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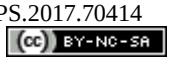
ISSN 2231-3354 

Identification, Biodegradation and bio-evaluation of biopolymer produced from *Bacillus thuringenesis*

Mostafa Abd El-Hameed El-Abd[1*], Hussein Hosny El-Sheikh[2,3], Saeid Desouky[3], Amr Shehab[3]

[1] National Research Centre, Dokki, Cairo, Egypt.
[2]The Regional Center for Mycology and Biotechnology, Al-Azhar University, Cairo, Egypt.
[3]Botany and Microbiology Department, Faculty of Science, Al-Azhar University, Cairo, Egypt.

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ABSTRACT

Poly3-hydroxybutyric acid (PHB) is a promising eco-friendly substance favorable for medical use. PHB was produced from local isolate *Bacillus thuringienesis* in a conc. 4.1 g/L using 30 g/L Sugar-cane molasses (SCM) at pH 7.5 and incubation temperature 35^{o}C. PHB biodegradation by soil microorganisms were completed after four weeks. Cell cytotoxicity testing is one of the critical factors affecting the biomedical application of polymers. 50% cell cytotoxic concentration (IC50) = 130 mg/mL while non-toxic concentration was 12.5 mg/mL studying of direct contact application between biopolymer and peripheral blood lymphocytes resulting monomers have no toxic effect on the cells.

INTRODUCTION

Plastic is one of the most widely daily use requirements, whereas their remains are in the long term the hardest to dispose and cause problems when entering into the waste stream. They are not broken down by the natural elements in the environment or in waste management infrastructures, such as composting to become a part of the biological carbon cycle of our ecosystem (Narayan, 1993). Incinerating plastics has been one option in dealing with non-degradable plastics, but other than being expensive it is also dangerous. (Johnstone, 1990; Atlas, 1993). Dangerous gases as carbon monoxide, dioxins and furans are emanated into the air during plastic burning. Microorganisms can generate renewable materials considering as alternatives to petroleum derived chemicals including polymers and having the same physical and mechanical properties of plastic but with the added property of biodegradability (Akaraonye *et al.*, 2010).

* *Corresponding Author*
Mostafa Abd El-Hameed El-Abd, Resarcher in National Research Centre Cairo, Egypt. Email: mostafaelabd77 @ yahoo.ca

The need of bioplastic is also linked to decline of petrochemical reserves (Zagar, 2000). Eco-friendly bioplastics can help to overcome the problem of pollution caused by non-degradable plastics.

Thus, it becomes obligatory to optimize the method of bioplastic production, selection of raw materials, conversion to suitable forms of certain wastes, so that we do not add any material waste into the environment which nature cannot recycle (Reddy *et al.*, 2003). The long-term growth of biobased industrial products will depend on the development of cost-competitive technologies and access to diverse markets. This has prompted many countries to start developing biodegradable plastic (Kalia *et al.*, 2000).

Bioplastics are produced by various microorganisms (Kragelund *et al.*, 2005; Akar *et al.*, 2006). These biopolymers accumulate as storage materials in microbial cells under stress conditions (Kadouri *et al.*, 2005; Berlanga *et al.*, 2006). The most widely produced microbial biopolymers are polyhydroxyalkanoates (PHAs) and their derivatives (Madison and Huisman, 1999; Kim and Lenz, 2001; Witholt and Kessler, 2002).

Microorganisms can assimilate various carbon sources to produce different types of PHA. Various metabolic pathways have been demonstrated to be involved in the generation of the monomers incorporated into PHA (Steinbüchel, 1991; Madison and Huisman, 1999). The physical and chemical properties, monomer composition and the number and size of the granules are varied according to the microorganism (Anderson *et al.*, 1990; Ha and Cho, 2002). PHA materials have the ability to biodegradation in various environments, including soil, sea water and lake water (Tokiwa and Calabia, 2007).

The PHAs are not only non-toxic but also biodegradable and can be produced from renewable resources. Also, they are biocompatible thermoplastics having a high degree of polymerization, high crystallinity and insoluble in water. These features make them highly competitive with the petrochemical-derived plastics. PHAs have a wide range of applications (Madison and Huisman, 1999) specially the medical applications of PHAs (Pankajakshan and Agrawal, 2010). At present, approximately 150 different constituents of PHAs have been identified (Sudesh and lwata, 2008). If all these different types of PHAs can be produced in a cost-effective manner, materials with an enormous range of properties can be obtained.

MATERIALS AND METHODS

Microorganism

A bacterial strain *Bacillus thuringenesis* was isolated from a Benzene stations in Giza and identified in a former study (Desouky *et al.*, 2014).

