Analysis of Polyhydroxyalkanoates Granules in *Bacillus Sp. MFD11* and *Enterobacter Sp. SEL2*

Nighat Naheed* and Nazia Jamil
Department of Microbiology and Molecular Genetics, University of the Punjab, Lahore-54590, Pakistan.
nighat_shabbir@yahoo.co.nz*

(Received on 21st July 2014, accepted in revised form 21st September 2016)

Summary: *Bacillus* sp. MFD11 (JF901809) and *Enterobacter* sp.SEL2 (JF901810) were isolated from agriculture waste contaminated sites. When fed with 2% glucose as a carbon source, these bacteria produced 75.26±0.45% and 76.61±0.28% PHA of their wet weight respectively. The accumulated PHA was extracted by direct addition of sodium dodysyl sulphate in the culture medium, which yielded 52.3±0.56 µg/l and 136.21±0.45 µg/l PHA respectively when assayed with Crotonic acid. The PHA detection medium (PDM) provided nutrient limitation condition which favored accumulation of PHA granules. A tremendous increase in cell size was observed when strain MFD11 was grown in PDM. The size of the granules as revealed by TEM micrographs spanned from 0.1 to 1.5µm which is quite large as compared to the size reported in the literature 0.2 to 0.5µm 18]. [PHA polymer was analyzed by FTIR, GC/MS and proton Nuclear magnetic resonance. The intense absorption band in the spectrum at 1724-1740 cm $^{-1}$ and 1215 cm $^{-1}$ to 1280 corresponding to C=O and C-O stretching group, respectively, indicated that the both strains were PHA producers. GC/MS analysis indicated that the polymer produced were copolymers of PHB-co-PHV. NMR also suggested that the extracted PHA was not a homopolymer but was the blend of copolymers with 3HV in lower abundance. Differential calorimetric thermal analysis showed melting temperature of 163 and 169 °C for PHA produced by both strains, respectively. However, the observed melting temperature was found to be lower than the standard PHB (Sigma-Aldrich). .

Key Words: Polyhydroxyalkanoates; TEM, FTIR, GC/MS, Proton NMR, Thermal analysis.

Introduction

Polyhydroxyalkanoates are water insoluble polymers composed of polyoxoesters and are stored inside the bacterial cells as granules. In the 21st century, researchers are concentrating on a category of polymeric material called Polyhydroxyalkanoates synthesized by microorganisms due to their biodegradability and biocompatibility. As petrochemical base plastics are non-degradable polymers which persist in environment and escalating with time, which aggravating the environmental concerns.

The resolution of light microscopy is not high enough to distinguish the granules inside the cytoplasm and produce a clear contrasting picture of the conditions of granules. Fortunately, Transmission Electron Microscope (TEM) has the ability to view the finest cell structures. Hence, TEM is been used in all fields of biological and biomedical research. Thus, in the current study, TEM analysis was also used to analyzed the nature of the PHA granules [1]. Previous reports on the Electron microscopic studies of PHB granules from *Chlorogloea fristschii, Rhodospirillum rubrum*, *Bacillus cereus* and *Bacillus megaterium* revealed a membranous structure surrounding the granule surface whose thickness ranged from 3-20 nm depending on the species [2]. The poly-β- hydroxybutyrate granules occur in two physical states *i.e*. amorphous and crystalline. The intracellular granules are generally amorphous and when extracted they become crystalline [3]. During the extraction the layer of the granules becomes damaged and the crystallinity increases. Thus, the PHB crystalline granules have an elevated melting temperature in the range between 170 and 180 °C [4-6].

Purified biopolymers (PHA) are diverse in their material properties and chemical composition due to the multitudinous of PHA monomeric units and their incorporation in varying amounts [7]. Characterization of PHA is indispensable to identify its suitable applications. Traditionally, crotonic acid assay was rapid and easy way to quantify PHB but presently, characterization and quantification of various PHA can also be accomplished using advanced analytical techniques like FTIR (Fourier transform infrared spectroscopy) [8]. FTIR has been applied to identify and differentiate between different types of purified PHA or PHA present within intact cells [9, 31]]. Well-established analytical methods can provide information on the molecular mass distribution and overall structures of the PHA. Coupling gas spectrometry to MS detector (GC-MS) ensures more reliable identity, detection, quantification and confirmation of PHA monomers, as well as in the absence of analytical standards, enables tentative detection of novel PHA. On the

*To whom all correspondence should be addressed.

other hand NMR could study the chemical composition of an intact PHA polymer and distinguish between PHA copolymers and PHA blends by providing details about functional group and the topology of the molecules. Two types of nuclear magnetic resonance (NMR) techniques are available *i.e*. ^{1}H-NMR and ^{13}C-NMR. ^{1}H-NMR (proton NMR) requires shorter analytical time as high proton is abundant in nature and is more sensitive. NMR could be applied to the analysis of novel PHA polymers as it is a powerful non-destructive tool for which analytical standards are usually not required. NMR together with GC-MS remains an excellent analytical tool in PHA investigations [10].

In addition, the PHA thermal properties such as melting temperature (Tm), thermodegradation temperature (Td) and glass transition temperature (Tg) were also examined using DTA (Differential thermal analysis) and DSC (Differential scanning calorimetry). DTA provides qualitative thermal information and is able to measure mass loss while direct heat flow measurement enables DSC to provide quantitative thermal information, making DSC the preferred method in PHA analysis [10, 11].

PHA produced by *Bacillus* sp.MFD11 and *Enterobacter* sp. SEL2 was analyzed by more advance and accurate techniques such as TEM, Crotonic acid assay, GC/ MS, FTIR and ^{1}H NMR. Thermal properties were also observed by DSC, DTA and TGA.

Results and Discussion

Bacillus sp.MFD11and *Enterobacter* sp. SEL2 produced 75.26±0.45% and 76.61±0.28% respectively by the direct addition of sodium dodesyl sulphate (SDS) in the culture medium [12]. Crotonic acid assay is an easy and common spectrophotometric assay of microbial poly-hydroxybutyrate. The PHB was quantitatively converted to Crotonic acid by heating in sulfuric acid (conc.) and its ultra violet absorption was measured [13]. PHA calculated from standard curve is shown in Fig 1. Bacterial strain *Bacillus* sp.MFD11 and *Enterobacter* sp. SEL2 produced 52.3±0.56 µg/l and 136.21±0.45 µg/l PHB respectively after 24 hours of incubation. The concentrations of PHA measured by Crotonic acid assay were a confirmation of the estimation done with the SDS method. *Bacillus sp.* was reported to produce PHB-co-PHV polymer from inexpensive carbon sources [14]. [15] Wu *et al*. (2001) also isolated *Bacillus sp.* JMa5 from molasses contaminated soil for PHB production using sugar cane molasses. It was not a generalized method for the measurement of all PHA content. The amount of PHA accumulation could further be increased by careful optimization of the assay techniques. The recovery method is also very important to obtain highest PHA concentrations without damaging the structure of the polymer [16].

