Iraqi Journal of Industrial Research (IJOIR)

Journal homepage: http://ijoir.gov.iq

Synthesis of Environmental Bioplastic Polyhydroxyalkanoate (PHA) from Waste Glycerol, Palm Oil and Different Concentrations of Glucose by A New Strain *Propionibacterium* Sp.

Khanssa Badie Jamil*

Ibn Sina Research Center/ Corporation of Research and Industrial Development – Iraq

Article information

Article history:
Received: June, 08, 2022
Accepted: September, 29, 2022
Available online: October, 20, 2022

Keywords:
Polyhydroxyalkanoate (PHA),
Waste glycerol,
Propionibacterium Sp.

Corresponding Author:
Khanssa Badie Jamil
alkaddokk@gmail.com

DOI:
https://doi.org/10.53523/ijoirVol9I2ID203

Abstract

Propionibacteria have mainly been found in dairy products and on the skin of humans and animals. This genus classified as non-producing PHA but in this study we show that *Propionibacterium* sp. can produce PHA. A member of the genus *Propionibacterium* was isolated from soil. The isolate was identified based on its gram positive pleomorphic rods. Cells occur short chains, singly or in pairs, in V or Y configurations. On mineral salt agar colonies are semi-opaque, convex, glittering and often pigmented in orange in color, biochemical tests and 16SrDNA also done. The 16SrDNA analysis confirmed 85% identity to *Propionibacterium* (accession number NR 074675.1). The presence of 3HB was identified, based on the analysis of NMR. ^{13}C and ^{1}H NMR analysis confirmed that *Propionibacterium sp* was able to produce PHA.polymer. These confirm the results of GC. The isolate was then grown on media with waste glycerol (WG), palm oil, different concentration of glucose and one stage and two stages of cultivation. This lead to the conclusion that *Propionibacterium* is able to grow utilizing waste glycerol, palm oil and different concentration of glucose as the sole carbon source under limited conditions. The PHA content 3 (wt%) and the composition (mol%) and (86% 3HB) when used glucose 2% at frist stage cultivation. while the PHA content 13 (wt %) when used palm oil and 5 (wt%) when utilized (WG), as sole carbon source.

1. Introduction

Propionibacterium is a Gram-positive, non-spore forming; non-motile and Facultative anaerobes but have variable aerotolerance. They belong to the class of Actinobacteria with high GC content (53 - 67 mol %) [1, 2] and to date, there is 14 species under order of Actinomycetales. In 1909, this genus was first described by Orla-Jensen. It is divided into two groups, the acne group strains or cutaneous *Propionibacteria* and the classical or dairy *Propionibacteria* [3]. Acne group strains is a normal existent of human skin and on the body related in causing acne vulgaris and other bacterial infections while, the classical group is often used in dairy production, specially swiss cheese and other natural fermentations [4, 5].

Propionibacteria sp. mainly *P. freudenreichii* and *P. shermanii* have a high ability to accumulate intracellularly vitamin B12 and as major fermentation products excreting acetic acid and propionic acid. An important cofactor

for the metabolism of carbohydrates is Vitamin B12 (cyanocobalamin) that can be used in chemotherapy like nucleic acids and amino acids and as significant additive for animal feed and Propionic acid is used to prevent mouldy food infection in chicken and other animal feed. It is used for manufacturing herbicides, perfumes and cellulose-based plastics. Propionic acid salts like potassium, sodium and calcium are used as preservatives [6].

Propionibacteria are pleomorphic rods, 0.5-0.8 µm in diameter and 1-5µm in length. Are often club- shaped with one end tapered and the other rounded, some cells may be coccoid or branched but not filamentous. Cells occur short chains, singly or in pairs, in V or Y configurations. Usually on blood agar colonies are semiopaque, convex, glittering and often pigmented in white, gray, pink, red, yellow, or orange in color. Have metabolism fermentative, producing large amounts of propionic acid and acetic acids from glucose and some other carbohydrates. The optimum growth temperature is 30 °C - 37 °C. Usually catalase positive. Easily confused with some species of *Clostridium* or *Corynebacterium.* [4].

Polyhydroxyalkanoates (PHAs) production by Propionibacteria sp. using inexpensive substrate isolated from soil. Polyhydroxyalkanoates (PHAs) are biodegradable, biocompatible and renewable polymers produced by numerous of bacterial groups and accumulated intracellularly as carbon and energy sources. These PHAs can harvest by humans to synthesis bioplastics, like polymers that bacteria produce resemble to the chemical structure of petroleum-produced plastics [7]. The accumulation of PHAs increases the surviving of bacteria under limited conditions such as high salinity, UV irradiation, desiccation, osmotic shock, oxidative and thermal stress [8]. Presently, more than 150 different hydroxyalkanoate units have been identified with various properties in up to 300 different microorganisms [9].
bioprospection approaches for New microbial have become significant way to find new synthesized polymers and new PHA producers. Over the past years, the bacteria that belonging to group of actinomycetes have become known as PHA producers, such as *Rhodococcus* and *Nocardia* species, *Kineosphaera limosa* [10] and lately, *Streptomyces* species. Now, we disclose that there are more PHA producers belong to actinobacteria. Some unusual genera, such as *Streptoalloteichus*, and some genera frequently present in soil, such as *Streptacidiphilus* and *Propionibacteria* have been found.

Thirty-four strains were able to accumulate poly (3-hydroxybutyrate) and some of these less produce of poly (3-hydroxyvalerate) when cultured on glucose and casein or glucose as carbon source. Moreover, some strains appeared traces of medium chain length PHA [11]. Ataei *et al*. [12] were carried out the production of poly (3-hydroxybutyrate – hydroxyvalerate) fermentation by using two bacteria (*Propionibacteria shemmanii* and *Bacillus* sp.) the main fermentation medium for PHA production was mixed inexpensive carbon source containing (2% cane molasses, 2% date syrup, 20% whey) supplemented with 3% corn steep liquor as source of nitrogen. This method permitted to production of 3- hydroxyvalarate 27 mol% in 2.4 g/L of total accumulated copolymer. Mostly phosphate accumulating organisms (PAOs) belong to Actinobacteria and proteobacteria are quite disparate under aerobic conditions they accumulate polyphosphate (polyp) intracellular while, typically under anaerobic condition they synthesize polyhydroxyalkanoates (PHAs) [6].

This study was carried out to isolate a new strain with the ability to produce PHA from inexpensive substrates (waste glycerol, palm oil, as sole carbon source and different concentration of glucose), to detect and identify these bacteria by utilizing both phenotypic and genotypic detection methods and to Confirm Producing PHA by using GC and NMR analysis.

