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Extraction and characterization of *β*-hydroxybutyrate from *Azotobacter chroococcum 9*

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Azotobacter chroococcum 9 was isolated from corn field in Agricultural College in Abu Ghrab , β-hydroxybutyrate was extracted from *A. chroococcum 9* by different methods including sodium hypochlorite , SDS and sodium hypochlorite and by NaOH. The optimum recovery of PHB was 39.5% by using sodium hypochlorite extraction method .The molecular weight of PHB extracted from *A. chroococcum 9* was 2.6×10^5 dalton and the melting point was 180 ˚C .The solubility of PHB in several solvents was studied . PHB was soluble in chloroform and in 1-2 dichloroethanol , while it was insoluble in methanol , ethanol , acetone , hexane , diethyl ether and carbon tetrachloride .The viscosity of *A. chroococcum 9* PHB was determined at different pH values , a decreasing in viscosity was detected at acidic pH (pH 4) and at alkaline pH (pH 10) which indicates a reducing in PHB MW , while at neutral pH (pH 7) there was no change in viscosity .

Keywords: Poly -*β*-hydroxybutyrate, *Azotobacter chroococcum*, HPLC, FTIR

INTRODUCTION

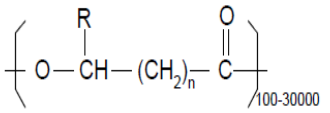
Poly -*β*-hydroxybutyrate (PHB) is the most abundant of a wide range of high-molecular-mass microbial polyhydroxyalkanoates(Henderson, R. A. and C. W. Jones ,1997) . It is a unique intracellular polymeric material accumulating under unbalanced growth conditions in a wide variety of bacteria. It is regarded as a source of potentially useful biodegradable natural plastics since its physical characteristics are similar to those of petrochemical polyesters such as polypropylene (Sujatha et.al.,2005) . Therefore, PHB and its copolymer can be used as Biodegradable plastic , which can reduce the current problems with decreasing fossil resources and environmental impact caused by plastic garbage (Sangkharak . and Prasertsan , 2008). In addition, it has a promising application in medicine, material science and agriculture, etc.

The homopolymer PHB is a stiff and relatively brittle thermoplastic . It has several useful properties such as moisture resistance, water insolubility and optical purity, this differentiate PHB from other currently available biodegradable plastics which are either water soluble or moisture sensitive, it also shows good oxygen impermeability (Ojumu et. al., 2004).

Generally ,Polyhydroxyalkanotes (PHA) homopolymers are classified into three groups based on the number of carbon atoms in the monomer units incorporated into the polymer chain: short - chain length (SCL) with 3-5 carbon atoms in length , medium – chain length (MCL) 6-14 carbon atoms in length and long – chain length (LCL) with more than 14 carbon atoms in length (SharifzadehBaei et.al.,2010).

The majority of PHAs are composed of R(-)-3-hydroxyalkanoic acid monomers ranging from C3 to C14 carbon atoms with variety of saturated or unsaturated and straight- or branched chain containing aliphatic or aromatic side groups , PHA is a polyester of repeating subunits (100-30000) (Figure 1) . The molecular weight of the polymers are in the range at 2×10^5 to 3×10^6daltons, based

on the type of microorganism and growth condition (Ojumu et. al., 2004).



n =1	R =	hydrogen	poly (-3-hydroxypropionate)
		methyl	poly (-3-hydroxybutyrate)
		ethyl	poly(3-horoxyvalerate)
		propyl	poly (-3-hydroxyhexanoate)
		pentyl	poly (-3-hydroxyoctanoate)
		nonyl	poly (-3-hydroxydodecanoate)
n = 2	R =	hydrogen	poly (-4-hydroxybutyrate)
n = 3	R =	hydrogen	poly (-5-hydroxyvalerate)

Figure(1) :The general structure of polyhydroxyalkanoate

Un saturation in PHAs increases their elasticity, and different functional groups change the physical and chemical properties of the polymer. PHAs with short pendant groups (SCL) are hard, crystalline materials, whereas PHAs with longer pendant groups are elastomeric. The length, saturation and functional group of the side chain influence the properties such as melting point (Tm), glass transition temperature (Tg) and crystallinity (Eggink et. al.,1995).

