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Study the characters of poly- β -hydroxybutyrate isolated from local sample in Babylon

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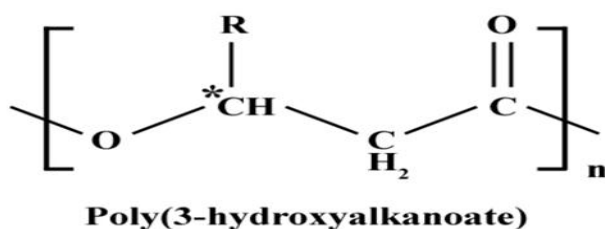
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1.Introduction

At the date 2017, the virgin plastics have been produced to reach about 8300 million metric tons (Mt). approximately 9% of this amount had been recycling, and 12% was incinerated, and 79% was aggregate in natural environments or the landfills (Geyer *et al.*, 2017). petroleum-derived polymers are alternatives by the biopolymers because of their non-toxic, eco-friendly producing processes and large ranges of applications in many sections of expendable product to medical field (Anjum *et al.*, 2016). Poly-hydroxalkanoates (PHAs) biodegradable and biocompatible polyesters family, production and storage in intracellular granules by microorganisms that use the PHB as carbon and energy supply below aerobically and stress conditions (Viloria *et al.*, 2017). several bacterial species are able to synthesize and accumulated PHB, among them *Cupriavidus necator* (formerly known as *Ralstonia eutropha*), *Pseudomonas sp.*, *P. denitrificans* and *P. extorquens*, as a results to limited nutritional conditions, such as

an carbon supply increase and decrease in other nutrient (nitrogen and phosphorus source) (Peña *et al.*, 2014; Chanprateep, 2010). monomer units of poly-hydroxyalkanoates molecule usually created from 600 to 35,000 (R)-hydroxy fatty acid (Khanna and Srivastava, 2005). The saturated alkyl groups are the most R group or side chain in the monomer unite of PHA (Figure 1). but the less common forms are of unsaturated alkyl groups, substituted alkyl groups, and branched alkyl groups (Lu *et al.*, 2009). The PHB molecule is amphiphilic- methyl groups are hydrophobic alternate with carbonyl ester are hydrophilic appropriately, the methyl groups may contract in hydrophobic cooperation, and the ester carbonyl oxygens may act as hydrogen-bond acceptors or as ligands for cations (Reusch, 2013). Bioplastics can be degraded and catabolized by a variety of microorganisms, including aerobes, anaerobes, photosynthetic bacteria, archaeobacteria and lower eukaryotic organism. These bacteria may grow in a wider range of environments, including soil and compost (Lee *et al.*, 2005; Kumaravel *et al.*, 2010; Accinelli *et al.*, 2012).



<i>R</i> group	Carbon no.	PHA polymer
methyl	C ₄	Poly(3-hydroxybutyrate)
ethyl	C ₅	Poly(3-hydroxyvalerate)
propyl	C ₆	Poly(3-hydroxyhexanoate)
butyl	C ₇	Poly(3-hydroxyheptanoate)
pentyl	C ₈	Poly(3-hydroxyoctanoate)
hexyl	C ₉	Poly(3-hydroxynonanoate)
heptyl	C ₁₀	Poly(3-hydroxydecanoate)
octyl	C ₁₁	Poly(3-hydroxyundecanoate)
nonyl	C ₁₂	Poly(3-hydroxydodecanoate)
decyl	C ₁₃	Poly(3-hydroxytridecanoate)
undecyl	C ₁₄	Poly(3-hydroxytetradecanoate)
dodecyl	C ₁₅	Poly(3-hydroxypentadecanoate)
tridecyl	C ₁₆	Poly(3-hydroxyhexadecanoate)

Figure (1): Poly(3-hydroxyalkanoate) chemical structure (PHA). The functional alkyl R group persists in the nomenclature, carbon number for PHA compounds (Khanna and Srivastava, 2005).

2. Material and Methods

2.1. Collection of soil samples

This study involved 35 Samples of soil were collected from altered land in Iraq/Babylon specially that with hydrocarbon polluted. All samples were kept separately in the sterile plastic bags and transferred to the laboratory. After a serial dilution of each sample, culture from dilution (10^{-4}), spread on nutrient agar then incubate at 37°C for 24 hours. The pure colonies of each sample were picked and subculture for several times and kept for screening of PHB production.