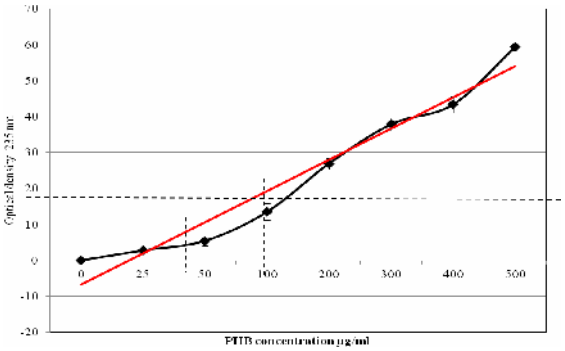

Fig. 1: The relationship between PHB concentration and absorbance at 235 nm. PHB polymer was heated in concentrated sulfuric acid for 10 minutes before absorbance was measured.

TEM images are not true reflection of the original materials as thin sections were cut randomly, some cells were cut diagonally and some were obliquely. So we can get an overall picture of the material [17]. PDM provided the nutrient limitation condition which favored accumulation of PHA granules. It was observed that in strain MFD11 there was a tremendous increase in the cell under PHA accumulating condition (Fig 2 A, B, C and D). In some of the cells a single central granule was observed. It seemed that smaller granules combined at the later stages to form a single large granule. The size and shape of the bacteria also seemed to be affected. The size of the granules as depicted in some pictures has a range from 0.1 to 1.5µm which is quite large as compared to the size reported in the literature 0.2 to 0.5µm. This reported size of the granules is more or less equal to the size of the bacteria as most of the cell reported to be ranged from 0.4 to 1.0 micron meter [18]. Surprisingly no discrete granules were visible in the strain *Enterobacter* sp. SEL2. The PHA was accumulated in the peripheral lumen of cell membrane and at poles some of the content was also visible in the central vicinity of the cytoplasm (Fig 2 E, F, G and H). According to some researchers PHA granules are spherical structures which can be maintained stably outside the microbial cell exhibiting a size range up to several µm [19]. The gram negative bacteria like *Ralstonia and Pseudomonas* exhibit the ability of using a range of substrate for the production of polyhydroxyal-kanoates. The gram positive bacteria like *Bacillus* has been successfully used in many industrial applications but yet to be considered for PHA

production at industrial scale [20].

In literature a large number of electron micrographs are available. The experimental pictures were compared and it was confirmed that the PHA granules were present in the cytoplasm as discrete granules. The bacterial strain *Bacillus* sp. MFD11 under PHA accumulation conditions revealed the range of granule size increased to 1.0 to 1.5 micrometer (Fig 2). [21]Tian *et al.,* in 2005a studied the effect of nutrient rich and nutrient limitation conditions on *W. eutropha*. It was observed that the cells grown under the first condition did not formed granules while the under the later conditions the size of the granules were significantly increased. In strain *Enterobacter* sp.SEL2 the results were also the same except that no discrete granules were observed but the accumulated PHA was observed in the lumen of the cytoplasm. The number of granules increased and that's why the accumulation percentage was high (76.61±0.28%).

SDS extracted and chloroform purified polyhydroxyalkanoates polymer from PHA detection media fermented broth was analyzed by GC/MS. The PHB was detected (Fig 3 and 4) by analyzing the mass spectra (M/Z). The peak 2 matches the retention time for 2-methyl 2-propenyl ester with the molecular formula $C_7H_{12}O_2$. Polymer obtained was dried and subjected to Fourier Transform Infra Red spectroscopy. The FTIR spectroscopic results obtained for strain *Bacillus* sp. MFD11is shown in Fig 5 and for strain *Enterobacter* sp. SEL2 is shown in Fig 6. In Fig 5, a peak at 2927.561represents the $–CH_2$ group, the next distinct peak was observed at 2857.329 which represents the –CH group, However the absorption band at 1741.44 cm^{-1} has been described to be a PHA indicator band allocated to carbonyl (C = O) ester bond according to Randriamahefa *et al*. [32]and then a peak at 1215.695 which represented the –C-O group. This peak would have been around 1280 so it meant it was in stretched form. In the Fig 6, a peak at 1724.933 represented the –C=O group and another important group observed was at 1215.749 which represented the-C-O group. A prominent peak at 4200 cm^{-1} was also observed in Fig 5 and 6.

