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Study of the Fermentation Conditions of the *Bacillus Cereus* Strain ARY73 to Produce Polyhydroxyalkanoate (PHA) from Glucose

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ABSTRACT

Polyhydroxyalkanoates (PHAs) have gained much attention as biodegradable polymers, many efforts are being made to minimize the cost of PHAs by finding cheap carbon source depending on the type of microorganism and fermentation conditions. The aims of this study were to evaluate the effects of different glucose concentrations and other important conditions on the PHA production by *Bacillus cereus* isolated from soil. Polyhydroxyalkanoates PHAs accumulated by soil microorganisms were examined by screening the isolated bacteria using Sudan B Black and Nile Blue staining process. A Gram positive strain was identified using the 16s rRNA gene, deposited in the NCBI GenBank sequence database. Different growth conditions (favorite glucose concentrations 1-8 % (w/v), temperatures and pH) were tested and the growth parameters (sugar consumption, cell counting and Cell Dry Weight CDW) were studied. The extracted polymers were analyzed and characterized using an FTIR spectrophotometer followed by a GC-MS analysis. The pure bacterial strain isolated from soil was deposited in the NCBI GenBank database *B. cereus* strain ARY73, which showed significant black colored granules (or dark blue) using Sudan B Black stain, it also showed positive to Nile blue A as a high indicator stain for PHA accumulation. *B. cereus* ARY73 showed high production of PHA using (w/v): 2% glucose and 1% nitrogen source at 35 °C and pH7 yields 79% per Cell Dry Weight and 96 h of incubation. The extracted polymers were analyzed and characterized using an FTIR spectrophotometer confirming the PHA structure. The FTIR spectrophotometer, followed by a GC-MS analysis indicated the Scl-co-mcl PHA structure. This research demonstrates that the isolated strain *B. cereus* ARY73 was a good candidate for PHA production with a better quality for use in biomedical and other applications. The use of biopolymer in soil, enhanced the accumulation of the microorganisms (such as bacteria) capable of degrading biopolymer or biodegradation by-products yields by other species which were isolated in this study and demonstrated their efficiency in producing biopolymers.

Keywords: Polyhydroxyalkanoates, PHA, *Bacillus cereus*, biopolymer, glucose.

INTRODUCTION

Polyhydroxyalkanoates (PHAs) as a biodegradable material are considered as alternative biopolymers for petroleum-derived plastics [Kourmentza et al., 2017]. Synthetic plastics which have been extensively used since many decades, known as non-degradable plastics, are potent environmental toxic pollutants and have accumulated in the environment. The most abundant member of the PHAs family, known as poly-3-hydroxybutyrate (P3HB), a homopolymer of 3-hydroxybutyrarte, similar in properties to polypropylene. Poly-3-hydroxyvalerate (PHV) is another member of the PHAs family with more flexible properties [Napathorn, 2014]. PHA are thermoplastic polyesters produced by bacteria intracellularly as carbon and energy storage and capsulated as granules in cytoplasm [Radivojevic et al., 2016]. PHA was found to accumulate in large number of bacteria e.g. *Ralstonia eutrophes, Pseudomonas putida*, *P. fluorescens*, *Bacillus spp.* and *Escherichia Coli* [Poltronieri and Kumar, 2017; Pagliano et al. 2017].

Meanwhile, PHAs have been attracting global attention as a biomaterial polymer, renewable, and biodegradable alternatives to a synthesized petrochemical plastics [Rhee and Park, 2021].

Many Prokaryotic organisms produce polyhydroxyalkanoates (PHA), including gram negative and gram positive bacteria, when carbon source (e.g. glucose, lactose, galactose) is available in excess as reserve material with a limitation of nutrients essential for growth such as nitrogen, phosphorous, magnesium or sulfur [Kamravamanesh and Herwig, 2018]. Under aerobic conditions these microorganisms decompose PHAs resulting in final products, i.e. carbon dioxide and water. Under anaerobic conditions, they result in carbon dioxide and methane [Shrivastav et al., 2011]. Under carbon limitation conditions, microorganisms use PHA depolymerase and other enzymes to hydrolyze accumulated PHA intracellularly for the growth and survival [Mitra et al., 2020].

PHA synthase is the key enzyme responsible for polymerization of PHA, and encoded by the phaC genes. PHA synthases are categorized into four main classes: class I, III and class IV prefer synthesizing scl-PHA, while Class II PhaC enzymes prefers synthesizing mcl-PHA (medium-chain-length). Some exceptions were observed; in literature it was reported that several class I PhaCs may produce scl-mcl-PHA. PhaCAc is considered as one of the most successful enzymes used for scl-mcl-PHA production, while Class IV PHA synthases from *Bacillus cereus* type (IVc) [Chee et al., 2012; Mezzolla and Poltronieri, 2018].

Recombinant microorganisms could allow high cell density and faster growth with high levels of PHA production The most standard organism used in genetic engineering is *E. coli* which has been shown to be advantageous for PHAs production [Mezzolla and Poltronieri, 2017]. Most PHA producing bacteria can use simple sugars. Generally, substrates can be divided into three categories which are simple sugars (monosaccharides), triacylglycerol and hydrocarbons. The cost of the carbon source estimated at approximately 50% for the entire process of PHAs production [Khosravi-Darani et al., 2013].

The PHAs products are not toxic to cells and soft tissues with some diverse properties to contribute in medical applications [Koller, 2018]. High cost of PHA production is considered as the main challenge for scientists. Many efforts of minimizing the cost of PHA have been made by finding a cheap carbon source, such as dairy waste, lubricating and edible oil waste and agriculture waste [Surendran et al., 2020]. From previous literature it is known that only few microorganisms are able to produce PHA from lactose. The purpose of this study was to evaluate the effects of different amounts of the carbon source (glucose) at different temperatures on the growth and PHAs yield by the isolated bacteria.