PHA production media and growth condition

Minimal salt medium (MSM)was used for both seed culture and fermentation medium with the following composition (g/L): $Na_2HPO_4.2H_2O$, 4.42; KH_2PO_4, 1.50; $MgSO_4.7H_2O$, 0.20; $CaCl_2$, 0.02; Ferric citrate, 0.0015; Trace element Solution, 0.1 mL; using different carbon and nitrogen sources with different concentrations. The pH was adjusted to 7.0. Flask cultures were carried out in 250-mL flasks containing 100 ml of MSM medium in a shaking incubator at different temperatures and agitation speeds to detect the most proper conditions for PHA production. The Trace element solution (Pfennig,1974) contains: (mg/l dist. H_2O) $ZnSO_4$. $7H_2O$, 10.0; $MnC1_2$. $4H_2O$, 3.0; H_3BO_3, 30.0; $CoC1_2$. $6H_2O$, 20.0; $CuC1_2$. $2H_2O$, 1.0; $NiC1_2$. $6H_2O$, 2.0; Na_2MoO_4. $2H_2O$, 3.0.

Characterization of PHA

Various methods such as NMR, FTIR, GC-mass, UV, and DSC were used for the characterization of this polymer as described below:

NMR analysis

The NMR spectra were recorded in a JEOL ECA 500 spectrometer. The 500 MHz 1H-NMR spectra were recorded from a $CDCl_3$ solution of the PHA (30 mg/ml) at 20°C, 1.30809856s acquisition times and 12.5250501 kHz spectral width. The ^{13}C-NMR spectra were recorded from a $CDCl_3$ solution of the samples, using ^{1}H decoupling, 0.83361792s acquisition time and 39.3081761 KHz spectral width. Analysis was done at National Research Center, Dokki, Egypt.

Fourier Transform Infrared Spectroscopy

Standard P3 (HB) was dissolved in chloroform and layered on KBr window, allowed to dry and were subjected to FT-IR analysis. Alternatively, sample (5 mg) was mixed with 100 mg of FTIR grade KBr and pelletized. The FTIR spectrum was recorded at 400-4000 cm^{-1}. This test was done in Faculty of Pharmacy, Al-Azhar University, Egypt.

Gas Chromatography

GC analysis was carried out using purified polymer after subjecting them to methanolysis. Analysis was done at National Research Center, Eldookki, Egypt.

Sample preparation

Air-dried biomass or purified PHA was weighed into a clean glass tube, to this 1 ml of chloroform, 850µl methanol and 150µl H_2SO_4 were added. The glass tube was sealed and kept for hydrolysis at 100 ^{o}C for 160 min. To the hydrolysed material equal volume of water was added, mixed thoroughly and 2µl of the sample was taken from the bottom layer of injection. Benzoic acid was used as internal standard.

GC conditions (Brandl *et al*, 1988):

The methyl esters formed were analyzed with a flame ionization detector in a 30 m DB^{-1} capillary column of 0.25 mm internal diameter and 0.25µm film thickness. The analysis parameters used were: injector 170^{o}C, detector 220^{o}C, and temperature Program of 55^{o}C for 7 min, ramp of 4^{o}C per minute to 100^{o}C and 10^{o}C per minute rise to 200^{o}C and hold for 10 min. High purity nitrogen (1 mL/min) was used as carrier gasat a static flow rate of 1.69 ml min^{-1}.

Scanning electron microscope (SEM) identification

The sample were fixated by glutheraldhyde 2.5% and dehydrated by serial dilution of ethanol using automatic tissue processor (Leica EM TP). Then the samples drying using CO2 critical point drier (Tousimis audosamdri-815) the samples coated by gold sputter. Coater (SP – Module) Finally the samples examined by scanning electron microscopy (JEOL-JSM-5500 LV) by using high vaccum mode at Regional Center of Mycology and Biotechnology, Cairo, Egypt.

Differential scanning calorimetry

Differential scanning calorimetric (DSC) experiments was performed using a Perkin Elmer DSC apparatus. Analysis was done at National Research Center, Eldookki, Egypt. The phase transition temperatures were reported as glass transition

temperature (Tg) and melting temperature (Tm) Approximately 4-5 mg of PHA samples were subjected to the following protocol: (1) heat at 106°C and hold for 1min, (2) heat at 170°C and hold for 1 min and (3) heat at 266°C and hold for 1min.

Film casting (Savenkova *et al.*, 2000)

Films were prepared by the solvent casting method 2% solution of PHA in chloroform was prepared. Uniform, flat glass plates were selected for casting. These plates were placed on a flat and levelled surface to get a film of uniform thickness. A spirit leveler was used to level the plates. The chloroform solution (80 - 100 ml) was poured on to plates (30 X 20 cm) and was left in a place without air turbulence for a minimum of 4 hours at room temperature. Care was taken not to disturb the plates during drying. After drying the films were peeled out of the plates.