2. Materials and Methods

2.1. Sample Collection

The samples of soil (0.5gm) were collected from the garden flower, tea farm and palm tree. From 20 cm depth at temperature (30-35) °C. The soil was taken and stored at room temperature (35-37) °C untile use.

2.2. Isolation of Bacteria

The isolation of bacteria were made by serial dilution and cultured on nutrient agar. Were taken 0.5 gm of soil sample was added in to 10 ml of sterile distilled water and mixed for 1 min with a vortex. This mixture is serially dilluted from 10^{-1} to 10^{-8}, 1 mL of each dilution (10^{-3} to 10^{-7}) was spread on nutrient rich (NR) agar (Hi-Media)

containing (per liter) 10 g meat extract, 10 g peptone, 2.0 g yeast extract and 15 g/L agar powder (pH 7.0) and incubated for 24 hour at 30 °C.[13]

2.3. Screening of Soil Bacteria for PHA Production

The bacterial colony that obtained from the nutrient agar plates medium were picked up, examind under microscope (gram positive pleomorphic rods) and purified. On mineral salt agar colonies are semi-opaque, convex, glittering and often pigmented in orange in color. The colonies were grown in 5 ml of nutrient broth and shaking over night at 30 °C [13]. These culture were used as inoculums (3% v/v) for 50 ml mineral salt media (MSM) contain: Na2HPO4 (3.32 g/L), KH2PO4 (2.80 g/L), NH4Cl (0.5 g/L), MgSO4.7H2O 1ml/L, trase element 1 ml/L [14]. MgSO4.7H2O and trase element were added together with inoculums to MSM with 20 g/L waste glycerol as the sole carbon source for 48 h at 30 °C with shaking at 180 rpm. The trace elements compositions per liter of 0.1 N HCl were as follows [15]: $CaCl_2$ 7.8 g/L, $CoCl_2.6H_2O$ 0.22 g/L, $NiCl_2.6H_2O$ 0.12 g/L, $FeCl_3$ 9.7 g/L, $CuSO_4.5H_2O$ 0.16 g/L, $CrCl_3.6H_2O$ 0.11 g/L (pH 7.0).

After increase of turbidity in the culture media, prepare slides smeared from these cultures to examine under phase contrast micrscope, fluorescence microscope and gram stain. On plate method the bacteria culured on MSM supplemeted with 0.5 µg/ml nile red [16]. Bacterial colony with pinkish color when observed under UV light (wavelength 250 nm) indicated a PHA (Polyhydroxyalkanoate) producer.

2.4. Phase–Contrast Microscopy

A single colony from bacteria was suspended in one drop of water or used one drop of culture on a slide. Then, the smear was covered with a cover slip. The slide was observed at 100x magnification with oil immersion under a phase contrast microscope, *Nikon LABOPHOT-2* (Nikon, Japan)

2.5. Fluorescence Microscopy

The method of Nile Blue A staining was utilized for the observation of PHA granules [17]. On a slide a single colony from bacteria was suspended in a drop of water and heat-fixed. The smear was flooded with Nile blue A stain and dried at 55 ºC for 10 min. then, 8% of acetic acid (8.0 mL of acetic acid was added to 92.0 mL distilled water) was used to de-stain the slide for 1 min to remove all the unbound stains. After that, the slide washed with water and was allowed to dry. Then, the slide was covered and observed using oil immersion 100x magnification under UV compatible lenses on *UV Olympus* microscope (Olympus, Japan) and the images were recorded using *Prog Res –C10, Size 5.0* software. The bright orange fluorescence was indicated the PHA granules inside the bacterial cells.

2.6. Identification of Propionibacterium sp. Using 16S rDNA Analysis

Identificaeion of the *Propionibacterium* was officiated based on morphological observation, biochemical characterization and *16S rDNA* analysis. The extensively used of *16S rDNA* gene for evolutionary analysis is due to a number of reasons: The gene exists approximately in all bacteria, the function of gene has not changed and the length of gene sufficient for the purposes informatics [18,19].

A single colony of *Propionibacterium sp.* from a plate that had been incubated for 20-24 h was transferred into NR medium and incubated overnight. The overnight culture was harvested and used for DNA extraction. genomic DNA was extracted from pure cultures following the manufacturer's instructions of the G- spin™ Genomic DNA Extraction Kit (for bacteria) (iNtRON Biotechnology, Inc., South Korea). DNA integrity and concentration were assessed by standard agarose gel electrophoresis and spectrophotometric reads using a NanoDrop Lite spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). The DNA concentration was 1.8-2.0 ng/µl, DNA samples were subjected to gel electrophoesis. DNA electrophoesis was carried out on 1.0% (w/v) agarose gel using 0.5 X Tris-acetate-EDTA (TAE) buffer as running buffer. Gel was soaked in ethidium bromide for 15 min and rinsed with distilled water for 5 min before viewing under Gel Doc XR system UV transilluminator (Bio-Red,USA). Amplification of the *16S rDNA* gene was officiated by PCR using universal primers F (5′-TNA NAC ATG CAA GTC GAG CG- 3′) and R (5′-ACG GGC GGT GTG TAC-3′) [20]. The PCR parameters were used as follows: Denaturation, 94 °C for 2 min: 30 thermal cycles including 94 °C for 45 sec, Annealing, 58 °C for 30 sec, 72 °C for 2 min and a final step for extension 72 °C for 2 min. The PCR products were purified and subjected to

agarose gel electrophoesis. The identity and similarity of the sequence were compared to another sequence in the database of Gene Bank using BLAST in (NCBI) National Center for Biotechnology Information. [21]

2.7. PHA Production of the Isolate in Shake Flask

In NR medium the isolate was grown in 50 mL of (NR) broth at 30ºC and 180 rpm for 24h then, 3% of the cell culture (OD600 = 4.5) was inculated in nitrogen–limiting MSM containing 20g/L waste glycerol, 1% palm oil and 1% and 2% glucose all using separately as sole carbon source. The incubation period was 48 h at 30 °C and 180 rpm to grow the cell of bacteria and accumulation of PHA. Later, the cell were centrifuged (8000 rpm, 10 min, 4 °C), washed one time with hexane when used palm oil and finally washed two times with distelled water. The cells were lyophilized before subjecting to gas chromotography (GC) analysis. [22]

2.8. Optimization of Carbon Source (Waste Glycerol, Palm Oil and Different Concentrations of Glucose) and Different Nitrogen Sources for PHA Production

The effect of waste glycerol, palm oil and different concentration of glucose on PHA production by *Propionibacteria* were performed in nitrogen limiting conditions. Sources of nitrogen such as ammonium chloride and ammonium sulphate in different concentrations were used for PHA production. The waste glycerol as the sole carbon source added at the rate of 20 g/L in MSM while the Palm oil when used as the sole carbon source was added at the rate of 10 g/L also 1% and 2% of glucose was utilized as the sole carbon source.