MATERIALS AND METHODS

Isolation of PHA producing bacteria

In order to isolate the PHA producing bacteria, bioplastic sheets made of PHA granules manufactured by BASF (ecovio, Germany), were buried in a garden soil located in the city of Baghdad/Iraq at the depth of (5-10 cm) and allowed to be degraded. Two months later, the samples of the degraded polymers mixed with soil were collected and transferred immediately by a sterilized plastic bags to a laboratory (department of Biology, college of science, University of Baghdad). A serial dilution technique was performed, aseptically a solution of 1 ml was poured on the nutrient agar medium (Oxoid, UK) contains 5% (w/v) of glucose and incubated (Precision, USA) at 37 °C for 3 days.

The bacterial colonies were subjected to a series of steps to obtain purified PHA producing strains by sub-cultured on polyhydroxyalkanoates medium (PM) agar by adding: 0.6 g of $K_2HPO_4.3H_2O$; 0.2 g of $MgSO_4.7H_2O$; 0.2 g of KH_2PO_4; 0.2 g of $(NH_4)2SO_4$; 20 g of agar-agar; 900 ml distilled water and 2.0 ml/L of the trace element solution (g l^{-1}): 0.10 $ZnSO_4.7\ H_2O$; 0.3 H_3BO_3; 0.03 $MnCl_2.4H_2O$; 0.2 $CoCl_2.6\ H_2O$; 0.01 $CuCl_2 \cdot 2H_2O$; 0.03 $Na_2MoO_4.2H_20$; 0.02 $NiCl_2.6H_2O$. pH adjusted to 7.2. The medium was autoclaved (LabTech, Indonesia) at 121 °C, 15 psi for 15 min, 5 g of glucose (as the only source of carbon) was suspended in 100 ml distilled water and separately autoclaved at 121 °C for 5 minutes (in order to prevent glucose from caramelization) [Javaid et al., 2020]. All cultures were incubated at 37 °C for 3 days.

Screening of PHA producing bacteria

Streaked isolates on the PM medium agar (glucose) plates were examined for PHA production by using Sudan B Black staining (Merck, Germany) as described by Kumar et al. [2018], smears were examined using light microscope (Olympus, Japan) under (100×) oil immersion objective. Another specific stain method was conducted using Nile Blue A stain (Merck, Germany) which was

performed according to Legat et al. [2010], all slides were examined at 460 nm using fluorescence microscope (ZEISS AXIOLAB, GERMANY) [Yasin and Al-Mayaly, 2020].

Sudan B Black staining solution was prepared by adding the dye in 70% ethanol mixed well and filtered. The staining procedure was as follows: 10 ml of sample was centrifuged, using s loop cell pellet was taken and placed on dry slide, dispersed in the clockwise and anticlockwise direction, and allowed to air dry, then it was fixed by heating. The fixed smear was flooded with Sudan B Black staining solution and allowed to react for 15 minutes. After draining off the excess stain and drying, it was rinsed with xylene and dried again. Then, it was counterstained by safranin for 10 seconds, immediately rinsed with water and left to dry. The slide was tested under oil immersion objective. The Nile Blue A staining procedure included: after fixed the smears (as mentioned above), the slides were stained with 1 % of Nile Blue A aqueous solution and kept at 55 °C for 10 minutes, washed with tap water. The smears were stained with 8% aqueous acetic acid for 1 minute, washed with water, dried, remoistened with water and covered with cover slip. The slides were examined at 460 nm using fluorescence microscope.

Characterization of the bacterial isolates

The morphological and physiological properties of the isolated bacteria (L1-a) were investigated according to Bergey's Manual of Determinative Bacteriology. The identity of the isolate was determined by sequence analysis of the 16s rRNA gene. Genomic DNA was isolated from bacterial growth according to the protocol of Wizard Genomic DNA Purification Kit, Promega. PCR product were processed for Sanger sequencing using an ABI3730XL automated DNA sequencer, by Macrogen Corporation – Korea. The results were analyzed using Geneious software version 11.1. [Kearse et al., 2012].

The amplification of 16s RNA gene of unknown bacterial species were fractionated on 1.5% agarose gel electrophoresis stained with Ethidium Bromide M: 100bp ladder marker. Lanes ARY73 resembles 1500bp PCR products.

27F Primer 5`-AGAGTTTGATCCTGGCTCAG-3`

1429R Primer 5`-TACGGTTACCTTGTTACGACTT-3`

The obtained sequence from strain was deposited in the NCBI GenBank (National Center for Biotechnology Information) sequence database under accession number ARY73 MW362363 and compared with those stored in the GenBank database by using the BLAST (Basic Local Alignment Search Tool) program [Johnson et al., 2008]. On the basis of THE Blast analysis, the phylogenetic tree was prepared and constructed using phylogeny [Vogel et al., 2021].

Effect of growth conditions on PHA production by selected Isolate

The effect of carbon source concentration (w/v): low conc.:1%, 1.5%, 2%, 2.5% and 3 %; high conc.: 4%, 6% and 8%, initial pH (5, 7 and 9), temperature (25, 30, 35 and 40 °C), and nitrogen concentrations (0.5, 1.0, 1.25 and 1.5 g/L) on the PHA production by the selected isolate was evaluated using a PM mineral medium according to Franz et al. [2012], (500 ml Erlenmeyer flask containing glucose), the addition of inoculum was 1% (v/v) after grown overnight at 37 °C, 150 rpm. All samples were incubated for 5 days in a shaking incubator (LabTech, Indonesia) 150 rpm, the results were then compared by measuring the sugar consumption (DNS), Cell counting, Cell Dry Weight (CDW) and the weight of extracted PHA, PHA% quantified according to Eq. 1.

$$\%\,PHA = \frac{PHA\ weight}{Biomass\ dried\ weight} \times 100 \quad (1)$$

Monitoring of bacterial growth conditions and PHA yield

Cell counting

Cell counting was performed by using the Hemocytometer method. Cell counting was measured every 24 h. 0.5 µl of cell suspension were stained by Trypan Blue (Sigma Aldrich, Germany) and tested on Hemocytometer, viable cells were counted under microscope.

Cell Dry weight

Aseptically, 10 ml of sample were taken in a glass centrifuge tube. The culture was centrifuged at 10,000 rpm at -4 °C for 15 min, and the pellet was then dried in an oven at 55 °C for 24 h and weighed [Getachew & Woldesenbet, 2016].