The majority of separation processes that have been proposed involve the extraction of PHB from the cells with solvents . For example, PHB can be extracted from bacterial cells with methylene chloride , propylene carbonate , dichloroethane , or chloroform . As an alternative to the solvent extraction, aqueous enzymatic digestion methods have been developed by Zeneca (Holmes and Lim ,1990 ; Hasan,,2017). Another separation process that involves a differential digestion method employing sodium hypochlorite (Ramsay et.al., 1990) . The aim of this study was to obtain an efficient isolate of *Azotobacter* producing PHB and determination the stability of PHB at different conditions.

MATERIALS AND METHODS

Activation medium (Thompson and Skerman ,1979).

This medium is composed of : 0.3 g K_2HPO_4,0.7 g KH_2PO_4 ,0.2 g $MgSO_4.7\ H_2O$, 0.1 g $CaCl_2.2H_2O$, 0.05 g $FeSO_4.9H_2O$, 0.005 g $Na_2MoO_4.2H_2O$, 20 g Sucrose and 5 g Yeast extract , pH was adjusted to 7.3

Culture medium and production medium of PHB:

Sucrose mineral salts (SMS) (Becking , J.H., 1981)

This medium is composed of 10g Sucrose , 3g $CaCO_3$, 0.5g K_2HPO_4, 0.2g $MgSO_4.7H_2O$, 0.1g $CaSO_4$, 0.02g $FeSO_4.7H_2O$, 0.02g $MnSO_4.H_2O$, 0.01g MoO_3, 0.01gKI**,** for preparing of solid medium 2% agar was added , pH was adjusted to 7.2-7.3.

Stockdale nitrogen free medium (production medium) (Stockdale, H. et. al., 1968)

This medium was used routinely as a liquid medium and had the following composition per liter of distilled water .

Solution A: 20g Glucose, 0.4g $MgSO_4.7H_2O$, 0.11g $CaCl_2.2H_2O$, 0.012g $FeSO_4.7H_2O$, 0.01g $Na_2MoO_4.2H_2O$.

Solution B: 2g K_2HPO_4, 0.4g NaCl

Solution A and B were autoclaved at 121 ˚C/15 pounds for 10 min. and equal volumes were mixed after cooling ,the pH of the medium was 7.7 . Solid medium was prepared by addition 2% agar was added to the combined medium .

Collection of sample :

Twenty soil specimens were collected by sterile instruments, these specimens were obtained from soil near to plant roots at depth 10-15 cm and mixed together in clean sterile sacs and transported to laboratory .The bacteria used in the experiments was an isolate of *Azotobacter chroococcum* isolated from corn field in Agricultural College in Abu-Grab.

Isolation of *Azotobacter* :

Soil dilutions were prepared by adding 10 g of soil to 90 ml of sterile D.W in 250 ml flask and shaked for 20 min , serial dilutions (10^{-1} – 10^{-8}) were made . One ml from each dilution was used to inoculate Sucrose mineral salts medium (SMS) (Thompson , J . P. & V. B . D. Skerman ,1979) , and incubated at 28-30 ˚C for 3 days.0.1 ml of liquid culture was spreaded out on plate medium (triplicate plates) , the plates were incubated at 28-30 ˚C for 3 days . the growing bacterial colonies were purified by streaking on SMS medium for several times.