2.9. GC Analysis

Quantification of PHA was done by GC analysis using the internal standard caprylic methyl ester (CME) for determination of PHA content and composition. Approximately 25 mg of freeze – dried cells were weighed into screw-cap test tubes and subjected for methanolysis (140 min in 100 °C). The methanolysis solution consists of 2 mL of 85% (v/v) methanol and 2 mL of 15% (v/v) sulphuric acid [21]. The resulting methyl esters were analyzed by GC (*Shimadzu GC-2010 AF 230 LV*) equipped with a capillary column SPB-1 (30 m length, 0.25 mm internal diameter, and 0.25 mm film thickness; Supelco, Bellefonte, PA, USA) connected to a flame ionization detector. The carrier gas that utilized was nitrogen (1 mL/min), and the chloroform-dissolved sample (1 mL) was injected utilizing an auto injector (*Shimadzu AOC-20i*). The injector and detector temperatures were set at 270 and 280 °C, consecutively. The temperature of column was increased from 70 to 280 °C at 10 °C/ min.

2.10. Extraction of Polymers

The PHA polymer was extracted from lyophilized cells by stirring around 1 g of freeze – dried cells in 100 mL of chloroform for 5 days at room temperature. Then, the chloroform extract was filtered using *Whatman No.1* filter paper in order to separate the debris of cell. After that, the filtered chloroform extract was concentrated by utilizing *Eyela N-1000* rotary evaporator (Eyela, Japan). Later, the solution of concentrated polymer was precipitated via drop wise addition into cooled methanol on a magnetic stirrer. Finally, the resulting precipitant was collected and dried [23, 24].

2.11. Nuclear Magnetic Resonance (NMR)

The total weight of polymer sample (25 mg) was dissolved in deuterated chloroform (CDCl3) (1mL). The NMR spectra was measured at 30 °C at 400 MHz on a *Bruker AVANCE* 300 (NC, USA) spectrometer. An internal chemical shift reference that used was tetramethylsilane (Me4Si). [23]

3. Results and Discussion

3.1. Screening of *Propionibacterium* sp. that Produces PHA Using Different Carbon Source as Sole Carbon Source

Propionibacteria sp. that isolate from soil were subjected for partial identification based on different biochemical tests according to Bergeys manual and also to see the characteristics differentiating of *Propionibacterium* sp. Table (1). In addition of that, KOH test (- ve) and catalase test (+ ve) were examined. PHA production was screened by plate and slide methods. In plate methods, the morphology of *Propionibacteria* on MSM supplemented e.g. with 2% waste glycerol are semiopaque, convex, glittering and often pigmented in orange color, Figure (1 A) and exhibit a bright fluorscence when grown on MSM in the presence of 0.5 µg/mL Nile red. Figure (1 B). Indicating that could used and efficiently transform waste glycerol into PHA inside the cell. In the slide method, using gram stain *Propionibacterium* sp. appearing as gram positive, pleomorphic rods or coccoid cells

occur singly, short chains, or in pairs, in V or Y configurations, or in clumps, branched but not filamentous as shown in Figure (2).

Under phase contrast microscope PHA can be observed as light–refracting intracellularly granules without any staining. Figure (3 A and B). Rapid staining methods by using lipophilic dyes are commonly used for the detection of PHA granules, during the screening of isolation of bacteria that producing PHA. Nile red and Nile blue A are lipophilic dyes used for the staining of bacterial cell that have PHA granules [17, 25, 16]. PHA granules are identified by the retention of the dye after staining and then bind onto the PHA granules which fluoresce under UV light. Figure (4) showed bright red flourescence *Propionibacterium* under fluorescent microscope. However, sometimes lipophilic dye can detect the presence of lipid granules that exist in the cells and produced bright orange fluorescence. Therefore, in order to get an accurate result GC and NMR analysis were needed in this study [26].

3.2. Differentiation of the Genus *Propionibacterium* Species

Table (1). Characteristics differentiating of *Propionibacterium* sp.

Characteristics	1	2	3	4	5	6	7	8
Usual pigmentation of colonies	W	W /gray	W/cream	Tan or pink	W/gray	W/pink	W	Orange/ red,brown
β-hemolysis	−	**d**	**(+)**	−	**(−)**	−	−	+
Acid produced from:								
Maltose	+	−	+	−	+	+	+	+
Sucrose	+	−	+	−	+	+	d	+
L-Arabinose	+	−	**d**	+	−	−	−	−
Cellobiose	+	−	−	−	−	d	−	−
Glycerol	+	**d**	+	+	+	+	−	+
Hydrolysis of:								
Esculin	+	−	+	+	−	+	−	+
Gelatin	−	+	+	−	d	−	d	−

Symbols: +, 90% or more of strains are positive; (**+**),80-90% of strains are positive;(**−**) ,11-20% of strains are positive;−, 90% or more of strains are negative; **d**, 21- 79% of strains are positive. W: White1: *Propionibacteria acidipropionici* 2: *P. acnes* 3: *P. avidum* 4: *P. freudenreichii* 5: *P. granulosum* 6: *P. jensenii* 7: *P. lymphophilum 8*: *P. thoenii* Bergeys manual, (1978).

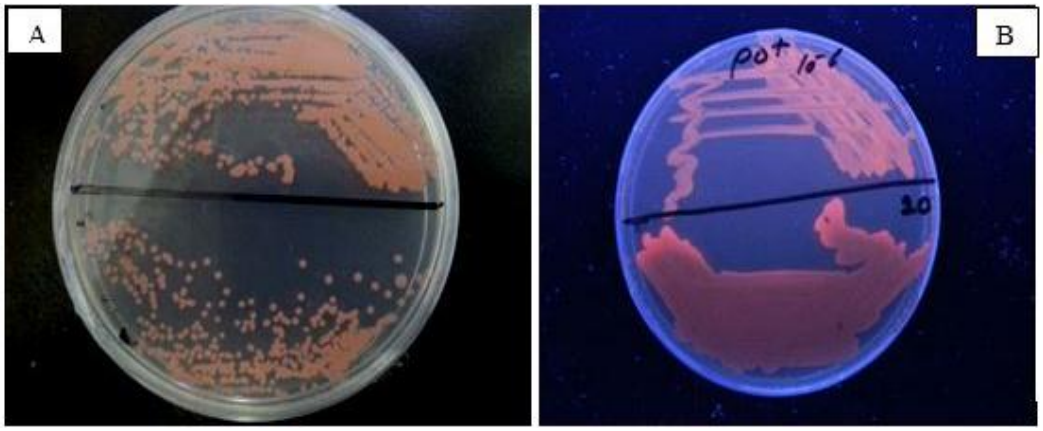


Figure (1). **(A)** Morphology of *Propionibacterium* sp. on mineral salt medium supplemented with 20 g/L waste glycerol as the sole carbon source incubated for 48h at 30 °C**. (B)**: *Propionipacterium sp.* on mineral salt medium with Nile red stain under UV light. Fluorescence of 48h incubation at 30 °C.

