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Characterization of polyhydroxyalkanoates (PHAs) biosynthesis by isolated *Novosphingobium* sp. THA_AIK7 using crude glycerol

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Abstract Biodiesel-contaminated wastewater was used to screen for PHAs-producing bacteria by using crude glycerol as the sole carbon source. A gram-negative THA_AIK7 isolate was chosen as a potential PHAs producer. The 16S rRNA phylogeny indicated that THA_AIK7 isolate is a member of *Novosphingobium* genus which is supported by a bootstrap percentage of 100% with *Novosphingobium capsulatum*. The 1,487 bp of 16S rRNA gene sequence of THA_AIK7 isolate has been deposited in the GenBank database under the accession number HM031593. Polymer content of 45% cell dry weight was achieved in 72 h with maximum product yield coefficient of 0.29 g PHAs g^{-1} glycerol. Transmission electron micrograph results exhibited the PHAs granules accumulated inside the bacterial cell. PHAs polymer production in mineral salt media supplemented with 2% (w/v) of crude glycerol at initial pH 7 was extracted by the sodium hypochlorite method. Polymer film spectrographs from Nuclear magnetic resonance displayed a pattern of signal virtually identical to spectra of commercial PHB. Thermal analysis by Differential scanning calorimeter showed a melting temperature at 179°C. Molecular weight analysis by Gel permeation chromatography showed two main peaks of 133,000 and 700 g mol^{-1} with weight-average molecular weight value of 23,800 and number-average molecular weight value of 755. Endotoxin-free of PHAs polymer was preliminarily assessed by a negative result of the gel-clot formation, Pyrotell® Single test vial, at sensitivity of 0.25 EU ml^{-1}. To our knowledge, this is the first reported test of endotoxin-free PHAs naturally produced from gram-negative bacteria which could be used for biomedical application.

Keywords 16S rRNA · Crude glycerol · Endotoxin-free · *Novosphingobium* sp. · Polyhydroxyalkanoates

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Introduction

Polyhydroxyalkanoates (PHAs) are a family of diverse biopolyesters in which currently approximately 150 different monomer units have been identified as constituents [30, 31], whereas homopolymer poly-β-hydroxybutyrate (PHB) is the most abundant [3]. PHAs have become the substitute materials for conventional plastic in response to the harmful effect of the plastic waste problem [8]. Interest in PHAs has been raised because they have properties

similar to various thermoplastics and elastomers, can completely degrade upon disposal, are nontoxic, are biocompatible, and can be made from renewable resources [30]. PHAs accumulation occurs naturally in bacteria to store carbon and energy, which is triggered by unbalanced nutrient supply in the presence of excess carbon source [3, 30]. The major obstacle of PHAs is the production cost, which mainly comes from carbon substrate cost [24]. Many efforts have been devoted to find an alternative cheaper carbon source to lower the total production price.

Crude glycerol, a byproduct of the biodiesel process, has now become a waste stream because of the worldwide production of biodiesel as an important source of alternative fuel [16]. The massive increase in global biodiesel production reached about 7.5 billion liters in 2006 [12] simultaneously with 750 million liters of crude glycerol, the major byproduct, being released based on a 10% ratio calculation from each process [10]. Biodiesel-derived crude glycerol contains many kinds of impurities, such as methanol and salts, which would obstruct its application in other industries [16]. Refining this waste is limited only to a large-scale firm, such as Cargill, with adequate financial capacity [5], which would in turn make the global amount of crude glycerol in the market rapidly accumulate [16]. These situations resulting in fall of crude glycerol price. It was suggested that the price of crude glycerol may be eventually stabilize as low as $0.05/lb [9]. A new utilization route has to be developed for this waste stream. Bioconversion process of glycerol has been reviewed for utilization to produce many kinds of products from microorganisms, including 1,3-propanediol, dihydroxyacetone, succinic acid, propionic acid, ethanol, citric acid, polyhydroxyalkanoates, pigments, and biosurfactants [10]. Using inexpensive waste as a substrate source, the cost for PHAs production can be decreased, and simultaneously, crude glycerol with associated disposal cost can be refined without extra cost [5, 8].

PHAs have promising medical and pharmaceutical applications due to their biodegradability and biocompatibility with immunological inertness and slow degradation in human tissue [2, 38]. PHAs have been investigated for medical uses such as wound management, drug delivery, heart valves, vascular graft, orthopedy, and urology [2, 40]. One important criterion for a biomedical product is endotoxin contamination. Endotoxin, an integral component of the outer membrane of gram-negative bacteria, is well known as an immune-activating agent [40]. It can be co-purified with PHAs [36]. According to US Food and Drug Administration (FDA) regulations, the endotoxin content of medical devices should not exceed 20 USP endotoxin units per device [40]. For biomedical use, FDA compliance must be achieved by an additional step in the purification process to remove endotoxins from the PHAs product [22, 40].