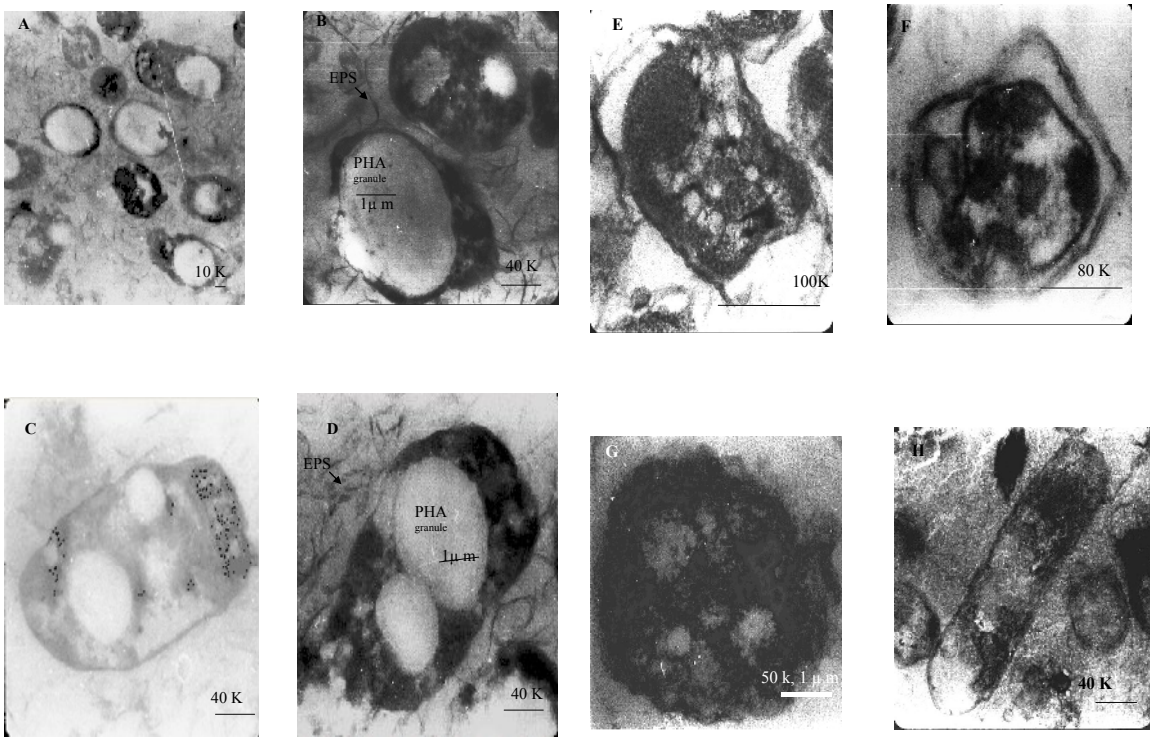

Fig. 2: Transmission electron micrograph of different cells of *Bacillus sp.* MFD11 (A, B, C and D) and *Enterobacter sp.*SEL2 (E, F, G and H) grown in PHA detection media at pH 7.0, 37ºC and 150rpm for 24 hours. Pictures were taken at different magnifications showing increased size of PHA granules. Fig. 1 B indicates a bulging cells having one granule of size >1 µm in diameter. Bar scale represents 1µm.

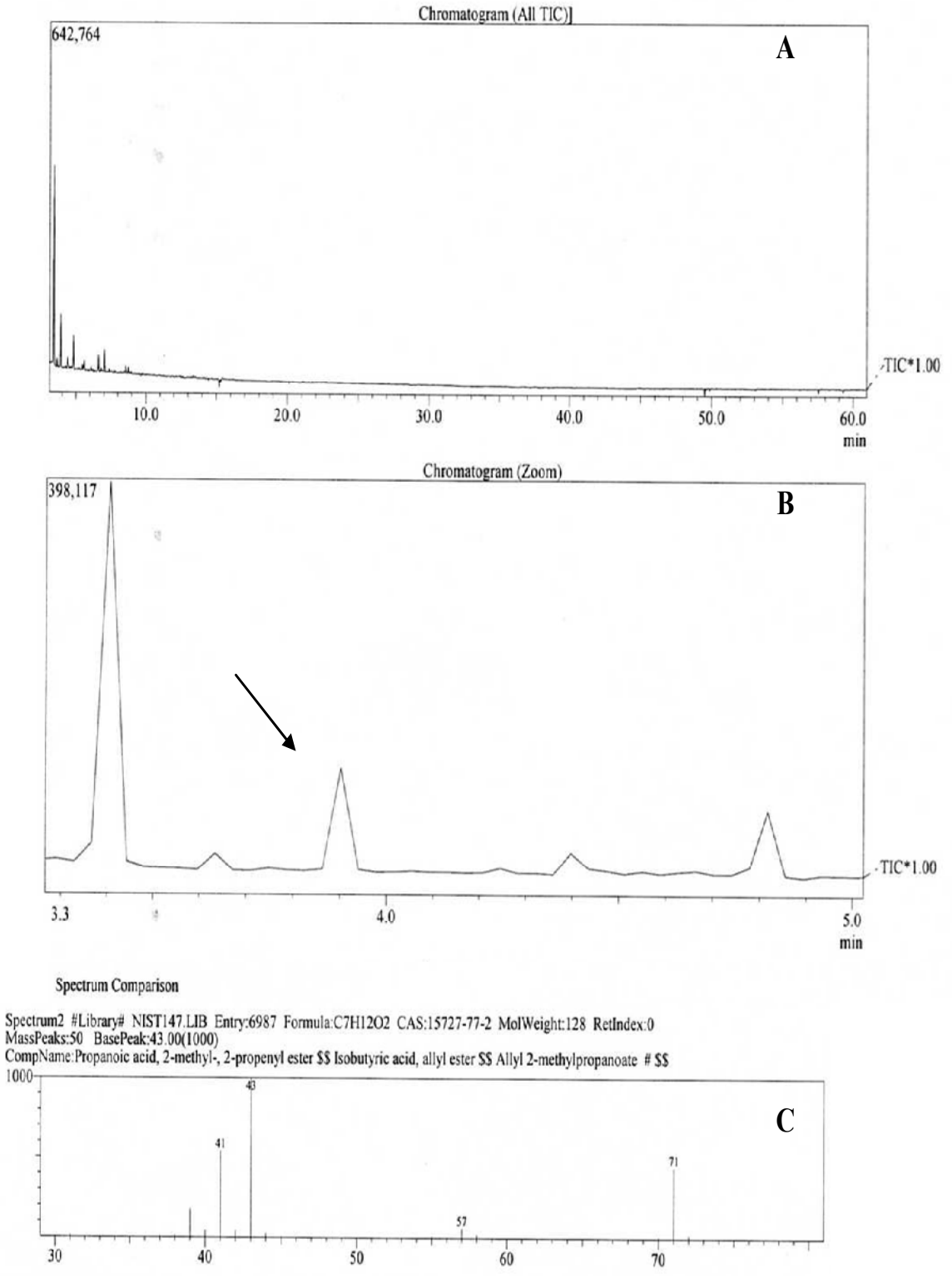

Fig 3: Gas chromatography and mass spectrometry of polyhydroxyalkanoates polymer produced by bacterial strain Enterobacter sp. SEL2 in PDM using molasses as carbon source. A). Chromatogram B). The peaks obtained at retention time 4.817 corresponding to methyl esters. C). Mass spectrum of peak 2 with one derivative.