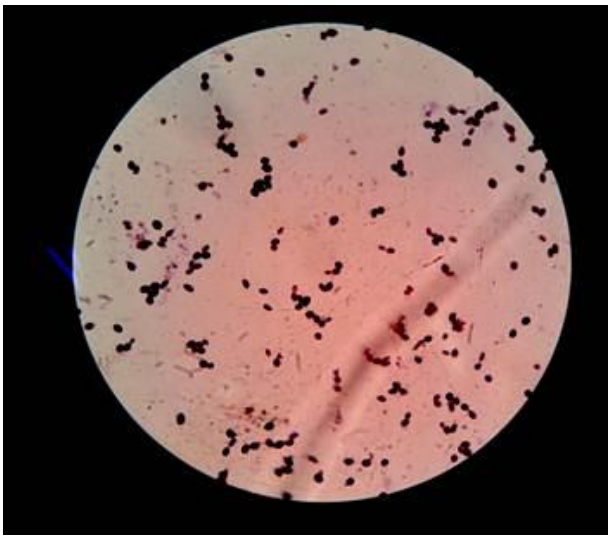


Figure (2). Morphology of *Propionibacterium* sp. under light microscope using gram stain, gram positive, pleomorphic rods or coccoid cells occur singly, short chains, or in pairs, in V or Y configurations, or in clumps, branched but not filamentous.

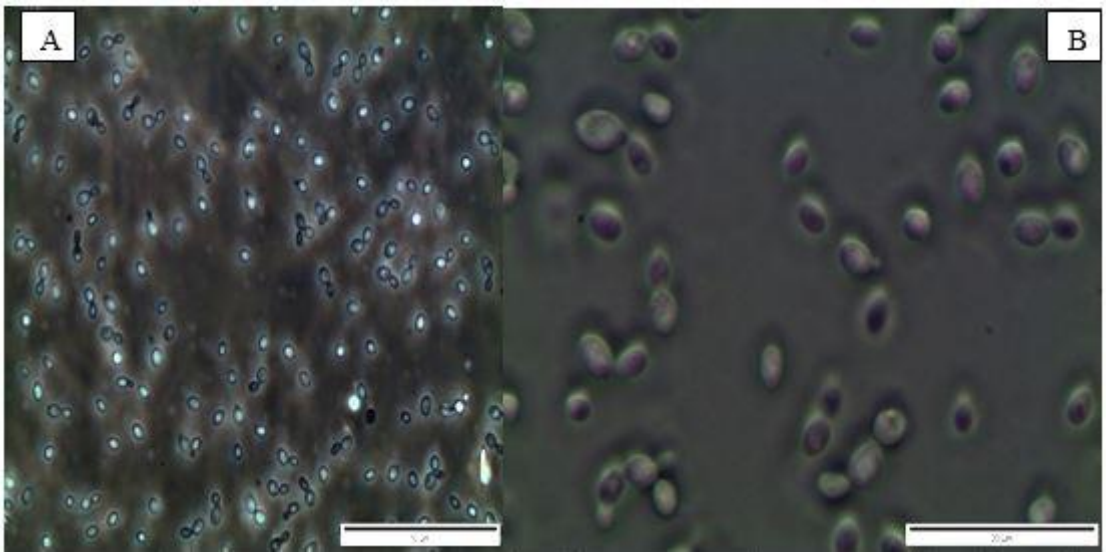


Figure (3). (A) *Propionibacterium* sp. under phase contrast microscope. Cells cultivated for 48hat 30 ºC using waste glycerol as the sole carbon source. **(B)**: *Propionibacterium* sp. under phase contrast microscope (dark field). Club-shaped with one end rounded and the other tapered.

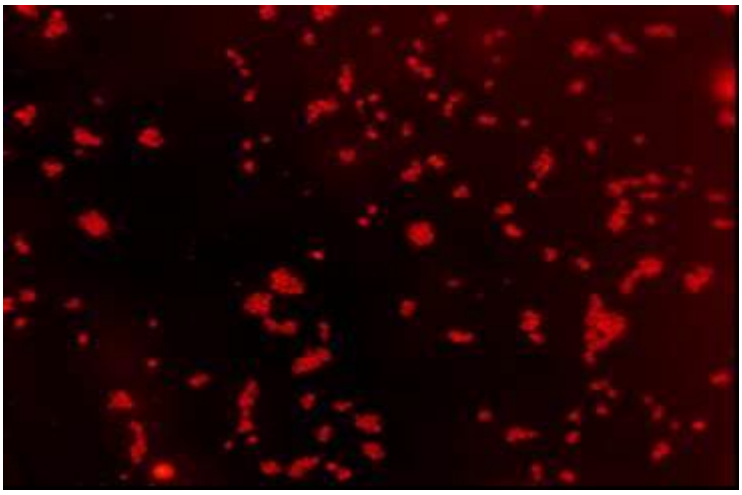


Figure (4). *Propionibacterium* sp. bright red flourescence under fluorescent microscope using Nile blueA lipophilic dye.

3.3. Identification by *16s rDNA* Analysis

A partial sequence of 16s rDNA of 1400bp was obtained by PCR. The BLASTX analysis revealed 85% identity and closely related and high similarity to a partial sequence of *16s rRNA* gene of *Propionibacterium* (accession number NR 074675.1) Figure (5). The *16S rDNA* sequencing method was used for bacterial species that cannot be identified confidently and to determine the phylogenetic relationships between bacteria. [18, 19].