The gram-negative bacteria in genus *Sphingomonas* were reclassified by Yabuuchi et al. in 1990 as a new member of the α-4 subgroup of *Proteobacteria* [32]. This genus was divided into 4 clusters, *Sphingomonas*, *Sphingobium*, *Novosphingobium*, and *Sphingopyxis*, on the basis of their 16S rRNA gene sequence and by combining the phylogenetic, chemotaxonomic, and phenotypic data by Takeuchi et al. [32]. One characteristic of the species of genus *Sphingomonas* is, unlike common gram-negative bacteria, the presence of glycosphingolipids (GSLs) as the cell envelope component instead of lipopolysaccharide (LPS) [19, 32].

This article reports the characterization by different techniques of the PHAs polymer produced by the newly isolated *Novosphingobium* sp. THA_AIK7 that used biodiesel-derived crude glycerol as a sole carbon source. In addition, this article reports the endotoxin investigation of the PHAs product to ensure that it is free from endotoxin.

Materials and methods

Batch cultivation

Mineral salt medium (MSM) was used in the experiment [25]. The 50% purity of the solidified vegetable-oil based biodiesel-derived crude glycerol (CG) was used as a carbon source. All chemicals used in the experiment were purchased from Wako chemical, Japan. The bacterial isolate THA_AIK7 (formerly AIK7) was isolated from biodiesel-contaminated wastewater and was selected as a high-potential PHAs-producing strain [33]. PHAs biosynthesis was studied in MSM supplemented with 2% (w/v) CG with initial pH of 7. Aerobic condition was maintained by shaking at 30°C and 150 rpm. PHAs and glycerol determination were described in Teeka et al. [33].

Bacterial identification

THA_AIK7 cell morphologies were observed under light microscope. Biochemical characterization such as gram staining, antibiotic test, enzyme activity test, motility test, and acid and gas production test were carried out according to Garrity et al. [13]. Bacterial identification was done by 16S rRNA gene amplification. Bacterial cells were cultured in nutrient broth (NB) (Cat. No. 234000, BD Difco™, USA) at 30°C for 24 h. The culture was then harvested and extracted with QIAamp® DNA mini kit (QIAamp, USA). The 16S rRNA cistron was amplified using universal bacterial primers pA (5′-AGAGTTTGATCCTGGCTCAG-3′) and pH (5′-AAGGAGGTGATCCAG CCGCA-3′) [11] by illustra™ puReTaq Ready-To-Go PCR Beads (GE Healthcare, UK). Purified 16S amplicons were subsequently

cloned for sequencing using a pGEM-T Easy kit (Promega, USA) and then transformed into competent *Escherichia coli* JM109. Plasmid DNA was isolated and the cloned inserts were sequenced using an ABI PRISM 310 instrument (Applied Biosystems, USA) according to the manufacturer's instructions. The 16S rRNA gene sequences were analysed with basic local alignment search tool (BLAST) function available on the National Center for Biotechnology Information (NCBI). CLUSTALX 2.0.11 was used for alignment of the different 16S rRNA gene sequences from the GenBank database with default settings. Phylogenetic trees were analysed by using Phylogenetic inference package (PHYLIP) version 3.69. Distances were calculated using the Kimura 2-parameter distance model. Trees were built by the neighbor-joining method [27]. The dataset was bootstrapped 1,000 times.

PHAs granule visualization

PHAs granule visualization was performed by cultivation of THA_AIK7 isolate on MSM agar supplemented with 1% (w/v) CG for 48 h. Bacterial colonies were taken from a plate and were fixed on a glass slide. Sudan Black B staining method was used to stain the lipid granules accumulated inside the bacterial cell [7]. Transmission electron microscopy (TEM) of the bacterial cell was conducted by washing with Millonig's phosphate buffer (Electron Microscopy Sciences, USA) and fixing in 3% glutaraldehyde (Wako, Japan) in buffer, and incubating at 4°C for 24 h. Secondary fixation was made in 1% osmium tetroxide, and then, the cells were incubated at 4°C for 1 h. Cells were suspended in 3% molten agar and were then sectioned with a blade. Tissue samples were successively dehydrated in a series of ethanol solutions of 50, 70, 90, 95, and 100% with each 5 min × 6 times. They were then immersed in propylene oxide for 5 min × 6 times. Tissue samples were infiltrated consecutively in a mixture of spurr and propylene oxide at 1:2, 1:1, and 2:1 ratio for 1 h in each step. Embedded tissue samples were made in 100% spurr and were incubated for polymerization at 70°C for 48 h. The resin blocks were cut using Leica EM UC6 Ultra-microtome and were transferred to copper grids. The sections were post-stained with uranyl acetate and lead citrate solution and were then observed with FEI Tecnai G^2 (Oregon, USA) TEM.