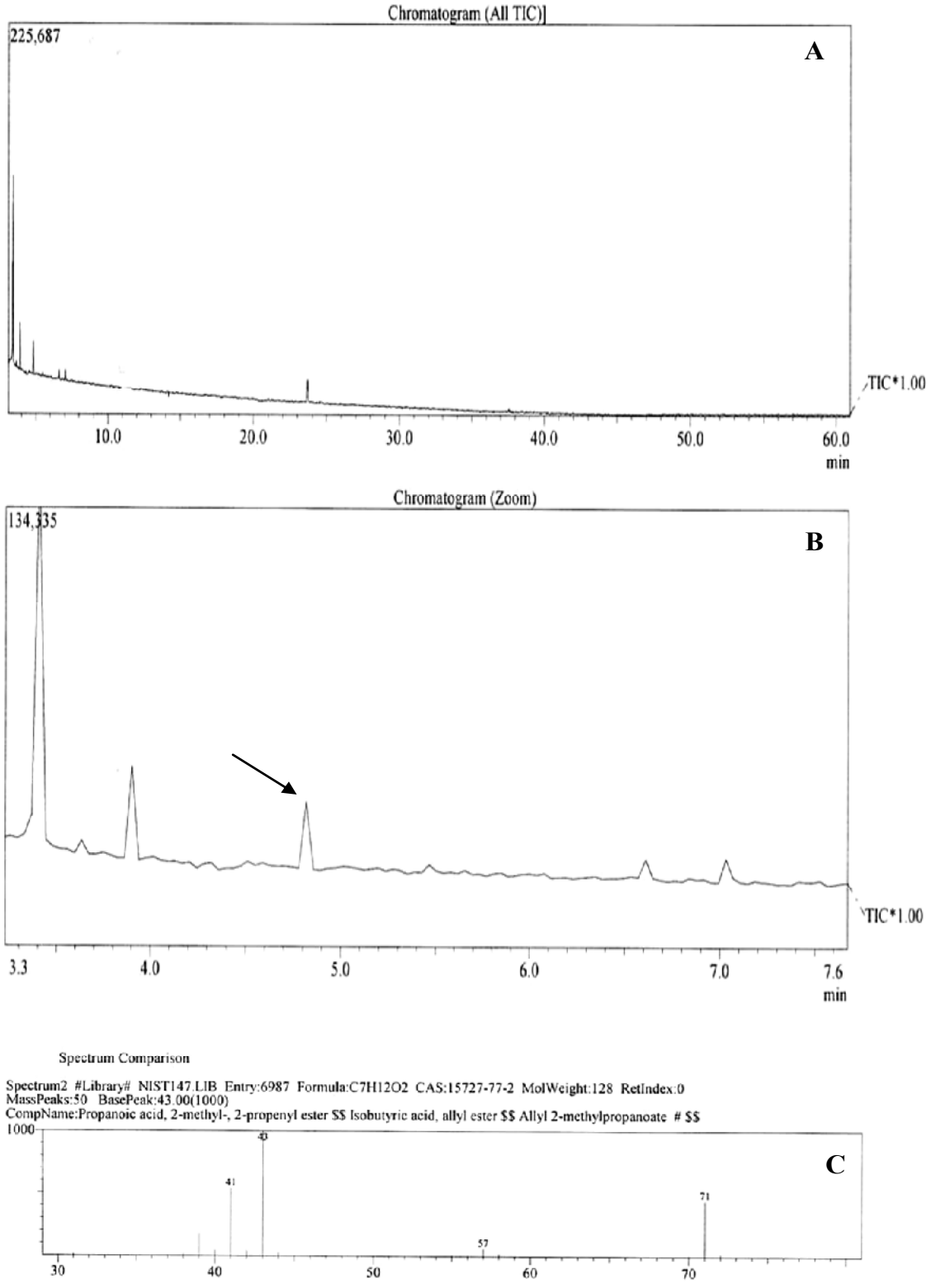

Fig. 4: Gas chromatography and mass spectrometry of polyhydroxyalkanoates polymer produced by bacterial strain Bacillus sp. MFD11 in PDM using molasses as carbon source. A). Chromatogram B). The peaks obtained at retention time 4.817 corresponding to methyl esters. C). Mass spectrum of peak 2 with one derivative.

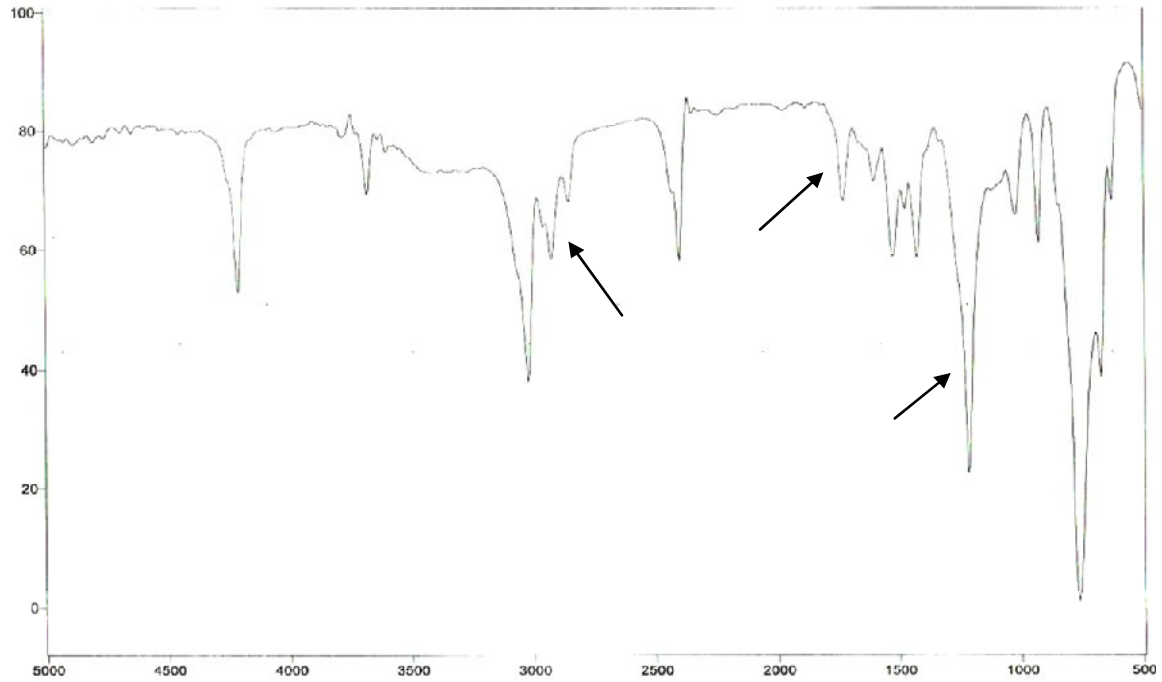

Fig 5: The FT-IR spectra of chloroform extracted PHB from Bacillus sp. MFD11. The arrows at 1724- 1730 cm^{-1} corresponding for ester carbonyl group and stretching at 1215 cm^{-1} attributed to C-O group respectively. The prominent peak at 4200 cm^{-1} was also observed.

