plastic usage increases every year. Bioplastics are increasingly being used instead of petroleum-based plastics, but the production and extraction costs are still high. The reduction in production costs is related to the study of the optimization of bioprocesses for bacterial growth and increasing PHA yields. The purpose of the present study is to investigate the optimal medium and conditions for PHA production with a newly isolated *Ensifer* sp. HD34 using the statistical approach and response surface methodology (RSM). RSM is an optimization method that improves production efficiency using several factors, and their interactions on responses are identified [13,14]. The best production conditions are predicted to achieve the highest result following experimental conditions [15]. Optimization is generally performed on various factors such as carbon source, nitrogen source, and culture conditions (such as incubation time, inoculum size, initial pH, temperature, etc.). The high production yield of PHA polymers from the optimal medium and conditions gives the opportunity to use them in many applications. Moreover, we consider enhancing the properties of PHA polymers to create a nanocomposite material.

2. Materials and Methods

2.1. Sample Collection and Bacteria Isolation

Forty soil samples were collected from three provinces in northern Thailand, Chiang Rai, Chiang Mai, and Pa Yao. The soil samples were collected at around 20 cm depth and then kept in zip-lock plastic bags at 4 °C until used in further studies. For each soil sample, 5 g was enriched with 45 mL of nitrogen-deficient minimal medium (NDMM) [16] containing 1% (*w*/*v*) non-chemical pretreated corncob at 120 rpm for 7 days. After 7 days of cultivation, 5 mL of culture was transferred to a new flask of 45 mL of the same medium and was shaken at the same conditions. This method was repeated four times. Thereafter, 1 mL of each four-week culture was diluted with 0.85% (*w*/*v*) NaCl and cultured on NDMM agar containing 1% (*w*/*v*) non-chemical pretreated corncob using the spread plate method. The agar plates were incubated at 30 °C for 3 days. The different character colonies were collected, and further sub-culturing was performed to obtain purified colonies. All selected pure cultures were characterized and kept in 20% (*v*/*v*) glycerol at −20 °C.

2.2. Screening of Polyhydroxyalkanoate (PHA)-Producing Bacteria

2.2.1. Observation of Fluorescence Intensity on Agar

Each strain was streaked on NDMM agar containing 1% (*w*/*v*) non-chemical pretreated corncob, which was spread over with 0.5 µg/mL final concentration of Nile red (Sigma-Aldrich, Burlington, MA, USA) [17] and incubated at 30 °C for 3 days. The positive strains showed red fluorescent colonies under ultraviolet (UV) light at 254 nm.

2.2.2. Determination of PHA Granules in Bacterial Cell

The positive bacteria were cultured in NDMM containing 1% (*w*/*v*) glucose. The 5:1 mixture of cultures and 1 µg/mL Nile red were dropped on agarose agar which was prepared on glass slides. The glass slide was then covered and the PHA granules were observed under a fluorescence microscope (Drawell, BK5000, Shanghai, China).

2.3. PHA Extraction and Determination

The 8 mg of dried cell samples was measured and treated with 1 mL of chloroform and 1 mL of solution A (15% sulfuric acid in absolute methanol). The mixture was vortexed and boiled at 100 °C in an oil bath for 2 h 30 min. After the solution was cooled, 1 mL of deionized water and 1 mL of solution B (0.2% benzoic acid methyl ester in chloroform) were added. The solution was separated into two layers. The bottom layers were collected. PHA yield was analyzed using gas chromatography analysis with a flame ionization detector (GC-FID) [18].

2.4. Identification of Selected Bacteria

The isolate HD34 was characterized using morphological, biochemical, and molecular methods. Morphological characteristics were studied using a stereomicroscope, light compound microscope, and scanning electron microscope (SEM). Colony forming was observed under a stereomicroscope after the isolate was cultured on nutrient agar. The bacterial strain was prepared using the Gram staining method and observed under a light microscope at 1000 × agnification. Moreover, the bacterial cell was prepared by the air-dried technique on the film surface and then coated with Au particles before SEM observation (Model JSM-5910LV, JOEL Ltd., Tokyo, Japan). For biochemical characteristics, carbon source utilization and enzyme production were tested using the API 20 E and API ZYM kits (bioMerieux, Singapore), according to the manufacturer's instructions. Other biochemical tests were prepared in the laboratory. The genomic DNA of the selected isolate was extracted by a Quick DNA universal kit (Zymo Research, Costa Mesa, CA, USA). The 16S rRNA gene was amplified using primers 27F (5′ AGAGTTTGATCMTGGCTCAG 3′) and 1492R (5′ TACGGYTACCTTGTTACGACTT 3′) [19]. One reaction for the polymerase chain reaction (PCR) contained 2 µL of a purified DNA template, 1 µL of each primer, 10 µL of Taq mix (PCRBio, London, UK), and 6 µL of deionized water. The condition of the thermal cycling program was the following: initial denaturation at 95 °C for five minutes, 30 cycles consisting of denaturation at 94 °C for one minute, annealing at 55 °C for one minute, extension at 72 °C for 1.5 min, and a final extension at 72 °C for one minute. The PCR products were analyzed by gel electrophoresis (Bio-Rad, Hercules, CA, USA), purified using the Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA), and analyzed by DNA sequencing (1st Base, Singapore). The 16s rRNA gene sequence was compared with 16s rRNA gene sequences available in the EzBioCloud Database. A phylogenetic tree was constructed using MEGA X software (Version 10.0, Masatoshi Nei, Harrisburg, PA, USA) with the neighbor-joining method. A bootstrap analysis was performed with 1000 repeats using the closely related strain *Methylobacterium organophilum* ATCC 27886^T as the outgroup.

2.5. Optimization of PHA Production

The selected isolate, *Ensifer* sp. HD34, was cultured in 50 mL of NDMM containing 1% (*w*/*v*) glucose in a 250 mL flask and horizontally shaken at 130 rpm at 30 °C for 18 h as the

inoculum. Then, 10% (v/v) of inoculum was added in 50 mL of production medium and incubated at 30 °C with 130 rpm shaking for 72 h. After that, cultures were centrifuged at 6000× g for 10 min and freeze-dried for collecting cell pellets. Each cell biomass was measured and then extracted for PHA content determination by gas chromatography [18].

2.5.1. Effect of Carbon Source

Ensifer sp. HD34 was cultured in 50 mL of NDMM containing 1% (w/v) of 10 various carbon sources (glucose, fructose, galactose, cellobiose, sucrose, lactose, xylose, mannose, arabinose, and xylan). Then, PHA content was determined.

2.5.2. Effect of Production Medium

Ensifer sp. HD34 was cultured in 50 mL of 12 various production media: nutrient broth (NB), casein yeast magnesium broth (CYM), tryptic soy broth (TSB), glucose broth (GB), Luria-Bertani broth (LB), peptone water (PW), potato dextrose broth (PDB), Sabouraud dextrose broth (SDB), yeast extract peptone dextrose broth (YPD), yeast malt broth (YM), peptone broth (PEP), and nitrogen-deficient minimal medium (NDMM)). All media contained the same carbon source concentration as 1% (w/v) glucose. PHA content was determined by gas chromatography.