PHAs polymer extraction

Polymer was extracted from THA_AIK7 cultivated in MSM supplemented with 2% (w/v) CG at an initial pH of 7 at 30°C and 150 rpm for 72 h. PHAs extraction was performed according to Shi et al. [28] modified method as follows: all of the culture medium was collected at 8,000 g × 10 min at 15°C and washed by distilled water. The cell pellets were re-suspended in 10 ml of 50% sodium hypochlorite solution (Wako chemical, Japan) and was incubated at 37°C for 1 h with shaking. The lysed cell pellet was centrifuged and was washed successively with 20 ml of water, acetone, and ethanol. Fifteen milliliters of chloroform solution was added to dissolve the polymer pellet, and the solution was transferred to a glass tube. Two milliliters of distilled water was added to the tube in order to separate cell debris from the solvent phase. The chloroform-water solution was then incubated at 55°C for 2 h with intermittent shaking. After cooling, the chloroform phase was transferred to a new glass tube and was evaporated under room temperature (20–25°C). PHAs THA_AIK7 films were used for polymer characterization.

Characterization of PHAs

The weight-average molecular weight (Mw), number-average molecular weight (Mn), and size-average molecular weight (Mz) of the polymer were determined by gel permeation chromatography (GPC) using GPC K-804L column (Shodex®) equipped with an ultraviolet (UV) SPD-10A detector, CTO-10AS column oven, LC-10AT pump, C-R8A recorder, and CHROMATOPAC C-R7A plus data processing (Shimadzu Corporation). $CHCl_3$ was used as eluent at a flow rate of 1.0 ml min^{-1} at 40°C. Molecular weights in the range of 10^6–10^2 g mol^{-1} of polystyrene were used for standard calibration. Differential scanning calorimetry (DSC) was performed with a Perkin-Elmer equipment. Sample was dried in vacuum for approximately 12 h at 60°C prior to thermal analysis. Sample was heated from 20 to 185°C at 20°C min^{-1}, held for 5 min to ensure melting, then cooled to 20°C at −20°C min^{-1}. Melting temperature (Tm) thermogram of the polymer was recorded from the first heating scan's data. The ^1H nuclear magnetic resonance (NMR) spectrum of the polymer was recorded using JEOL GSX500 (JEOL, Tokyo, Japan) spectrometer. Three milligrams of the polymer was dried at 40°C for 72 h in order to eliminate the solvent and humidity and then dissolved in 1 ml of deuterated chloroform ($CDCl_3$). The mixture was incubated at 45°C with shaking to enhance solubility. Spectra were recorded at a frequency of 500 MHz at 25°C. The chemical shifts (δ) were referenced against a tetramethylsilane (TMS) internal standard. Fourier transform infrared spectroscopy (FTIR) was analysed using a Perkin-Elmer FTIR spectrometer with a wave number range of 4,000–400 cm^{-1}.

Endotoxin test

All glassware used in the experiment was depyrogenated at 250°C for 1 h. *Escherichia coli* ATCC 11303 was cultured in NB medium for 24 h at 37°C and washed with LRW

(Limulus amebocyte lysate reagent water, Associates of Cape Cod, Inc., USA). One milliliter of LRW was added and transferred into a glass tube. Twenty milligrams of PHB standard (Sigma-Aldrich) and PHAs from THA_AIK7 were put into a glass tube. One milliliter of $CHCl_3$ was added to dissolve the polymer, and 1 ml of LRW was subsequently added. The negative control was prepared according to the polymer preparation procedure. All samples were placed in a water-bath type ultrasonicator and were sonicated in cooled water for 60 min [34]. *E. coli* sample was centrifuged, and the supernatant was transferred into a new glass tube to use it as the positive control. Polymer samples were vortexed for 1 min before the non-solvent phase was transferred into a new tube. Two hundred microliters of *E. coli* extract, polymer extract, and blank were added into a Pyrotell® Single test vial (STV) (Associates of Cape Cod, Inc., USA) at a sensitivity of 0.25 EU ml^{-1}. All samples were vortexed for 1 min and were incubated at 37°C for 60 min. The tube was gently inverted and then the result was read.

Results and discussions

PHAs biosynthesis

PHAs production from THA_AIK7 isolate in MSM supplemented with 2% (w/v) CG was investigated in batch cultivation. The MSM supplemented with 2% (w/v) CG contained 1.44 g l^{-1} of total nitrogen, 1.28 g l^{-1} of total phosphorus, 27.5 g l^{-1} chemical oxygen demand (COD), and 1.384 g l^{-1} of sodium element [33]. It appeared that highest PHAs content of 45% cell dry weight (CDW) was achieved in 72 h (Fig. 1) with biomass yield 3.5 g l^{-1}. The pH of culture medium fell down slightly from 7 to 6.3 in 120 h. At the end of cultivation, residual glycerol in culture medium of 4 g l^{-1} indicated that THA_AIK7 can utilize glycerol for their growth and polymer production. The maximum product yield coefficient of 0.29 g PHAs g^{-1} glycerol was attained. Up to now, the highest PHAs content of 75%CDW was reported from batch culture of *Halomonas hydrothermalis* utilizing 2% *Jatropha* biodiesel byproduct [29]. To date, production yields of PHAs from THA_AIK7 have not been optimized. However, this work establishes that CG can be utilized by THA_AIK7 as a carbon substrate for PHAs production without the need of refining.