2.2. Media of fermentation

The bacterial isolates were grown in 250 ml conical flasks, containing 50 ml of MA medium with inoculum size 5%, pH 8, incubation time 48 hours and 150 rpm. MA medium include (g/L): NaCl, 30; NaBr, 0.06; CaCl₂, 0.09; KCl, 0.5; KH₂PO₄, 0.25, MgSO₄·7H₂O, 0.25; yeast extract, 2.0; glucose, 20 sterile by autoclave for 15 minute (Thurc and Vóc, 2016). 20 FeCl₃O·6H₂O, 10CaCl₂·H₂O, 0.05MnCl₂·4H₂O and 0.10gZnSO₄·7H₂O, and 0.03CuSO₄·5H₂O are included in the trace element solution (g/l) (Yousif *et al.*, 2016). filtration used to sterilized trace element solution.

2.3. Poly-β-hydroxybutyrate Extraction: (Getachew and Woldesenbet, 2016)

From culture media centrifuged 10 ml at 10,000 rpm / 15 min. scrapped the supernatant and treated pellet with 10 ml sodium hypochlorite, after that incubated combination at 37 °C for 1 h (converted). Centrifuged combination at 5000 rpm / 15 min after that washed with distilled water, acetone and methanol (replace by ethanol) respectively. Dissolved the pellet in 5 ml boiling chloroform and filter by Watman filter NO.1, evaporated by streaming the solution on a sterile glass tray finally preserved at 4 °C and weighed. The corresponding PHB agglomeration by the altered isolates was compared to help in identification of the high production. The amount of PHB agglomeration was predicted as the percentage composition of PHB found in the dry cell weight, which was estimate using the following formula.

$$\text{“PHB accumulation (\%)} = [\text{Dry weight of extracted PHB (g/L)} / \text{DCW (g/L)}] \times 100\text{”}$$

2.4. Measurement of cell dry Weight (CDW) (Yousif et al., 2016)

After centrifuge at 14000 rpm/minute for 15 minutes of culture medium, the supernatant was scrapped and the pellet was washed with distilled water. washed pellet was re-suspended in 1 mL DW, transferred the pellet to pre-weighed test tube and dried to constant weight at 60°C. The CDW was estimate as in the formula:

$$\text{CDW (g/L)} = [(\text{Final weight of test tube} - \text{Initial weight of test tube}) / 50 \text{ ml}] \times 1000$$

2.5. PHB spectro photometer assay

A mixture of polymer and chloroform is transmitted to a test tube. After final evaporated of the chloroform, adding 10 ml of concentrated H₂ SO₄. capped tube with a glass marble and heated for 10 min at 100 °C in a waterbath. after hard mixing a sample, the solution is cooled and transmitted to a silica cuvette. Absorbance at 235 nm in a UV spectrophotometer is estimated contrast to a sulfuric acid blank (Belal and Farid, 2016)

3. analysis of Poly-β-hydroxybutyrate .

3.1. Differential Scanning Calorimetry Analysis (DSC)

The thermal properties of extracting polymer was assay by instruments Pyris 1 equipped without cooling component. The apparatus was balanced at room temperature before test in apparatus, at heating rate 10 °C /min. 7 mg of the polymer was enclosed in an aluminum pan and maintained at about 0 °C for 1 minute followed by heating to 600 °C at heating rate 10 °C/min. The second heating was prepared below the similar manner except for the heating which started from 0 °C to 200 °C. Melting temperature (T_m) were estimate from DSC thermogram.

3.2. Thermo gravimetric Analysis (TGA)

This apparatus used to estimate the thermal stability of PHB, it was resolved by the instrument STA 6000 (Perkin Elmer, USA). About 10 mg of PHB product was weighted with an aluminum pan and after that heated from

0 °C to 600 °C at a heating range of 10 °C/min below argon atmosphere. This assay was estimate the decomposition temperature (*TD*) which means as a temperature for 5% weight decrease of the PHB sample.