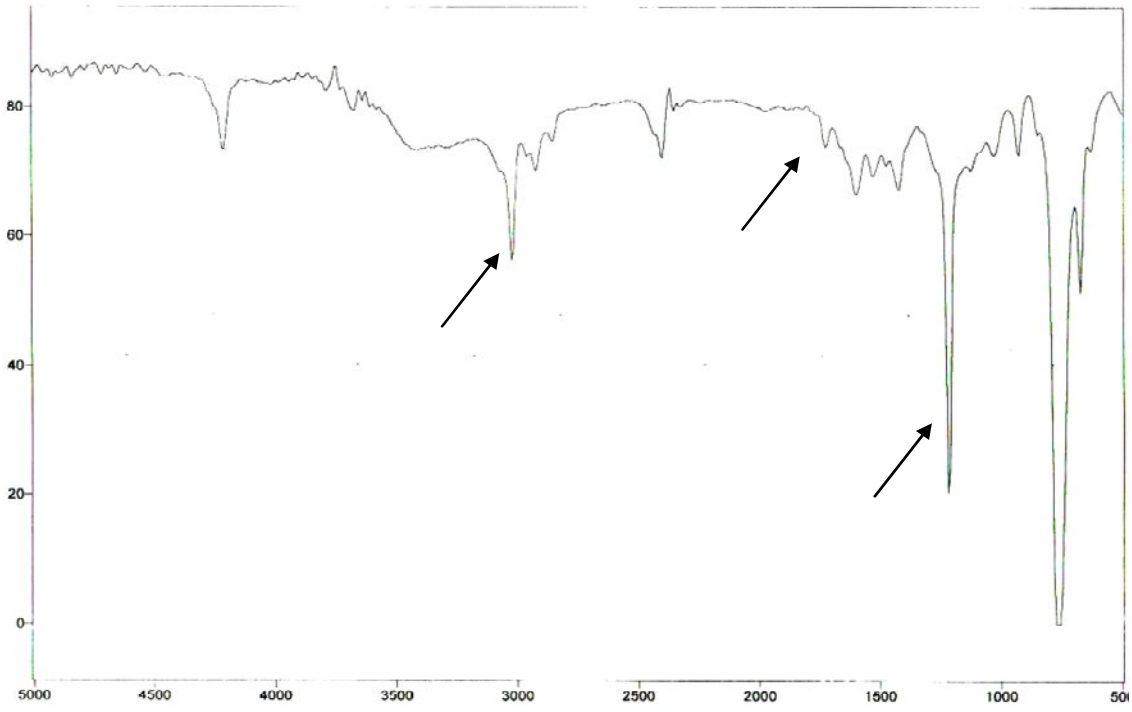

Fig. 6: The FT-IR spectra of chloroform extracted PHB from Enterobacter sp. SEL2. The arrows at 1724-1730 cm^{-1} and 1215 cm^{-1} corresponding for ester carbonyl group and stretching for C-O group respectively.

The experimental data was compared to the published data for the purpose of confirmation. [22] Shamala *et al.,* in 2003 described the intense absorption in this region was typical to polyhydroxyalkanoates at 1724 to 1740 cm $^{-1}$ and 1280 cm $^{-1}$ corresponding to C=O and C-O groups respectively which is stretched somewhat [23]. Otari and Ghosh in 2009 described the peak at 1732.13 corresponded to –C=O group and a peak at 2956.97 represents the –CH group. So on the bases of this comparison it was safely concluded that the extracted polymer was mostly PHB [32].

SDS extracted and chloroform purified PHA polymer was analyzed by 500-MHz ^{1}H NMR spectrometer at 300 K. The ^{1}H NMR spectra of the polymer produced by two maximum PHA accumulating strains *Bacillus* sp. MFD11 and the *Enterobacter* sp.SEL2 were given in Fig 7 and Fig 8 respectively. A copolymer of HB and HV was obtained. In both Figs peak number 3 could be of -CH group. A chemical shift at 0.87 (first biggest peak from the right) could be assigned to the protons connected with methyl group of HV (CH3, C5); also a peak at 1.65 (CH2, C4), a third at 2.74 (CH2, C2) and lastly a small one at 5.15 (CH, C3). All these chemical shifts are with a smaller area but can be assigned for a small fraction of HV in the polymer. The ^{1}H NMR spectra also showed the presence of three groups of other signals characteristics of PHB; a doublet of resonance at 1.28 ppm was observed which is associated to methyl group, it was found linked to one proton, two quadruplet at 2.30 ppm which are characteristic of methylene group next to an asymmetric carbon atom having a single proton and a multiplet at 5.35 ppm characteristic of methyne group. Two more signals are also observed one at 7.24 ppm which was attributed to chloroform. The ^{1}H NMR spectrum of polymer produced by strain *Bacillus* sp. MFD11 in Fig 8 showed the chemical shift in the region 1.27 this is also ascribed to methyl group linked to one proton of PHB. Other strong resonance signals observed were same in both spectra. The results obtained were confirmed by comparison with data reported in the literature [24, 11]. So in the light of above discussion it can be said that *Bacillus* sp. MFD11 and *Enterobacter sp.*SEL2 produces PHA in the form of copolymer PHBV.

In thermal analysis, change of material with temperature is studied. Several methods are commonly employed which are different from one another by the property which is measured. Polyhydroxyalkanoates polymer exhibit different mechanical and thermal properties depending on the types of constituents monomer attached in the side chain [25].