Extraction of PHB from bacterial cells:

Three methods for extraction of PHB from bacterial cells was performed:

1-Extraction with sodium hypochlorite solution (Aslim , B. et.al., 2002)

Ten ml of bacterial culture was centrifuged at 6000 rpm for 15 min , 10 ml of 5.64% sodium hypochlorite solution was added to the precipitate and incubated at 37 ˚C for 1h. The suspension was centrifuged at 6000 rpm for 15 min , the precipitate was washed with sterile D.W. Ten ml of ethanol was added to the precipitate and centrifuged and ten ml of acetone was added and centrifuged to obtain the precipitate. Ten ml of hot chloroform was added and dried by evaporation, then PHB was washed with D.W and dried at 45 ˚C for 2h.

2-Extraction with sodium hypochlorite and SDS (Tamer et.al., 1998)

Ten ml of bacterial culture was centrifuged at 6000 rpm for 20 min , then 5 ml of phosphate buffer pH 7.1 was added to the collected cells .Sterile glass beads (0.5 mm) diameter were added to the cells suspension and vortexed for 15 min in discontinuous periods. The suspension was centrifuged at 6000 rpm for 20 min and 5 ml of 1.5% SDS at pH 11 was mixed with the precipitate for 1h at 50 ˚C. The solution was centrifuged at 5000 rpm for 20 min and the precipitate was washed with deionized D.W, 5.64% sodium hypochlorite and with D.W respectively and finally dried at 45 ˚C.

3- Extraction with NaOH (Lee et.al., 1999)

Ten ml of bacterial culture was centrifuged at 6000 rpm for 20 min and the precipitate was separated and washed with D.W.Ten ml of 2N NaOH was mixed with the precipitate and heated at 60 ˚C for 1h.The solution was centrifuged at 25000 rpm for 30 min and the precipitate represents PHB.PHB was washed with D.W and dried at 45 ˚C for 2h.

Estimation of PHB % (Henderson and Jones ,1997) :

Total dry weight (total biomass) was determined by harvesting, washing, drying to constant volume and weighting , PHB content was estimated as % of biomass after calculated of PHB and bacterial cells dried weight.

$$\text{PHB \%} = \left(\frac{\text{Weight of PHB}}{\text{Weight of dry cell mass}} \right) \times 100$$

Qualitative Analysis of PHB

1- Fourier Transform Infrared (FT-IR) analysis:

One mg of the extracted PHB was ground well with 10 mg of spectral pure anhydrous potassium bromide crystals. The powder was made into a pellet for IR analysis. The relative intensity of transmitted light energy was measured against the wavelength of absorption on the region 400-4000 cm^{-1} using FTIR spectrometer.

2- High Performance Liquid Chromatography (HPLC) analysis:

PHB analysis was carried out by liquid chromatography using Shimadzu 2010 LC equipped with binary delivery pump model 2010 Shimadzu, the eluted peaks were monitored by CD- Refractometer detector 2010.

Sample: 5 mg of standard polyhydroxy butyrate was dissolved in HPLC grad chloroform to prepare standard 25 µg /ml by serial dilutions.

Separation: The sample of *Azotobacter* isolate PHB and PHB standard were separated under the same optimum separation conditions as below:

Column: Gel permeation column styragel type HR3-6(10×4.6 mm I.d) with quard column styragel (4.0×4.6 mm I.d).

Mobile phase: methylene chloride.

Detection: differential refractometer model (410).Determination of PHB properties

Melting point: Melting point of PHB was measured by using capillary tube , inserted in a melting point apparatus .

Solubility : The extracted PHB (0.05g) was dissolved in 10 ml of different solvents: (acetone , hexane , ethanol ,chloroform, tetra carbon chloride , methanol ,1-2 dichloroethanol , diethyl ether) and noting the solubility of the polymer in these solvents throughout the solutions homogenization.

Viscosity : The viscosity of PHB solution was measured according to the following equation (AL-Ani,K.A.R. , 1990):$\eta = t\,d$ (η =absolute viscosity, t = flow time , d = density)

Molecular weight estimation (Savenkova, L. et.al., 2000)

Molecular weight of PHB was estimated by viscometer. PHB dilutions were dissolved in 100 ml of chloroform . The mean of molecular weight is calculated at 30 ˚C according to the Mark–Houwink equation : $[\eta] = K\,Mr^{\alpha}$, where $[\eta]$ is the intrinsic viscosity , K and α the constants

for the given polymer–solvent system (K= 1.18 ×10^{-4}, α= 0.78) .