3.3. Fourier Transform Infrared Spectroscopy (FTIR)

The chloroform extract of PHB (4 mg) was fused efficiently with KBr (Spectroscopic grade) and dried at 100 °C for 4 h. Infrared spectra of the PHB sample was recorded and assay on a individual beam Perkin Elmer (Spectrum BX series, Japan), with the sequent scan parameters: scan in the range of 4000–400 cm⁻¹; number of scans, 16 and resolutions, 4.0 cm⁻¹ (Patel *et al.*, 2015).

3.4. Determent the Molecular Weight of the polymer.

The assay of PHB molecular weight, the PHB was returned by applying organic solvents as previously (James and Prichard, 1974). The returned polymer were dissolved in 100 ml of chloroform at a concentration of (0.05, 0.2, 0.4, 0.7, 0.9) gm and measurement of viscosity and density

$$\eta_2 = \frac{\eta_1 \rho_2 t_2}{\rho_1 t_1}$$

Here t_1 and t_2 are the time of flow of the and ρ_1 and ρ_2 are the corresponding densities, and η_1 is the coefficient of viscosity of water. liquid η has a particular value at the similar temperature. From the Mark-Houwink equation the relationship among the molecular weight and viscosity are given below

$$[\eta] = KM^\alpha$$

Where $[\eta]$ is the intrinsic viscosity, M is Molecular weight, $K = 7.7 \times 10^{-5}$ and $\alpha = 0.74$ are constants for a particular polymer solvent system.

3.5. GC Assay

To analysis the cellular polyester content and polymer composition, approximately 15 mg dry cells was exposed to methanolysis with a solution include 1.7 ml methanol, 0.3 ml 98% sulfuric acid and 2.0 ml chloroform at 100 °C for 140 min to change the component to their methyl esters (Braunegg *et al.* 1978). Add 1 ml water to the reaction combination convinced phase separation. lower chloroform layer was used for gas chromatography (GC) analysis. the GC analysis took about 30 minutes and the program was set as follows: **Injector:** Carrier gas : Nitrogen, Injection temperature : 280°C, Pressure : 100 kPa, Purge flow : 14.0 ml/min. **Column Oven (SE-30):** Initial temperature : 100°C, Final temperature : 300°C. **Flame ionization detector:** Temperature : 330 °C, Hydrogen flow rate : 40 ml/min, Compress air flow rate : 400 ml/min

4. Results and Discussion

4.1. Isolation and determent the high production isolate.

This study involved thirty-five soil sample polluted by hydrocarbon materials from a different position of Babylon province. Forty-five (Table (1)) isolates were different in morphology, size, and type of edge of the colony, this isolates were sup-culture on nutrient agar. This isolates were separation depending on gram stain, spore formation, morphology on nutrient agar, Blood agar, Oxidase, Catalase, growth on Macconkey agar, mannitol salt media, and egg yolk agar.

Table (1): Different genus of bacteria isolated from hydrocarbon soil in Babylon Province.

Isolates	No. of isolating
<i>Pseudomonas sp.</i>	2
<i>Micrococcus sp.</i>	1
<i>Staphylococcus sp.</i>	15
<i>Streptococcus sp.</i>	7
<i>Bacillus sp.</i>	20
Total	45

Only three isolate were positive when analysis by UV-Spectrophotometer as describe below. The percentage of extract PHB differs between isolates return to *Bacillus* species range from 1.4 - 4.3 % on MA medium for 48 hours, glucose (1%), and 37 °C (Table (2)). The average of production differs depending on species, also the media were used to produce the polymer effect on the production. Terrestrial Bacteria *B. cereus* SE-1 appear much PHB agglomerate cells (40%) than that of the marine *Bacillus* sp. CS-605 (33%) on Minimal Davis broth (Bhagowati *et al.*, 2015). While, the maximum production of PHB from *Lactobacillus plantarum* was (4%) on nitrogen limited minimal medium (NLMM) (Natarajan *et al.*, 2017)

Table (2): Comparison between PHB extracted from different *Bacillus* isolates at 37°C, 48 hours, glucose (1%), and 100 rpm.

S.D			
Isolation NO.	CDW (g/l)	PHB (g/l)	PHB%
Bacillus (1)	0.44±0.02 b	0.0192±0.00 a	4.3±0.20 c
Bacillus (2)	0.46±0.02 b	0.0176±0.09 a	3.8±0.04 b
Bacillus (11)	0.25±0.04 a	0.0035±0.00 a	1.4±0.10 a

various letters in the one bollard refer to significant difference ($p \leq 0.05$) .M (mean). S. D Stander deviation.