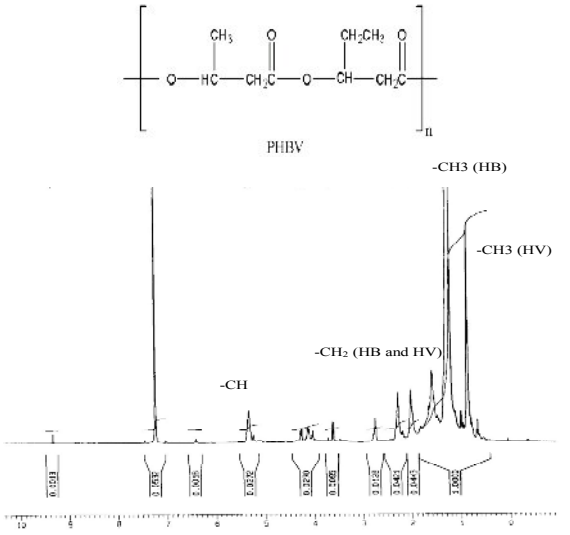

Fig. 7: The 500-MHz ^{1}H NMR spectrum obtained in d-chloroform of the PHA polymer produced by strain *Enterobacter sp.* SEL2 using optimal growth conditions in molasses based PHA detection media.

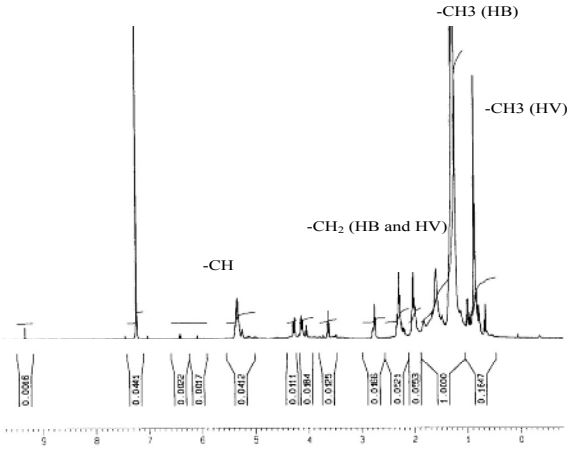

Fig. 8: The 500-MHz ^{1}H NMR spectrum obtained in d-chloroform of the PHA polymer produced by strain *Bacillus sp.* MFD11 using optimal growth conditions in molasses based PHA detection media.

The results of thermal analysis of polymer produced by strains *Enterobacter* sp. SEL2, *Bacillus* sp. MFD11 and PHB (sigma) are shown in Fig 9 A, B and C. Thermal degradation of extracted polymer proceeded in one-step with a highest decomposition temperature at 275, 245 and 300°C for bacterial strains MFD11, SEL2 and PHB (sigma) respectively. This thermal breakdown at highest degradation

temperature of about 300°C is mainly attributed to the ester cleavage of PHA polymer by β-elimination reaction [26]. The lower degradation temperature observed in case of extracted polymer (Fig 9 A and B) as compare to PHB (sigma) (Fig 9 C) suggested the existence of a copolymer with another monomer other than HB in low abundance. The residual weight of polymers beyond 250°C is given in Table-1.

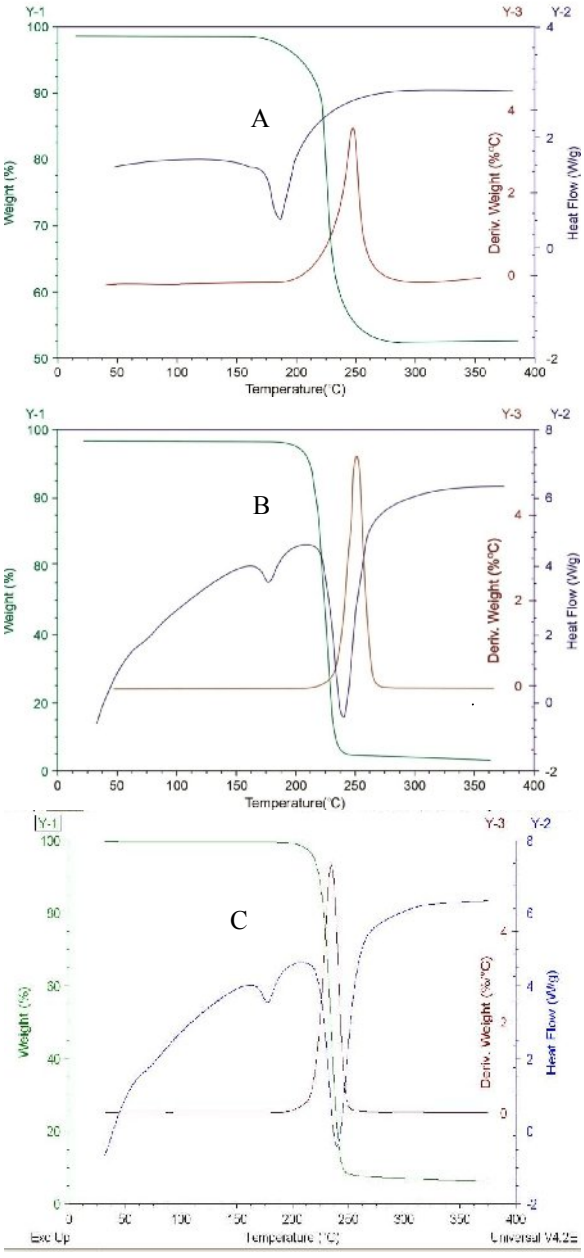

Fig. 9: Thermogram for thermal analysis A) *Bacillus sp.* MFD11 B).*Enterobacter sp.* SEL2 C): PHB (sigma). {TGA (green); DSC (Blue); DTA (Brown)}.

Table-1: Initial and maximum temperature evaluated from TGA.

Polymer	Ti (°C)*	T_{max} (°C)	Residual weight (%)at 250 °C
***Bacillus sp.* MFD11**	**170**	**274**	**67.5**
***Enterobacter sp.* SEL2**	**191**	**245**	**7.5**
PHB (Sigma)	**215**	**300**	**5.0**

*Value determined at a 5% weight loss on the TGA thermogram

DSC studies (non-isothermal) of extracted polymer and PHB (sigma) exhibited the crystallinity of polymer. Two peaks were observed for extracted polymer and PHB (sigma) in between 140 and 250 °C (Fig. 9). The peak observed at the higher temperature is associated with the melting of the polymer. Another one noticed at a lower temperature is probably due to the melting of the imperfect crystals formed during the sample preparation. The melting enthalpy (ΔHf) was obtained from the area of the two endothermal peaks. The crystallinity degree (Xc) was calculated based on the melting enthalpy of 146 J/g of 100% crystalline PHB (Table-2) The enthalpy of melting (ΔHf) is 36.5 J/g for standard PHB and PHB (sigma) was 37.5 J/g. The experimental results were compared to the published results for the purpose of confirmation. The results were quite in accordance with the PHBV type of copolymer. So the thermal analysis follows the similar pathway of PHA [4, 5, 6, 27].

