

# ISOLATION AND CHARACTERIZATION POLYHYDROBUTYRATE (PHB) PRODUCING BACTERIA FROM WASTE COOKING OIL USING POMEGRANATE MOLASSES AS CARBON SOURCE

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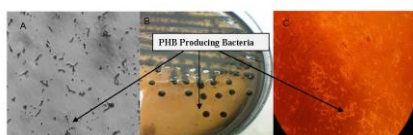
Laila Muftah Zargoun<sup>a</sup>, Nor Azimah Mohd Zain<sup>a,b\*</sup>, Shafinaz Shahir<sup>b</sup>

\*Corresponding author  
azimah@fbb.utm.my

<sup>a</sup>Department of Biosciences & Health Sciences, Faculty of Biosciences and Medical Engineering, Universiti Teknologi Malaysia, 81310 UTM Johor Bahru, Johor, Malaysia

<sup>b</sup>Water Research Alliance, Universiti Teknologi Malaysia

## Graphical abstract



## Abstract

In this study, a polyhydroxybutyrate (PHB) producing bacterium was isolated from waste cooking oil and characterized for its morphological and biochemical properties. Staining methods utilizing Sudan Black B and Nile Blue A were used on isolated bacterium to demonstrate good capability for synthesizing PHB. It was shown that the isolated bacterium species was related to *Bacillus thuringiensis* LMA by using 16S rRNA gene sequences analysis. During the stationary phase, the *Bacillus* strain was subjected to 10 % (w/v) of pomegranate molasses as a carbon source and 5 g/L of peptone as a nitrogen source. 2 ml of batch fermentation was collected. Samples were collected twice during the incubation period for detection of PHB using Sudan Black B. The PHB production accounted for up to 57.45% of the cell dry weight. The PHB produced was characterized using Fourier Transform Infrared Spectroscopy (FTIR) and Nuclear Magnetic Resonance Spectroscopy (NMR). The drastic absorption band at approximately 1717  $\text{cm}^{-1}$  indicated the stretching vibration of C=O group in PHB polyester, while the functional groups of PHB were identified methyl (-CH<sub>3</sub>) at 1.28 ppm, methylene (-CH<sub>2</sub>) 2.0 and 2.5 ppm, and methylene doublet group (CH<sub>3</sub>) at 5.3 ppm.

Keywords: PHB, waste cooking oil, FTIR, NMR, pomegranate molasses

## Abstrak

Dalam kajian ini, bakteria yang menghasilkan polihidroksibutirat (PHB) telah dipencilkan daripada sisa minyak masak dan dicirikan dari segi morfologi dan biokimia. Hanya satu strain Berjaya diasingkan dari sisatersebut. Bakteria yang dipisahkan telah menunjukkan kemampuan yang baik untuk menghasilkan PHB selepas ditentukan dengan menggunakan kaedah pewarnaan Sudan Black B dan Nil Blue A. Dengan menggunakan urutan gen 16S rRNA, spesies bakteria yang dipisahkan itu adalah berkaitan dengan *Bacillus thuringiensis* LMA. Semasa fasa pegun, strain *Bacillus* itu diletakkan dalam keadaan 10% (w/ v) sirap pekak buah delima (sumber karbon) dan 5 g/L pepton sebagai sumber nitrogen. Sampel dikumpulkan sebanyak dua kali sepanjang tempoh pengeraman untuk mengesan PHB menggunakan pewarnaan Sudan Black B. PHB yang terkumpul adalah sehingga 57.45% daripada berat sel kering PHB yang dihasilkan dicirikan menggunakan spektroskopi inframerah transformasi Fourier (FTIR) dan spektrokopi resonans magnet nucleus (NMR). Jalur serapan yang mendadak pada kira-kira 1717 $\text{cm}^{-1}$  menandakan getaran meregang olehkumpulan C=O pada polyester PHB, sementara kumpulan berfungsi yang telah dikenali pada PHB; metil (-CH<sub>3</sub>) pada

1.60 ppm, metilena (-CH<sub>2</sub>) 2.0 and 2.5 ppm, dan kumpulan metilena berganda (CH<sub>3</sub>) pada 5.3 ppm.

**Kata kunci:** PHB, sisa minyak masak, FTIR, NMR, sirap pekat buah delima

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## 1.0 INTRODUCTION

Due to the issue of environmental sustainability, attention has been drawn on the intense use of products that are environmentally not friendly. The use of plastics that are basically petroleum based products have been discouraged since the plastics are not biodegradable, thus, caused damages to the natural environment [1]. The damages inflicted as among others are high toxicity after incineration and huge accumulation into the landfill, which subsequently affects aquatic lives when drained into water ways. Recent guesstimate on the earth's mineral resources, including petroleum resources suggesting alarming rates of depletion of these valuable natural resources [2]. Research has also shown that the petroleum may likely diminish with time of usage [3], as the time taken for the formation is not proportional to the depletion. For this reason, the need for available and alternative technology to produce Bio plastics has become relevant.