4.2. Analysis of polymer extracted.

4.2.1. UV-Spectrophotometer.

The extracted polymers (50 µg) when hydrolysis by H_2SO_4 (99 %) converted to crotonic acid, analysis by UV-spectrophotometer shows a peak at 235 nm in Figure (2). This peak proves the occurrence of PHB. This result was similar in previously reported by Getachew and Woldeesenbet (2016).

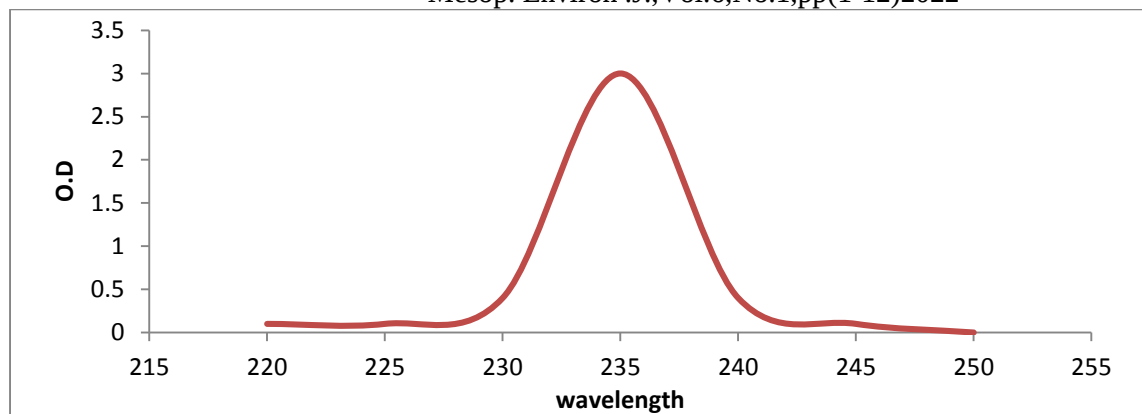


Figure (2): UV-spectrophotometer spectrum of PHB compounds extracted from *Bacillus cereus* strain M13.

4.2.2. Fourier Transform Infrared Spectroscopy (F.T.I.R) of PHB.

The PHB that extracted from isolate from positive Nile blue A and Nile red was scanned between a wave number of (400 and 4,000 cm^{-1}) the band at 1743.65 cm^{-1} refer to C=O this band at this point refer to the amorphous phase of PHB (Zhang *et al.*, 2005). The bands 2926.01 at and 2854.65 cm^{-1} evidence the existence of an alkyl-CH₃ group. The band at 1460.11 cm^{-1} is correlate to the asymmetric bending of -CH₂ or -CH₃ (Ramezani *et al.*, 2015). Whereas, bands at 1384.89, 1315.45 cm^{-1} refer to C-OH bond. range of bands from 1020.34-1238.30 cm^{-1} showed the C-O bonding (Figure (3)). The results coordinate with the article of Alarfaj *et al* (2015). Bagheriasl (2013) absorbed that the band at 1047.35 cm^{-1} and 1089.76 cm^{-1} related to C-CH₃ stretching and C-O-C stretching, respectively.