Table-2: DSC results of extracted polymer and PHB (sigma).

Polymer	Tm (°C)	ΔHf (J/g)	Xc (%)
***Bacillus sp.* MFD11**	**163**	**29.5**	**40.41**
***Enterobacter sp.* SEL2**	**169**	**34.5**	**47.26**
PHB (Sigma)	**174**	**37.5**	**51**

Experimental

Bacterial Strains

The bacterial strains *Bacillus* sp. MFD11 (JF901809) and *Enterobacter* sp.SEL2 (JF901810) were highest PHA producers isolated from agriculture waste contaminated sites and were identified from Macrogen In. Korea.

Growth Conditions

Both bacterial strains were grown in PHA detection media [28] to estimate the polyhydroxyalkanoate production. The inoculums were prepared in 50 ml of LB broth in 100 ml flask incubated overnight at 37°C and 150 rpm. The 150 ml of the PHA detection media broth with 2% glucose (carbon source) was taken in 250 ml conical flask [28]. The glucose used was of analytical grade. The media were inoculated such as to get the O.D 0.05 at 600 nm. The cultures were incubated for 24

hours at 37 ºC and 150 rpm. The 5 ml sample was used for the preparation of TEM sample.

Crotonic Acid Assay

The selected bacterial strains were grown under the optimized growth conditionsmention above. The cells were centrifuged and pellet was re-suspended in commercial grade sodiumhypochlorite solution equal to the original volume of medium. After 1 hour incubation at 37°C the PHA granules were centrifuged, washed with water, and then washed with acetone and alcohol. Finally, the granules were dissolved in chloroform, boiled and filtered. The filterate was used for Crotonic acid Assay. A 5 to 50µg PHA sample in chloroform was taken in a test tube. Evaporated the chloroform and added 10 ml of concentrated H_2SO_4, capped the tubes with glass marbles and heated for 10 minutes at 100°C in a water bath. After cooling and mixing, a sample was transferred to a silica cuvette. The absorbance was taken at 235 nm against a H_2SO_4 blank [13].The standard curve was constructed by using standard PHB purchased from Sigma.

Sample Preparation for TEM Analysis

Reagents

All the reagents were purchased from Sigma and were of analytical grade.

Fixation

Bacterial cell pellet was prepared from five milliliters of the cell culture in PHA detection media with 2 % glucose after 48 hours of incubation. It was dispersed in 5% Glutaraldehyde fixative prepared in 0.2 M Pipes Buffer and pH was set to 6.8. To complete the fixation process samples were placed for 18 hours on a 55^0 fixed angle specimen rotator at 5-7 rpm at room temperature. Fixative was removed by rinsing with 0.2 M Pipes buffer (pH 6.8) for three times each with an interval of 15 minutes. Post fixation was done with 1% Osmium Tetroxide for 18 hours at room temperature [21].

Dehydration

After uranyl acetate staining, the pellet was rinsed with 1.5 ml of distilled H_2O immediately and was then repelleted by centrifugation. Dehydration was done with increasing amounts of ethanol. The pellet was subjected to 30% , 50% and 70% (vol/vol) ethanol water for 15 minutes in each grade and then in 100% ethanol for overnight [21]. The pellet was left in absolute Acetone 2 X 30 minutes as transitional solvent because the ethanol doesn't mixes well with spur Resin.

Embedding

The cells were infiltrated with a mixture of Spur embedding media. The ratio of resin to acetone was 1:3 for 18 hours followed by 1:1, 2:1 and 3:1. Each fraction was left overnight. Finally a 100% resin mixture was added to the samples and vacuum infiltration was carried again for 5 hours. The samples were oriented in Moulds and resin cured at 70 ^{0}C for 48 hours [21].

Sectioning and Microscopy

The polymerized resin blocks were trimmed and faced with fine scalpel blade and glass knife before ultra thin serial sections of approx.120 nm, cut with RMC MT 7000 ultra microtome and placed on 200 mesh nickel grid, these sections were stained with 5% Uranyl Acetate for 30 minutes and then washed twice with distilled water and again stained with Lead Citrate for 10 minutes in NaOH chamber [21]. Examination of sections was performed with JEOL JEM1010 Transmission Electron microscope operating at 80 kv available at National Institute for Biotechnology and Genetic Engineering (NIBGE) Faisalabad.

Structure Elucidation of PHA

The extracted polymer produced by the selected bacterial strains was subjected to following tests.

Gas Chromatography and Mass Spectroscopy (GC/MS)

Samples of PHA polymer were analyzed on a J & W Scientific GC-2010, MS QP-2010 series gas chromatograph equipped with a 30-m by 0.25-mm ,film 0.25 µm DB-5 column (Shimadzu Japan) operating in split mode (split ratio, 5:1) with temperature programming (60°C for 2 minutes, increments of 5°C/minutes up to 200°C, increments of 40°C/minutes up to 280°C, and 5 minutes at 280°C). The injection volume was 1 µl. For peak identification, PHB standard from Sigma was used [29].

Fourier Transform Infra Red Spectroscopy (FT-IR)

The polymer obtained after extraction was dissolved in chloroform and placed on KBR window.

After evaporation of the solvent, the film spectrum was taken between 500 to 5000 cm^{-1} on FT-IR spectrophotometer (Model 2000, MIDAC Corporation, USA) to characterize banding pattern of extracted polymer [22]..

Proton NMR

^1H- NMR was carried out from extracted and purified PHA dissolved in CD_3Cl from HEJ Karachi on Bruker AVANCE-NMR-500 spectrometer. Standard PHB (Sigma) proton NMR spectrum was used for comparison [30].

Thermal Analysis

The thermal properties of the PHA extracted were determined on TA Instruments Systems.

Differential Scanning Calorimetry (DSC)

The differential scanning calorimetry (DSC) data of the polymer was recorded on a SDT Q600 V8.2 Build 100. A 15-20 mg of the sample was encapsulated in hermetic aluminium pans and heated from 0 to 400 °C at a heating rate of 10 °C per minute under nitrogen conditions. The melting temperature and melting enthalpy (ΔH_f) was determined from DSC endothermal peakes. In the presence of multiple endothermal peaks, the maximum peak was taken as Tm.The crystallinity (Xc) of polymer was calculated by the equation [27].

$$Xc = \Delta H_f * 100 / \Delta H_o * w$$

where,

ΔH_f = melting enthalpy of the sample (J/g).
ΔH_o = melting enthalpy of the 100% crystalline PHB which is assumed to be 146 J/g
w is the weight fraction of PHB in the sample.