Biodegradability of PHAs in soil

The ability to degrade PHAs depends on the secretion of specific extracellular PHA depolymerases that hydrolyze the polymer to water-soluble products. The PHAs film produced from the fermented broth of BC11 isolate was subjected to biodegradability test.

The PHAs film was buried into the soil to check its biodegradation rate and was maintained for 21 days. Biodegradability of PHA films in Arakawa river water (Japan) at 25ºC was reported by Doi and coworkers(1996) the degradation of PHA films was initiated after around 4 days, and then the biodegradation of PHA increased with time to reach 80% biodegradability in 28 days.

Cytopathic effect assay against VERO cell line (Vichai *et al.*, 2006)

The cytotoxicity of biopolymer extracts were measured by the cytopathic effect assay. The assay was carried out using 100µl of cell suspension, containing 10,000 cells seeded in each well of a 96-well microliter plate. Fresh medium containing different concentrations of the test sample was added after 24 hr of seeding.

Then, serial two fold dilutions of polymer extract were added to the confluent cell monolayer. The microliter plates were incubated at 37ºC in a humidified incubator with 5% CO_2 for a period of 48 hr. Six wells were used for each concentration of the test sample. Control cells were incubated without test sample after the incubation period, media were aspirated and the cells were fixed with 10% formalin solution for at least 20 min. The fixed cells were rinsed with phosphate buffer solution (PBS) then stained with a 1% crystal violet for 1 hr. The stain was removed and the plates were rinsed using tap water until all excess stain is removed.

The cell cultures were examined for evidence of the cytopathic effect that observed microscopically as detectable alterations. The stained cells were lysed by using glacial acetic acid solution and then plates were read on ELISA reader, using a test wavelength of 490 nm. This test was done at Regional Center of Mycology and Biotechnology, Cairo, Egypt.

Haemocompatibility and biocompatibility in vitro

Haemolytic assay and the effect of the polymer on human blood

The haemolysis tests were performed as described in American society for testing and materials (ASTM) (ASTM F 756-00, 2000) with slight modification. Samples (5mg of polymer) were put in polypropylene test tubes and 7 ml of PBS were added after 72 hrs of incubation at 37° C, the PBS was removed and 1ml of diluted human venous blood was added to each sample and maintained at 37° C for 3 hrs. Positive and negative controls were prepared by adding the same amount of blood to 7 ml of water and PBS, respectively. Each tube was gently inverted twice each 30 min to maintain contact of the blood with the material. After incubation, each fluid was transferred to a suitable tube and centrifuged at 2000 rpm for 15 min. Haemoglobin released by haemolysis was measured by optical densities (OD) of the supernatants at 540 nm. The percentage of haemolysis was calculated as follows:

$$\textbf{Haemolysis (\%)} = \frac{\text{ODsample} - \text{ODnegativecontrol}}{\text{ODpositivecontrol} - \text{ODnegativecontrol}}$$

RESULTSAND DISCUSSION

Fourier Transform-Infrared spectroscopy (FTIR)

Fig.1 illustrating FTIR data for biopolymer produced by *B. thuringienesis* showed characteristic absorption bonds for esters and the presence of C=O and C-O were obtained at 1735 cm^{-1} and 1287 cm^{-1} respectively. CH_2 or methylene group was observed at 1449 cm^{-1} and methine or CH peak was at 3440 cm^{-1}. The presence of a strong peak at 2400 cm^{-1} was probably due to OH of carboxylic acid such as poly β–hydroxybutyric acid along with the ester in the sample. The results are in agreement with previous study reporting the presence of similar functional groups in PHB correspond to the characteristic ones of PHB homopolymer, a short-chain-length (scl) (Oliveira *et al*., 2007).

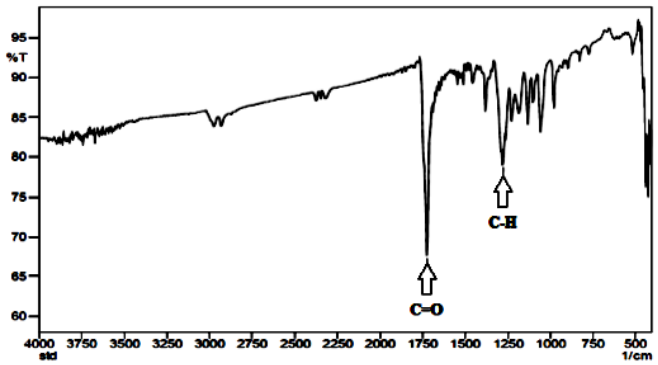

Fig. 1: FTIR for biopolymer extracted by direct Chloroform.