Effect of pH on molecular weight (Marchessault , R.H. *et.al.,* 1994)

PHB was added to different buffers included acetate buffer (pH 4), phosphate buffer (pH 7) and carbonate - bicarbonate buffer (pH 10) and incubated at 30 ˚C for two weeks, then centrifuged at 4000 rpm for 15 min. PHB precipitate was washed with D.W and the viscosity was calculated. The viscosity is proportion to the molecular weight; thus it was used as indicator for changing in molecular weight.

RESULTS

Isolation of *Azotobacter*:

Twenty samples were collected from different places in corn field in Agricultural College in Abu-Grab near the plant roots. Eighteen bacterial isolates were grown on Sucrose mineral salts medium (selective medium for *Azotobacter* genus which contain mineral salts without nitrogen source that facilitate the growth of this bacteria). Twelve isolates were belonged to *Azotobacter* depending on the morphological and microscopic examination (Garrity, G.M. ,2005).

PHB extracted by different methods

The results showed that the optimum method for PHB extraction was by digestion the bacterial cells with sodium hypochlorite, the percentage of PHB was 39.5%, followed by extraction with NaOH which gave 28% PHB content, and the lowest PHB percentage 8% was obtained by SDS and sodium hypochlorite (Figure 2).

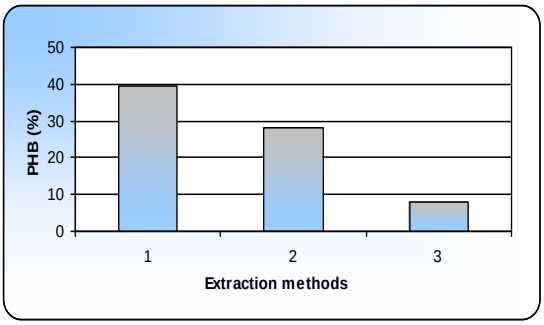


Figure (2): PHB yield extracted from *A. chroococcum*9 by different methods

1-Digestion with sodium hypochlorite 2- Extraction with NaOH 3-Extraction with SDS and sodium hypochlorite

PHB extracted from *A. chroococcum*9 by three methods in this study was a white powdery mass. The difficulty of PHB recovery from microorganisms has been the primary technical obstacle to its commercial exploitation. A number of different methods for the recovery of PHB, which is formed within a cell's cytoplasm as granular inclusions, have been suggested. Microbial PHB has often been recovered by treating with alkaline hypochlorite solution, a reagent that digests most of other cellular macromolecules except PHB granules (Yu, and Chen, 2006). This method is simple, effective and easy to scale up. However, this method causes degradation of PHB molecules, which is a major detrimental effect. To minimize the problems associated with conventional PHB digestion with hypochlorite, chloroform was used with hypochlorite (Hahn et.al., 1995). Molecular weight can be controlled by changing the hypochlorite concentration and treatment time. It gaves a high purity of over 97% and a high recovery of about 90%, by this method, high purity levels of PHA: 86% was obtained from *R. eutropha* and 93% from recombinant *E. coli* (Hahn, S. K. et.al., 1994). NaOH digestion method has several advantages: (i) NaOH is inexpensive and much more environmentally friendly, (ii) a high degree of purity (98%) of PHB can be obtained, and (iii) there is no degradation of PHB during recovery (Lee et.al., 1999).