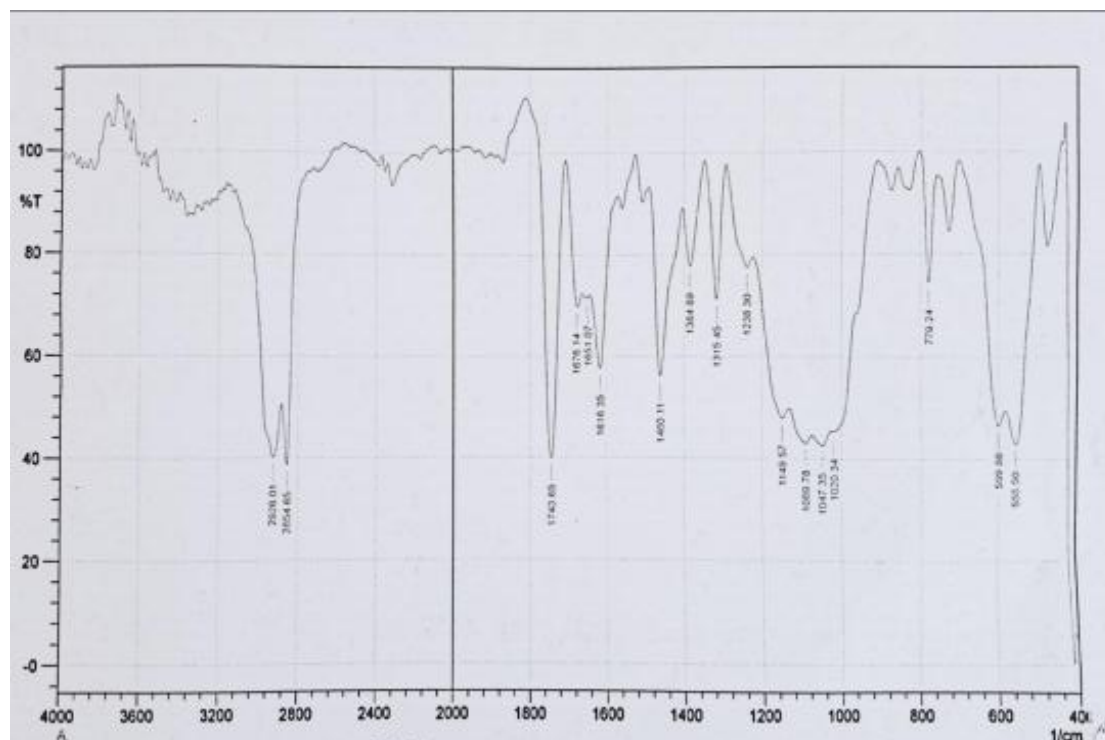


Fig.(3):- FTIR analysis Of extracted Poly- β -hydroxybutyrate.

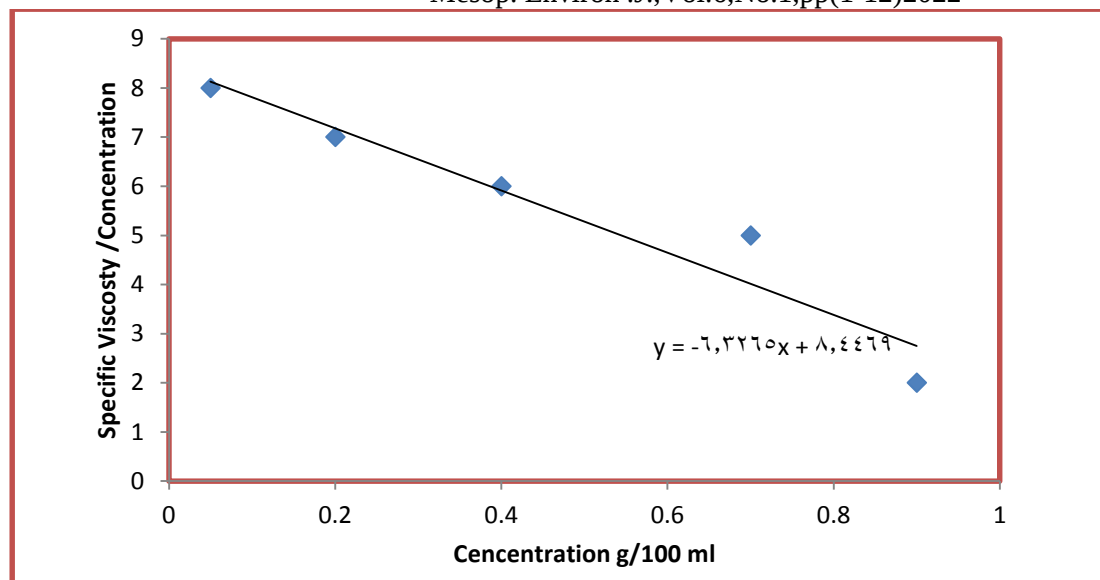


Fig. (4): specific viscosity of PHB extracted from *Bacillus cereus* strain M13.