2.5.3. Central Composite Rotatable Design (CCRD)

The CCRD experiments were obtained by 2 factors with 5 replicates at center points using Design Expert version 7.0. Thirteen experiments were applied to optimize the effect of PDB as a production medium and glucose as a carbon source on PHA production. The medium was prepared following the experiment's design and 3 replicates of each experiment were conducted in a 250 mL flask adding 10% (v/v) inoculum. The flasks were horizontally shaken at 130 rpm at 30 °C for 72 h, and then the PHA content of freeze-dried cells was determined and reported as PHA yield (g/L). The results were plotted as a 3D response surface which was statistically analyzed with ANOVA and fitted to the model equation (Equation (1)). The model should be exhibited as significantly different with 95% confidence with a not significantly different lack of fit value.

$$Y = \beta_0 + \Sigma\beta_i X_i + \Sigma\beta_{ij} X_i X_j + \Sigma\beta_{ii} X_{i^2} \quad (1)$$

From the equation, the best composition of the production medium was prepared in 3 replicates in 250 mL flasks adding 10% (v/v) inoculum and shaking at 30 °C for 72 h. The suspension was centrifuged and freeze-dried then the cell pellet was measured and extracted for determining PHA yield (g/L). For validation, the result was compared with a predicted result from CCRD optimization.

2.5.4. One Factor at a Time Experiment for the Optimal Conditions of PHA Production

Following the result of the CCRD experiment, the best composition of the production medium is 3.99% (w/v) PDB with 1.54% (w/v) glucose used as an optimal medium. Glucose and PDB medium were used in evaluation of the incubation time, initial pH, inoculum size, and temperature using the one factor at a time method with 3 replications for the highest result of PHA production by *Ensifer* sp. HD34.

- Effect of incubation time.

Ensifer sp. HD34 was cultured as an inoculum, then 10% (v/v) was added to the optimized medium and shaken at 30 °C for 5 days. Every 12 h, 50 mL of culture was collected to be representative of each time growth. Each sampling was centrifuged and freeze-dried, then the cell pellet was measured and extracted for determining PHA yield (g/L).

- Effect of initial pH.

An amount of 50 mL of the optimal medium was prepared in a 250 mL flask, which adjusted the initial pH range from 5.0 to 12.0. Ten percent (v/v) of inoculum was added

to the medium and incubated by shaking at 30 °C for 108 h (the optimal incubation time). The cultures were centrifuged and the cells were collected for PHA yield determination.

- Effect of inoculum size.

The seed cultures in amounts of 1, 2.5, 5, 7.5, 10, 12.5, and 15% (*v*/*v*) were added into each 50 mL of the optimized medium, which adjusted the pH to 9.0. The incubation conditions were 130 rpm at 30 °C for 108 h. After incubation, the cell pellets were collected by 6000× *g* centrifugation and freeze-dried for PHA yield determination.

- Effect of temperature.

The optimized medium consisting of 3.99% (*w*/*v*) PDB and 1.54% (*w*/*v*) glucose was adjusted to a pH of 9.0 and then 2.5% (*v*/*v*) of inoculum was added into the prepared medium. Each flask was incubated at various temperatures—28 °C, 30 °C, 37 °C, and 45 °C—for 108 h. The cell biomasses were collected and freeze-dried for PHA yield determination.

2.5.5. Time-Course Culture under Optimal Medium and Conditions for PHA Production

After optimization, the best composition of medium and optimal conditions of PHA production were selected. For inoculum preparation, *Ensifer* sp. HD34 was inoculated in 50 mL of PDB containing 1% (*w*/*v*) glucose in a 250 mL flask and incubated in an incubator shaker under 130 rpm at 30 °C for 18 h. Then, 2.5% (*v*/*v*) of inoculum was added into the 50 mL optimal medium (PDB 3.99% (*w*/*v*) and glucose 1.54% (*w*/*v*) with adjusted initial pH of 9.0) and incubated at 28 °C for 7 days, as shown in Figure 1. Every 12 h, 50 mL of culture was collected to be representative of each time growth. Each sampling culture detected growth rate, pH, glucose concentration, cell dry weight, PHA content, and PHA yield. Growth of the bacterium was detected by measuring optical density at 600 nm absorbance (OD_{600}) of suspension using a spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The culture broth with OD_{600} above 3.0 was diluted with the medium before measurement, and the values were calculated with a dilution factor as the approximate results of bacterium cell growth. Then, the culture broth was centrifuged at 6000× *g* for 10 min to separate the cell and supernatant. The pH and glucose residue were detected in the supernatant using high-performance liquid chromatography (HPLC) 1260 with a refractive index detector (Agilent Technology, Böblingen, Germany) and nitrogen residue was detected using the Kjeldahl method based on the Association of Official Analytical Chemists (AOAC) and Official Methods of Analysis (OMA). The freeze-dried cell part was measured and extracted for PHA content determination by gas chromatography [20].

2.6. Characterization of PHAs

The selected isolate *Ensifer* sp. HD34 was cultured in optimal medium and conditions. After cell culturing, freeze-drying, and extraction [16], the PHA powder was brought to characterization, including distinguishing types of PHAs, PHA polymeric composition, thermal properties, and crystallinity. The result was compared with former studies.

2.6.1. ^{1}H Nuclear Magnetic Resonance (NMR)

The purified PHA sample was dissolved in deuterated chloroform ($CDCl_3$) with a 10 mg/mL final concentration. The ^{1}H NMR spectra were obtained using 400 Ultrashield™, Bruker, Billerica, MA, USA. Chemical shifts were referenced to the residual proton peak of $CDCl_3$ and compared with the results of a PHB standard (Sigma-Aldrich, Burlington, MA, USA) [20].

2.6.2. Fourier-Transform Infrared Spectroscopy (FTIR)

The extracted PHA sample was mixed homogeneously with potassium bromide (KBr) in a 1:10 ratio and scanned using a Nicolet 6700 FT-IR spectrometer (Thermo Scientific, Waltham, MA, USA) under a spectral range of 4000–400 cm^{-1}. Each peak of determined results was shown as functional groups of polymers and compared with the prior studies [21].

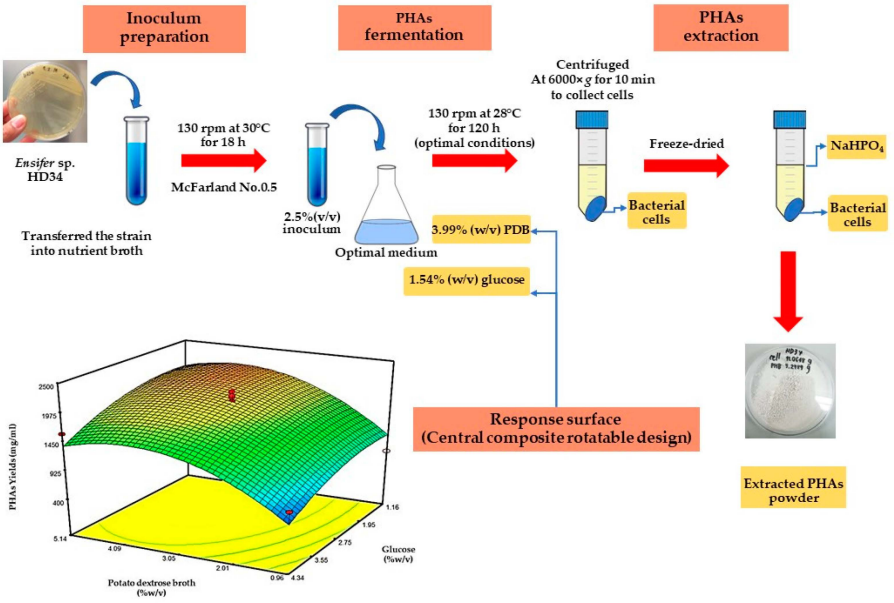

Figure 1. Schematic illustration of PHA production and extraction.

