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

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Abstract:

The most of *Bacillus* species was able to produce poly-β-hydroxybutyrate isolated from soil. Use of MA media and provide the necessary elements by used trace elements for bacterial growth and Poly-β-hydroxybutyrate production in addition to using glucose as carbon source (1%).Gas chromatography(GC) state that obtain biopolymer was homopolymer poly-β-hydroxybutyrate (PHB) composition.Fourier Transform Infrared Spectroscopy(FTIR)FTIR show that, the functional group of extracted polymer, peak at 1743.71 cm^{-1} be related to the (C=O) characters to the amorphous phase of the extracted polymer. In addition, thethermal properties of the extracted polymer were studied using ofDifferential ScanningCalorimetry Analysis(DSC) and Thermo gravimetric Analysis(TGA), the temperature melting(T□)was (142-182°C), While the degradation occur in two reactions from (210.6-447.9°C). Molecular weight was 6.4* 10^6 Dalton.

Keywords:poly-β-hydroxybutyrate, FTIR, Differential Scanning Calorimetry Analysis, Thermo gravimetric Analysis.

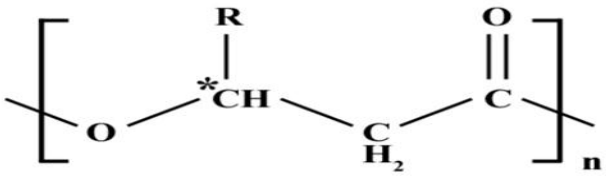
1.Introduction

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 naturalenvironments or the landfills [1]. 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 [2].

Poly-hydroxalkanoates (PHAs) biodegradable and biocompatible polyestersfamily, production andstorage in intracellular granules by microorganisms that use the PHB as carbon and energy supply below aerobically andstress conditions [3].several bacterial species are able to synthesized and accumulated PHB, among them *Cupriavidus necator* (formerly known as *Ralstonia eutropha), Pseudomonas sp., P. denitrificans* and *P.extorquens*, as a results to limited nutrimental conditions, such as an carbon supplyincrease and decrease in other nutrient (nitrogen and phosphorus source) [4] [5].

monomer units of poly-hydroxalkanoatesmoleculeusually created from 600 to 35,000 (R)-hydroxy fatty acid [6]. The saturated alklyl groups are the most R group or side chain in the monomer unite of PHA (Figure 1).but the less common forms areof unsaturated alkyl groups, substituted alkyl groups, and branched alkyl groups [7].

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 actas hydrogen-bond acceptors or as ligands for correlative bonds to cations [8].Bioplastics can be degraded and catabolized by a variety of microorganisms, including aerobes,anaerobes, photo syntheticbacteria, archaebacterialand lowereukaryotic organism. These bacteria may grow in a widerange of environ ments, includingsoil and compost [9] [10] [11]. The aim of this study to determent the poly-β-hydroxybutyrate extracted from *Bacillus cereus* strain M13.



Poly(3-hydroxyalkanoate)

R 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)

Fig.1:Poly(3-hydroxyalkanoate) chemical structure (PHA). The functional alkyl R group persists in the nomenclature, carbon number for PHA compounds [6].

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^{-}□), spread on nutrient agar thenincubate at 37°C for 24 hours. The pure colonies of each sample were pickedand subculture for several times and kept for screening of PHB production**.**

2.2.Preparation of Fermentation Medium.

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 (medium for PHA production - MA)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[12]. 20 FeCl3O·6H2O,10CaCl2·H2O,0.05MnCl2·4H2O and 0.10gZnSO4·7H2O, and 0.03CuSO4·5H2Oare included in the trace element solution (g/l) [13]. filtration used to sterilized trace element solution.

2.3.Poly-β-hydroxybutyrate Extraction:

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). Centrifugedcombination at 5000 rpm /15 min after that washed with distilled water, acetoneand methanol (replace by ethanol) respectively [14]. Dissolved the pellet in 5 ml boiling chloroformand 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.

"PHB accumulation (%) = [Dry weight of extracted PHB (g/L) / cell dry weight (g/L)]× 100"

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 distilledwater. washedpellet was re-suspended in 1 mL DW, transferee the pellet to pre-weighed test tube and dried to constant weight at 60ºC. The CDW was estimate as in the formula:

CDW (g/L) =[(Final weight of test tube-Initial weight of test tube)/50 ml]*1000

2.5.PHB spectrophotometer assay

A mixture of polymer and chloroform is transmitted to a test tube. After final evaporated of the chloroform, adding 10 ml ofconcentrated H_2 SO_4 . 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 [15].

3.analysis of Poly-β-hydroxybutyrate .