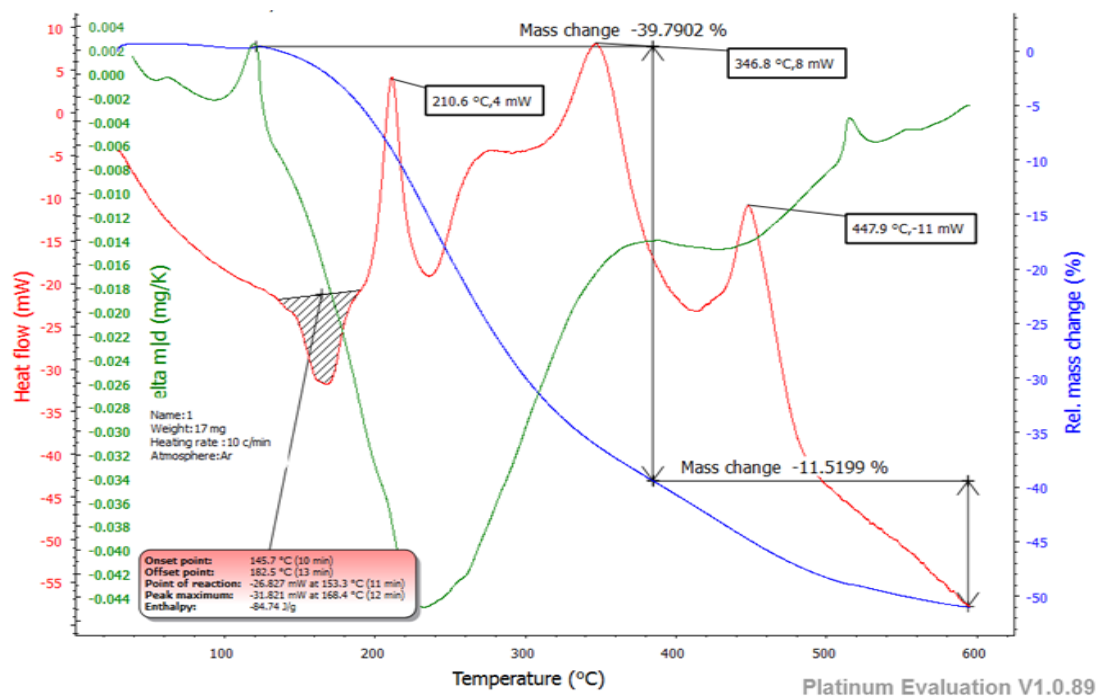


Fig.(5): Thermogravimetric (TGA) and Differential Scanning Calorimetry (DSC) of pure poly-β-hydroxybutyrate.