2.6.3. X-ray Diffraction (XRD)

XRD is commonly used for diffraction intensity correlated to crystallinity determination (Rigaku, Tokyo, Japan). A diffractogram of 2 g of the extracted PHA powder was performed at room temperature with nickel-filtered Cu-K^{α} radiation (wavelength = 0.1542 nm) in the scattering angle range of 2θ = 2–50° at 25 °C at a scan speed of 10° min^{-1} [20].

2.6.4. Thermogravimetric Analysis (TGA)

For thermal degradation temperature (T_d), 5 mg of the extracted PHA powder was packed in an aluminum pan and the decomposition temperature was determined. The sample was heated from 27 °C to 450 °C at a heating rate of 10 °C min^{-1} under nitrogen gas atmosphere [22].

2.6.5. Differential Scanning Calorimetry (DSC)

The extracted PHA powder was measured (3 mg) to perform the DSC method, which was used for melting temperature (T_m) determination. The PHA powder was heated from −20 °C to 250 °C at a heating rate of 10 °C min^{-1} under purified air [20].

2.7. Statistical Analysis

These experiments were performed in triplicate and data were statistically analyzed with one-way ANOVA and Duncan's comparison using SPSS statistics software version 24 (SPSS Inc., Chicago, IL, USA). The results were cut-off under 95% for statistical significance. Central composite rotatable design (CCRD) was obtained by Design Expert software version 7.0 (Minneapolis, MN, USA) and analyzed with ANOVA statistics.

3. Results

3.1. Sample Collection and Isolation of Bacteria

Soils are good resources for isolating microorganisms that could accumulate PHA granules in their cells. Many kinds of Gram-negative bacteria, especially rhizobacteria, can accumulate PHAs as their reserve energy in both free-living and legume-nodule-associated bacteria [5]. Rhizobacteria are nitrogen-fixing bacteria and produce enzymes to degrade organic compounds for food. Hence, 40 soil samples were collected from the agricultural areas in Phayao, Chiang Rai, and Chiang Mai Provinces. Three hundred and forty-one bacteria were isolated using NDMM agar containing 1% (*w*/*v*) non-chemical pretreated corncob (Table 1).

Table 1. Number of PHA-producing bacteria isolated from different soil samples.

Soil Sources	Number of Isolates	Number of PHA-Positive Isolates
7 soil samples from Phayao Province	64	10
3 soil samples from Chiang Rai Province	11	2
30 soil samples from Chiang Mai Province	266	91
Total **40**	**341**	**103**

3.2. Screening of Polyhydroxyalkanoate (PHA)-Producing Bacteria

After the primary screening, 103 isolates exhibited positive results in accumulating PHA granules with the Nile red staining method. The PHA granules were present in the bacterial cytoplasm as an inclusion body. The granules could be detected on plates under UV light and with a fluorescence microscope at 1000×magnification using the Nile red staining method (Figure 2). The results showed that 15 isolates could grow in the selective medium and accumulate many Nile red-dyed granules in their cells. Then, each isolate was cultured, collected, freeze-dried, extracted, and analyzed for PHA quantification. Among 15 isolates, isolate HD34 showed the highest result of PHA content at 26.79% (*w*/*w*) per cell dry weight, with positive Nile red dyed detection containing three PHA granules per cell (Table 2). Hence, strain HD34 was selected for further studies. Nile red/Nile blue A was utilized as an indicator to interact with phospholipids of storage compounds which are present in the outer layer of PHAs and appear pink/red/yellow/orange under UV illuminance. This technique is very easy to use for detection, so it was broadly used to screen PHAs in microbial cells. From previous reports, the bacterial colony which had the greatest intensity of fluorescence could express maximum PHA production, such as 11.50 (*Bacillus subtilis*), 76.61 (*Enterobacter* sp. SEL2), and 39.90% PHA content (*Bacillus* sp. NG05) [23–25].

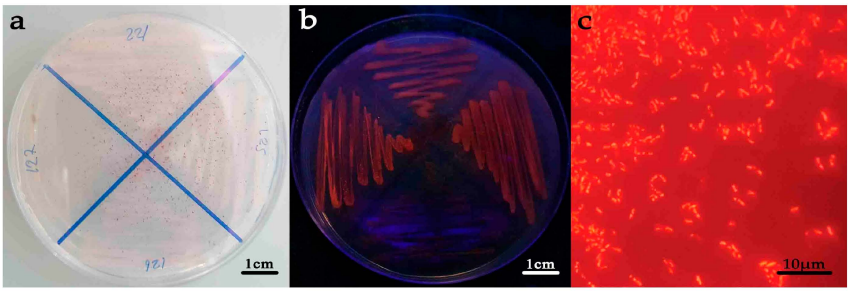

Figure 2. The selected isolates were cultured on NDMM + 1% (*w*/*v*) corncob agar (**a**) and PHA granules could be detected on plates under UV light (**b**) and under fluorescence microscope 1000× magnification using Nile red staining method (**c**).

Table 2. Amount of accumulated PHA granules per cell and overall PHA content in positive isolates.

Isolate	Amount of PHA Granules per Cell	PHA Content (%)
EP07	5	0
IK01	3	3.89
HD11	3	0
HD22	4	0
HD33	5	0
HD34	3	26.79
HD103	3	0
HD501	5	1.96
HD701	7	1.71
HD121	4	0
HD221	4	1.66
HD224	2	0
HD324	2	2.36
HD523	7	0
HD722	4	1.82

3.3. Identification of Selected Bacteria

The selected strain HD34 was morphologically and biologically examined, as presented in Table 3. Microscopic examination of isolate HD34 cells exhibited that colony forming on the nutrient agar was white, with circular form and convex elevation. Gram staining was Gram-negative bacteria with short-rod cells that were 2–3 µm length (Figure 3). Furthermore, the selected isolate showed motility under a light compound microscope. Following biochemical analysis, strain HD34 was positive for Voges-Proskauer, nitrogen production, and L-phenylalaninase and catalase activity. On the other hand, the strain exhibited negative reactions for indole production, citrate utilization, H_2S production, starch hydrolysis, and nitrate reduction. Moreover, it could grow at different concentrations of NaCl in the range of 0–3% (*w*/*v*), with a pH between 6.0–10.0. The isolate can utilize and ferment D-glucose, D-mannitol, inositol, D-sorbitol, L-rhamnose, D-sucrose, D-melibiose, amygdalin, L-arabinose, dextrin, and D-melezitose. The 16s rRNA gene sequence of strain HD34 showed a nucleotide sequence of 1354 base pairs, and it was deposited in the DNA Data Bank of Japan (DDBJ) under accession number LC277187. Following the EzBioCloud Database, the 16s rRNA sequence revealed maximum similarity with *Ensifer adhaerens* LMG20216^T (99.6%), which is a type strain of genus *Ensifer* (formerly *Sinorrhizobium*), *E. sesbaniae* CCBAU 65729^T (99.5%), and *E. molerensis* Lc04^T (99.4%). The 24 referenced sequences were collected from the EzbioCloud Database and the phylogenetic tree was structured adopting the neighbor-joining tree method with 1000 bootstrap values using MEGA software (Version 10.0, Masatoshi Nei, Harrisburg, PA, USA). The neighbor-joining phylogenetic tree exhibited that *Ensifer* sp. HD34 was clustered with the clade of genus *Ensifer* with 73% bootstrap support. The result proved that isolate HD34 is the most evolutionally close to *Ensifer morelensis* Lc04^T with 67% bootstrap support (Figure 4).

Table 3. Morphological and biological characterization of bacterium strain HD34.