The term bio plastics refer to special type of biomaterial, which are polyester and are produced by a range of microbes, cultured under different nutrient condition [4]. The most widely produced microbial bioplastics are PHB and PHA (polyhydroxyalkanoates) [5]. Nonetheless, other polyesters can also be produced by microorganisms. The majority of them either requires similar biosynthetic enzymes or lack modern industrial applications [6]. Polyhydroxyalkonates (PHAs) was first isolated and characterized in 1925 in *Bacillus megaterium* [6, 7, 8]. The bio-plastic have attracted considerable interest in recent years because they are easy to obtain, and by using alternative biological processes, they exhibit biodegradability and biocompatible properties [2].

The production of PHAs by bacteria bridged over the Gram's division, with several species of bacteria from both positive and negative have been isolated from various sources including soil, wastewater, industrial effluents, dairy waste, and domestic sewage [3]. *Bacillus cereus* and *Bacillus mycooides* were able to produce 75% of PHB were isolated from garden soil [4, 5]. PHA producing bacteria were divided into two groups based on media nutrient compositions. The first group of bacteria requires limitation of nitrogen and phosphorus for PHB production, example are *Ralstonia eutropha* and *Pseudomonas oleovorans*. The second group has the ability to produce PHB without the need for nutritional limitation. They are

the *Alcaligenes latus*, *Bacillus thuringiensis*, *B. subtilis*, and *B. megaterium* [9, 10, 11].

The carbon source or substrate used by PHB producing bacteria is an important factor in accumulating a high level of PHB and to lessen the cost of PHB production. Many studies have used different types of carbon sources from agro-industrial wastes [12], corn oil, molasses, fermented mash and spent wash [6], and vegetable oil, heated vegetable oil, and waste frying oil [13]. Fukui and Doi (1998) used olive oil, corn oil, palm oil, and oleic acid as a carbon sources for producing PHA which resulted in up to 81% of PHA production, after 72 hours of incubation [14].

In this study, polyhydroxybutyrate accumulating bacterial strain was isolated from waste cooking oil in order to discover new source of PHB producing bacteria and to identify a new carbon source for PHB production. The identification and characterization for its morphological, biochemical properties was conducted to recognize the species of bacteria. The strain isolated from waste cooking oil was confirmed by using molecular biology methods. The PHB production was carried out using peptone and pomegranate molasses as nitrogen and carbon sources respectively. These were added in high concentration to the media. The biodegradable microbial bioplastic produced was characterized using Fourier Transform Infrared Spectroscopy (FTIR) and Nuclear Magnetic Resonance Spectroscopy (NMR).

## 2.0 EXPERIMENTAL

### 2.1 Collection of Waste cooking Oil Sample

Waste cooking oil (WCO) was obtained from Restaurant Hadramot at Taman Universiti, Skudai, Johor, Malaysia. The oil sample was collected in sterile glass container then kept at 4 °C till use.

### 2.2 Isolation of PHB Producing Bacterium

Waste cooking oil sample (1 ml) was pipetted into 100 ml of nutrient broth. After that it was incubated at 37 °C in shaker incubator 180 rpm for 24 hours. The spreading and streaking plates methods were used to isolate bacteria from the sample. The enrichment media was diluted up to 10<sup>-6</sup> dilution. Then, 0.1 ml of each mixed culture samples from 10<sup>-1</sup> to 10<sup>-6</sup> serial dilution were spread on nutrient agar. All plates were sealed and kept at 37 °C for 24 hours. The bacterial

colony obtained was streaked on agar medium, this procedure was repeated until only a single bacterium exists on agar plate. All steps were done under sterilize condition. Physical morphology of each bacterial colony obtained was observed. The pure colony was maintained at 4 °C on nutrient agar slant for use experiment.

### 2.3 Screening Bacterium for PHB Production

Bacterial cell was screened using two different stains, Sudan Black B and Nile Blue A to detect the accumulation of PHA granules in the bacteria.

#### 2.3.1 Sudan Black B Stain

The bacteria colony on the agar plate was stained with Sudan Black B to detect the presence of PHB granules. The dye solution was prepared by dissolving 0.3g of Sudan powder in 100 ml alcohol. The prepared solution was spread over the plate and then left for 30 minutes. The plate was washed with ethanol (96% v/v) to remove the excess stain [15]. For more confirmation, the same of colony of bacteria was smeared onto clean slid, then stained with 0.3 g of Sudan black B in 70% (v/v) (ethanol) for 10 minutes. Subsequently, the smear was immersed with xylene to decolorize the cells, after that, 5 % (w/v) Safranin water solution was used for 10 seconds as counter stain. Finally, the slide was washed with distilled water and dried before observation under an optical microscope [16].

#### 2.3.2 Nile Blue A Stain

An aqueous solution of 1 % (w/v) of Nile Blue A was prepared after dissolving at 50 °C, and then filtered before use. Bacterial smear was fixed on the slide by heating, before adding the Nile Blue A. The slide was inserted into a coplin staining jar filled with aqueous solution at 55 °C for 10 minutes. Afterwards, the slide was washed with tap water to remove excess stain and then with 8 % (w/v) acetic acid for 1 minute. The smear was washed again with tap water and dried with bibulous paper. Finally, the smear was covered with a cover slip and then examined under a Nikon fluorescence microscope [17].