DNS Method (sugar consumption)

The determination of sugar consumption was estimated by Di-Nitrosalicylic acid (DNS method) throughout each fermentation period according to the method described by Miller [Blandón et al., 2020]. This method gives a rapid estimation by measuring the total amount of reducing sugars in the hydrolysate.

Briefly, the calibration curve was done using glucose as standard (0 to 2 g/L). 500 µl of supernatant, obtained by centrifugation, was added to 500 µl of the DNS reagent (Sigma Aldrich, Germany) [DNS 1% (w/v), phenol 0.2% (w/v), Rochelle salt 40% (w/v), and potassium disulfide 0.5% (w/v) mixed with sodium hydroxide at 1.5% (w/v)]. These solutions were heated for 5 min in boiling water. Absorbance was measured at 540 nm.

PHA Extraction

After being weighed, the dried pellet was washed once with ethanol and distilled water. PHAs were extracted by adding a mixture of 7 mL of hot chloroform (POCH, Poland) and 3 mL aqueous NaOCl solution (4%) (Peros, Turkey), mixed vigorously by vortex (Labco, Germany). The mixture was centrifuged to obtain three separated layers. The lowermost layer was the PHA dissolved in chloroform. Nine-fold amount of ice-cold methanol was added for PHA precipitation and then allowed to evaporate at the room temperature [Ojha and Das, 2020].

Analytical procedures

FTIR analysis for PHA detection and characterization

FTIR spectroscopy is considered one of the most powerful techniques to be used to follow the kinetics of PHA production. Major functional groups and moieties in the PHB polymer were identified using a KBr disc on FTIR 8300 (Shimadzu spectrophotometer, Japan). Standard PHB and sample of the extracted PHA was used for comparison, 5 mg of each were mixed with 100 mg of FTIR grade KBr and pelletized. The FTIR spectra were recorded in the range of 4000–400 cm^{-1}.

GC-Mass

PHA was qualitatively characterize using gas chromatographic analysis to monomers after methanolysis of the polymer by adding 10 mg of dry cell samples to (2 mL) of chloroform and (2 mL) of 15% sulfuric acid/methanol in a screw-capped tube, heated to 100 °C for 120 min. After cooling, 1mL of demineralized water was added, vortexed and centrifuged for 30 min at 13,000 rpm, the organic phase containing methylester derivatives was analyzed by Agilent Technologies 7820A GC-MS System (Agilent Technologies, CA, USA) equipped with MS. The analysis was performed using a HP-5ms Ultra Inert 30 m x 250 µm x 0.25 µm column. Helium carrier gas was set at a flow rate of 1.2 mL/min. The oven temperature was programmed at 35 °C as an initial temperature, and subsequently raised at rate of 15 °C /min to 280 °C and held for 2 min, the total-ion scan mode at a mass-to-charge ratio (m/z) = 40–600 [Devi et al., 2015; Tan et al., 2014b].

RESULTS AND DISCUSSIONS

Isolation and screening of the PHA producing bacteria

A gram positive isolate (L1/a) were selected to study its ability to produce PHA. This isolate showed high growth on the PM agar medium supplemented with glucose as the only source of carbon. (Fig. 1a and Fig. 1b). The PHA detection was screened using Sudan B black staining. The L1/a bacterium showed granules filled up with black or dark blue stain. Under microscope light, the PHA granules were observed as dark spot and had a potential for PHA production (Fig. 1c and Fig. 1d). Nile Blue A staining which is another specific dye, were performed for the detection of PHA granules within the cells, a fluorescent orange granules were observed under fluorescence microscope, the intensity increased with the increase of the PHA content, indicating the presence of PHAs in the cells (Fig. 1e and Fig. 1f). Tan et al. [2014a] demonstrated that, Nile blue staining provides an evidence for the production of PHAs which enabled microbe producers to be isolated and identified. Ratnaningrum et al. [2019] stated that all the studied colonies confirmed to be able to present the cells that fluoresce under a fluorescent microscope.

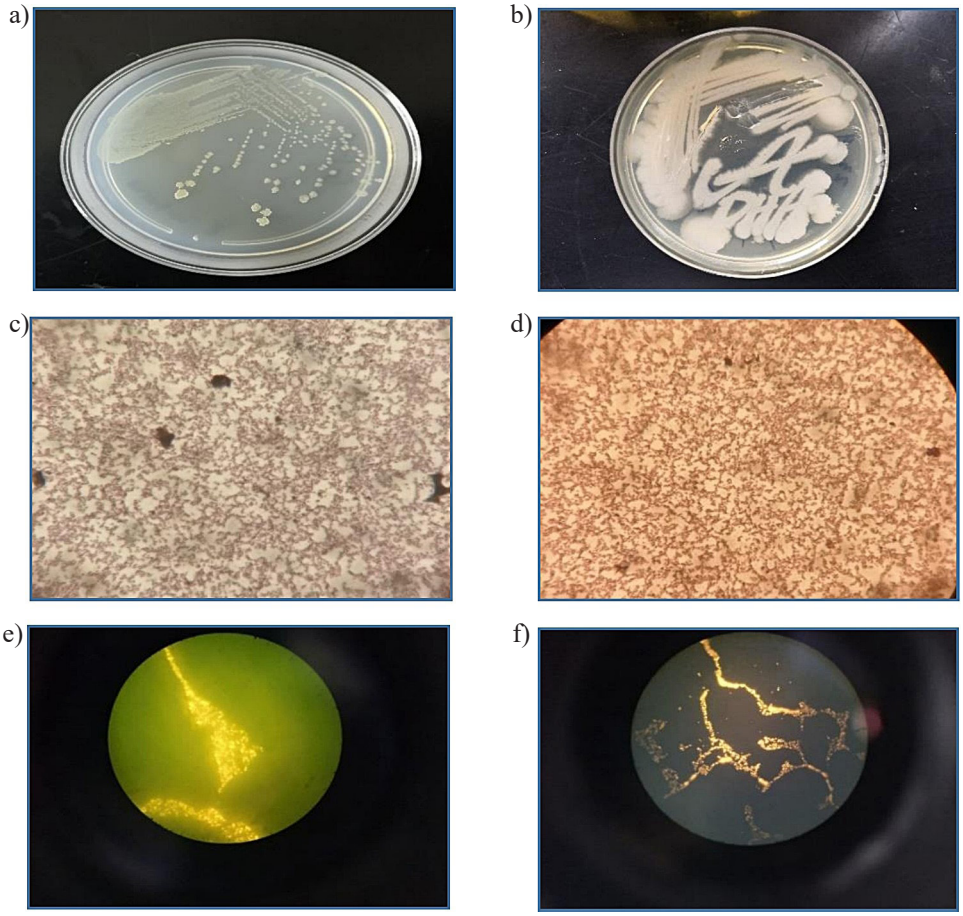

Figure 1. Staining methods for PHAs detection: a, b) Isolated bacteria (L1/a) subcultured on the PM agar supplemented with 5% (w/v) glucose at 37 °C, pH 7.2 for 3 days. C, d) Sudan Black B staining observed on PHAs granules. c, d) Fluorescence microscopy of Nile Blue A, PHAs accumulation