Characteristics	Results
Gram stain	negative
Indole production (IND)	−
Acetoin production (VP)	+
Citrate utilization (CIT)	−
H_2S production (H_2S)	−
Fermentation/oxidation:	
D-Glucose (GLU)	+
D-Mannitol (MAN)	+
Inositol (INO)	+
D-Sorbitol (SOR)	+
L-Rhamnose (RHA)	+
D-Sucrose (SAC)	+
D-Melibiose (MEL)	+
Amygdalin (AMY)	+
L-Arabinose (ARA)	+
Dextrin	+
D-Melezitose	+
Starch hydrolysis	−
NO_2 production (NO_2)	−
N_2 production (N_2)	+
Nitrate reduction	−
Catalase	+
L-Phenylalaninase	+
β-Galactosidase (ONPG)	+
Arginine dihydrolase (ADH)	−
Lysine decarboxylase (LDC)	−
Ornithine decarboxylase (ODC)	−
Urease (URE)	+
Tryptophan deaminase (TDA)	+
Gelatinase (GEL)	−
Growth at NaCl (% *w*/*v*)	0–3
Growth at different pH	6.0–10.0

(+) Indicates positive and (−) indicates negative reactions.

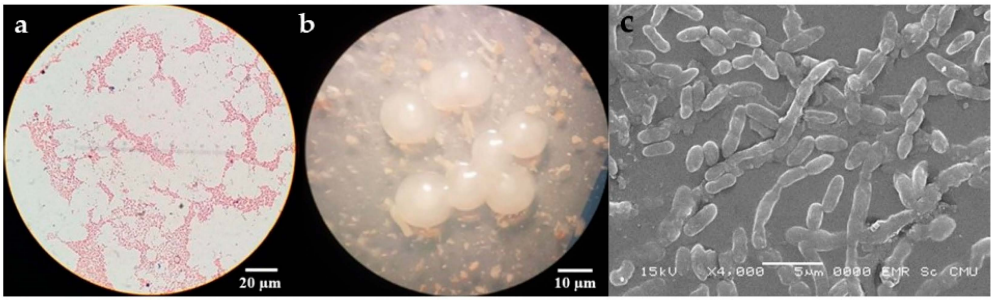

Figure 3. Cell characterizations of bacterium strain HD34 under microscopic techniques including Gram staining at 1000× magnification under light compound microscope (**a**), colony forming on agar at 250× magnification under stereomicroscope (**b**), and cell characterization at 4000× magnification under scanning electron microscope (**c**).

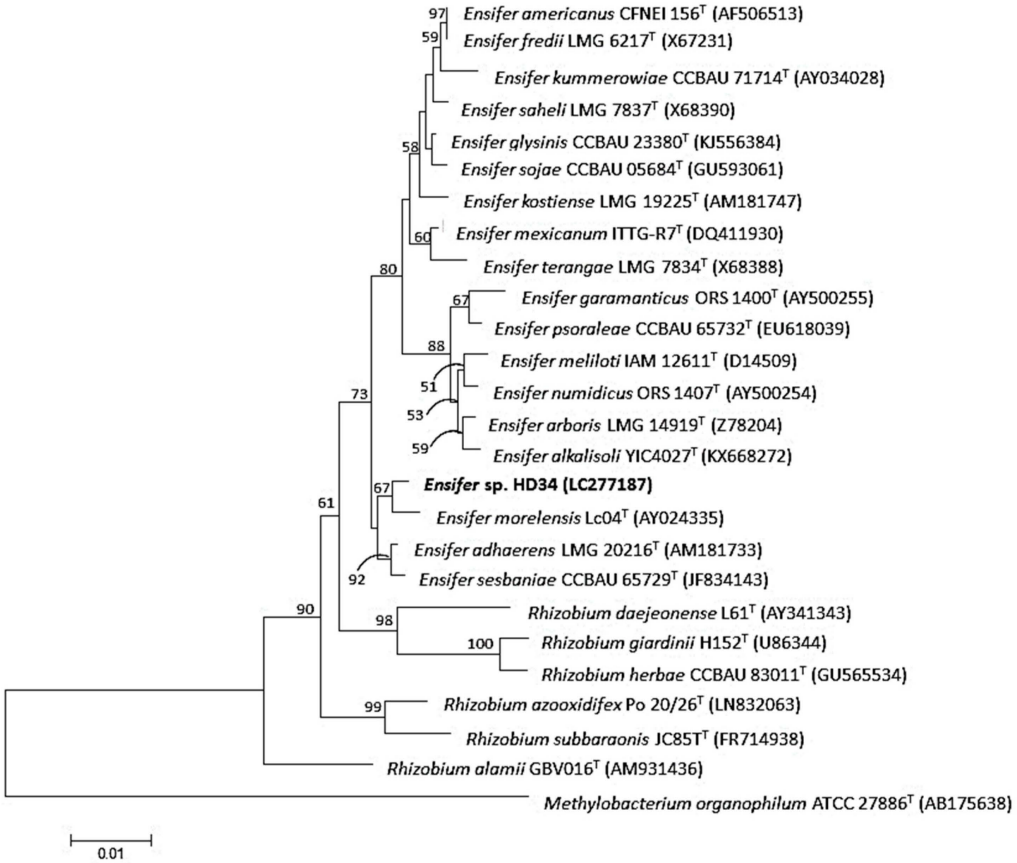

Figure 4. Neighbor-joining phylogenetic tree based on 16s rRNA sequences of *Ensifer* sp. strain HD34 (LC277187) within closely related strains and *Methylobacterium organophilum* ATCC 27886^T as an outgroup. Only bootstrap values above 50% (percentages of 1000 replications) are shown. Bar 0.01 nucleotide substitutions per site.

From these results, it could be indicated that the bacterium strain HD34 belongs to the genus *Ensifer*, with morphological evidence via microscopic analysis, as well as biochemical evidence, information from the Bergey's Manual of Systematic Bacteriology [26], and the use of molecular techniques. However, some characteristics of strain HD34 were not similar to the closely related strains that were reported in previous studies [27] (Table 4), which may be tested by other methods to confirm in further studies. That being said, *E. adhaerens*, *E. sesbaniae*, and *E. molerensis* have not been reported in PHA production. It could be interesting for more studying in the future.

Table 4. Comparison of selected isolate HD34 and various strains in the genus *Ensifer* sp. which is closely related by phylogenetic data. Strains: 1, strain HD34 (LC277187); 2, *Ensifer morelensis* Lc04^T (AY024335); 3, *Ensifer adhaerens* LMG 20216^T (AM181733); 4, *Ensifer sesbaniae* CCBAU 65729^T (JF834143).

Characteristics	1	2	3	4
Carbon source utilizations:				
Dextrin	w	+	+	−
D-Melezitose	+	+	+	+
Sodium acetate	−	+	+	w
Growth at conditions:				
1% (*w*/*v*) NaCl	+	−	+	+
2% (*w*/*v*) NaCl	+	+	+	−
Ampicillin (5 µg/mL)	+	+	+	+
L-Phenylalaninase activity	+	+	−	+
Catalase activity	+	+	+	−
Nitrate reduction	−	+	+	+
Growth in nutrient broth	+	+	+	+
Hydrolysis of starch	−	+	+	+

(+) Positive, (−) negative, and (w) weak positive reactions.