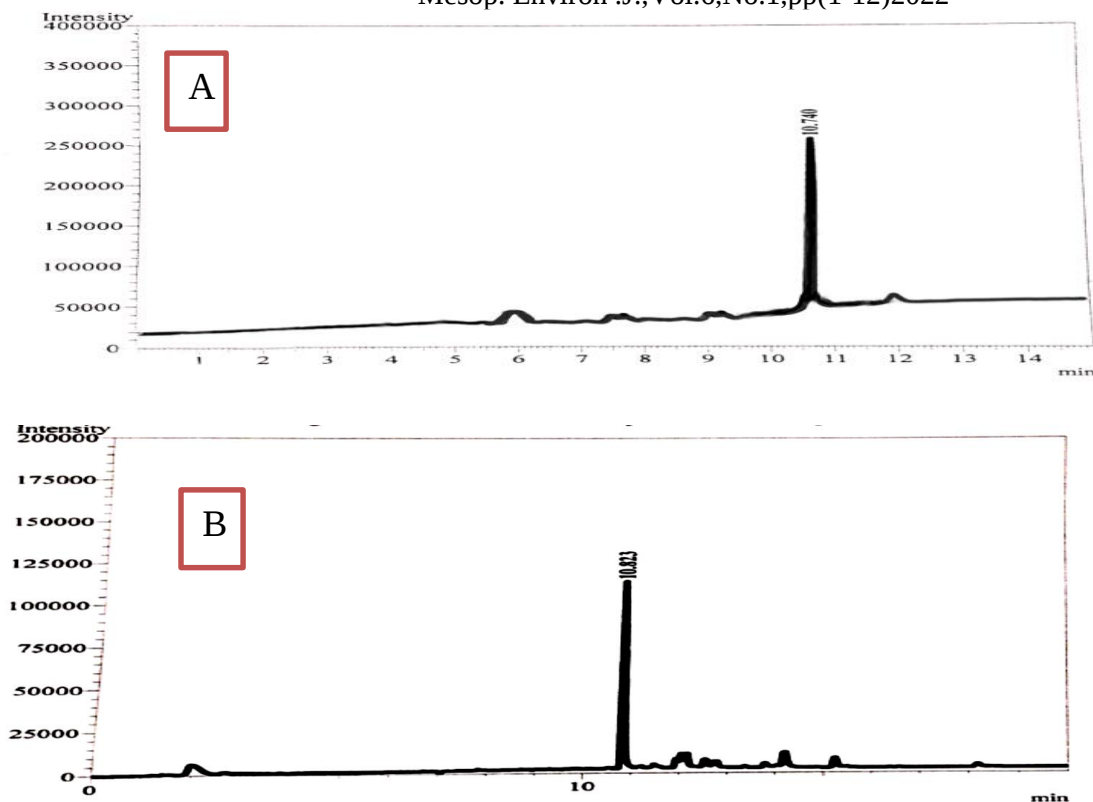


Fig. (6): GC-MS of PHB extracted from *Bacillus cereus* strain M13 at peaks 10.740(A). and PHB from sigma company (B).

4.2.3. Molecular Weight.

Low in Mw (< 1000 kDa) of PHB is much brittle and low elastic in comparison to PHB of above Mw (≥ 1000 kDa) or ultrahigh Mw (≥ 4000 kDa). (Peña *et al.*, 2014; Singh *et al.*, 2015). The molecular weight was 6.4×10^6 Dalton of polymer extracted in this study figure (4). So, the producer of PHB in high Mw is accepted for its application in the biomedical range, as surgical implants, scaffolding in tissue engineering, where a tensile strength and high elasticity are requiring (Peña *et al.*, 2014; Chen and Wang, 2013). In this study, the molecular weight of poly- β -hydroxybutyrate extracted from *Bacillus cereus* strain M13 was measured by viscosity use viscometer at 30°C.

4.2.4. GC-MS assay.

This analysis is important in illustrating the design of the components. The key composite of concern were studied depended on their retention peak. PHB extracted from *B. cereus* strain M13 culture in HM-media when use of glucose as a carbon source was analyzed by GC-MS appear a monomer chains of PHB without dimer in retention time (10.740) min Figure (6). The peak at 10.740 min was related to Methyl ester. This result is the same as of GC-MS observed by Devi *et al.*, 2015. The result also was similar to standard poly- β -hydroxybutyrate from (Sigma) company. GC-MS of standard shows peak at retention time 10.823 that provides the polymer extract was poly- β -hydroxybutyrate.

4.2.5. Thermal Properties of Polymer.

The thermal properties of extracted PHB considered by (DSC and TGA). The results were showed the melting temperature (T_m) of the extracted PHB usage of glucose as a carbon supply was between the onset and offset degree

(145.7° C-182°C) figure (5). This results were reported in previous study (Medvecky, 2011). The thermal degradation of pure PHB proceeds by two reaction. The first reaction occurs at 210.6 °C and the second reaction was at 364.8 °C. Between 153.3°C and 380 °C the mass change was (-39.7902 %) and between 380 °C and 600 the mass change was (-11.5199 %), while at 600 °C was occurring as a final reaction of polymer. The total mass loss was 51.3101% figure (3-12) . Dhangdhariya *et al* (2015) the PHA was degraded in two phase and completely degrade at 300°C, while our study revealed that the PHB was degraded in three stages and resist till 446.9°C. On the other hand, PHB from *Bacillus shackletonii* K5 was degraded totally in two phase and reach to 280°C as high temperature (Liu *et al.*, 2014).

Conclusion

Bacillus sp. were the most genus from soil bacteria had ability to produce of poly- β -hydroxybutyrate in this study. The primary screened of polymer was study by UV-Spectrophotometer. The functional group of the polymer was study by use FTIR, in addition the content of polymer were determent by GC and thermal properties also has study . The high molecular weight of obtained PHB from this study can be recycled as a alternate for manufactured polymers. so, make it used in commercial operations.

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