Physiological and biochemical characterization

Conventional biochemical tests were used for bacterial characterization. Bacterial isolate THA_AIK7 was gram-negative. Colonies were 2 mm in diameter with whitish color, circular form, convex elevation, opaque, glistening surface, and smooth edges. Cells were motile, bacilli form, 0.5 μm wide, and 2.5 μm long. Cells had no catalase activity, no nitrate reductase activity, no caseinase activity, and no amylase activity. Cells could not grow on 5% NaCl. Cells produced acid and gas from glucose. Cells were susceptible to penicillin at 10 IU. The presence of intracellular lipid granules was confirmed by Sudan black B staining (Fig. 2a). TEM exhibited a white fraction of PHAs granules inside the THA_AIK7 isolate (Fig. 2b–d). The black fraction in the Sudan black B pictures revealed that the THA_AIK7 isolate could accumulate a lipid sink that was more than 70% of the cell content.

16S rRNA gene sequence and phylogenetic analysis

The 1,487 bp of the THA_AIK7 isolate's 16S rRNA amplicon was aligned and compared with the GenBank database. The BLAST result showed that THA_AIK7 is identical to *N. capsulatum* (99%), *N. aromaticivorans* (98%), and *N. hassiacum* (96%). *N. capsulatum* and

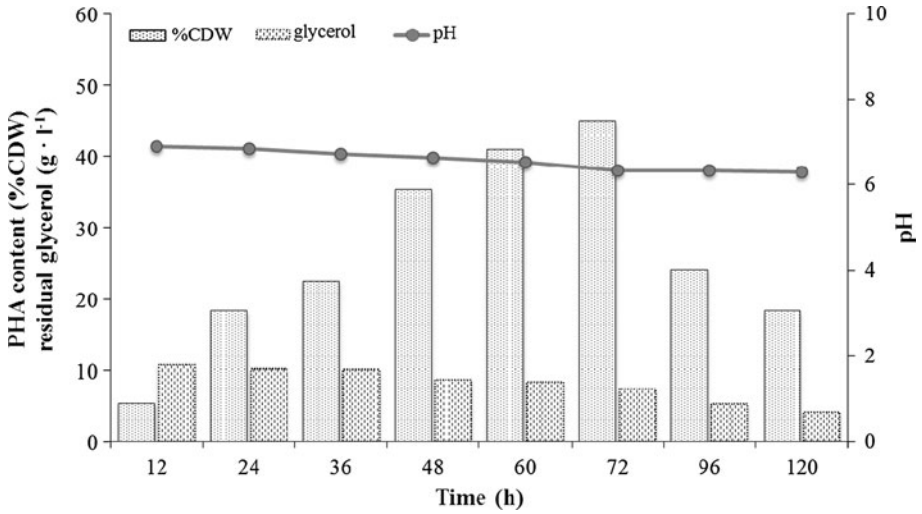

Fig. 1 Time course of PHAs content, residual glycerol, and pH trend of THA_AIK7 isolate cultivated in MSM supplemented with 2% CG (w/v) with initial pH of 7. Aerobic condition was maintained at 30°C and 150 rpm