3.4. Optimization of PHA Production

From the previous study, *Ensifer* sp. HD34 was cultured in agricultural waste (corncob) medium which could grow and accumulate PHA granules in its cells. When the acid pretreatment method was employed to prepare the production medium, both solid and liquid pretreatment substrates caused higher results of PHA content compared with non-pretreatment substrates [28]. However, the bacterium could not be completely separated from the substrates, which caused an error in determining PHA yield calculation and purification. A prior study focused on optimized production medium and conditions. Types of carbon sources and production media were determined that influenced the PHA production. The one factor at a time optimization was employed, and the results are shown in Table 5. The effect of carbon source was studied in NDMM medium containing 1% (*w*/*v*) of each different carbon source. Glucose was observed to be the best carbon source for PHA production by the strain (1.60 g/L), followed by galactose (1.37 g/L), lactose (1.24 g/L), and sucrose (1.09 g/L). Because of nutrient-limited and constant ingredients, NDMM was selected to be a basal medium. Previous studies supported the result that microorganisms accumulate energy sources in granules in their cells under scarce conditions, such as limited amounts of nitrogen and phosphorus or a high concentration of carbon sources. It caused acetyl-CoA to be synthesized through the PHA production pathway [29,30]. According to a previous report, glucose is easily accessible in the Krebs cycle and the process of PHA synthesis. *Ensifer* sp. HD34 utilized five to six carbon atoms of monosaccharides and disaccharides as a sole carbon source to produce PHAs. Regarding the effect of production medium, *Ensifer* sp. HD34 produced the highest PHA yield in PDB, at 2.75 g/L. However, NDMM exhibited PHA production of 1.17 g/L. The bacterium strain grew quite well in CYM, TSB, LB, and PW broth, but PHA polymer was not accumulated. It can be said that the mentioned media affected the growth of cells and could be a good inoculum media to use for enhancing cell volume in production methods. However, these complex media have a high organic nitrogen concentration compared with PDB; therefore, they might not enhance PHAs accumulated in HD34 cells. The PDB medium consists of a carbon and organic nitrogen source. It could cause higher PHA production when compared with NDMM, which contains an inorganic nitrogen source. Penkhrue and colleagues (2020) used various types of complex medium with 1% (*w*/*v*) glucose testing of growth and PHB production from *Bacillus drentensis* BP17. The result showed that TSB gave the best result of PHA yield at 3.53 g/L [20]. Following the media and carbon source optimization results in this study, PDB medium and glucose were selected for the central composite rotatable design (CCRD). PDB was not directly reported to be a medium for PHA production. Still, a

publication reported using potato waste as a carbon source in fed-batch fermentation for PHA production by *Ralstonia eutropha* [31]. Notwithstanding, glucose is a primary carbon source used in PHA production. There are many Gram-negative bacteria using glucose for PHA production, such as *Azohydromonas lata*, *Azotobacter beijerinckii*, *Burkholderia cepacia*, and *Novosphingobium nitrogenifigens*, which could produce 79.4, 24.8, 59.0, and 81.0% (*w*/*w*) PHA content, respectively [32–34].

Table 5. Effect of production media and carbon sources on PHA production by *Ensifer* sp. HD34.

Nutrient Sources	PHAs Yield (g/L)
Carbon sources	
Glucose	1.604 ± 0.109^{a}
Cellobiose	0.394 ± 0.265^{c}
Fructose	0.107 ± 0.037^{c}
Sucrose	1.096 ± 0.426^{ab}
Galactose	1.372 ± 0.322^{a}
Lactose	1.239 ± 0.436^{ab}
Xylose	0.461 ± 0.096^{c}
Xylan	0.180 ± 0.068^{c}
Mannose	0.537 ± 0.202^{c}
Arabinose	0.618 ± 0.167^{bc}
Culture media	
Nutrient broth (NB)	0.34 ± 0.013^{d}
Casein yeast magnesium broth (CYM)	N/A
Tryptic soy broth (TSB)	N/A
Glucose broth (GB)	1.34 ± 0.119^{b}
Luria-Bertani broth (LB)	N/A
Peptone water (PW)	N/A
Potato dextrose broth (PDB)	2.75 ± 0.274^{a}
Sabouraud dextrose broth (SDB)	0.40 ± 0.041^{d}
Yeast extract peptone dextrose broth (YPD)	0.38 ± 0.093^{d}
Yeast malt broth (YM)	0.59 ± 0.179^{cd}
Peptone broth (PEP)	0.68 ± 0.017^{c}
Nitrogen-deficient minimal medium (NDMM)	1.17 ± 0.027^{b}

N/A indicates not detected. Lowercase letters represent significant differences at 5% probability level. Significant differences were analyzed by the Duncan test using SPSS software (SPSS Inc., Chicago, IL, USA).

For the optimum composition of the production medium, CCRD was employed as a statistical tool. The designed experiments were calculated to find the optimal volume of two factors, PDB and glucose, and their interaction. Thirteen runs were performed and cultured with biological replicates. The regression equation of the highest result of PHA production by *Ensifer* sp. HD34 was derived from statistical analysis as follows in Equation (2).

$$\text{PHAs yield (g/L)} = -258.36 + 893.59X_i + 519.31X_j - 8.6X_iX_j - 109.40X_{i^2} - 109.46X_{j^2} \quad (2)$$

From the equation, X_i stands for the amount of PDB medium (% *w*/*v*), and X_j stands for glucose concentration (% *w*/*v*). The capability of the model was analyzed using ANOVA. The results of PHA production showed the model was significantly fitted at 95% reliability, and that the value of prob > *F* of model terms are significant ($p < 0.05$) with $R^2 = 0.9088$ and insignificant lack of fit (Table 6). The 3D response surface was plotted and exhibited the optimum composition of PDB medium and glucose concentration (Figure 5). According to the statistical analysis, the optimum composition of PDB medium and glucose was 3.99% (*w*/*v*) and 1.54% (*w*/*v*), respectively, which was a carbon: nitrogen ratio of 15:1. From the equation, it predicted that *Ensifer* sp. HD34 could produce a PHA yield of 2.05 g/L using the optimal medium. To verify the model, the experiment was validated. The result indicated that *Ensifer* sp. HD34 provided 1.96 g/L PHA yield at 72 h of incubation time, which is 95.3% of the predicted value from the equation.

Table 6. Statistical analysis using ANOVA from experimental design of central composite design between potato dextrose broth (PDB) and glucose on PHA production of *Ensifer* sp. HD34.

Source	Coefficient Estimate	Sum of Square	df	Mean Square	*F*-Value	*p*-Value Prob > *F*
Model		3.576×10^{-6}	5	7.152×10^{-5}	13.96	0.0016
X_i-PDB	422.58	1.429×10^{-6}	1	1.429×10^{-6}	27.88	0.0011
X_j-Glucose	−173.41	2.406×10^{-5}	1	2.406×10^{-5}	4.70	0.0669
X_iX_j	−28.57	3.266×10^{-3}	1	3.266	0.064	0.8079
X_i^2	−476.02	1.576×10^{-6}	1	1.576×10^{-6}	30.76	0.0009
X_j^2	−277.08	5.341×10^{-5}	1	5.341×10^{-5}	10.42	0.0145
Residual		3.587×10^{-5}	7	51,237.33		
Lack of fit		2.205×10^{-5}	3	73,493.37	2.13	0.2395
Pure Error		1.382×10^{-5}	4	34,545.30		
Cor total		3.935×10^{-6}	12			

Standard deviation = 226.36; mean = 1514.04; coefficient of variance (C.V.%) = 14.95; PRESS = 1.784×10^6; R-squared = 0.9088; Adj R-squared = 0.8437; Pred R-squared = 0.5466; Adeq Precision = 10.077.