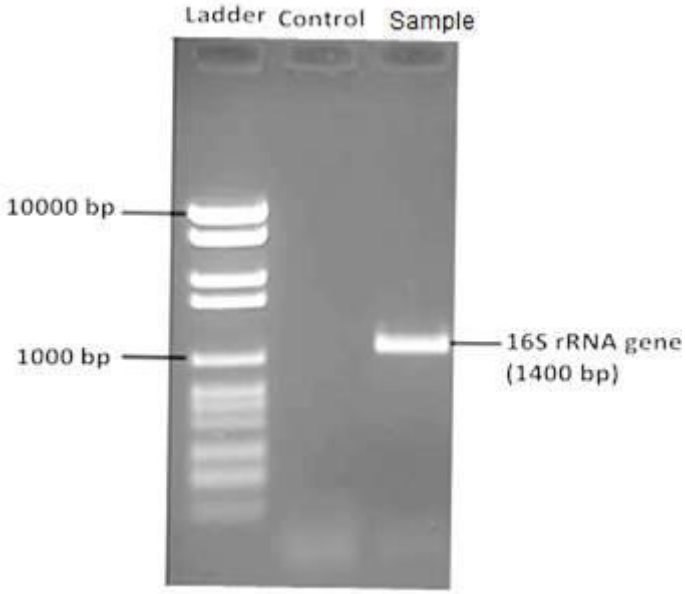


Figure (5): *Propionibacterium* sp. *16S rDNA* result. Agarose gel electrophoresis of PCR products (1.0% agarose,75 volts for 90 min) for *16SrDNA* gene product using DNA template of *Propionibacterium* sp. Lane 1: DNA Ladder:(10000 bp). lane 2: Negative control. Lane 3: bacterial sample. The bacterial sample showed positive result (amplified size 1400bp).

3.4. Optimization of Nitrogen Limitation for PHA Production

Propionibacterium sp. showed that cannot grow in nitrogen free medium but can grow in medium supplemented with little amount of nitrogen source (0. 25 g/L) more growth than (0. 5 g/L) and NH4Cl was the best nitrogen source Figure (6).

3.5. Extraction of P(3HB) Homopolymer

P(3HB) homopolymer biosynthesized via *Propionibacterium* using palm oil as carbon source was extracted and subjected to GC analysis again to reconfirm the ability of this strain to produce PHA.

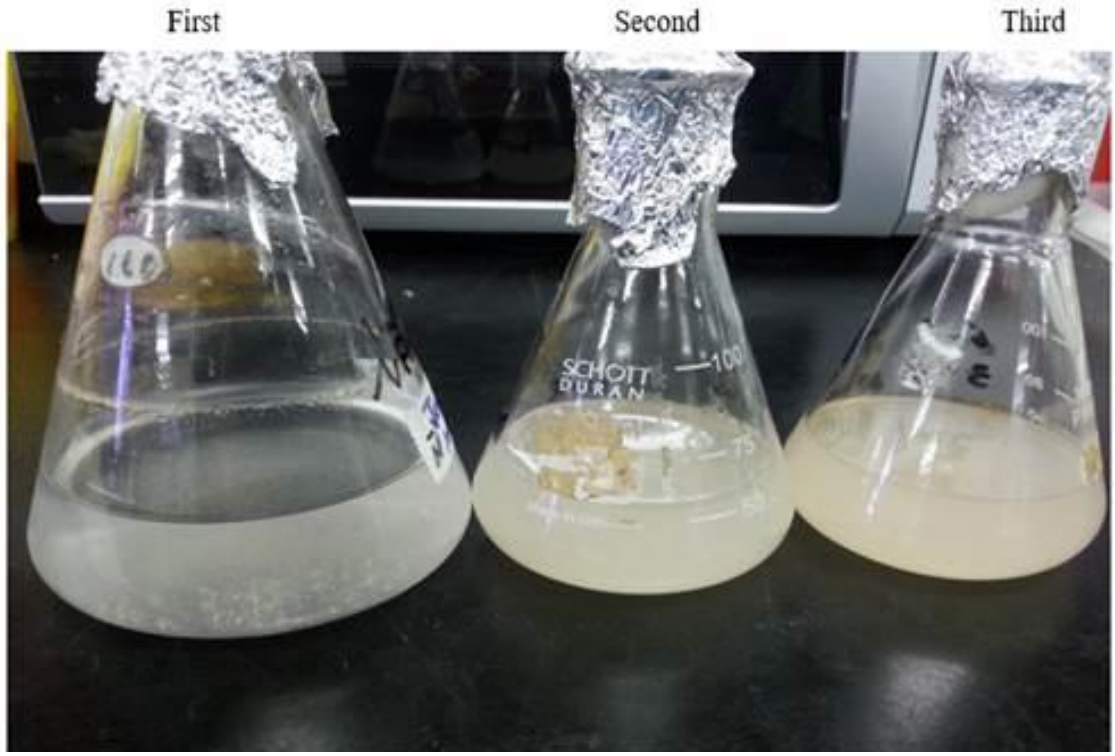


Figure (6). *Propionibacterium* sp. cultured in mineral salt media supplemented with 2% waste glycerol as sole carbon source and NH4Cl as nitrogen source incubated for 48h at 30 °C. **First flask**: free nitrogen source (no growth); **second flask**: 0.05g/50 ml NH4Cl (growth); **third flask**: 0.025g/50 ml NH4Cl (more growth).

3.6. GC Analysis

GC analysis revealed that *Propionibacterium* sp. can produce PHA and it can produce co – polymer, the PHA composition (mol %) for *Propionibacteria* sp. are listed in Table (2) and Figure (7). The high PHA content production for the isolate was might refer to the bacteria able to utilize waste glycerol, palm oil and glucose readily as their sole carbon source for accumulation of PHA. Probably, these bacteria able to produce acetyl - coenzyme A (acetyl- CoA), which is converted into PHB by utilizing three biosynthetic enzymes. Glycerol is metabolized, through glycolysis pathway. First, glycerol is converted into glycerol-3-phosphate with aid of ATP molecule to activate the reaction. It is followed by converting to dihydroxyacetone phosphate before entering the glycolysis pathway to produce acetyl- CoA and, which is diverted into PHB via three steps of synthesis pathway.

Table (2). Production of PHA by using various carbon sources in *Propionibacterium* sp.