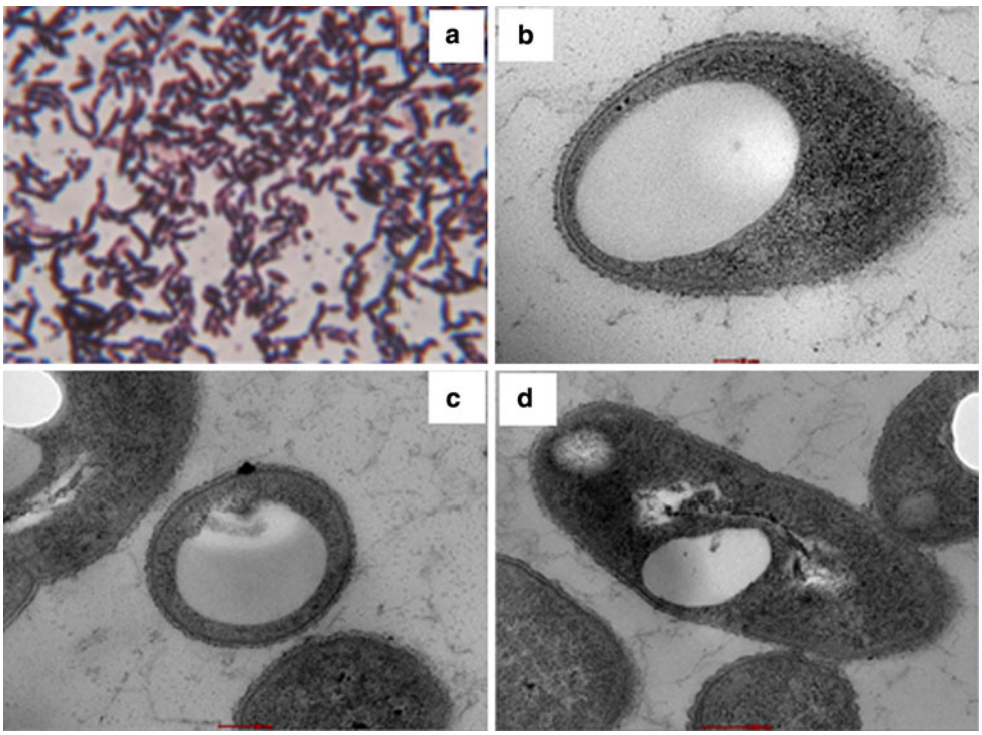

Fig. 2 Sudan black B staining of the THA_AIK7 isolate exhibiting a black fraction of lipid storage granules under a light microscope (**a**) and transmission electron micrographs showing a white fraction of PHAs granule accumulation after 2 days of incubation (**b–d**; *bar* = 50, 100, 200 μm)

THA_AIK7 showed 99% identity with each other with 1,356 bp coverage. The phylogenetic tree was constructed using the consensus sequence of THA_AIK7 and other members belonging to the *Novosphingobium* genus for which information was acquired from the Ribosomal database project and GenBank. A neighbor-joining tree (Fig. 3) indicated that the bacterial isolate THA_AIK7 clustered closely with *N. capsulatum* (FJ266331) with a bootstrap percentage of 100%. Nevertheless, conventional biochemical test showed that some features of the THA_AIK7 isolate were dissimilar to *N. capsulatum* ATCC 14666. They differed in gas production from glucose, nitrate reduction, and catalase activity [1]. However, it was also observed that THA_AIK7 had a variance characteristic in glucose utilization. The 16S rRNA gene sequence of the THA_AIK7 isolate used in this study has been deposited in the GenBank database under the accession number HM031593 and referred to as *Novosphingobium* sp. THA_AIK7. Most of the bacteria of the genus *Novosphingobium* have been of special interest because of the ability to degrade toxic compounds and other chemicals such as polycyclic aromatic hydrocarbons (PAHs), polychlorophenol, carbofuran, and carbazole [15, 35, 42, 44]. Till now, only 1 species of *Novosphingobium* has been reported as a PHAs producer. *Novosphingobium nitrogenifigens*, a nitrogen-fixing species, can produce PHAs from a glucose substrate and is capable of accumulating PHAs up to 41% of its cell content [1]. In our study, a spectrophotometric method was routinely used to quantify the PHAs content. Valappil et al. [36] noticed that crotonic acid assay method is known to overestimate the actual PHAs content in a sample. In the present study, bacterial cell staining with Sudan black B, a lipid dye, revealed that *Novosphingobium* sp. THA_AIK7 could accumulate lipid granules inside their cell more than 70% of the cell content. Apart from PHAs, bacteria can also store intracellular polymeric lipid triacylglycerols (TAGs) and wax esters (WEs) as their energy and carbon pool under imbalanced growth [39]. Although the white fraction seen by TEM of PHAs and the TAGs granular structure looked similar, about 1 mg ml^{-1} of polymer film was extracted from the culture medium at the end of a 72-h cultivation and was used to confirm its property.

PHAs polymer characterization

Differential scanning calorimetry

The melting temperature (Tm) point of the THA_AIK7 polymer was taken at the summit of the endothermic peak in the DSC heating thermogram at 179°C (Fig. 4). The melting point of the polymer was in the common range reported by many research groups [14, 24, 45]. The melting temperature was not affected by the low molecular mass of

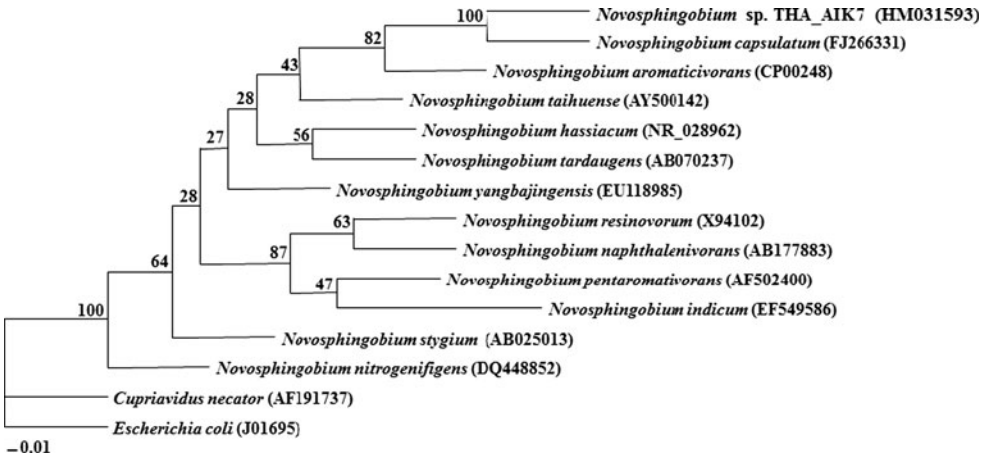

Fig. 3 Phylogenetic tree indicating the estimated relationship between the strain *Novosphingobium* sp. THA_AIK7 and other members of the genus *Novosphingobium* based on 16S rRNA sequence. *E. coli* was chosen as an outgroup. The *numbers* at the nodes represent the bootstrap percentages of 1,000 replicates. The *scale bar* indicates the substitutions per nucleotide position. Nucleotide accession numbers are given in *parentheses*