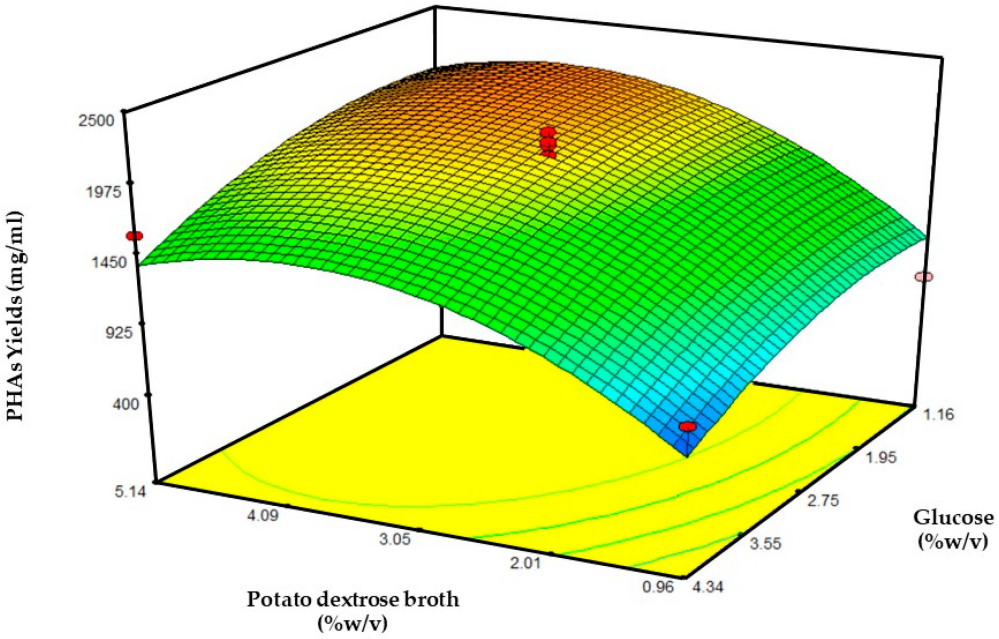

Figure 5. The 3D response surface plot of interactive effect of potato dextrose broth (PDB) and glucose concentration for PHA production by *Ensifer* sp. HD34.

3.5. One Factor at a Time Experiment for the Optimal Conditions of PHA Production

After the experiment under optimal medium was performed, the effects of incubation time, initial pH, inoculum size, and temperature were investigated.

3.5.1. Effect of Incubation Time

The results exhibited that longer incubation time caused more cell growth of *Ensifer* sp. HD34. At 108 h of incubation, the highest PHA content of 45.54% (*w*/*w*) of cell dry weight, which is 3.82 g/L PHA yield (Figure 6a), was produced. It indicated that *Ensifer* sp. HD34 could increase the amount of PHA yield twice at 108 h compared with 72 h. PHA producers mostly accumulate PHAs during the optimal incubation time in their stationary phase, which is different depending on the properties of microorganisms. The result from this study was consistent with a previous report that the highest PHA production was performed at around 90–108 h of incubation time from *Bacillus cereus* [35]. Nevertheless, there are bacteria which produced PHAs during the exponential phase and diminished during the stationary phase [36]. Following the growth curve, PHA production was decreased because of nutrient deficiency and increasing metabolites, toxins, and inhibitors, which can cause a negative effect on PHA synthesis [37].

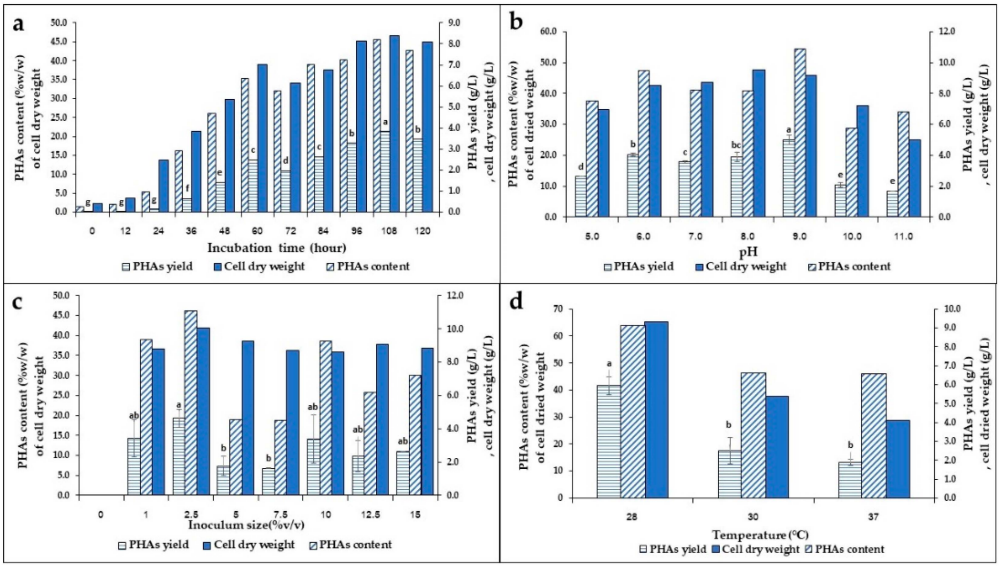

Figure 6. Effect of incubation time (**a**), initial pH (**b**), inoculum size (**c**), and temperature (**d**) on PHA production by *Ensifer* sp. HD34. Lowercase letters represent significant differences at 5% probability level. Significant differences were analyzed by the Duncan test using SPSS software (SPSS Inc., Chicago, IL, USA).

3.5.2. Effect of Initial pH

Furthermore, the initial pH of the medium is also important, as the result that *Ensifer* sp. HD34 could produce the maximum PHA yield of 5.0 g/L in the optimal medium, which adjusted the initial pH to 9.0 (Figure 6b). Even the cell dry weight of each culture at an initial pH of 6.0, 7.0, and 8.0 was higher than at an initial pH of 9.0. PHA content at an initial pH of 9.0 was the highest at 54.49% (*w*/*v*), which was the best result compared with the others. Compared with previous optimization studies of PHA production, many cases were reported that various bacteria could produce PHAs over a pH range between 6.0 to 9.0, while the result of this study is that the optimal initial pH of 9.0 is similar to the study of the PHA production from *Pseudomonas pseudoalcaligenes* and *Bacillus* spp. [38,39]. Since pH affects the enzymes involved in PHA biosynthesis, a neutral pH is commonly used for the initial pH of the medium for bacterial PHA production. In this study, an initial pH of 9.0 was adjusted and gave the highest result of PHAs production, because the high pH may cause the induction of high energy requirements for substrate uptake at the beginning of the bacterium culture [38]. A wider range of the optimal pH level can cause lower PHAs production, because pH values possibly affect the degenerative enzymes of biopolymer-producing bacteria [40].

3.5.3. Effect of Inoculum Size

When the effect of inoculum size was investigated, the cell dry weight of all samples was not significantly different. However, a 2.5% (*w*/*v*) inoculum size was the highest of PHA content at 46.14% (*w*/*v*) and PHA yield at 4.62 g/L (Figure 6c). It could be assumed that using a lower concentration of inoculum than 2.5% (*w*/*v*) might take a longer period for a large amount of PHA production, and using a high concentration of inoculum might cause rapid nutrient consumption [41]. Compared with a former study, there is a similar report of inoculum size for PHA production from *Lysinibacillus* sp. [42]. There were many studies that reported using a larger inoculum size such as 5%, 6%, and 7% (*v*/*v*) as an optimal value for PHA production [23,43,44].