### 2.4 Identification and Characterization of Isolated Bacterium

The morphology of bacteria was observed using Gram stain. The bacteria smear was fixed onto clean slide and treated with three types of stains; crystal violet, gram's iodine and Safranin. The slide was dried and observed under a light microscope at 1000x magnification [18]. In addition, conventional biochemical test was conducted to identify the bacteria according to previous studies [18].

### 2.5 Endospore Staining

The method utilized malachite green to stain the endospore and Safranin to stain the vegetative portion of the cell. The bacteria was smeared, fixed by heating, and then covered with a small piece of filter paper which was saturated with malachite green. The slide was steamed over boiling water for 5 minutes, then, allowed to cool at room temperature. Afterwards, the paper was removed and the slide was rinsed with water before being counter stained with Safranin for 20 seconds. Excess stain was washed off and the air-dried smear was examined under oil immersion at 100x magnification [19].

### 2.6 Bacteria Identification by 16S rRNA Gene Method

Promega kit was used to extract the genomic DNA of bacteria. The 16s rRNA gene amplification was achieved using two universal primers which were forward 5'-AGA GTT TGA TCC TGG CTC AG-3' and reverse primer 5'-ACG GTC ATA CCT TGT TAC GAC TT-3'. PCR amplification was carried out at 95 °C/1min, 55 °C/1 min; 72 °C/2 min with final extension at 72 °C/5 min. The PCR products were purified using Qiagen PCR purification kit [21]. The purified PCR was sent to First BASE Laboratories Sdn Bhd, Malaysia to obtain the DNA sequence. The received sequences were analyzed using NCBI-BLAST (National Center for Biotechnology Information <http://www.ncbi.nlm.nih.gov>) program. Molecular Evolutionary Genetics Analysis version 5 (MEGA5.2) software was used for building sequence alignments, and phylogenetic trees of isolated bacteria.

### 2.7 Carbon Substrate

The pomegranate molasses was purchased from an Albatol Arabic shop in Skudai, Johor Bahru, Malaysia. The stock of pomegranate molasses was diluted to 10 % (w/v), and then analyzed by High Performance Liquid Chromatography (HPLC) to detect the sugar content. The sugars were measured by (HPLC), using a 300 mm x 7.8 mm, Rezex RCM-Monosaccharide column (Phenomenex) with RI detector. Five percent of pomegranate molasses was then filtered through 0.2µm Milipore membrane filters into HPLC vials. The mobile phase used 100% Nanopure water at a flow rate of 0.6 ml per minute, 20 µl of injection volume and ambient temperature, and the carbohydrate was identified relative to the standard carbohydrates (Association of Official Analytical Chemists (AOAC), 1984).

### 2.8 Culture Medium and PHB Production

Liquid cultures were obtained by inoculating loopful bacteria to 250 ml Erlenmeyer flasks containing 50 ml of nutrient broth media composed of (g/L): peptone 5, yeast extract 1.5, sodium chloride 5 [11]. The pomegranate molasses used as a carbon source was added to the media with 10 ml of 10% (w/v). The pH

was adjusted to 7.0 with a solution of 1 M NaOH. The bacterium was incubated at 37 °C for 48 hours with continuous shaken 150rpm. The optical density of bacteria (OD) was measured using a spectrophotometer at 600 nm 6 hours interval.

## 2.9 PHB Extraction

A modified Selvakumar method (2011) was used to extract PHB from bacteria. The sample was collected at stationary phase and centrifuged at 10000 rpm for 15 minutes. The supernatant was discarded and the pellet was suspended in distilled water with EDTA pH 5 at 5°C for 24 hours. This condition ensures the cell lysis. The lysed suspensions were centrifuged at 10,000 rpm for 15 minutes. The pellet was then washed repeatedly with distilled water to remove the excess carbon source. The final pellet was dried in the oven at 80°C until it reached a constant weight. The white dusts resulting from this treatment were dissolved in chloroform for 24 hours. After the chloroform was evaporated at room temperature [20], a white film of PHB was obtained which was sent for characterization to determine its functional groups by FTIR and NMR.

## 2.10 Total Cell Dry Weight

Total cell dry weight (CDW) measurement was carried out after centrifugation of the fermentation broth at 10,000 rpm for 15 minutes and washing 5-6 times. The bacteria pellet was dried in the oven at 80 °C until a constant weight was achieved [21].

## 2.11 Characterization of the Produced PHB

The PHB polymer was analyzed using Fourier Transform Infra Red Spectroscopy. The FTIR was recorded on a Thermo Scientific Nicolet I S 5 spectrometer to identify the functional groups structure, and <sup>1</sup>H and <sup>13</sup>C Nuclear Magnetic Resonance (NMR) spectra was recorded on a Bruker AV-400 NMR spectrometer at 400 MHz to identify the polymer chemical structure.