Gas chromatography mass chromatography

Similarly, the results of GC-MS was matching with the results from the study of polymer characterization with *B. megaterium* strain uyuni S29 (Rodrı-guez-Contreras *et al*., 2013).

The molecular fragments obtained are as shown in (Fig.2) The major molecular fragment obtained was Butanoic acid, 3-Hydroxy, methylester ($C_5H_{10}O_3$) at Retention time 7.59 min.

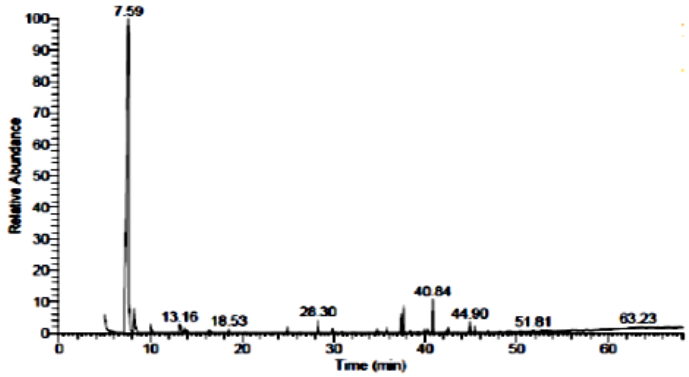

Fig. 2: Gas mass chromatography of PHB sample extracted from *Bacillus thuringienesis* Bt407.

Characterization of biopolymer by NMR

^{1}H NMR spectroscopic analysis:

The ^{1}H NMR spectrum of PHA (Fig.3) was matched with the former results indicated three groups of signals characterizing PHB, a single of quadruplet at 1.26 ppm which is attributed to the methyl group $-CH_3$, a single of quadruplet at 2.5 ppm which is attributed to methylene group $-CH_2$ and a multiplet at 5.25 ppm, which is characteristic of methine group $-CH$. Two small additional peaks at $\delta = 0.8$ and $\delta = 1.6$ were found may be due to impurities present.

^{1}H-NMR spectrum of PHA isolated from glucose or molasses media indicated characteristic signals of PHB, namely a doublet at 1.26 ppm, which is attributed to the methyl (CH_3) group coupled to one proton while a doublet of quadruplet at 2.51 ppm due to the methylene (CH_2) group adjacent to an asymmetric carbon atom bearing a single proton.

The third signal at 5.25 ppm, which was attributed to the methine (CH) group. ^{1}H-NMR is a very sensitive method for determining the domain size and miscibility, which is difficult to identify by conventional microscopic or thermal analysis (Kichise *et al.*, 2002). The values of the chemical shifts as well as the assignments of the ^{1}H-NMR signals, which appeared in the spectra are in agreement with results obtained by Kichise *et al*. (2002) and typically identical to peaks of the authentic PHB sample produced from Aldrich Company, which clearly shown that the extracted biopolymer from the *B. thuringienesis* in this study was poly-3-hydroxybutyric acid. These results were also confirmed by ^{13}C NMR spectrum (Fig.4).