Characterization and identification of the bacterial isolates

The bacterial isolate was initially characterized using various microbiological and biochemical tests and identified to be gram positive *Bacillus* spp. The data analysis performed using BLAST program to compare the 16s rRNA sequence of the isolate with the database sequences indicated that the (L1/a) bacterium gives 100% identical to that of the model strain *Bacillus cereus*, deposited in the NCBI GenBank database ARY73 Code MW362363. (Fig. 2a).

The phylogenetic tree was prepared based on the 16S ribosomal RNA sequences of the bacterial strain. Evolutionary analyses were conducted in MEGA-X application (Fig. 2b).

Monitoring of bacterial growth and PHA yield at different carbon concentrations

In order to study the effects of the glucose concentration over the growth of *B. cereus* strain ARY73 and PHA production, low concentrations were performed of (w/v) 1.0%, 1.5%, 2.0%, 2.5%, and 3.0%, as well as higher concentrations of (w/v) 4%, 6% and 8%. Growth was monitored every 24 h by measuring optical density (600nm) and cell counting using the hemocytometer method, while the PHA yield was monitored after 48 and 96 h of incubation, respectively. (Fig. 3)

Table 1 shows a high absorbance for the low concentrations of glucose medium, 2.0% (w/v) glucose showed highest growth OD 2.275 and cell count was 2.45×10^9 cells/ml at 48h of incubation time (stationary phase), PHA yield was 1.78 g/L (49% per gram CDW). The growth continued to increase slowly after 96h, OD was 2.586, while cell count was 2.91×10^9 cells/ml and maximum yield of PHA 1.98 g/L (74% per gram CDW), (Fig. 3c). *B. cereus* ARY73 gave stable growth after 48 h, with an increase in PHA production in the glucose containing medium as the only source of carbon. A similar observation was also reported by Thammasittirong et al. [2017]. They showed that PHB accumulation was observed after 72 h as it served an alternative as energy source.

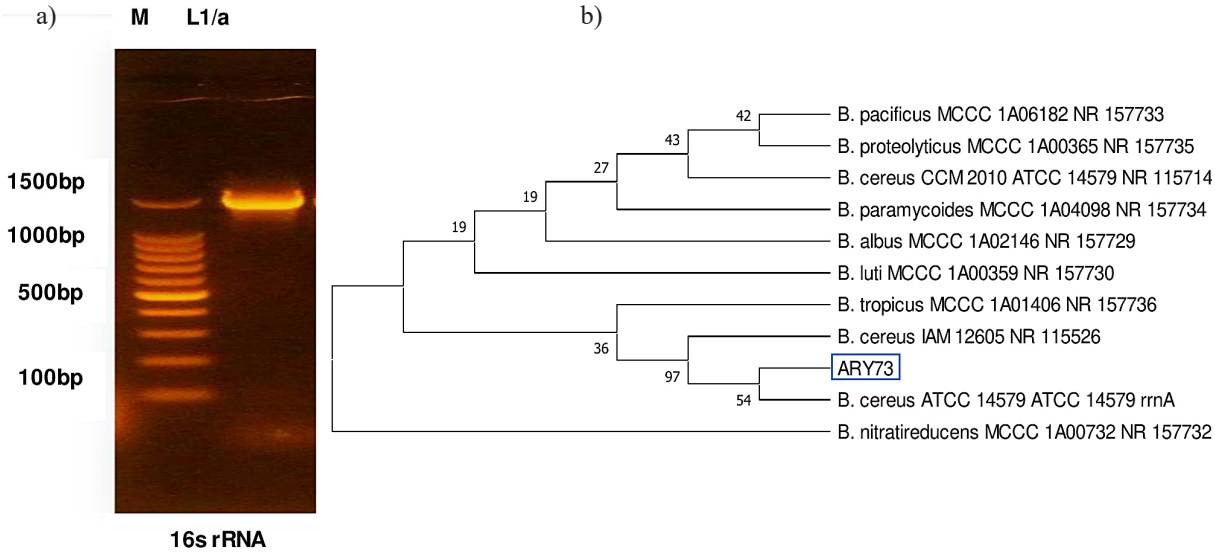

Figure 2. PCR Result. a) 16s rRNA gene with universal primers in L1/a (*B. cereus* ARY73). b) Phylogenetic tree of *B. cereus* strain ARY73. The bootstrap values are shown at branch nodes

For higher glucose concentrations, 4 % (w/v), 6 % (w/v) and 8 % (w/v), *B. cereus* ARY73 showed higher growth at 4% and maximum PHA production of about 0.64 g/L (40% gm of CDW) in the glucose medium 4% (w/v) with its ability to produce lesser quantity of PHA at 6% and 8% (w/v) concentrations. This may give an explanation that the highest concentrations of sugar can be inhibitory to the culture, decreasing its metabolic rates and affect the ability of isolate to produce PHA [Al-Kaddo et al., 2020]. Blandon et al. [2020] observed that the high values of glucose concentration give a low productivity and growth for the *Bacillus* sp.

Glucose consumption (DNS) by *Bacillus* sp. ARY73 increased rapidly after 24h of incubation to record a significant decrease at 48 h of 1.16%; in this cultivation time PHA polymerase activates as the growth and CDW increased (Fig. 3d). The glucose consumption decreased continuously in a constant rate recording lowest value of 0.95% after 96 h with high production of PHA (1.98 g/L). The PHA production decreased as nutrients depletion in medium resulting in the exhaustion of PHA using PHA depolymerase.