# 3.0 RESULTS AND DISCUSSION

## 3.1 Isolation and Identification of PHB-Producing Bacterium

The bacterial strain was successfully isolated from waste cooking oil by serially diluted sample from 10<sup>-2</sup> and 10<sup>-3</sup> concentration. All the colonies on the agar plates were similar morphologically, with white creamy color, round shaped and raised elevation. Therefore, only a colony was selected to obtain pure bacterium by sub culturing more than three times. The gram stain has been carried out to identify the group of bacteria which belonged to gram positive category. The isolate was a spore forming bacterium which was observed after 7 days, the

observation was done by using a light microscope at 100X magnification. The biochemical tests were used to characterize the bacterium based on Bergey's Manual of Systematic Bacteriology [22]. The results were summarized in Table 1. The oil was basically used in the restaurants for chickens and chips frying. The number of cycles used for frying the oil was not known, however, study from Verlinden *et al.* (2011), showed that few spores were present in waste cooking oil that had gone through prolonged frying. In this work only a single colony was observed. This work supports the finding by Verlinden *et al.* (2011). Moreover, Verlinden *et al.* (2011) screened 3 oils (pure vegetable oils, heated vegetable oil, waste cooking oil) as carbon sources for PHB production, the result showed that waste cooking oil was a good carbon source for PHB production [13,23]. The mechanism of utilizing the waste cooking oil by bacteria can be described in terms of lipolytic activity (triglyceride degradation). Since *Bacillus thuringiensis* was reported to possess a lipolytic activity, its presence in waste cooking oil unveiled that the oil can support its carbon energy requirement [24]. Haba *et al.* (2010) stated that waste cooking was a high energy source for microbial growth [25].

**Table 1** Showed the result of biochemical tests that confirmed the isolated strain to be from the genus *Bacillus*. The activity test revealed that the bacterium was *Bacillus thuringiensis* according to Bergey's Manual of systematic Bacteriology [22]

Test	Results
Spore	+
Gram stain	+, purple-blue color, rod-chain
Motility test	+
Catalase test	+
Citrate test	+
MacConky agar	-
Urease test	-
Nitrate	+
T.S.I	Fermentation of glucose
Of glucose	Aerobic
Methyl red	+
Casein hydrolysis	+
Gelatin	+



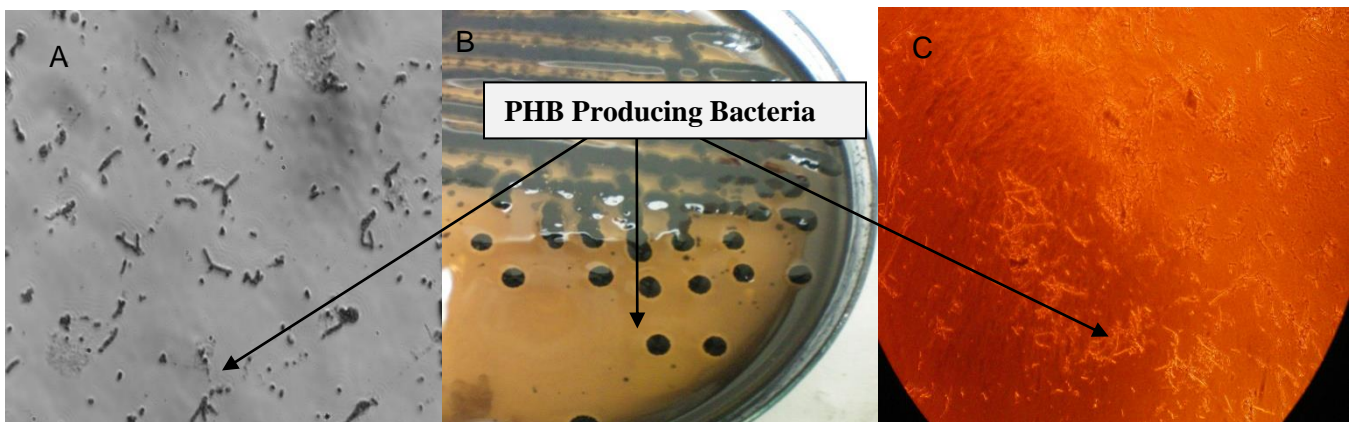
### 3.2 Screening of PHB Producing Bacteria

The isolated bacterium was screened for PHB production using Sudan Black B and Nile Blue A to detect PHB accumulating bacteria on agar plate staining methods. First, Sudan black B stain was used and by using heat fixation on a slide, which revealed intracellular PHB granules accumulated in bacteria. The visual and microscopic observations of the plate and the slide respectively, showed the bacteria to have a black colour, which was an indicator of positive PHB producing bacteria [16, 17]. The Figure 1(A, B) showed screening images of the PHB producing bacteria. Nile Blue A as a sensitive dye for detecting PHB granules was also used [26, 27]. The orange fluorescence of the PHB granules was investigated under UV-light as seen in Figure 1(C). Similar image was also observed by (Ostle and Holt, 1982) [18].