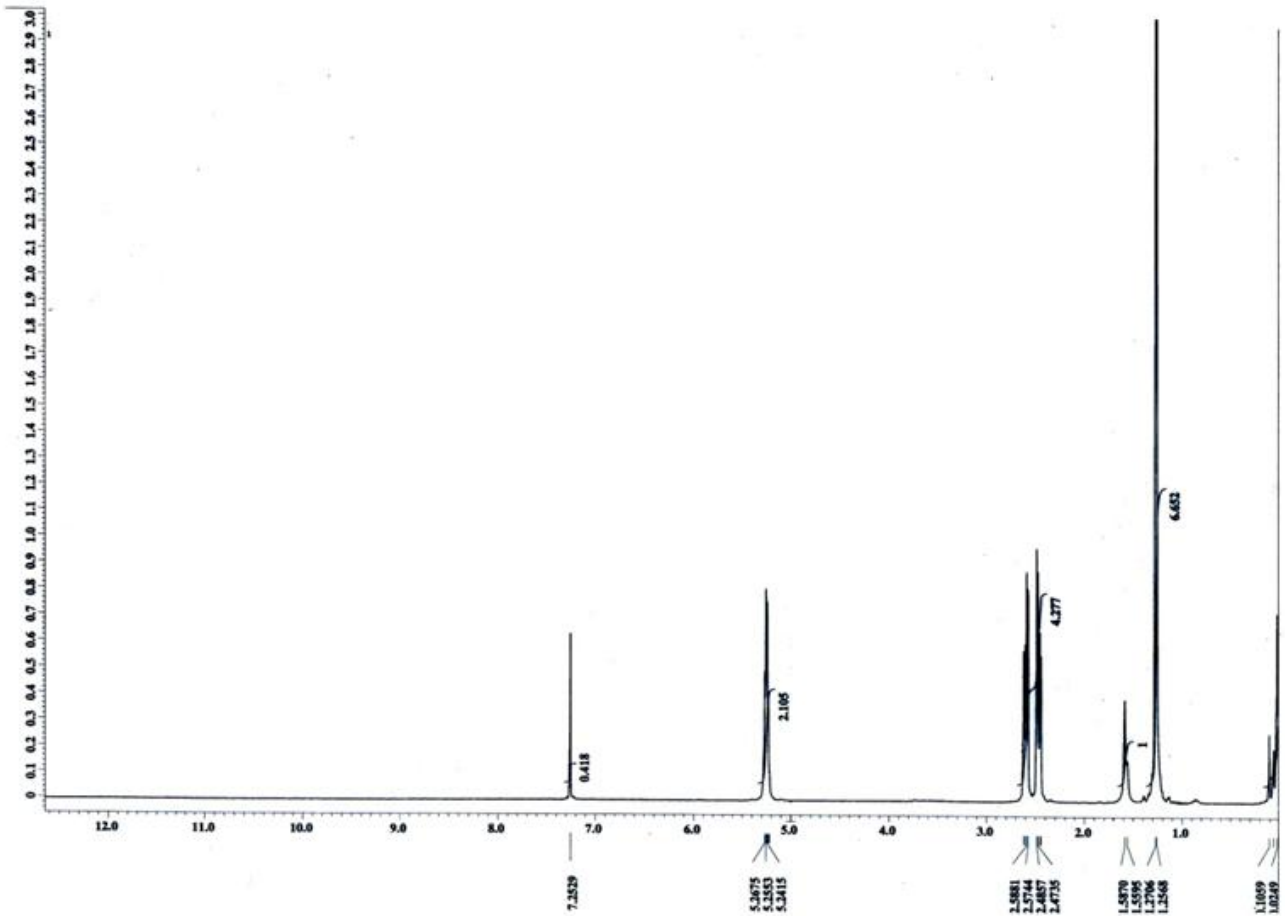

Fig.3: ^{1}H chemical shifts of constituent monomers of purified PHA produced by *B. thuringienesis.*