Effect of temperature and initial pH on the PHA production

The PHA production extracted from dry biomass and glucose consumption (Fig. 3a and Fig. 3b) was measured after 48 and 96 h of incubation at an optimized glucose concentration (2%). *B. cereus* ARY73 showed a clear PHA production in the range of 30 to 35 °C, the highest PHA content was obtained at 35 °C of 2.61 g/L (PHA 76%) after 96 h of

Table 1. Growth kinetics of *B. cereus* ARY73 isolate at low and high glucose concentrations during 24-96 h of incubation and CDW at 48 and 96 h. Illustrate the maximum yield of PHA production at 2.0% of glucose concentration. (Control OD = 0.1)

w(Glucose)/ $(\%)_{(w/v)}$	*t*/24 h	*t*/48 h				*t*/72 h	*t*/96 h			
	OD (600nm)	OD (600nm)	(CDW)/ (g/L)	(PHA)/ (g/L)	(PHA)/ (%)	OD (600nm)	OD (600nm)	(CDW)/ (g/L)	(PHA)/ (g/L)	(PHA)/ $(\%)_{(w/v)}$
1.0	1.868	2.636	3.84	-	-	1.556	1.476	1.16	-	-
1.5	1.678	2.157	3.05	1.18	38	2.308	2.143	1.83	0.98	53
2.0	1.849	2.275	3.61	1.78	49	2.644	2.586	2.67	1.98	74
2.5	1.766	1.772	1.65	0.61	37	2.036	2.243	1.90	1.08	57
3.0	1.532	1.482	1.27	0.39	31	1.566	1.572	1.22	0.42	34
4.0	1.187	2.445	1.61	0.64	40	2.508	2.215	1.33	-	-
6.0	1.222	2.343	1.28	0.41	32	2.343	1.954	1.21	-	-
8.0	1.410	2.403	1.0	-	-	2.271	1.843	0.76	-	-

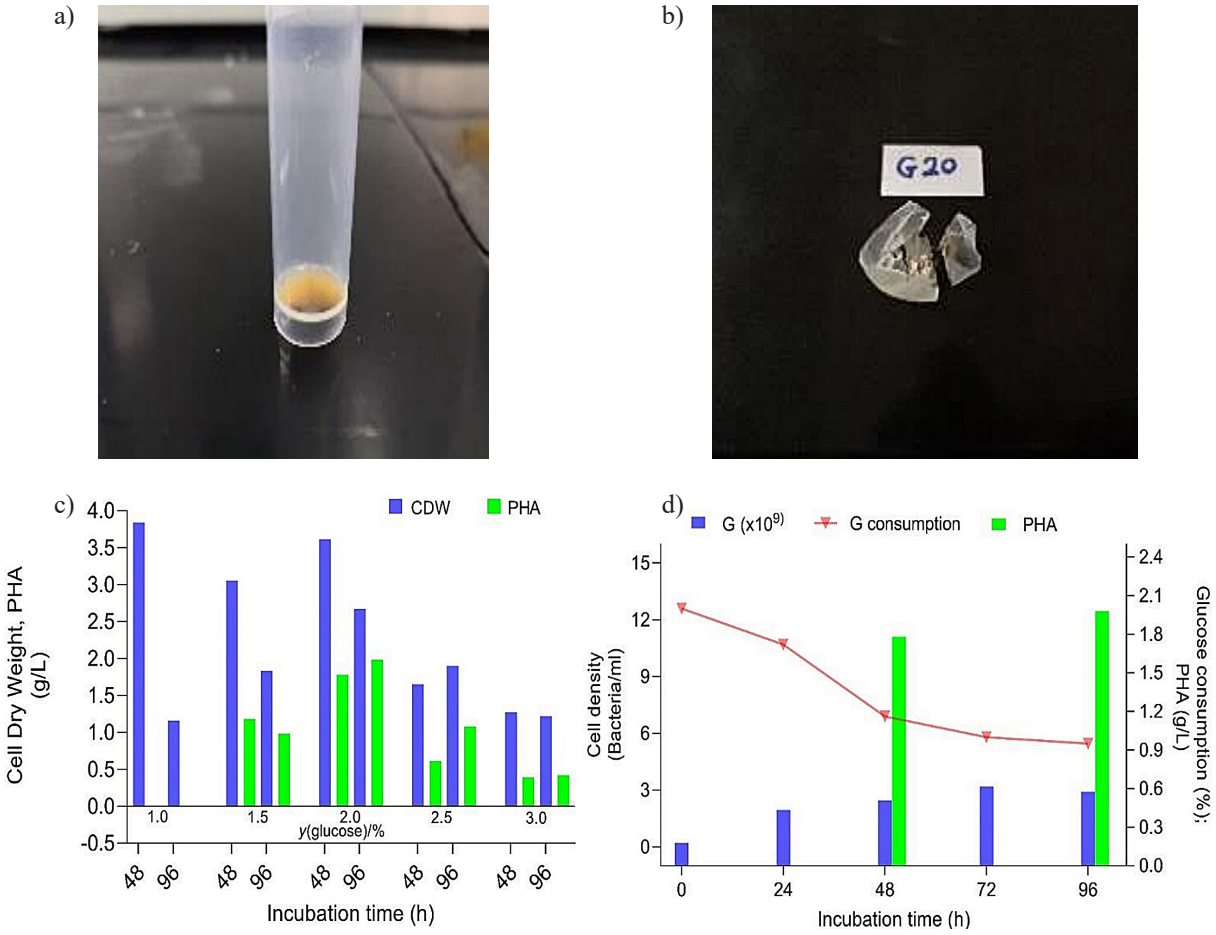

Figure 3. Effects of different glucose concentrations on the growth and PHA production by *B. cereus* ARY73: a) Cell biomass, b) Extracted PHA, c) Optimized glucose concentration, PHA yield for 48 and 96 h, d) Sugar consumption (DNS) with highest PHA yield using 2% (w/v) glucose

incubation, DNS of glucose was 1.36% (Fig. 4a). No PHA production occurred at 40 °C, while the strain was able to grow with a Cell Dry Weight of 3.41 and 2.51 g/L at 48 and 96 h, respectively, this indicates that the higher the Cell Dry Weight does not necessarily imply an increase in PHA production. Javaid et al. [2020] demonstrated that the *Stenotrophomonas maltophilia* strain IAM 12423 showed poor decline in PHA yield at a temperature above 37 °C, while still recorded high amount of CDW.