3.5.4. Effect of Temperature

For the other factor, the effect of temperature at 28, 30, 37, and 45 °C was studied. The best result was shown at 28 °C, with PHA yield and PHA content being 5.94 g/L and 63.75%

(*w*/*v*), respectively. The result corresponded to a former study of the PHA production from *B. Megaterium* JHA, which was statistically optimized from one-way ANOVA analysis [43]. *Ensifer* sp. HD34 could not grow well or be extracted to determine any amount of PHA content at 45 °C, which may be due to the effect of temperature on PHA-producing enzyme activity. The data showed that the temperature was an inverse variable to cell growth and PHA accumulation, as higher temperatures caused a lower PHA content of *Ensifer* sp. HD34 (Figure 6d). Inoculum size, initial pH of medium, incubation time, and temperature are important parameters in the metabolite's synthesis and the growth of bacteria because they affect the metabolic processes.

3.6. Time-Course Culture under Optimal Medium and Conditions for PHA Production

Ensifer sp. HD34 was batch cultivated in the optimal medium including PDB and glucose to investigate PHA production. The optimal medium was prepared containing 3.99% (*w*/*v*) PDB and 1.54% (*w*/*v*) glucose with an adjusted initial pH of 9.0, then shaken in an incubator for 168 h. The log phase appeared at 12 h of culturing, the growth still consistently increased until the end of the culturing experiment, and the result is directly proportional to PHA yields, PHA content, and cell dry weight. At 108 h of incubation (the optimal incubation time), *Ensifer* sp. HD34 appeared to have the highest PHA content and PHA yield, at 74.34% (*w*/*w*) and 6.31 g/L, respectively. The highest result of PHA production was shown at 120 h of incubation with 6.78 g/L PHA yield, while the pH and glucose concentration of the culture medium decreased from 9.0 to 6.8 and 5.23 to 1.49 g/L, respectively, which is an inversion with PHA production. This can be explained by *Ensifer* sp. HD34 utilizing sugar reduction for PHAs production (Figure 7). At the beginning of PHA production, the initial pH of 9.0 of the optimal medium was adjusted and induced substrate uptake for cell division and growth of bacterium cells. Then, an acid compound was produced during PHA production and caused the pH to decrease to neutral pH, which is suitable for the enzymes involved PHA production. Previous research reported that PHB accumulation was found at its maximum at a pH of 7.5 to 8.5 in *Synechocystis* sp. [45]. This bacterial strain adaptation is consistent with the result of the pH decreasing to neutral and PHA content increasing at around 50 h of incubation. Compared with other previous studies of the genus *Ensifer* for PHA production, *Ensifer* sp. HD34 produced a PHA content of 74.34% (*w*/*w*), which is less than *Ensifer fredii* AB92049 (79.2% (*w*/*w*)) using glucose as a carbon source [46] but higher than *Ensifer meliloti* PTCC 1684 (43.10% (*w*/*w*)) using sucrose as a carbon source [47]. For further study, it could be interesting to optimize the conditions of PHA production using *Ensifer* sp. HD34 under fed-batch fermentation and to use potato waste instead of commercial potato dextrose broth to reduce production costs.

3.7. Characterization of PHAs

Ensifer sp. HD34 cells were collected after culturing in an optimized medium and then extracted using sodium hypochlorite solution, and the result was white powder. It was brought to PHA characterization using ^{1}H NMR, FTIR, XRD, TGA, and DSC analysis.

The extracted PHA polymer was tested using ^{1}H NMR and FTIR for polymer structure determination. PHA monomers can be quantitatively estimated using the intensity ratio of the signals performed with NMR. This method is broadly used for saturated and unsaturated PHA analysis to determine functional groups such as methane and methylene. The result of ^{1}H NMR spectra shows peaks at 1.2, 2.5, and 5.2 ppm which are attributed to a methyl group (-CH_3), methylene group (-CH_2), and characteristic of methylene group (-CH), respectively (Figure 8). The other two signals present in the chart were also determined. The peak at 1.6 ppm is water and at 7.2 is chloroform, which were used as solvents in this method. When comparing the result with ^{1}H NMR spectra of previous studies, the spectra pattern of the sample is similar to the PHB standard and PHB accumulated in bacteria cells [48–50].

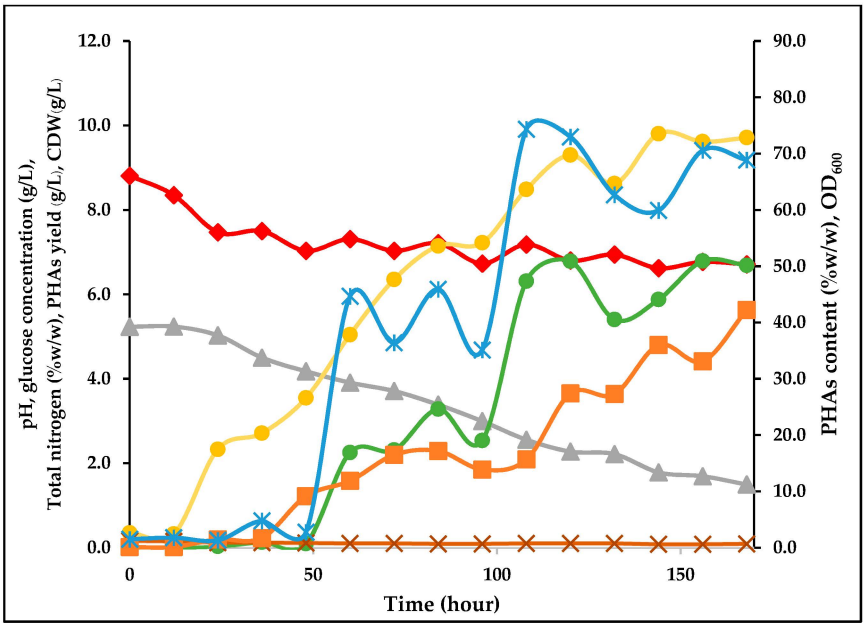

Figure 7. Time course of cell culture and PHA production of *Ensifer* sp. HD34 which is incubated in an optimized medium containing 3.99% (*w*/*v*) potato dextrose broth and 1.54% (*w*/*v*) glucose: (◆) pH; (■) OD_{600}; (▲) glucose (g/L); (×) nitrogen (% *w*/*w*); (∗) PHAs content (% *w*/*w*); (●) cell dry weight (g/L); (●) PHAs yields (g/L).

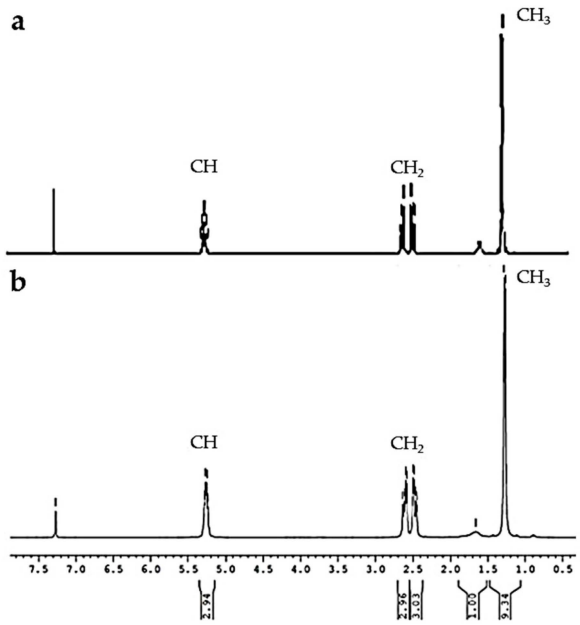

Figure 8. The ^{1}H nuclear magnetic resonance (NMR) signals of standard PHB (Sigma-Aldrich, Burlington, MA, USA) (**a**) and PHAs produced by *Ensifer* sp. HD34 using glucose as the sole carbon source (**b**).