3.1. Differential ScanningCalorimetry Analysis (DSC)

The thermalproperties 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 byheating 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. Thermogravimetric 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 panand 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, Japain), with the sequent scan parameters: scan in the range of 4000–400 cm^{-1}; number of scans, 16 and resolutions, 4.0 cm^{-} 1 [16].

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 [17]. 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[η]is the intrinsic viscosity, M is Molecular weight, K =7.7 * 10 $^{-5}$ and α =0.74 are constants for a particular polymer solvent system.

3.5.Gas chromatography (GC)

To analysis the cellularpolyester content and polymercomposition, approximately 15 mg dry cells wasexposed to methanolysiswith 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 [18]. 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 (Bacillus cereus) were culture on nutrient agar. This isolates were separation depending on gram stain,

spore formation, morphology on nutrient agar, Blood agar, Oxidase, Catalase, growth on Maconkey 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**
Pseudomonas sp.	2
Micrococcus sp.	1
Staphylococcus sp.	15
Streptococcus sp.	7
Bacillus sp.	20
Total	45

Only three isolate (*Bacillus* No.1,2, 11) 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 isolate Bacillus No. 1 was choose to extracted poly-β-hydroxybutyrate .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 [19]. While, the maximum production of PHB from *Lactobacillus plantarum* was (4%) on nitrogen limited minimal medium [20].

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. UV-Spectrophotometer.

The extracted polymers (50 µg) when hydrolysis by H_2 SO_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 [6].

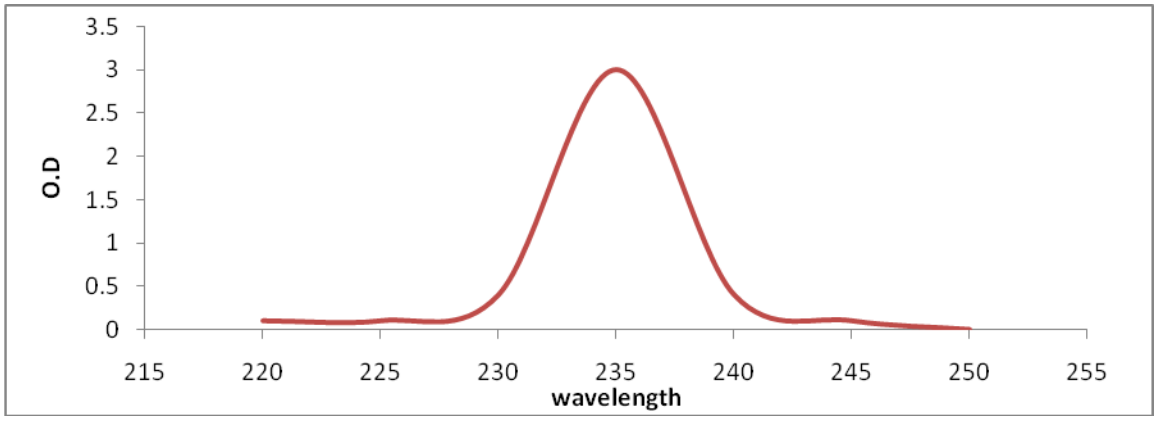


Fig.2: UV–spectrophotometer spectrum of PHB compounds extracted from *Bacilluscereus* strain M13.

4.3. 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 [21]. 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 [22]. 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 [23]. [24] 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.