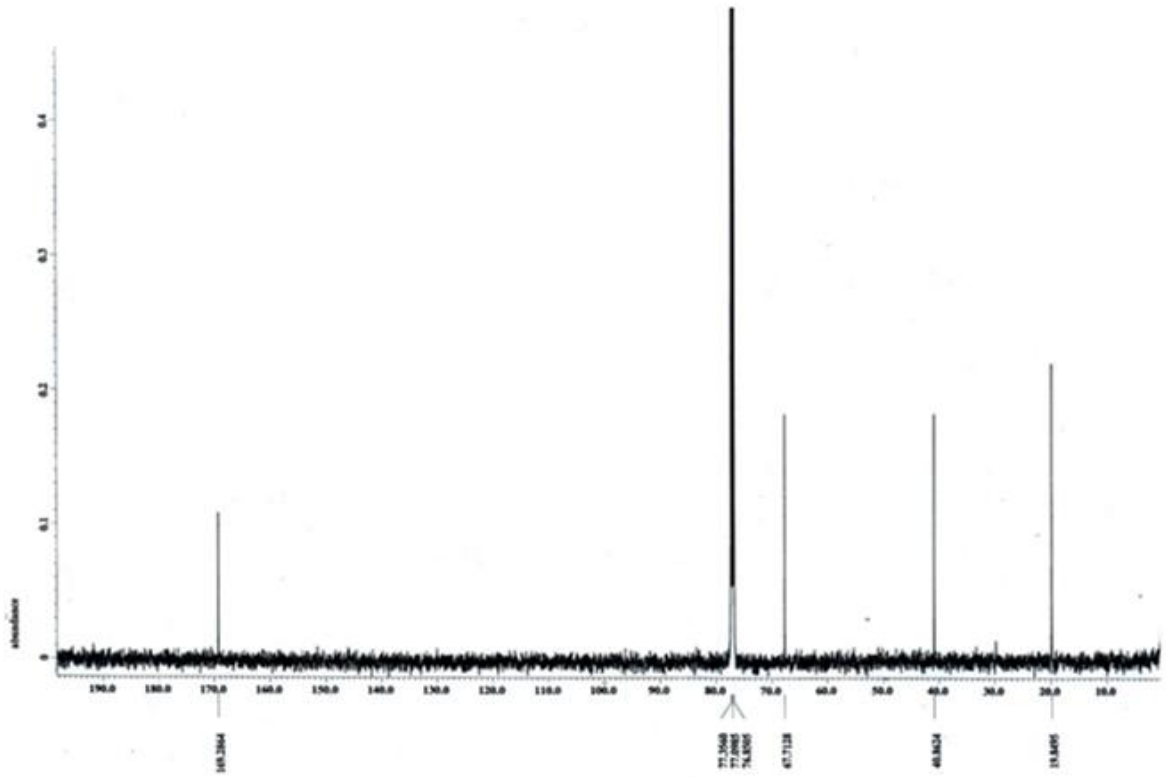

Fig.4: ^{13}C chemical shifts of constituent monomers of purified PHA produced by *B.thuringienesis*

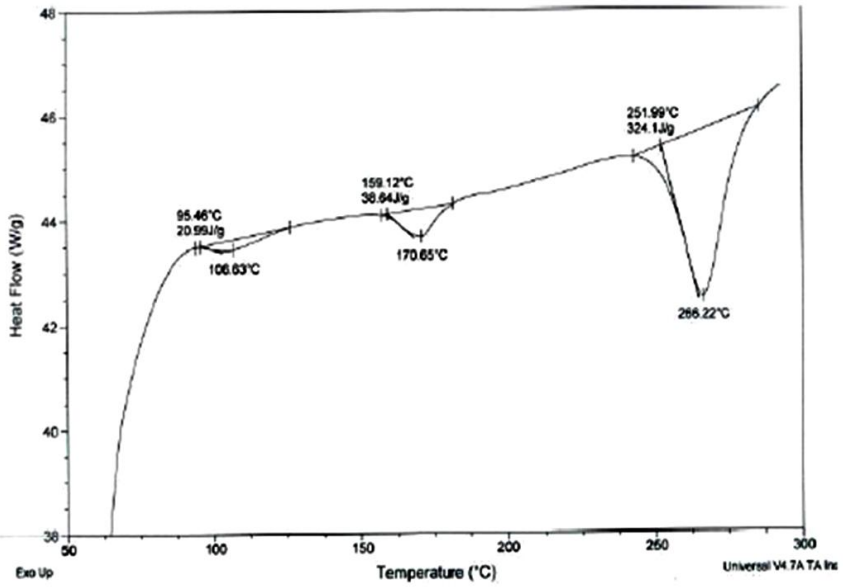

Fig. 5: Differential scanning calorimetry showing melting temperature of PHA sample extracted from *B.thuringienesis.*

Characterization of biopolymer by differential scanning calorimetry

Studies on the DSC (Fig.5) gave details about the melting temperatures of the samples. This was compared with the temperatures of standard PHB, which confirms the presence of this polymer. The standard (PHB) from Sigma showed a melting temperature (Tm) value of 176^{o}C PHA samples from *B.thuringienesis*showed a peak value of 168.8^{o}C (Fig.5). Melting temperature is an important feature since the temperature at which the polymer degrades has to be higher than the temperature at which it melts.

Lower Tm will make the polymer better for a varied number of applications. Hence a decrease of 7^{o}C in the melting temperature is of great significance.

Scanning electron microscope for *B. thuringienesis*

The morphological characteristics of *B. thuringienesis* showed that it has a rod-shape (Fig. 6a), the cells occurred singly or in clusters and accumulate massive amounts of PHB and became swollen (Fig. 6b).

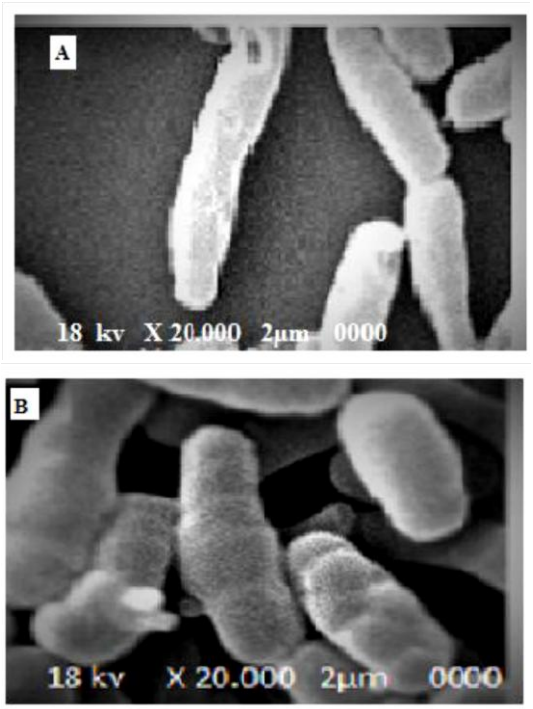

Fig. 6: Scanning Electron Microscopy photographs for *B.thringienesis*

Biodegradability of PHB in soil

Degradability is the main difference between the synthetic plastics and bioplastics. Hence, the degradability of the PHB films produce by the *B. thuringienesis* was tested in soil. The PHB film of *B. thuringienesis* was degraded completely within 28 days. After three weeks decrease in weight of sheet from 50 mg to 12 mg was observed (Fig.7, 8). The most attractive property of PHB with respect to ecology is that it can be completely degraded to CO_2 and H_2O by microorganisms.