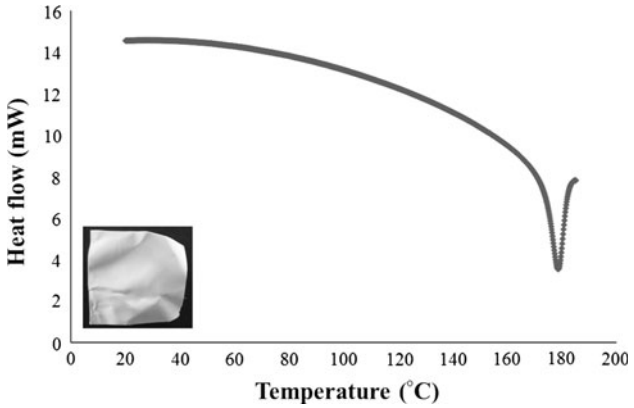

Fig. 4 Differential scanning calorimetry heating thermogram obtained from the first scan. The melting point of 179°C obtained from the THA_AIK7 polymer was in the common range of PHAs. The polymer film used in the experiment is shown in the *picture*

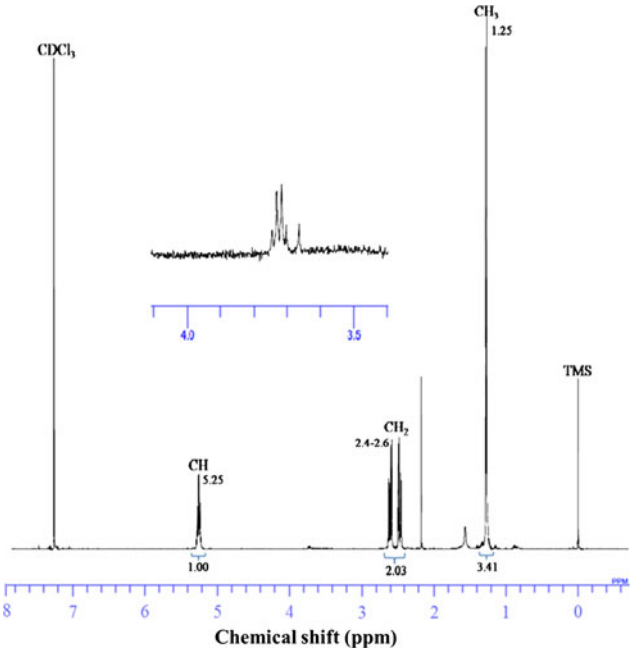

Fig. 5 500 MHz ^{1}H nuclear magnetic resonance spectrum of the THA_AIK7 polymer. The main resonance at 1.25, 2.4–2.6, and 5.25 ppm of CH_3, CH_2, and CH end groups, respectively, are identical to PHB homopolymer. The glycerol end-capping resonance, in the range 3.5–4.0 ppm, is shown in the expanded region

the polymer conforming to the previous result of Zhu et al. [45]. The cooling thermogram obtained after the first heating scan at a high temperature of 131°C (data not shown) may predict a fast crystallization rate for the polymer as discussed by Hong et al. [14]. The narrow peak of DSC trace may also indicate a highly crystalline polymer as those results were shown by Hong et al. [14]. However, Xu et al. [41] mentioned that the crystallization of a polymer could spontaneously increase with storage-time at room temperature.

Nuclear magnetic resonance

Under the culture conditions for bacteria at initial pH of 7 and 30°C, NMR spectrographs displayed a pattern of signal virtually identical to the spectrum of the PHB homopolymer [5, 24] with main resonance at ~1.25, 2.4–2.6, and 5.25 ppm of CH_3, CH_2, and CH end groups, respectively (Fig. 5). The expanded region of the NMR spectrum revealed the presence of a glycerol end-capping resonance in the range of 3.5–4.0 ppm as described by Zhu et al. [45] and Ashby et al. [5]. The spectrum derived from NMR strengthened that the typical properties of the THA_AIK7 polymer were identical to those of PHB published elsewhere [5, 24, 43].