The other important factor affecting the growth of *Bacillus* spp. and its ability to produce PHA is the pH. In order to study the response of isolate to pH, *B. cereus* ARY73 was grown at different pH (5, 7 and 9) and best temperature (35 °C) for 96 h, then the PHA production was measured (Fig. 7b). This strain produced maximum amount of PHA at pH 7 on the days 48 and 96 h which was 2.65 g (71% of CDW) and 2.74 g (74% of CDW), respectively (Fig. 4b). Increasing pH to 9 decreased the production of PHA, the PHA production amounted to 0.48 g (33% of CDW) and 0.44 g (36% of CDW) for 48 and 96 h, respectively. On the other hand, decreasing pH to 5 prevented *B. cereus* ARY73 producing PHA, which may indicate that this strain could grow and produce PHA in neutral pH as a favored pH, while it can also produce it in less amounts in the alkaline medium (pH 9) in contrast to an acidic medium [Tan et al., 2014]. It is clear from the data in Figure 4a and Figure 4b that the glucose concentration was decreased as the PHA production increased during incubation time, this gives a fact that *B. cereus* ARY73 exploited abundance glucose to produce the PHA polymer and for other metabolic requirements.

Effect of Nitrogen Source Concentration on PHA production

The effect of various nitrogen concentrations in both media on the PHA production by the two isolates were investigated. Figure 5 illustrates that the, *B. cereus* ARY73 biomass showed higher PHA production at the concentrations (w/v) of 0.75% and

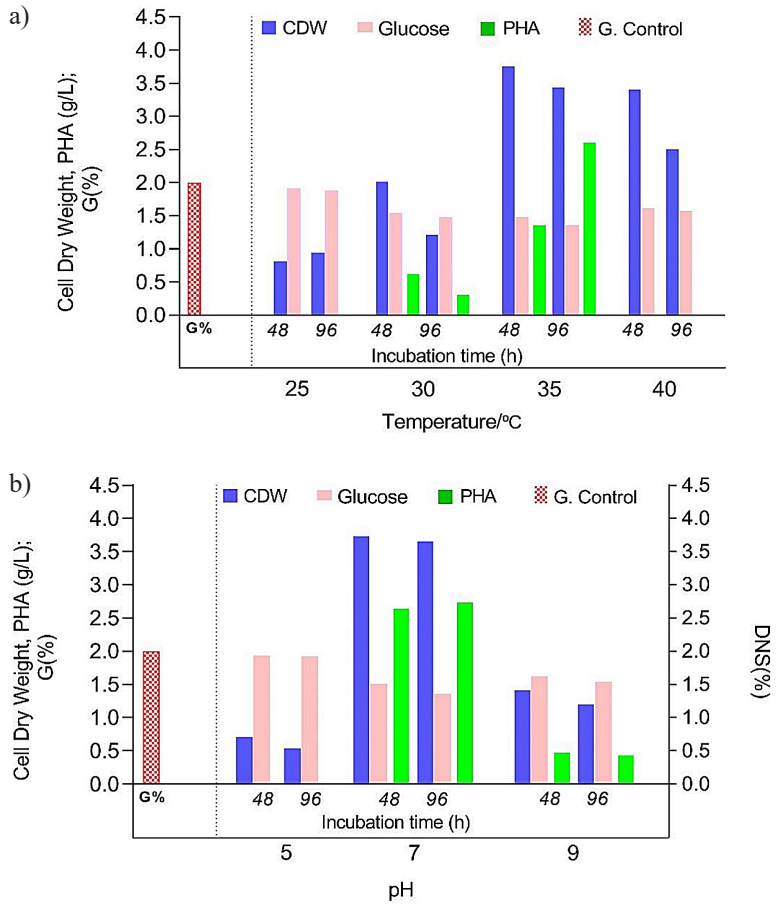

Figure 4. PHA production, CDW (g/L) and glucose content values (DNS%) by *B. cereus* ARY73 during 48 and 96 h of incubation, in response to: a) Different temperatures 25, 30, 35 and 40 °C, glucose 2% (w/v). b) pH 5, 7 and 9 at favorite temperature 35 °C and glucose 2% (w/v)

1% of nitrogen tested 2.87 g/L (PHA 77%) and 2.34 g/L (PHA 79%) respectively. The low nitrogen concentration (0.5% w/v) decreased the PHA production and limit the amount of CDW. This indicates that the nitrogen concentrations over the average (0.5-1% w/v) decreased the PHA production.

These results also supported by Campos et al. [2014], who studied the influence of crude glycerin and nitrogen concentrations on the PHA production by *Cupriavidus necator*; they found that the limited nitrogen concentration is essential for PHA production while low and high concentrations lowered it. The extracted PHA by the *B. cereus* strain ARY73 was cultivated under favorite conditions [2% (w/v) G, pH7, 35 °C, 1% N] is shown in Figure 5b.

PHA Characteristics

FTIR analysis for PHA detection and characterization

All samples were analyzed by FTIR spectroscopy to characterize the PHA by identifying the functional groups. PHB standard (BASF ecovio, Germany) and the PHA extracted from *B. cereus* ARY73 were used for comparison (Fig. 6). Strong peaks were at 1726.17 cm^{-1} indicates the presence of C=O ester bond. Similar results were reported by Paul et al. [2017], they mentioned that the peaks between 1718 and 1735 cm^{-1} are for ester stretch bands (C=O) [Sasikumar and Balakumaran, 2017]. In addition, the bands between ~1643.24 and 1278.72 cm^{-1} are due to the presence of C=O and C-O stretching groups. The peaks obtained between 1456.16 and 1380.94 cm^{-1} correspond to the asymmetric and symmetric stretching C–H bond in the methylene group, also the peaks between ~2962.46 and 2877.60 cm^{-1} originated by C-H in methylene group O-H group [Sawant et al. 2017]. Compared with PHB standard, the strongest vibrations near 2933 cm^{-1} indicated to Scl-co-mcl PHA [Shamala et al. 2009]. MCL-PHA can offer a wide range of applications due to their physical and chemical properties that come from the fact they can bear different functional groups in the side chains [Rigouin et al. 2019].