Second stage carbon sources[a] (vol/ vol)	First stage carbon sources (vol/vol)	Cell Dry Weight (g/l)	PHA content[b] (Wt%)	PHA composition [c] (mol %)		
				3HB	3HV	3H4MV
Glucose 2%	Nutrient broth only	2.1±0.1	2±1	55	33	12
-	Glucose 2%	2.2±0.1	3±2	86	7	7
Glucose 1%	Nutrient broth only	2.1±0.1	1±1	31	47	22
-	Glucose 1%	2.3±0.1	2±2	35	49	16
Waste glycerol 2%	Nutrient broth only	3.2±0.1	4±2	100	-	-
-	Waste glycerol 2%	3.4±0.1	5±1	100	-	-
Palm oil 1%	Nutrient broth only	3.1±0.2	11±1	100	-	-
-	Palm oil 1%	2.4±0.2	13±2	100	-	-

[a]Second stage culture consisted of Mineral salt media +(vol/ vol) different carbon sources and incubated for 48 h at 30° C , PH 7.[b] Polymer content in lyophilized cells .[c] Determined by GC analysis. 3HB, 3-hydroxybutyrate; 3HV, 3-hydroxyvalerate; 3H4MV, 3-hydroxy-4-methylvalerate

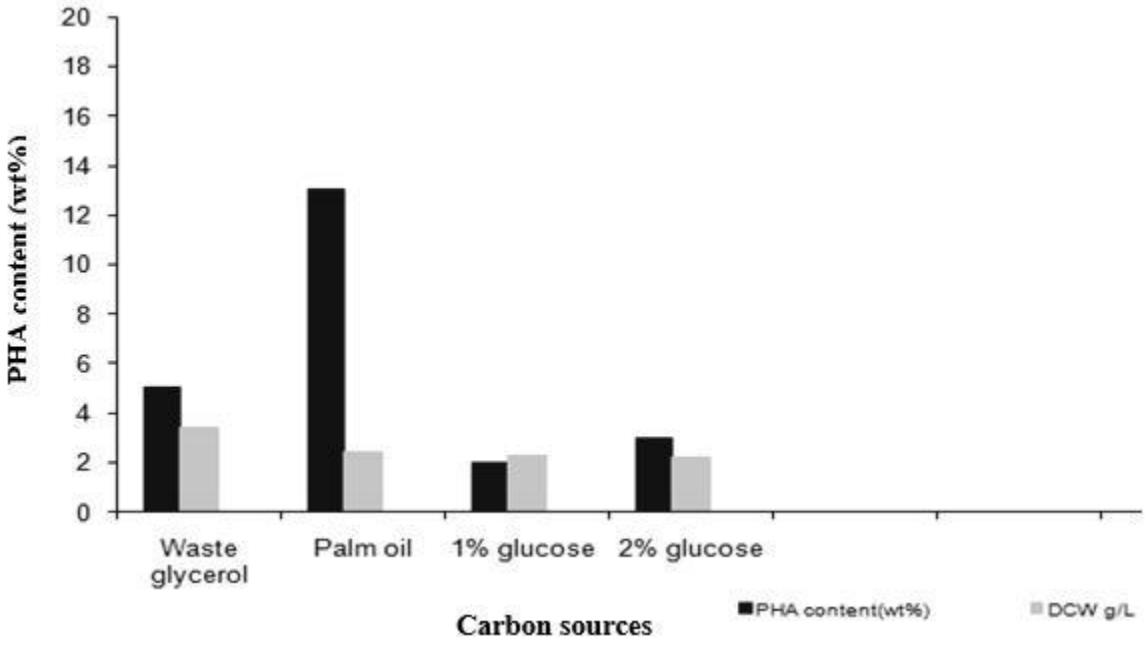


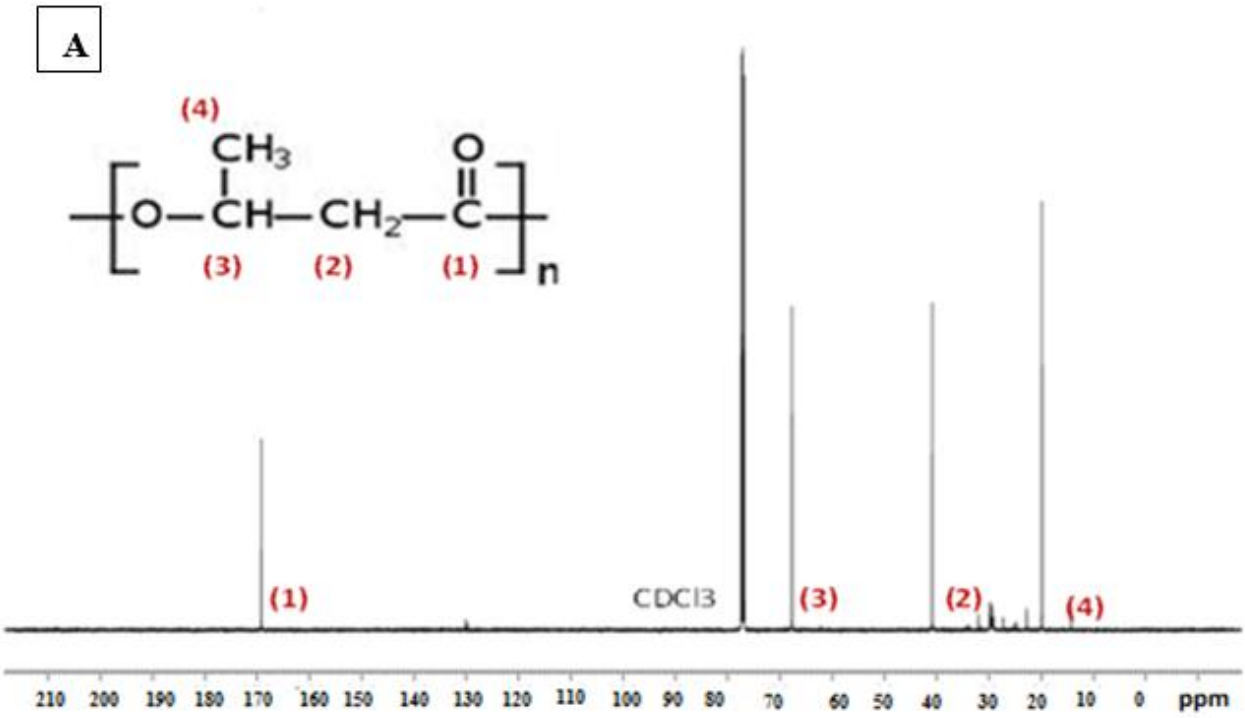
Figure (7). Production of PHA by using different carbon sources for *Propionibacterium* sp.