Fourier transform infrared spectroscopy

The distinct band at 1,735 cm^{-1} in the FTIR spectrum (Fig. 6) corresponds to the ester functional group of PHB as explained by Kansiz et al. [17]. The series of band in the range of 900–1200 cm^{-1} correspond to C–O–C bond stretching, which could be contributed from PHB as well [17]. Xu et al. [41] demonstrated that the wave number of

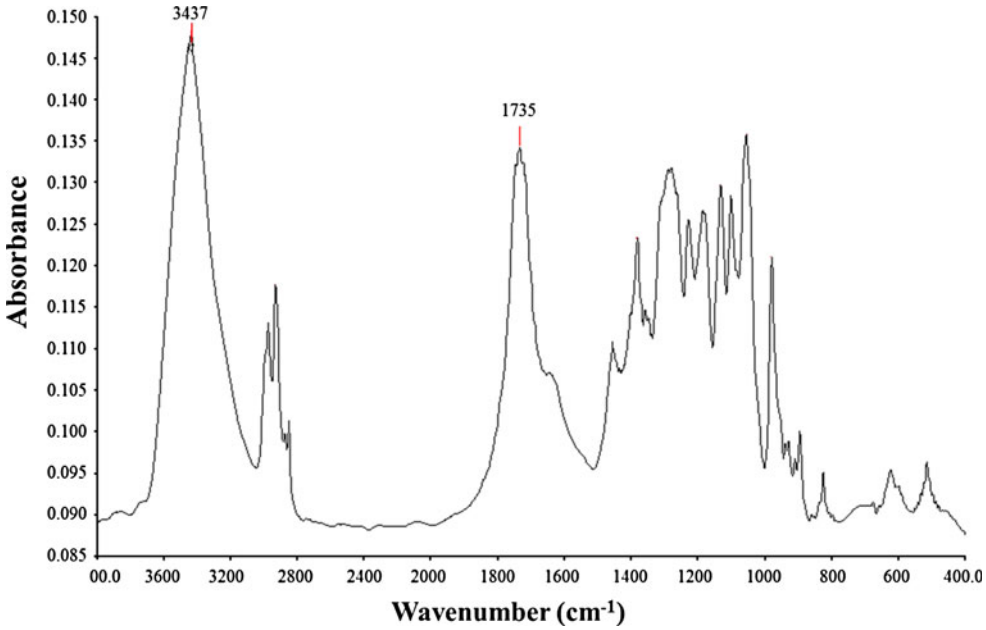

Fig. 6 Fourier transform infrared spectrum of the THA_AIK7 polymer. The peak at 1,735 cm^{-1}, characteristic for PHB, corresponds to the ester functional group. The high intensity at 3,437 cm^{-1} may have corresponded to the degradation of the carboxylic group of the polymer and showed the increment of hydrogen-bonded groups at this band

the ester bond was affected by the dipole moment. Their result showed that the wave number could vary in the range of 1,741–1,724 cm^{-1} if the temperature for FTIR analysis was changed. The high intensity of the 3,437 cm^{-1} band may have corresponded to the degradation of the carboxylic group and showed an increase of hydrogen-bonded groups at that distinct band as discussed by Hong et al. [14]. The degradation of polymer shown by FTIR was in agreement with a large PDI value with 2 main peaks for inhomogeneity in polymer seen in the GPC curve.

Gel permeation chromatography

The calibration curve was generated using a polystyrene standard (Fig. 7a). Two main peaks of molecular weights were found at 133,000 and 700 g mol^{-1} (Fig. 7b). Mn of 755, Mw of 23,800, Mz of 416,000, and polydispersion index (PDI) (Mz/Mw) of 17.5 were derived from the GPC analysis. The GPC result showed a large PDI value, which reflected the inhomogeneity of the polymer chain. Patel et al. [24] mentioned that the molecular weight of PHAs produced by microorganisms could be in the range of 20×10^6 and 75×10^3 g mol^{-1}. Differing from the other carbon sources that have been studied for PHAs biosynthesis, both pure and crude glycerol used as carbon sources for PHAs production were reported with significantly low Mw PHAs because of glycerol esterification of PHAs resulting in chain termination (end-capping) [5, 45]. It has been reported that the PHAs produced from biodiesel-based glycerol showed Mw ranging from 786×10^3 to 87×10^3 g mol^{-1} and Mn ranging from 215×10^3 to 29×10^3 g mol^{-1} for different types of microorganisms and culture conditions [4, 5, 8, 20, 21, 23]. Among these, the lowest Mn PHAs was derived from *Halomonas* sp. KM-1 cultivated in a medium supplemented with 3% of waste glycerol at 29×10^3 g mol^{-1} [20]. Some researchers have published data that we will compare with our observation. Yu and Marchessault [43] mentioned that PHB solubility in an organic solvent is poor because of high molecular weight and high crystallinity. They explained that, in their experiment, low molecular weight PHB could be dissolved in dioxane and THF (tetrahydrofuran).