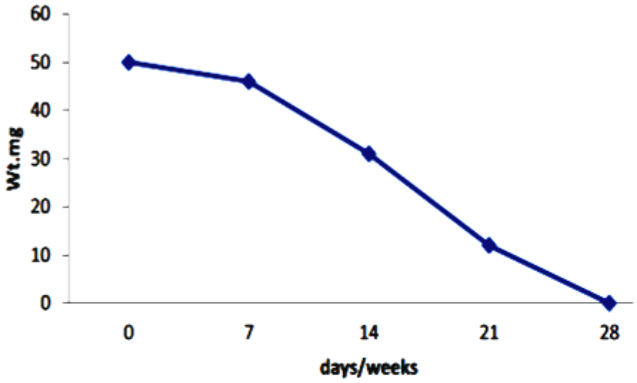

Fig. 7: Biodegradation of PHB.

A wide range of PHA-degrading microorganisms have been isolated. PHA-degrading microorganisms are widely distributed in various ecosystems such as soil, sewage sludge, compost, and marine water (Mergaert *et al.*, 1993). The percentages of PHA- degrading bacteria in soil environment have been estimated to be 0.8 to 11.0% of the total colonies by Nishida and Tokiwa (1993) and 2 to 18% by Suyama *et al.*, (1998). The rate of biodegradation of PHAs is dependent upon a number of factors such as microbial activity of the environment, exposed surface area, temperature, pH, and properties of the plastic material to be degraded (Jendrossek *et al.*, 1996). PHB biodegradation obtained from soil were completely degraded for seven days. Both the film weight and Mw of PHB decreased with time. PHAs contain amorphous and crystalline regions, of which the former are much more susceptible to microbial attack (Sudesh *et al.*, 2000**).** In this study PHB biodegradation obtained from soil were complete degradation after three weeks.

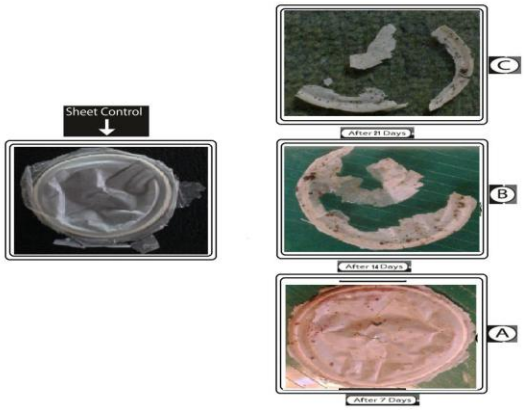

Fig. 8: Photo showed Biodegradation of PHB film (A) after 7 days, (B) 14 days and (C) 21 days.

Evaluation of cytotoxicity against VERO cell line

Cell cytotoxicity testing is one of the critical factors affecting the biomedical application of polymers. Cytotoxicity activity against Mammalian cells from African Green Monkey kidney (Vero) cells was detected under these experimental conditions with 50% cell cytotoxic concentration (IC50) = 130 mg/ml while non-toxic concentration was 12.5 mg/ml (Fig.9).

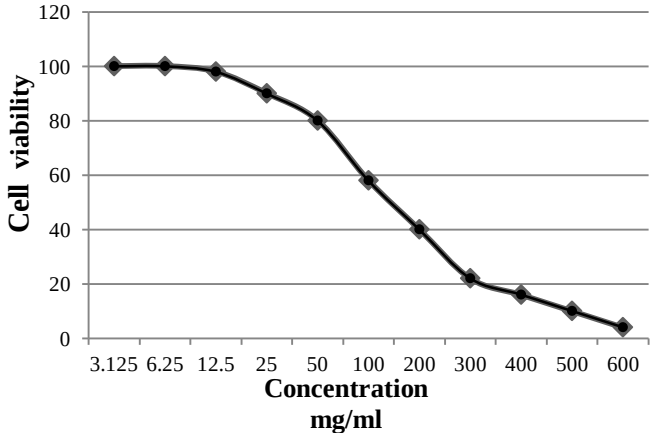

Fig. 9: Cytotoxicity evaluation of biopolymer extracted on against VERO cell line viability.