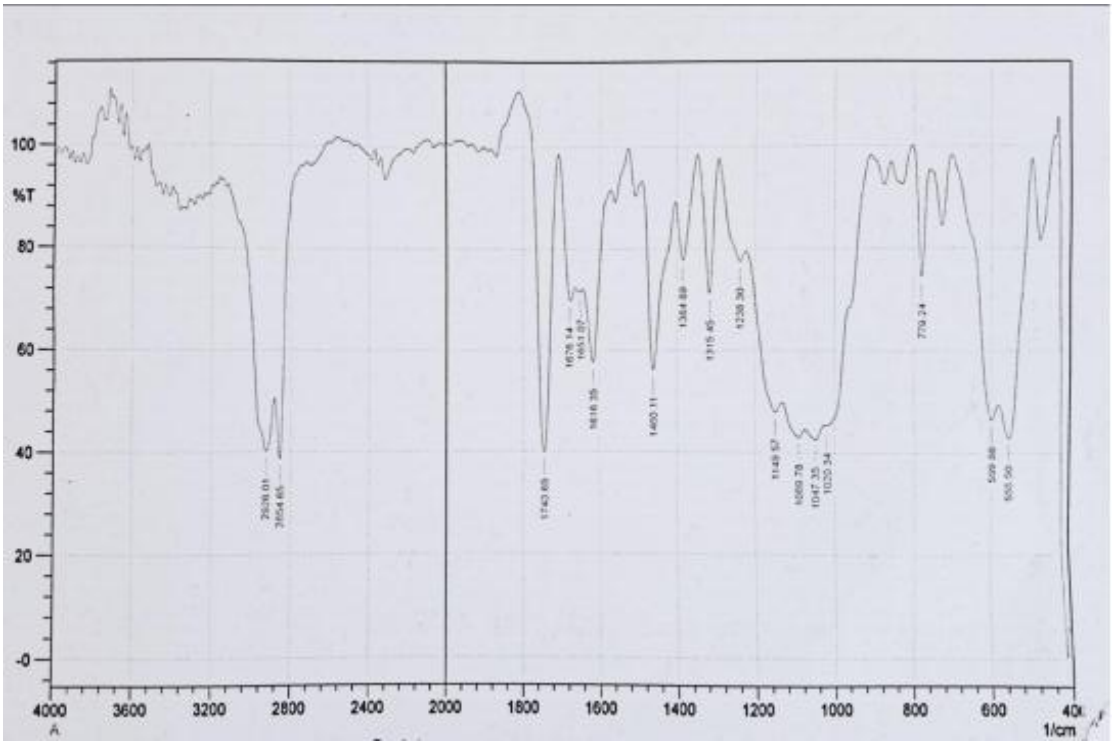


Fig.3: FTIR analysis Of extracted Poly-β-hydroxybutyrate.

4.4.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). [4][25]. The molecular weight was 6.4 $*10^6$ Dalton of polymer extracted in this study figure (4).So, the producer of PHB in highMw is accepted for its application in the biomedical range, as surgical implants, scaffolding intissue engineering , where a tensile strength and high elasticity are requiring [4][26] .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.

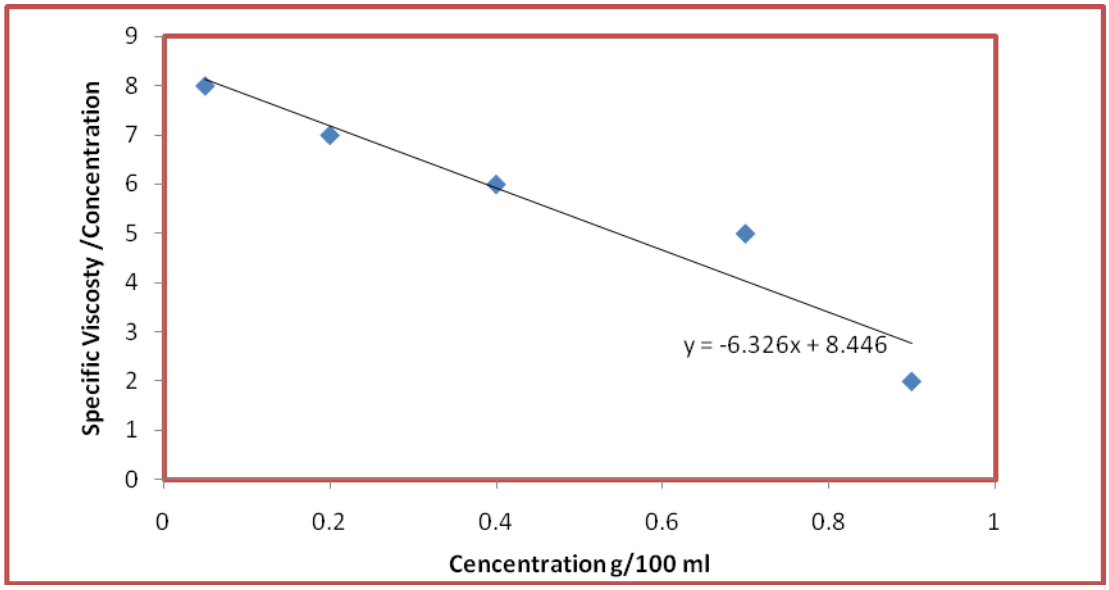


Fig.4:specific viscosity of PHB extracted from *Bacillus cereus* strain M13.

4.5. GC-MS assay.

This analysis important in illustration the design of the components. The key composite of concern were study depended on their retention peak. PHB extracted from *B. cereus* strain M13 culture in MA-media when use of glucose as a carbon source was analysis by GC-MS appear a monomer chains of PHB without dimer in retention time (10.740) min Figure (5). The peak at 10.740 min was related to Methyl ester. This results same as of GC-MS observed by [27]. The result also was similar to stander poly-β-hydroxybutyrate from (Sigma) company. GC-MS of stander show peak at retention time 10.823 that provide the polymer extract was poly-β-hydroxybutyrate.