The pyruvate is the yield of glycerol metabolism, which is utilized by TCA cycle. When waste glycerol was utilized as the sole carbon source most of the carbon used for the biomass and few carbons used as PHA precursors and because of that few amount of PHA accumulation was seen. Also, the low percentage of PHA content of its cell dry weight (CDW) refer to the complex structure of oils that caused the difficulty for isolate to degrade it and

unable to utilize it as their carbon source. As well as the PHA contents decrease when the propionic acid produce in the media by *Propionibacteria* and this acid is toxic for growth of bacteria when produce in high amounts in media. We can be hypothesized that production of hydroxyvalerate (HV) in *Propionibacteria* was from the amino acids that excretion into the media**.** It is reported that in *Alcaligenes* SH 69, the addition of amino acids into culture medium like isoleocine, threonine and valine increased the molar fraction of 3-hydroxyvalerate [27]. The cells was synthesized Propionyl- CoA by themselves from intermediates of amino acids metabolism. There is a possibility of excreation of some of the essential amino acids for production of PHA by *Propionibacterium*, which further, may be assimilated to produce a copolymer. *Propionibacterium* can produce considerable amount of copolymer in the absence of any other precursor, the synthesis of copolymer can be clarified depend on the above facts. In *Alcaligenes*, the produced PHA from oils and fats did not yield any copolymer [28] and that's agreed with our results.

3.7. Nuclear Magnetic Resonance (NMR)

The presence of 3HB could be identified, depended on the spectrum and NMR analysis was carried out, Figure (8), (A): displays four strong signals assignable to the carbon resonances of 3HB monomer. The corresponding peaks were assigned to methyl (-CH3) **(4)** at 19.770 ppm, methylene (-CH2) **(2)** at 40.796 ppm and methine (-CH) **(3)** at 67.618 ppm in carbon groups and the carbonyl group (C=O) **(1)** at 169.149 ppm. (B) show the peak assignments of the ^{1}H NMR spectrum (proton groups), high peak **(3)** (three H) at 1.213 ppm, peak **(1)** (two H) at 2.548 ppm and peak **(2)** (one H) at 5.209 ppm. These further confirm the results of GC analysis and showed the ability of this isolate to produce P (3HB) polymer [29].



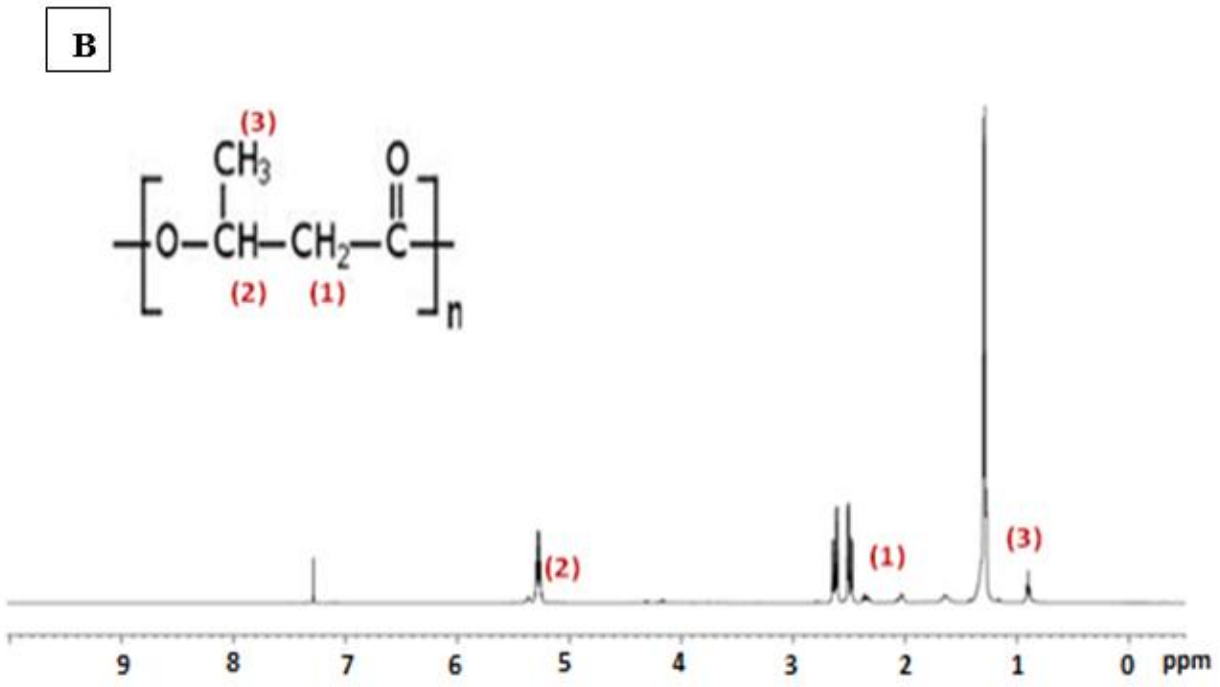


Figure (8). (A)^{13}C NMR and (B) ^{1}H NMR spectrum confirms the presence of P (3HB) polymer produced by *Propionibacterium sp.*

4. Conclusions

A new PHA producing strain was isolated and identified and this genus classified as non-producing PHA but in this study, we show that *propionibacterium* sp. can produce PHA from inexpensive substrates (waste glycerol, palm oil and different concentrations of glucose) and the ability of this strain to produce copolymer without using precursors.

References

[1] K. Kuasano, H. Yamada, M. Niwa, et al., "Propionibacterium cyclohexanicum sp. nov., a new acid-tolerant - cyclohexyl fatty acid-containing Propionibacterium isolated from spoiled orange juice," *Int. J. Syst. Bacteriol*, vol. 47, p. 825-831, 1997.

[2] E. Sophie, C. Carine, R. Didier, et al., "Mass Spectrometric Identification of Propionibacterium Isolates Requires Database Enrichment, " *Advances in Microbiology*, vol. 2, p. 497-504, 2012.

[3] M. Gautier, A. de Carvalho and A. Rouault, "DNA fingerprinting of dairy *propionibacteria* strains by pulsed-field electrophoresis, " *Curr. Microbiol*, vol. 32, p. 17-24, 1992.

[4] C. S. Cummings and J. L. Johnson, "Genus I. Propionibacterium Orla-Jensen 1909," Bergey's manual of systematic bacteriology," 2 nd ed., vol. 2, p. 1346-1353, Williams & Wilkins, 1986.

[5] A. L. Jeremiah, A. B. David, J. B. Mary, A. K. Stephen, A. Mohamed, et al., "Isolation of *Propionibacterium acnes* from a case of placentitis and abortion in a cow," *J. Vet. Diagn. Invest*, vol. 21, p. 274–277, 2009.