The accumulation of the black granules as well as the orange fluorescence which was a major indicator, prove the PHB producing activity of the isolated bacterium

### 3.3 16S rRNA Gene Sequence Analysis

For more reliable results 16S rRNA gene sequencing was used to identify the isolated bacteria. DNA template for 16S rRNA was successfully extracted and amplified for the purification to obtain a high purity of PCR product. In order to achieve an accurate result for DNA sequencing, the purity of PCR product was ascertained by running gel electrophoresis. The nucleotide length of the bacterium was 1365 bp, which was used to identify the bacteria gene. The identification of the bacteria was determined by comparing the 16S rRNA sequence with *Bacillus* strains sequences available in the public nucleotide databases at the National Centre for Biotechnology Information (NCBI) BLAST. The similarity index of the analysis revealed 95% with *Bacillus thuringiensis*. The nucleotide sequence data reported in this paper have been deposited at GenBank (GenBank, EMBL Europe and the DNA Data Bank of Japan - <http://www.ncbi.nlm.nih.gov>) with an accession number of KJ628093.

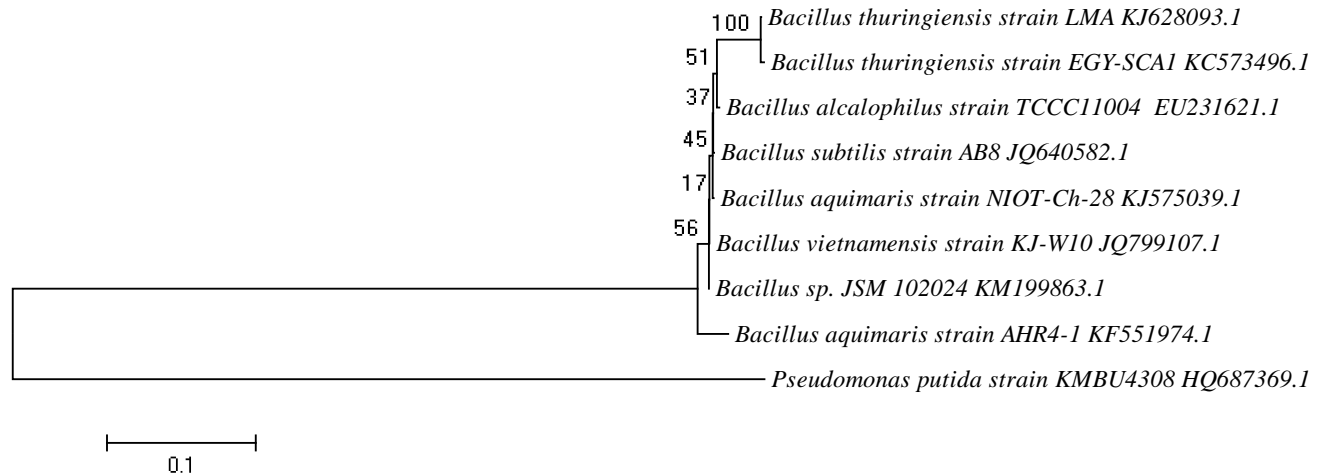


**Figure 1** Shows the results of the screening of PHB producing bacteria appearing with different stains, (A, B) illustrated that the isolated bacterium sustained dark black color with Sudan Black B on the slide and agar plate respectively. While (C) showed the PHB producing bacteria exhibited a yellow-orange color under a fluorescent microscope after stained with Nile blue A. All the images were positive PHB producing bacterium. The results were similar with previous studies [17, 16, 18] respectively

### 3.4 Phylogenetic Tree Analysis

Different species from BLAST analysis related to the bacterium *Bacillus* were chosen together with a sequence of *Bacillus thuringiensis* isolated in this work to perform alignment using MEGA5.2 software. Figure 2 shows the *Bacillus thuringiensis* colours and HSV within its range. Besides the HSV range, the result will display unknown or not detected. LMA was clustered

with value of 500 and scale bar 0.1 substitutions per site with a clad consisting of *Bacillus thuringiensis*. This suggested that isolated bacterium was closely related to the respective *Bacillus thuringiensis*. The isolated bacterium can be divided into two major groups based on the phylogenetic analysis. *Bacillus thuringiensis* formed a group with a bootstrap value of 99% similarities within each morphotype, which were relatively high.