Qualitative analysis of PHB extracted from *Azotobacter chroococcum9* :

1- Fourier Transform Infrared (FT-IR) analysis:

PHB extracted from *A. chroococcum* 9 was analyzed by FTIR at a wavelength of absorption on the region 400-4000 cm^{-1} to detect the functional groups in the chemical structure of PHB

Results showed the presence of methyl and methylene deformation of aliphatic compounds and C-O stretch vibrations in 1000-1465 cm^{-1} (Jarute, G. et.al., 2004), strong absorption band of aliphatic carbonyl (ester bond) C=O in 1728 cm^{-1} , 2854-2923 is the stretching of C-H group and the stretching of O-H group in 3448-3487 (Sujatha , K. et.al., 2005;Luo, R. et al., 2006 ; Abd-El-Haleem, D. A.M. et al., 2007) (Figure 3).

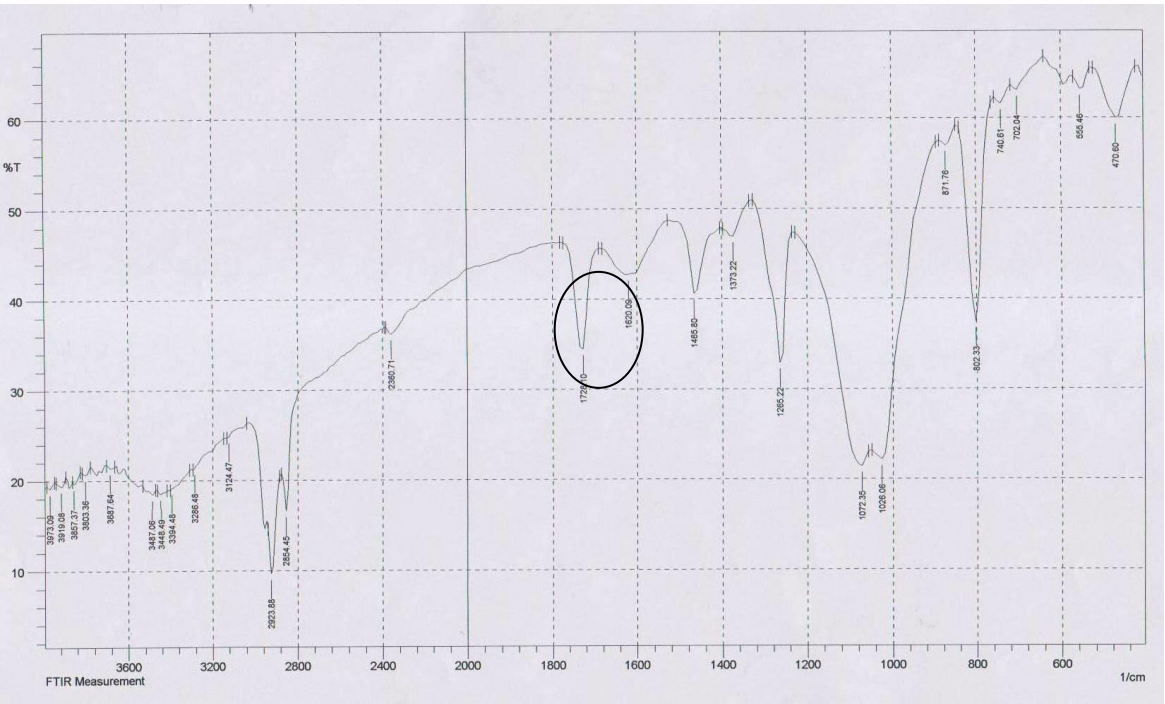


Figure (3) : FTIR analysis for PHB extracted from *A. chroococcum* 9 <u>black cycle</u> refers to the ester bond of PHB structure