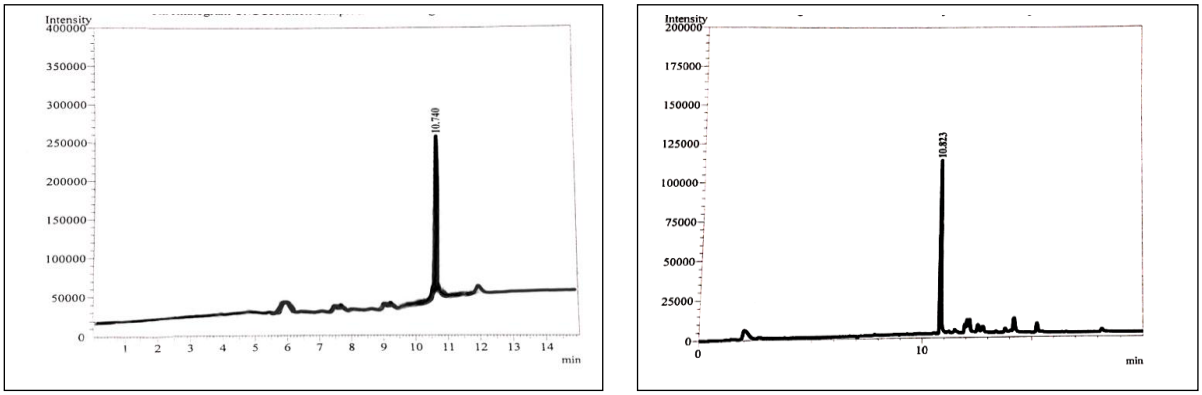


Fig.5:GC-MS of PHB extracted from *Bacillus cereus* strain M13 at peaks 10.740(A). and PHB from sigma company (B).

4.6. Thermal Properties of Polymer.

The thermal properties of extracted PHB considered by (DSC and TGA). The results were showed(figure (6)) 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). This results were reported in previous study [28]. 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 (6). [29] 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 degradedtotally in two phase and reach to 280°C as high temperature [30].

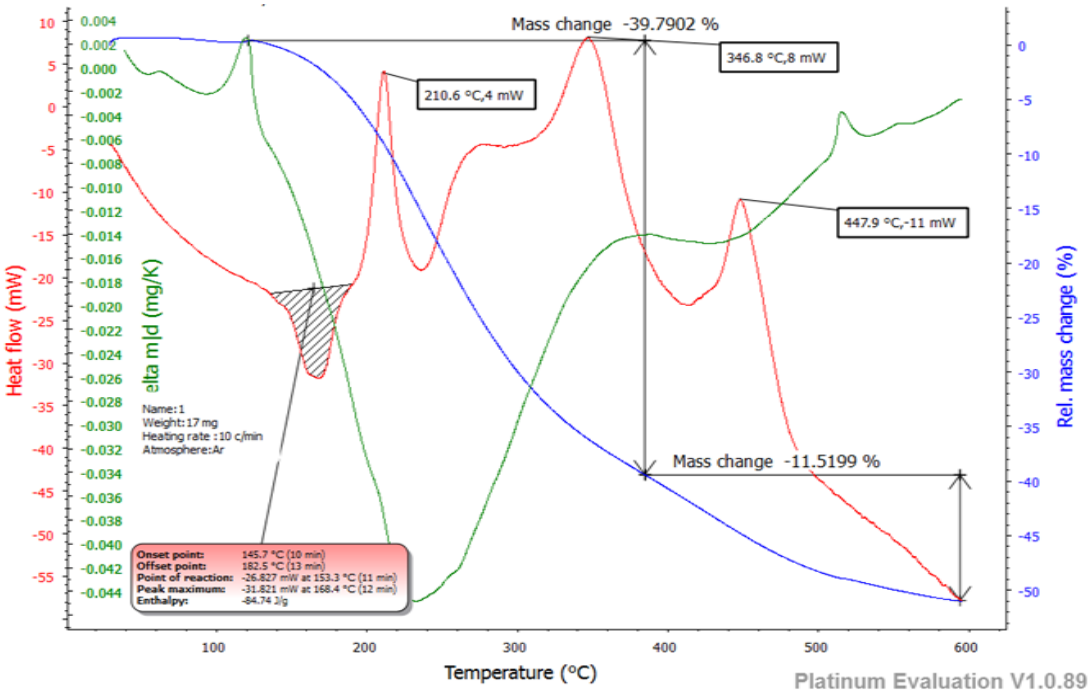


Fig.6: Thermogravimetric (TGA) andDifferential Scanning Calorimetry (DSC) of pure poly-β-hydroxybutyrate.

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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