**Figure 2** Phylogenetic analysis of partial 16S rRNA sequences of *Bacillus thuringiensis* LMA, mapped together with homology sequencing obtained from Gen Bank (NCBI)

### 3.5 PHB Production

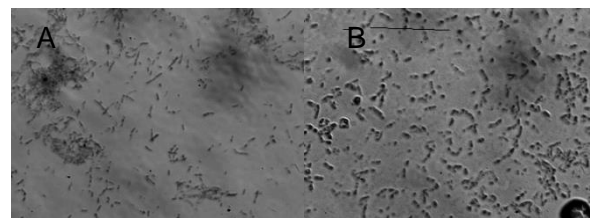
PHB biosynthesis was carried out in 250 ml conical flask with 10 ml of 10% (w/v) pomegranate molasses in modified NB media. The growth (OD) of bacteria in the media increased after 6 hours of incubation at 37 °C, which declined after 72 hours. The PHB granules of bacteria were optimum around 48 hours as showed in Figure 4 (B), due to utilization of carbon source in the metabolism. A fermentation process conducted with *Bacillus thuringiensis* LMA using pomegranate molasses as a carbon source in NB achieved a PHB production 57.45%. The result in this study using pomegranate molasses as carbon source was higher in comparison to previous study [11], where *Bacillus thuringiensis* D3 was used to achieved 29.41% PHB using glucose as a carbon source in NB media. Pal *et al.* (2009), used *Bacillus thuringiensis* IAM 12077 to produce PHB under nutrient limitation condition, the strain had ability to accumulate 24% and 43.37% PHB when nutrient broth and nitrogen-deficient medium were used respectively [28]. Another study used cane molasses with *Bacillus megaterium* to produce 46.2% of PHB when 3% of sugar cane molasses was added to the media [29]. Beet molasses were also used as a carbon source instead of glucose to produce 80 % (w/w) PHB by a recombinant *Escherichia Coli* strain (HMS174) [30]. Page (1992) mentioned that nitrogen source in PHB fermentation media played important role in PHB accumulation in bacterial cell [31]. *Bacillus thuringiensis* IAM 12077 produced 60.3% PHB with carbon and nitrogen sources in the ration 4:1 (glucose: sodium nitrate) [28].

### 3.6 Analysis of Sugar in the Pomegranate Molasses

High performance Liquid Chromatography (HPLC) has been developed recently to determine the sugar concentration of molasses. It was used to detect and measure the sugars of 10% (w/v) of pomegranate molasses samples. The composition and quantities of pomegranate sugars was showed in Table 2.

**Table 2** The contents of 10% (w/v) pomegranate molasses analyzed by HPLC

Sugar	% (w/v)
Sucrose	6.8
Glucose	7.4



**Figure 3** (A) and (B) show the results of PHB production during 24 and 48 hours, respectively. The PHB granules in the bacteria were stained with black color when Sudan Black B used as indicator stain. The maximum PHB granules appeared after 24 hours which were observed under Nikon inverted microscope

### 3.7 Extraction PHB from Bacterial Cells

The extraction of PHB from the bacteria cells was carried out after the cell lysis. The dry pellets were

suspended in chloroform which was twice the volume of the pellets. The solutions were left overnight at room temperature to enable chloroform evaporation. After 24 hours, white PHB appeared at the bottom of the cylinder. The PHB was measured after weighing the extracted PHB film, which was subjected to NMR and FTIR analysis.