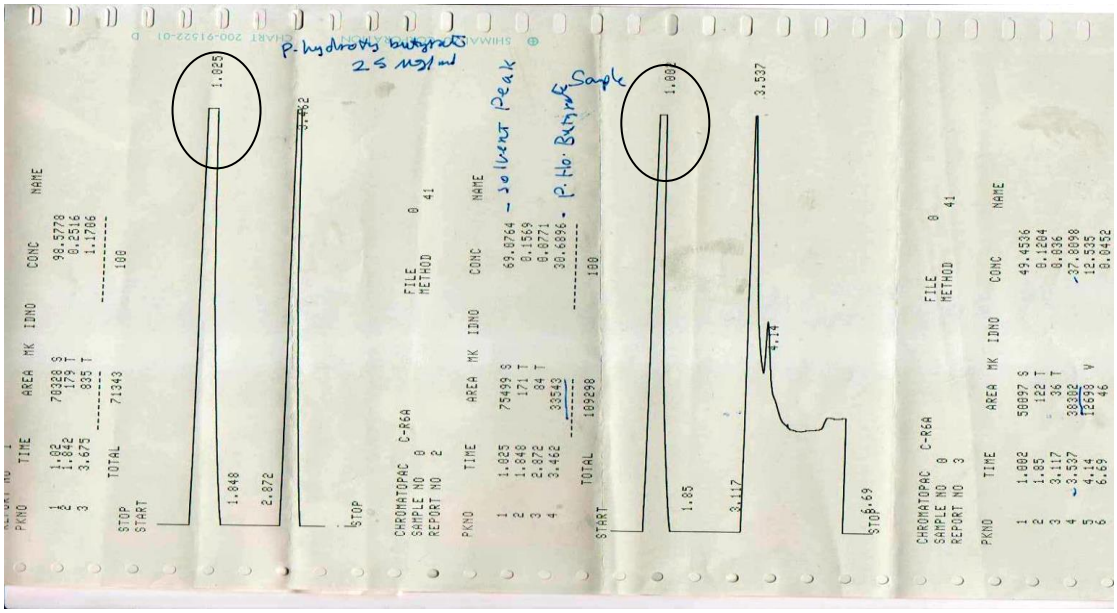


Figure (4) : HPLC analysis for PHB extracted from *A. chroococcum* 9 <u>black cycles</u> represent retention time

Sujatha et al., (2005) reported that the band between 1728 cm^{-1} and 1744 cm^{-1} is characteristic of PHA. But the presence of band 1728 cm^{-1} means that bacteria accumulate PHB not other type of PHA_s (Hong et.al., 1999)

2- High Performance Liquid Chromatography (HPLC) analysis:

HPLC analysis of *A.chroococcum* 9 PHB revealed one peak with the same retention time, the retention time of standard PHB peak was 3.46 min while the retention time of *A. chroococcum* 9 PHB was 3.54 min , this means that the extracted polymer of *A. chroococcum* 9 is PHB (Figure 4) . (Karr et al., 1983) reported that PHB can also be measured by HPLC after sulfuric acid digestion of *R. japonicumbacteroids*, PHB converted to crotonic acid .The standard crotonic acid and the crotonic acid resulted from sulfuric acid digestion of PHB revealed the same peak with retention time of 29 min.

PHB characterizations

Melting point

Melting point of PHB extracted from *A. chroococcum* 9 in this study was 180 ˚C , PHB is a highly crystalline thermoplastic polymer with a relatively high melting temperature (in the range of 171-182 ˚C) (Chaijamrus , S. & N. Udpuay ,2008). Melting temperature of *C . necator and Azotobacter sp*. FA8 PHB was 175 ,176 ˚C, respectively , and for recombinant *E.coli* was 172 ˚C and for *Bacillus megaterium* ATCC 6748 was 177 ˚C (Nikel et.al., 2006 ; Chaijamrus , and Udpuay ,2008).

Solubility

The results showed that this polymer is soluble in chloroform and 1-2 dichloroethanol ; it was insoluble in methanol , ethanol , acetone , hexane , diethyl ether and carbon tetrachloride .

In the extraction processes of PHB from different bacteria the solvents used for this purpose were chloroform, methylene chloride, propylene carbonate, and dichloroethane and others (Mohammad et.al., 2009).