In addition, FTIR was used to confirm the information on polymer structure presented as the contribution of the transmittance spectrum. The results are shown in Figure 9. The FTIR spectrum revealed the presence of intense absorption bands at 1720 cm^{-1} which correspond to the stretching vibration of the ester carbonyl group (C=O). Transmittance bands located at 2980 cm^{-1} and 2930 cm^{-1} are attributed to the stretching vibration of C-H bonds of a methyl group (-CH_3) and methylene group (-CH_2), respectively. In addition, the band which is found at 3440 cm^{-1} originated by terminal hydroxyl groups (-OH) or water absorption on the sample [49,51,52]. Moreover, the asymmetrical methyl group's

transmittance band at 2870 cm^{-1} is the result of conformational disorder during the crystallization process [53]. In addition, other transmittance bands from 1380 cm^{-1} to 970 cm^{-1} are attributed to the C-C, C-O, and C-H group. From these results, it can be clarified that the bacteria *Ensifer* sp. HD34 can produce PHAs in the form of polyhydroxybutyrate (PHB) in their cells.

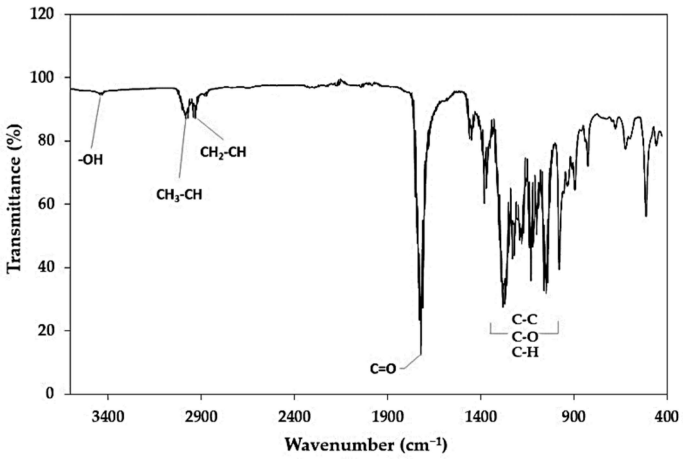

Figure 9. FTIR spectrum of PHB produced by *Ensifer* sp. HD34.

The X-ray diffractograms of the polymers from the strain HD34 provided seven significant peaks, which are corresponding 2θ values at 13.61°, 17.04°, 20.18°, 21.73°, 22.63°, 25.58°, and 27.2° shown in Figure 10. The diffractogram result is closely related with previous reports [20,54–56]. The most intense peaks at 13.61° and 17.04° indicated that the crystallinity nature of the polymer and sharper peaks could demonstrate higher crystallinity.

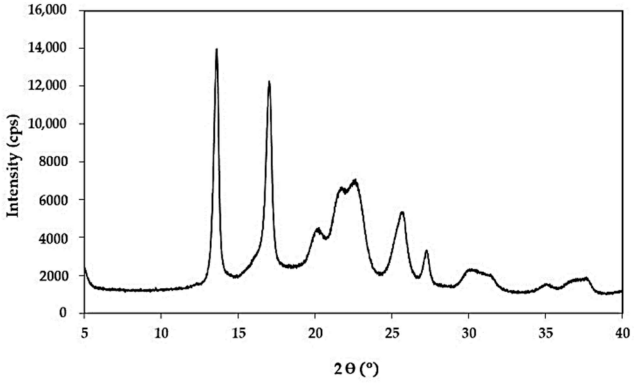

Figure 10. X-ray diffractogram of PHB produced by *Ensifer* sp. HD34.

Thermal properties were investigated using DSC and TGA analysis. TGA analysis was used to determine the temperature at which the polymer was degraded. At the above melting point temperature, the thermal degradation of PHB occurred by non-radical random chain scission reaction. The reaction is cis-elimination, has a six-membered ring transition state, and causes rapid decreasing molecular weight due to the fragmentation into the more diminutive form with a carboxylic and olefinic terminal group [57]. The resulting graph was plotted and the characteristic decomposition of polymers was determined, including initial degradation temperatures at 2% weight loss (T_{onset}), the degradation temperature (T_d), and the temperature of the maximum rate of degradation (T_{max}). The extracted PHB was slightly decomposed at T_{onset} = 88.15 °C. Then, weight loss percentage rapidly increased at T_d = 260.8 °C until total degradation, with the highest rate of degradation of 59.87%/min at T_{max} = 289.2 °C (Figure 11a). Endothermic heat flow was performed in the DSC process which presents the curve as shown in Figure 11b. As revealed by XRD analysis, the synthe-

sized PHB has high crystallinity as indicated by narrow peaks of full width half maximum value (FHWM), which essentially indicates a more ordered arrangement of polymer chains. The melting point of the PHB synthesized from *Ensifer* sp. HD34 is 173.4 °C, which is in the range of the PHB standard melting temperature (160–177 °C) [9].The degradation temperature results of extracted PHB matched well with the standard PHB [58,59]. As demonstrated, it could be summarized that the polymers produced by *Ensifer* sp. HD34 are PHB with high crystallinity and resistance for thermal degradation.

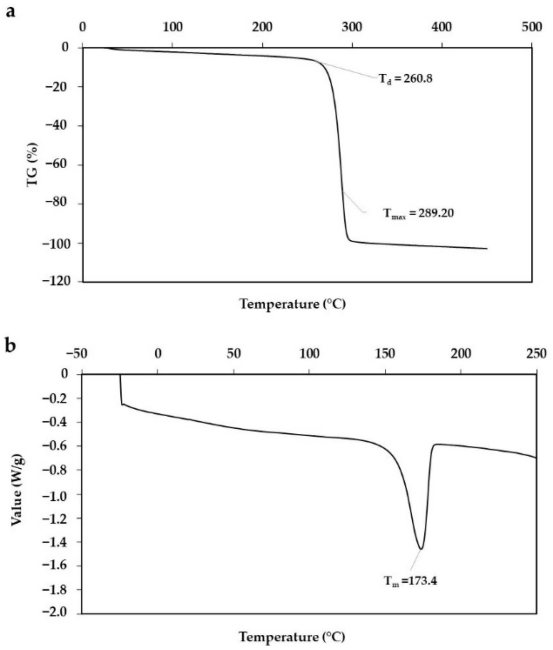

Figure 11. Thermal properties of PHB produced by *Ensifer* sp. HD34: thermogravimetric analysis (TGA) (**a**) and differential scanning calorimeter (DSC) (**b**).

4. Conclusions

A PHB-producing bacterium was newly isolated from decomposed soil and identified using morphological, biochemical, and molecular methods. The strain was Gram-negative bacterium which was classified as genus *Ensifer*. After optimization by RSM, the optimized nutrient consisted of 3.99% (*w*/*v*) PDB and 1.54% (*w*/*v*) glucose which could produce 6.78 g/L of PHA yields (increased 5.8 folds) in the best culture conditions. Furthermore, the extracted PHB product is a thermopolymer. In the future, it will be interesting to examine more about this strain using industrial potato wastes and fed-batch cultivation methods for further study.

Author Contributions: Conceptualization, T.K., W.P. and S.L.; methodology, T.K. and W.P.; software, T.K.; validation, T.K., W.P. and S.L.; formal analysis, T.K.; investigation, T.K.; writing—original draft preparation, T.K.; writing—review and editing, T.K., W.P. and S.L.; supervision, T.K.; project administration, T.K., W.P. and S.L.; funding acquisition, T.K. and S.L. All authors have read and agreed to the published version of the manuscript.

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