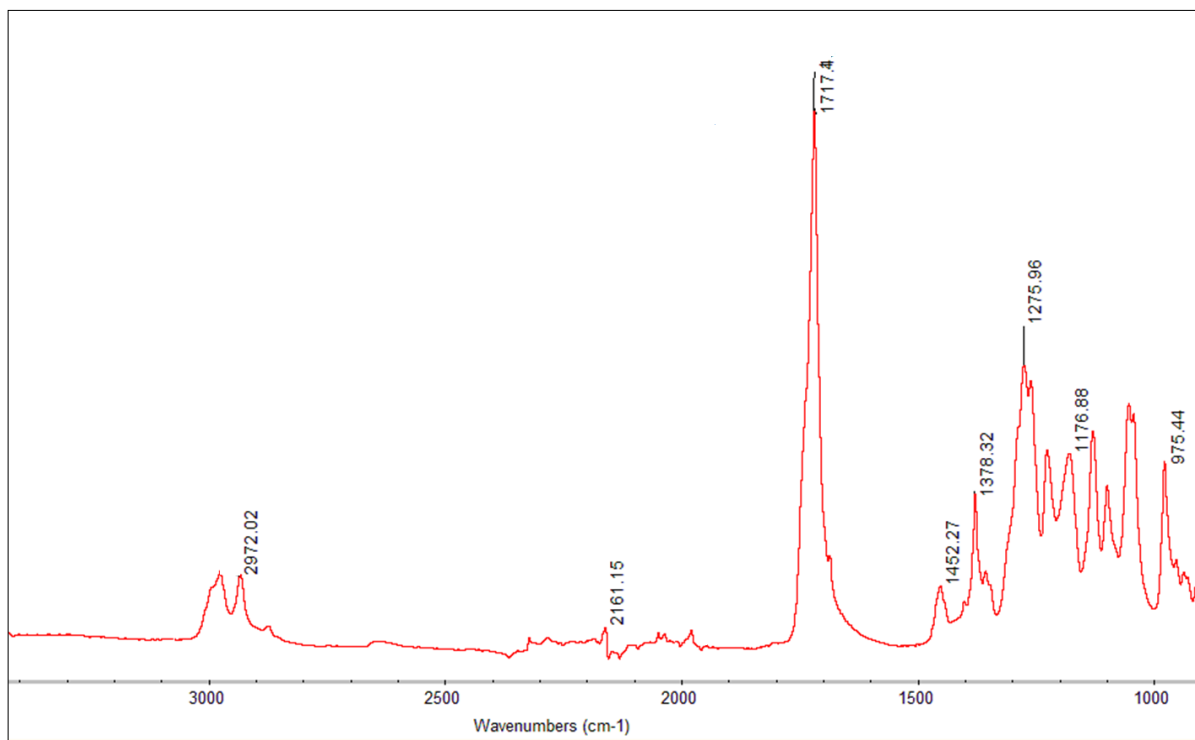
### 3.8 Analysis PHB Production

#### 3.8.1 Fourier Transform Infrared Spectroscopy (FTIR)

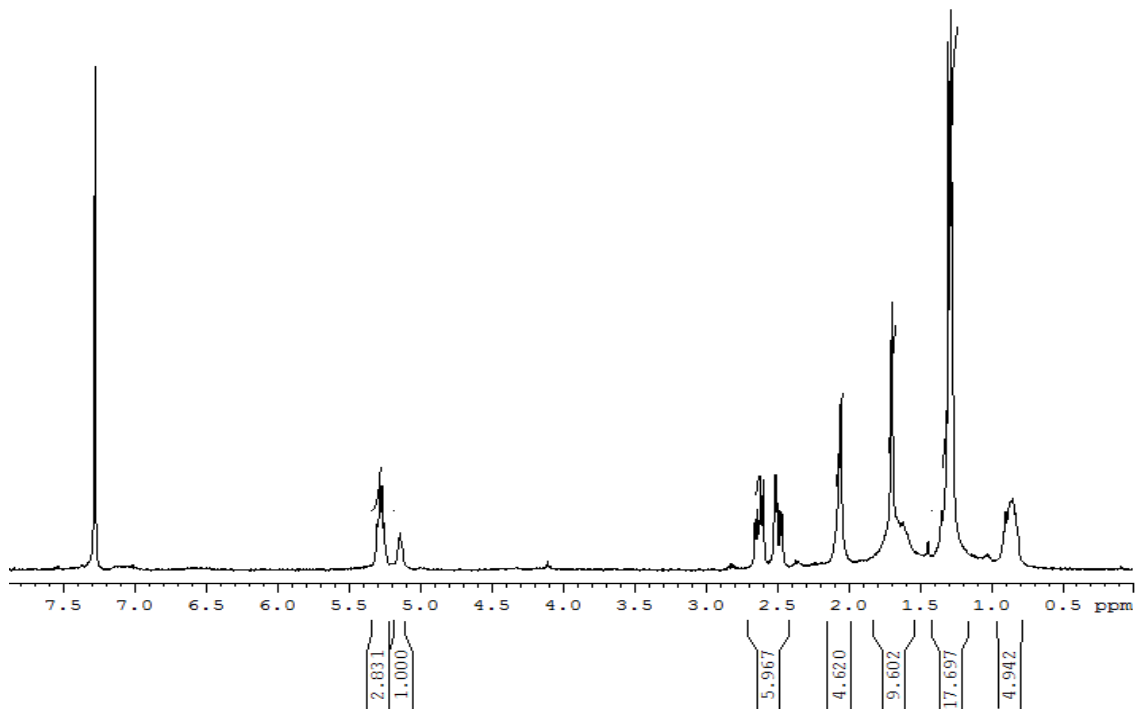
In this study, the functional groups of the polymer PHB was confirmed as C=O groups by FT-IR spectroscopy. FTIR analysis carried out at a range of 4000-400  $\text{cm}^{-1}$ . Figure 4 revealed the presence of different conformational bands from mixed culture in the extracted PHB. The figure showed the specific character of PHB molecules in the presence of carbonyl bands C=O at 1717  $\text{cm}^{-1}$  absorption band. The band at 1378  $\text{cm}^{-1}$  was assigned to methyl (-CH<sub>3</sub>) groups and the bands at 1176 and 1275  $\text{cm}^{-1}$  were characteristic of the stretching of the C-O groups, respectively, while the band at 1452  $\text{cm}^{-1}$  was assigned to the methylene group (CH<sub>2</sub>). Figure 4 shows the FTIR Spectrum of extracted PHB.