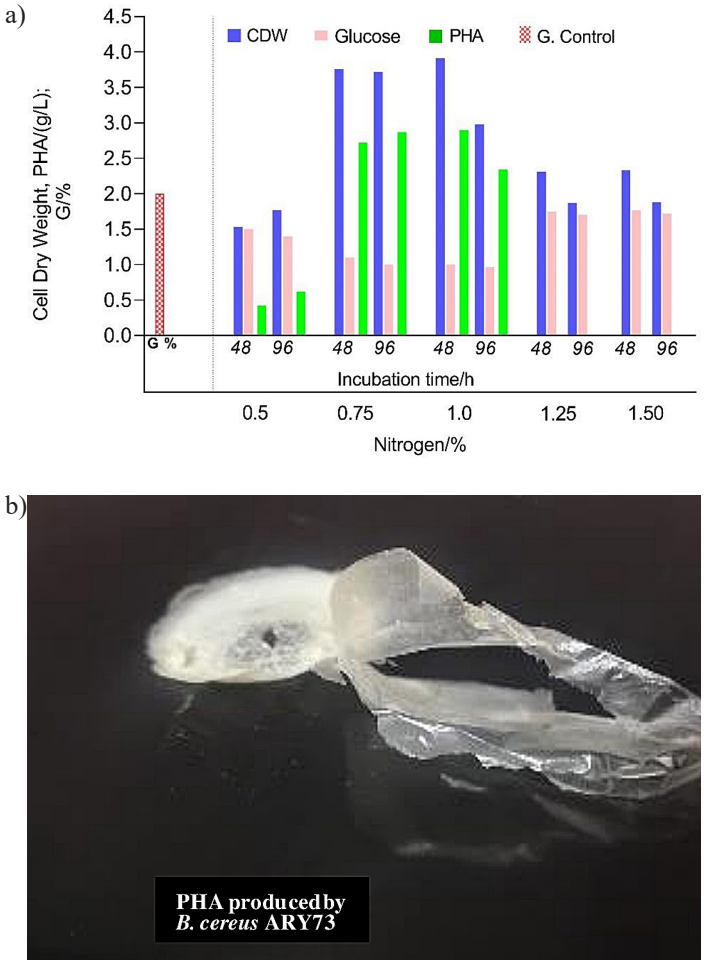

Figure 5. a) Favorite nitrogen concentrations (0.75 and 1.0%) for the PHA production by the *B. cereus* ARY73 strain, illustrating the amount of Cell Dry Weight (CDW) and glucose consumption (DNS) on incubation time 48 and 96 h. [Glucose 2% (w/v); 35 °C; pH 7], b) the PHA extracted from *B. cereus* ARY73 cultivated under favorite conditions

The strong peak between 1278.72 and 1228.57 cm^{-1} corresponding to asymmetric stretch of C-O-C group indicated the presence of an ester bond due to the C-H group in biopolymer [Vieira et al., 2011]. On the other hand, the peaks at 1182.28 and 1056.92 cm^{-1} are expected for the ether stretch (C-O-C) and dioxane bands, respectively, in which the presence of 3-hydroxybutyrate units (scl-PHA) may be confirmed. The bands of dioxane were observed at 1056.92 cm^{-1} C-O-C symmetric stretching. The presence of the peak at 3434.98 cm^{-1} may be due to the terminal O-H bonding or water adsorption on the PHB [Hassan et al., 2016].

GC-Mass

In order to determine the PHA content and monomeric composition within the *B. cereus* ARY73 isolate, the GC-MS data was evaluated after acidic methanolysis of dried cells. Figure 11 showed that, two peaks were detected with a retention time (Rt) values of 6.305 and 6.185 min (Area 1) corresponding to PHB (3-hydroxybutyric acid) as scl-PHA monomers and the Rt values of 9.718 and 9.873 min (Area 2) as mcl-PHA (3-hydroxyoctanoic acid) with some derivative products – methyl ester, 2-pentenoic acid, trifluoroacetate, hydroxyoctanoic acid, 2,3,4-Trimethyl-2-pentene (which is formed during the copolymerization). *Bacillus* spp. are among a few (wild-type) bacteria that can synthesize and accumulate P(3HASCL-co-3HAMCL), this research proved that the *B. cereus* ARY73 strain was able to produce scl-mcl-PHA as a rare strain comparing to the same species mentioned in previous publications. Gram-positive bacteria lack lipopolysaccharides (LPS) endotoxin which may make them better sources of PHAs for use in biomedical applications [Sawant et al., 2017].