Haemocomptaiblity and biocompatibility in *vitro*

Hemolytic assay and the effect of the polymer on human blood:

Hemolytic index for biopolymer (PHB) extracted by different methods using chloroform, ammonium hydroxide and sodium hypochlorite with chloroform were 2.94%, 3.51% and 3.22% respectively as shown in Table 1. Hemolysis of all the extracted biopolymer samples were in safety between 2 – 5 % so act as slightly hemolytic but better polymer for application was extracted by direct chloroform.

Table 1: Hemolytic index of biopolymer:

Sample	Hemolytic index %
Polymer extracted by chloroform method	2.94 %
Polymer extracted by ammonium hydroxide method	3.51 %
Polymer extracted by dispersion of sodium hypochlorite and chloroform	3.22 %

Biopolymer culturing in mononuclear cells:

This experiment aimed at studying the effect of direct contact between prepared biopolymer and human peripheral blood mononuclear cells in order to indicate any signs of toxicity and the viability in comparison with control one. The result indicated that; there is no change in cell morphology after 48 hours of contact and viability was about 90% (Fig.10). The lymphocyte cells after staining by trypan blue was shown in Fig.(11), blue cells are dead while shiny cells are viable.

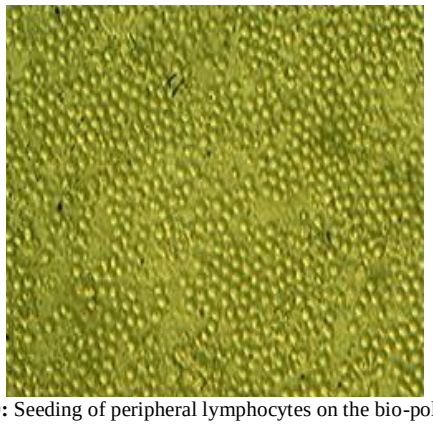

Fig. 10: Seeding of peripheral lymphocytes on the bio-polymer.

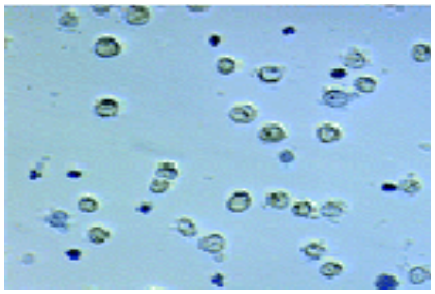

Fig. 11: Lymphocyte cells under light microscope.

The result of direct contact application between biopolymer and peripheral blood lymphocytes is considered a preliminary data studying the biopolymer characteristics including biocompatibility of polymer with the biological matter (Peripheral lymphocyte), biodegradable through enzymatic activity of cells and the resulting monomers have no toxic effect on the cells(Fig.12). Sevastianov *et al*. (2003) reported that PHB have neither effect on blood though heam-biocombatibility studies nor polymer activate complement system.

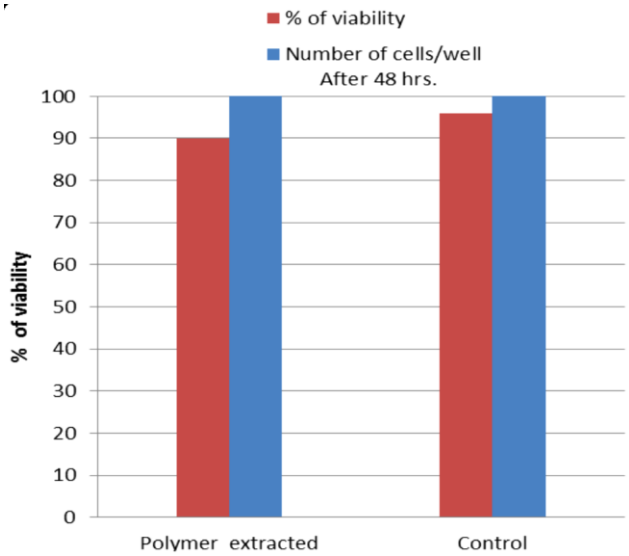

Fig.12: Relation between biopolymer sample and number of living and dead lymphocyte cells.

CONCLUSION

A massive yield of PHB (4.1 g/L) was achieved by the local bacterial isolate *B. thuringienesis*using a cheap agro-industrial waste (SCM) as a sole carbon source.PHB showed biocompatibility with the biological matter (Peripheral lymphocyte) with a biodegradability through enzymatic activity of cells and the resulting monomers have no toxic effect on the cells. This mean that PHB produced can be used in medical field.

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Conflict of Interests: There are no conflicts of interest.

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