#### 3.7.2 Nuclear Magnetic Resonance (NMR)

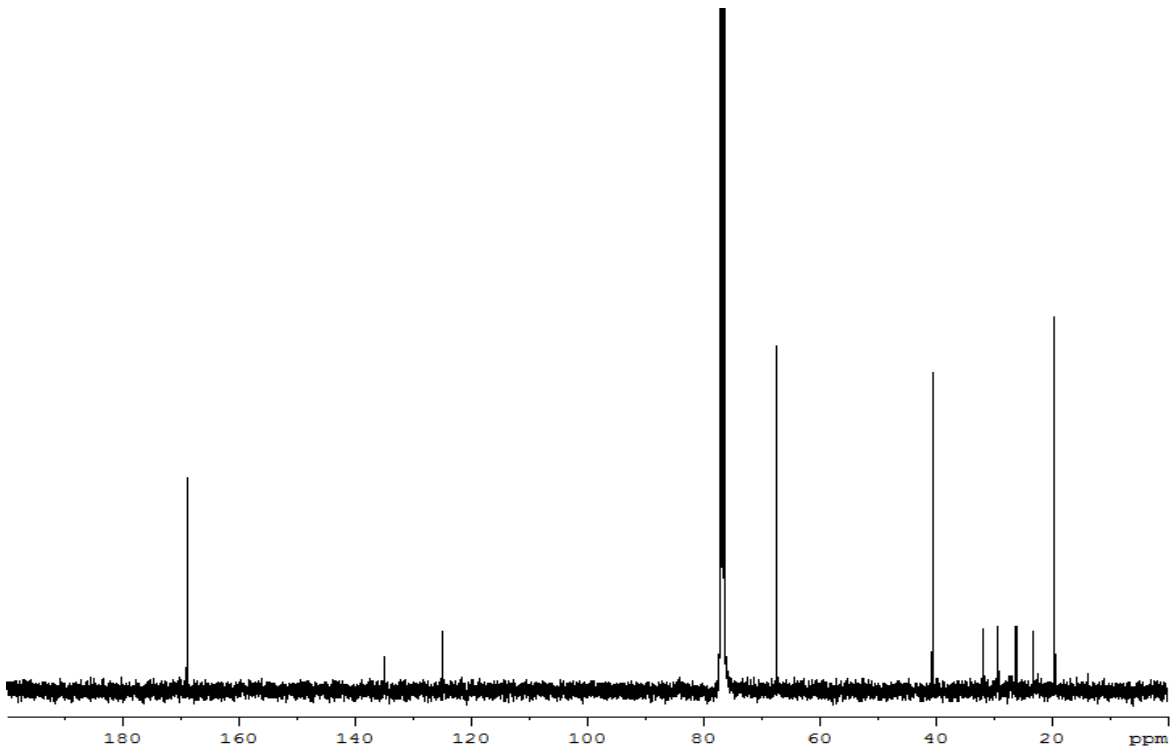
NMR analysis was used to determine the functional groups of PHB when strain LMA was grown in a high nitrogen media with pomegranate molasses as a cheap carbon source. The chemical structure of the polymer was confirmed by dissolving PHB with deuterated chloroform (CDCl<sub>3</sub>). The <sup>1</sup>H and <sup>13</sup>C spectra results were displayed in Figure 5 and 6 respectively. The <sup>1</sup>H NMR spectral analysis revealed the presence of methyl protons (-CH<sub>3</sub>) which appear to have a resonance of 1.27 ppm, and that methylene protons (-CH<sub>2</sub>) which appeared singly at 2.00 and 2.520 ppm were adjacent to a double bond, methine proton (-CH) of bacterial polyhydroxybutyrate, which also has a multiple resonance at 5.260 ppm and a terminal -CH<sub>3</sub> at 0.90 ppm. The <sup>13</sup>C NMR spectra showed chemical shifts at 19.74, 40.84, 67.64 and 169.07 ppm, which showed the presence of CH<sub>3</sub>, CH<sub>2</sub>, CH and C=O groups, respectively. The chemical structure of PHB was consistent with other description for this type of polymer; the peaks were assigned similarly [32].



**Figure 4** FTIR Spectrum of Extracted PHB, The figure shows the absorption at 1717  $\text{cm}^{-1}$  for carbonyl group (C=O), at about 1378  $\text{cm}^{-1}$  for methyl (-CH<sub>3</sub>) groups. Absorption at 1176 and 1275  $\text{cm}^{-1}$  were characteristic of the stretching of the C-O groups, respectively. And at 1452  $\text{cm}^{-1}$  for methylene group (CH<sub>2</sub>). This result was compared with a previous study that stated absorption C=O group at 1720  $\text{cm}^{-1}$  [4]



**Figure 5**  $^1\text{H}$  NMR of PHB produced by *Bacillus thuringiensis* LMA grown on 10% pomegranate molasses as a carbon source. The regions at 1.70 and 7.28 indicate the impurities from a carbon source and chloroform respectively



**Figure 6** shows the results of  $^{13}\text{C}$  NMR spectra of PHB produced with pomegranate molasses a carbon source. The expanded region indicates chloroform that used for dissolving the PHB extracted



## 4.0 CONCLUSION

A type of bacterium was successfully isolated from waste cooking oil. The isolated bacterium was identified and characterized for its morphology and molecular properties. In addition, the strain was confirmed as a positive PHB producer. Based on the 16S rRNA gene analysis, the strain is linked to *Bacillus thuringiensis* LMA. This isolated strain produces PHB when pomegranate molasses was used as a carbon source in an excess nitrogen supplementation. The results indicated that the possibility of strain LMA to produce PHB without the need of nutritional limitation.

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