[6] A. Kawakoshi, H. Nakazawa, J. Fukada, M. Sasagawa, Y. Katano, S. Na kamura, et al., "Deciphering the genome of polyphosphate accumulating Actinobacterium microlunatus phosphorus," *Oxford Journals, DNA research*. Online ISSN 1756-1663 - Print ISSN 1340-2838, 2012.

[7] D. F. Gilmore, "PHAs: Chemistry, History, and Biology," *Environmental Microbiology Research Lab. Arkansas State University*, 2012.

[8] S. Castro-Sowinski, S. Burdman, O. A. Matan, et al., "Natural functions of bacterial polyhydroxyalkanoates, " *Plastics from Bacteria*, p. 39-61, 2010.

[9] Y. Kim Do, H. W. Kim, M. G. A. Chung, et al., "Biosynthesis ,modification and biodegradation of bacterial medium - chain - length polyhydroxyalkanoates," *The Journal of Microbiology*, vol. 45, p. 87-97, 2007.

[10] W. T. Liu, S. Hanada, T. L. Marsh, Y. Kamagata, et al., "*Kineosphaera limosa* gen. nov., sp. nov., a novel

Gram-p polyhydroxyalkanoate accumulating coccus isolated from activated sludge," *International Journal of Systematic and Evolutionary Microbiology*, vol. 52, p. 1845–1849, 2002.

[11] M. Fernanda, B. Diego, P. Gabriel, et al., "Polyhydroxyalkanoates production by actinobacteria isolated from soil," *Canadian Journal of Microbiology*. vol. 55 (7), p. 790, 2009.

[12] S. A Ataei, E. V. Farahani, S. A. Shojaosadati, et al., "Poly(hydroxybutayrate- Co –hydroxyvalerate) production in batch fermentation of cheap substrates with Co- cultures of *Propionibacterium shermanii* and a Bacillus strain isolated from date syrup waste," 2014.

[13] M. U. Arshad, N. Jamil, N. Naheed, et al., "Analysis of bacterial strains from contaminated and non-contaminated sites for the production of biopolymers," *The African Journal of Biotechnology*, vol 6, no. 9, p. 1115-1121, 2007.

[14] Y. Doi, S. Kitamura, and H. Abe, "Microbial synthesis and characterization of poly(3- hydroxybutyrate-co-3-hydroxyhexanoate), " *Macromolecules*, vol. 28, p. 4822-4828, 1995.

[15] P. Kahar, T. Tsuge, K. Taguchi, et al., "High yield production of polyhydroxyalkanoates from soybean oil by Ralstonia eutropha and its recombinant strain," *Polymer Degradation and Stability*, vol. 83, no. 1, p. 79-86, 2004.

[16] P. Spiekermann, B. H. A. Rehm, R. Kalscheuer, D. Baumeister, et al., "A sensitive, viable – colony staining method using Nile red for detection screening of bacteria that accumulate polhydroxyalkanoic acids and other lipid storage compounds," *Arch. Microbiol*, vol. 171, p. 73-80, 1999.

[17] A. G. Ostle, and J. G. Holt, "Nile blue A as a stain for poly-β-hydroxybutyrate,"*Appl.Environ.Microbial*, vol. 44, p. 238-241, 1982.

[18] M. J. Janda and S. L. Abbott, "*16S rRN A* gene sequencing forbacterial identification in the diagnostic laboratory: pluses, perils, and pitfalls," *J. Clin. Microbiol*, vol. 45, no. 9, p. 2761–2764, 2007.

[19] J. B. Patel , "*16S rRNA* gene sequencing for bacterial pathogen identification in the clinical laboratory," *Molecular Diagnosis*, vol. 6, no. 9, p. 313-321, 2001.

[20] M. G. Wise, J. V. McArthur and L. J. Shimkets, "Bacterial diversity of a Carolina bay as determined by *16S rDNA* gene analysis: confirmation of novel taxa," *Appl Environ Microbiol*, vol. 63, p. 1505, 1997.

[21] A. Carolina G. Martı´nez, L. Hever, Q. Jose, G. Maldonado, et al., "Screening of polyhydroxyalkanoateproducing bacteria and PhaC-encoding genes in two hypersaline microbial mats from Guerrero Negro, Baja California Sur, Mexico," *Peer J*, DOI 10.7717/peerj.4780. 2018.

[22] G. Singh, A. Mittal, A. Kumari, V.Goel, N.K. Aggarwal, et al.. "Optimization of poly-B-hydroxybutyrate production from *Bacillus* species. *European Journal of Biological Sciences*," vol. 3, no. 4, p. 112–116. 2011.

[23] A. K. Bhuwal, G. Singh, N. K. Aggarwal, et al., "Isolation and screening of polyhydroxyalkanoates producing bacteria from pulp, paper, and cardboard industry wastes," *International Journal of Biomaterials*, Hindawi Publishing Corporation. 2013.

[24] W Alcaraz-Zapata, A Acosta-Cárdenas and A. F. Villa-Restrepo, "Evaluation of polyhydroxyalkanoate (PHAs) production with a bacterial isolate using cassava flour hydrolysates as an alternative substrate," *DYNA*, vol. 86, p. 75-81, 2019.

[25] G. Braunegg, B. Sonnleitner and R. M. Lafferty, "A rapid gas chromatographic method for the determination of poly- β-hydroxybutyric acid in bacterial biomass," *European Journal Applied Microbiology Biotechnology*, vol. 6, p. 29–37, 1978.

[26] K. Nasir, R. Batool and N. Jamil, "Scale-Up Studies for Polyhydroxyalkanoate and Halocin Production by *Halomonas* Sp. as Potential Biomedical Materials, " *Journal of Biomimetics, Biomaterials and Biomedical Engineering*, vol. 56, p. 49-60. 2022.

[27] S. Kitamura and Y. Doi, "Staining method of poly (3- hydroxyalkanoic acids) producing bacteria by Nile blue, " *Biotechnol.Tech*, vol. 8, p. 345-350, 1994.

[28] J. S. Yoon, J. Y. Kim and Y. H. Rhee, "Effects of amino acid addition on molar fraction of 3- hydroxyvalerate in copolyester 3-hydroxybutyrate and 3- hydroxyvalerate synthesized by *Alcaligenes* sp. SH-69," *Journal of fermentation and bioengineering*, vol. 80, no. 4, p. 350-354, 1995.

[29] N. Sinaei1, D. Zare1 and M. Azin, "Production and characterization of poly 3-hydroxybutyrate-co-3-hydroxyvalerate in wheat starch wastewater and its potential for nanoparticle synthesis," *Brazilian Journal of Microbiology*, 2021.