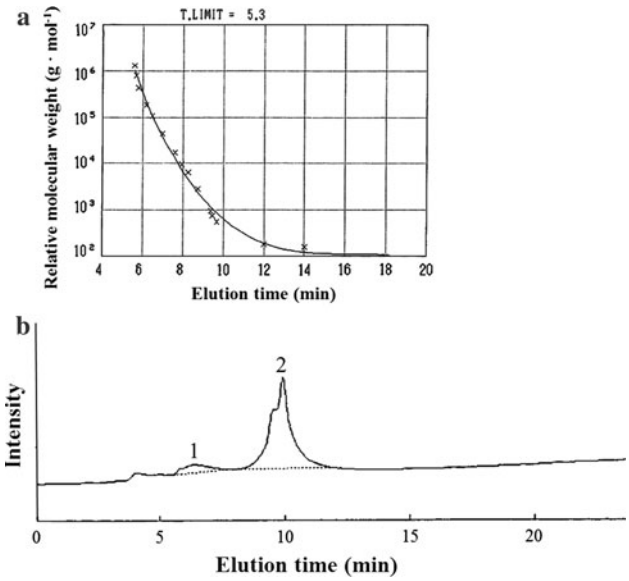

Fig. 7 Gel permeation chromatography analysis showed a calibration curve analysed in $CHCl_3$ against a polystyrene standard (**a**) and GPC curve of the THA_AIK7 polymer; the relative weight average molecular mass for the peak 1 = 133,000 and 2 = 700 g mol^{-1} (**b**)

Valentin et al. [37] found that PHAs in the THF-insoluble portion had greater molecular weight than the THF-soluble portion at 1,180 × 10^3 g mol^{-1} compared to 94.9 × 10^3 g mol^{-1}, respectively. In this instance, the molecular mass of the THA_AIK7 polymer might not have been as low as it was shown because it did not dissolve well in THF when we tried for GPC analysis (data not shown), even though we tried incubation at 40°C. The Mz of 416,000 g mol^{-1} derived from the GPC result shows long-chain polymer synthesizing capability as well. We also found a small peak, when we changed the cultivated condition to an initial pH of 6, at a molecular mass over the range (10^6) of the standard calibration curve (data not shown). Therefore, we guess that the THA_AIK7 isolate could synthesize a longer chain polymer. One cause-effect in the polymer mass decrease may come from the extraction method in which sodium hypochlorite was used. As was found by Berger et al. [6], they summarized severe degradation of the PHB polymer due to sodium hypochlorite digestion resulting in about 50% molecular weight reduction. The actual molecular mass of the THA_AIK7 polymer remained in doubt. The GPC analysis will be done in the next step with a mild extraction method.

Endotoxin evaluation

A bacterial endotoxin test for the polymer was done by Pyrotell® Single test vial at a sensitivity of 0.25 EU ml^{-1}. The polymer produced by the *Novosphigobium* sp. THA_AIK7 isolate and a PHB standard from Sigma-Aldrich showed a negative result for gel formation. The reliability of the test was shown by the positive and negative results of the *E. coli* extract and blank sample, respectively (Table 1).

Due to the environment problem arising from petroleum-based plastic, biodegradable plastic has become the alternative choice. Nevertheless, PHAs price is still high and cannot compete with synthetic polymers [30]. Since 1996, PHAs have been raised in biomedical field study [46]. It has been noted that D-β-hydroxybutyrate, the monomer of PHB, is a common intermediate metabolite in human plasma [26]. Therefore, PHAs implantation in mammalian tissue will not cause any problems [22], and in addition, the cost factor that hampers PHAs use becomes less important for biomedical application [36]. Several gram-negative bacteria, such as *Wautersia eutropha*, *Pseudomonas oleovorans*, and recombinant *E. coli*, have been used for industrial PHAs biopolymer production [36]. It is well known that gram-negative bacteria contain endotoxin, in the form of LPS, in their outer membrane [36]. As mention above, endotoxin is prohibited for biomedical use according to the US FDA regulations. The polymer derived from *Novosphingobium* sp. might be the alternative choice for biomedical use because it has GSLs in the cell membrane instead of LPS. GSLs is normally found in eukaryotic cells, whereas bacterial GSLs is uncommon [18]. Williams et al. [40] mentioned that commercial PHB contained endotoxin greater than 120 EU g^{-1}, whereas PHB from Sigma-Aldrich in our experiment showed a negative result. The purification procedure might be improved in order to remove more impurities by using depyrogenating agents such as NaOH, hydrogen peroxide, and supercritical CO_2 [22, 40]. In our study, the negative result of endotoxin in the THA_AIK7 polymer product at a sensitivity of 0.25 EU ml^{-1} by gel-clot formation could be preliminarily assessed as the absence of bacterial endotoxin in agreement with the data of LPS lacking, which were obtained by Takeuchi et al. [32] and Kawahara et al. [19]. This could verify that the polymer produced by *Novosphingobium* sp. THA_AIK7 was endotoxin-free.

Table 1 Endotoxin test at a sensitivity of 0.25 EU ml^{-1}

Specimen	Result
PHAs THA_AIK7	Negative
PHB (Sigma-Aldrich)	Negative
E. coli extract (ATCC 11303)	Positive (undiluted, 1:4, 1:16)
Blank	Negative

Conclusions

In our study, using *Novosphingobium* sp. THA_AIK7 for PHAs biosynthesis from crude glycerol could be a benefit for 3 reasons: (1) crude glycerol could be refined without disposal cost, (2) PHAs production cost could be lowered because of use of inexpensive carbon source, and (3) THA_AIK7 polymer could be the alternative choice of PHAs for biomedical application.

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