The observed RTs peaks of Area (1 and 2) methyl esters derived from both PHA and

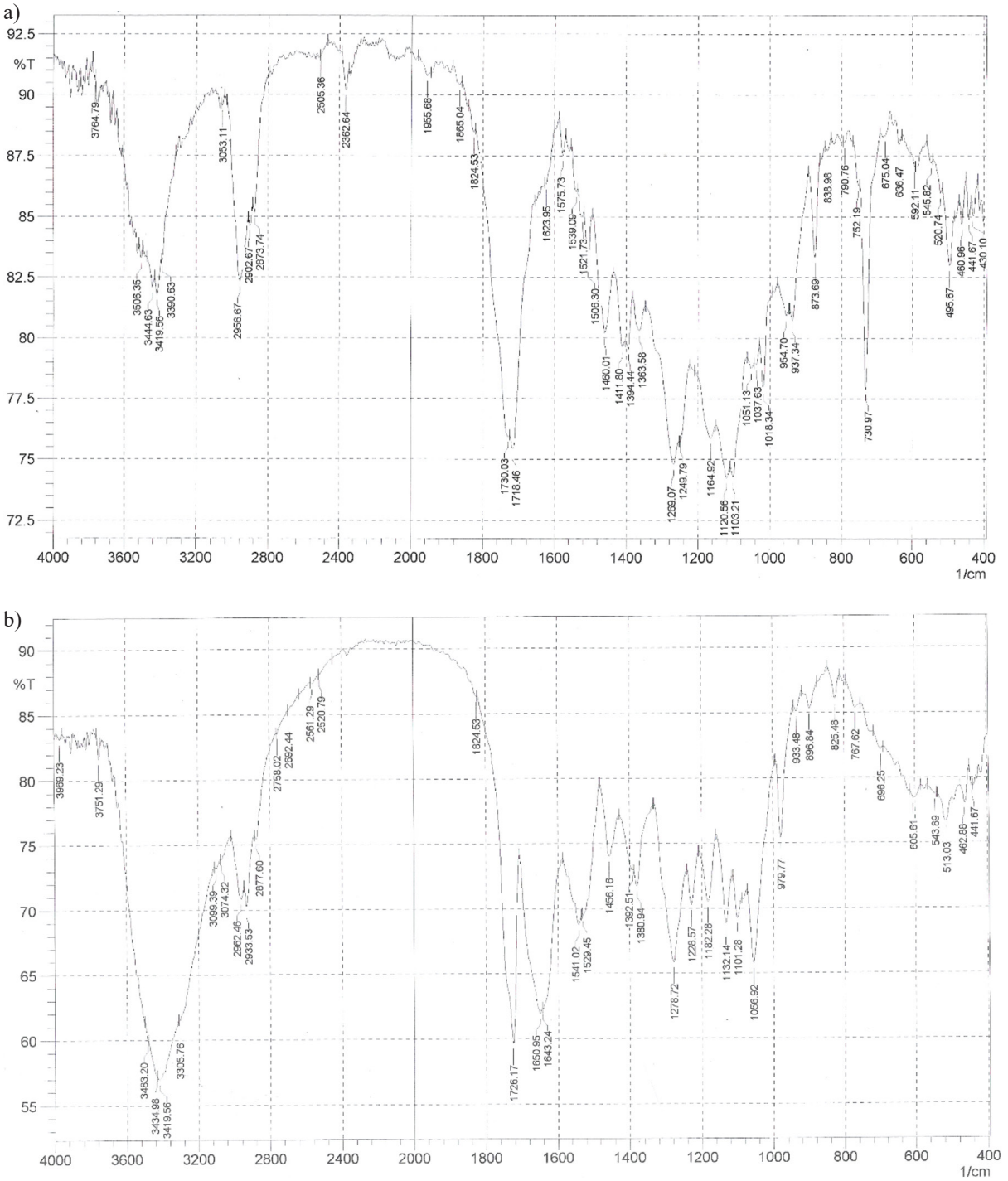

Figure 6. FTIR spectrum of PHAs: (a) Standard PHB; (b) PHA extracted from *B. cereus* ARY73

PHAHV were similar comparing to that observed from the PHA monomer of synthesized sample (control), with a strong peak noticed at Area 1.

CONCLUSIONS

In the current study, a bacterial strain was isolated from a garden soil located in the city of Baghdad-Iraq, the ability of this isolate to produce PHA was determined using staining methods (Sudan B Black stain and Nile Blue A stain). The results showed abundance of accumulated intracellular PHA granules in bacteria. The best conditions for the growth and PHA production by *B. cereus* ARY73 were examined, 2% (w/w) glucose was the best concentration (yield 74% PHA per gram CDW). It was also found that this isolate was able to grow and produce polymer at concentrations ranging between

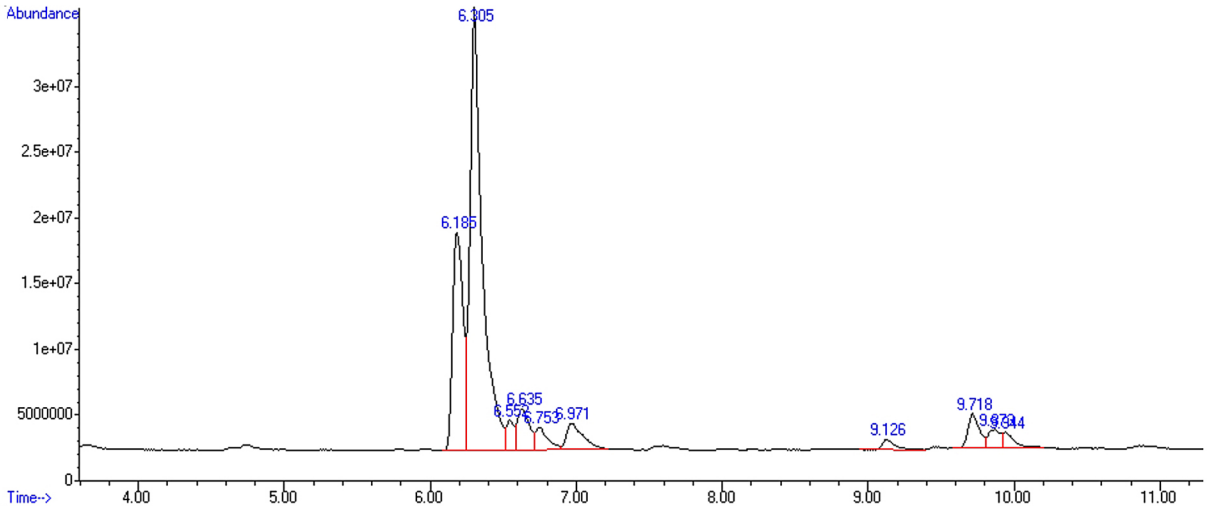

Figure 7. GC-MS analysis result of PHA obtained from *B. cereus* ARY73

4-6%, but in lesser amounts, together with other best fermenting conditions (temperature 35 °C, pH7, and 1% N). The PHA produced by *B. cereus* ARY73 was analyzed by FTIR and GC-MS, indicating the (Scl-co-mcl) co-polymer PHA structure which is suitable for diverse applications. The strain ARY73 was submitted to the NCBI GenBank database with the accession number of MW362363. Further extensive research into using variant biopolymer products within different site of soils for the isolation of PHA producing bacteria, and investigate its ability to produce PHA (individually or mixed bacteria) are highly recommended. Genetic modification of bacteria to improve the production of biopolymers is recommended for further study.

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