PHB was recovered from Z. denitrificans MW1 by using different organic solvents , only chloroform and methylene chloride showed remarkable efficiencies in PHB recovery , but no detectable PHB was extracted from the cells of Z. denitrificans MW1 with carbon tetrachloride, diethyl ester, ethyl acetate, or mixtures of chloroform and acetone under the studied (Mohammad et al., 2009).

PHB from *A. chroococcum* MAL-201 was soluble in acetic acid, butanol, chloroform, dichloromethane, dimethyl formamide , ethylene carbonate . It was insoluble in Water, methanol, ethanol, propanol, hexane, benzene (Pal and Paul, 2002).

Viscosity and molecular weight

The molecular weight of PHB extracted from *A. chroococcum* 9 was measured depending on the viscosity. The results showed that the intrinsic viscosity was 2 (dl g^{-1}), and the molecular weight was 2.6×10^5daltons.

The molecular weight of PHA is one of the most important factors affecting the mechanical properties and biodegradability of the polymer (Korsatko et.al., 1983). It is important that the polymer has a high molecular weight in order to retain its strength during processing (Zhang, H. 1994).Substrates and culture conditions affect the molecular weight of *A. vinelandii* PHB.The method of PHB isolation may causes severe damage of granules and led to loss of molecular mass of polymer (Chen and Page. 1994). Savenkova et al., (2000) reported that the intrinsic viscosities of the PHB samples from *A. chroococcum*23 ranged between 7.2-12.3 (dl g^{-1}), and the MW ranged between 1365-2713 KD . Bacteria of the genus *Azotobacter* can synthesize polymer at a wide range of molecular weight. PHB extracted from *A. vinelandii* UWD with chloroform had a MW of (1.7×10^6 to 2.0×10^6) daltons (Pageand Cornish. 1993) while the polymer isolated from *A. chroococcum*MAL-201 has Mw of 1.5×10^6daltons (Pal. and Paul ,2002).

Effect of pH on PHB molecular weight

To study the effect of pH on the molecular mass of PHB extracted from *A. chroococcum*9, the viscosity of the polymer was measured as indicator for molecular weight of the polymer since the viscosity increases with MW increasing. It has been observed that the polymer at pH 7 has higher viscosity than in alkaline or acidic buffers, and the alkaline pH reduced the viscosity more than the acidic pH (Figure 5)

Both polymers PHB and PHBV from *R. eutropha* showed a more rapid loss of mass in pH 10 and 13 at 37 ˚C than in pH 7.4which indicating greater degradation in alkaline medium than in neutral medium. In this case,PHB and PHBV were totally degraded after 20-30 days of ageing in highly alkaline media (Muhamad et.al.,2006).

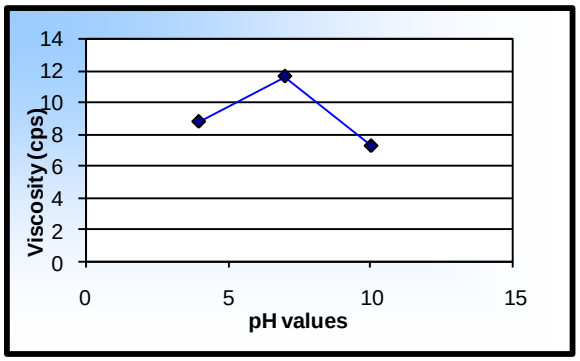


Figure (5) : Viscosity of PHB of *A. chroococcum* 9 at different pH values after incubation at 30 ˚C for 7 days.

CONCLUSION

β-hydroxybutyrate was extracted from *A. chroococcum 9* by different methods The optimum extraction of PHB by using sodium hypochlorite extraction method. PHB was soluble in chloroform and in 1-2 dichloroethanol. The molecular weight of PHB extracted from *A. chroococcum 9* was 2.6×10^5dalton and the melting point was 180 ˚C .

CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

AUTHOR CONTRIBUTIONS

SSH designed the experiments. SAA performleed the experiments and also wrote the manuscript. All authors read and approved the final version.

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