Thermogravimeteric analysis (TGA) and Differential Thermal analysis (DTA)

Thermogravimetric analysis was performed on the same instrument (SDT Q600 V8.2 Build 100) with different sample temperatures. The temperature was built up at a heating rate of 10°C/minutes under nitrogen conditions to a temperature well above the degradation temperature of PHA [27].

Conclusion

The polymer produced by strains SEL2 and MFD11 was analyzed by FTIR, GC/MS and proton Nuclear magnetic resonance. The intense absorption band in the spectrum at 1724-1740 cm^{-1} and 1215 cm^{-1} to 1280 corresponding to C=O and C-O stretching group, respectively, indicated that the both strains were PHA producers. GC/MS analysis indicated that the polymer produced were of PHBV copolymers. Further analysis by proton NMR strongly suggested that polymer was of PHBV type. The melting temperature was lower as compare to PHB (sigma). The medium chain length polyhydroxyalkanoates have melting temperature ranged from 170 to 180 °C. The experimental data obtained the melting temperature of strains *Enterobacter* sp. SEL2 and *Bacillus* sp. MFD11 from differential calorimetric thermo analysis were 163 and 169 °C respectively. Comparing it with the literature it was confirmed that the compound was not a single polymer but was the blend of other polymers may be HV in lower abundance as suggested by NMR.

Acknowledgement

This work is a part of Ph.D. Dissertation submitted by Nighat Naheed, Department of Microbiology and Molecular Genetics, University of the Punjab, Lahore, Pakistan to the Higher Education of Pakistan.

References

1. D. Jendrossek, O. Selchow and M. Hoppert, Poly (3-hydroxybutyrate) granules at the early stages of formation are localized close to the cytoplasmic membrane in Caryophanon latum. *Appl Environ Microb* **73**, 586 (2007).
2. J. Tian, J. He, A. G. Lawrence, P. Liu, W. Watson, A. J. Sinskey and J. Stubbe, Analysis of transient polyhydroxybutyrate production in Wautersia eutropha H16 by quantitative Western analysis and transmission electron microscopy. *j bacteriol*, 3825 (2005b).
3. M. Porter and J. Yu, Monitoring the in situ crystallization of native biopolyester granules in *Ralstonia eutropha* via infrared spectroscopy. *J Microbiol Meth,* **87**, 49 (2011).
4. W. P. Xie and G. Q. Chen, Production and characterization of terpolyester poly (3-hydroxybutyrate-co-4-hydroxybutyrate-co-3-hydroxyhexanoate) by recombinant Aeromonas hydrophila 4AK4 harboring genes phaPCJ. *Biochem Eng J,* **38**, 384 (2008).
5. Y. Takagi, R. Yasuda, A. Maehara and T. Yamane, Microbial synthesis and characterization of polyhydroxyalkanoates with fluorinated phenoxy side groups from

Pseudomonas putida. *Eur Polym J,* **40**, 1551 (2004).

6. R. J. Sánchez, J. Schripsema, L. F. da Silva, M. K. Taciro, J. G. Pradella and J. G. C. Gomez, Medium-chain-length polyhydroxyalkanoic acids (PHA mcl) produced by Pseudomonas putida IPT 046 from renewable sources. *Eur Polym J* **39**, 1385 (2003).
7. L. Tripathi, L. P. Wu, M. Dechuan, J. Chen, Q. Wu and G. Q. Chen, *Pseudomonas putida* KT2442 as a platform for the biosynthesis of polyhydroxyalkanoates with adjustable monomer contents and compositions. *Bioresource Technol,* **142**, 225 (2013).
8. M. V. Arcos-Hernandez, N. Gurieff, S. Pratt, P. Magnusson, A. Werker, A. Vargas and P. Lant, Rapid quantification of intracellular PHA using infrared spectroscopy: an application in mixed cultures. *J Biotechnol,* **150**, 372 (2010).
9. T. Shamala, M. Divyashree, R. Davis, K. L. Kumari, S. Vijayendra and B. Raj, Production and characterization of bacterial PHA copolymer and evaluation of their blends by Fourier transform infrared spectroscopy and scanning electron microscopy. *Indian J Microbiol,* **49**, 251 (2009).
10. G. Y. A. Tan, C. L. Chen, L. Li, L. Ge, L. Wang, I. M. N. Razaad, Y. Li, L. Zhao, Y. Mo and J. Y. Wang, Start a research on biopolymer polyhydroxyalkanoate (PHA): a review. *Polymers* **6**, 706 (2014).
11. J. Tao, C. Song, M. Cao, D. Hu, L. Liu, N. Liu and S. Wang, Thermal properties and degradability of poly (propylene carbonate)/poly (β-hydroxybutyrate-co-β-hydroxyvalerate)(PPC/PHBV) blends. *Polym Degrad Stabil*, **94**, 575 (2009).
12. N. Nighat, J. Nazia, H. Shahida and A. Ghulam, Biosynthesis of polyhydroxybutyrate in Enterobacter sp. SEL2 and Enterobacteriaceae bacterium sp. PFW1 using sugar cane molasses as media. *Afr J Biotechnol,* **11**, 3321 (2012).
13. J. H. Law and R. A. Slepecky, Assay of poly-β-hydroxybutyric acid. *J Bacteriol,* **82**, 33 (1961).
14. P. K. A. Kumar, T. R. Shamala, L. Kshama, M. H. Prakash, G. J. Joshi, A. Chandrashekar, K. S. carbohydrate-rich mahua (Madhuca sp.) flowers. *J Appl Microbiol,* **103**, 204 (2007).
15. Q. Wu, H. Huang, G. Hu, J. Chen, K. P. Ho and G. Q. Chen, Production of poly-3-hydroxybutyrate by Bacillus sp. JMa5 cultivated in molasses media. *A Van Leeuw J Microb* **80**, 111 (2001).
16. J. Choi and S. Y. Lee, Factors affecting the economics of polyhydroxyalkanoate production by bacterial fermentation. *Appl Microbiol Biot,* **51**, 13 (1999a).
17. K. Sudesh, H. Abe and Y. Doi, Synthesis, structure and properties of polyhydroxyalkanoates: biological polyesters. *Prog Polym Sci,* **25**, 1503 (2000).
18. C. Y. Loo, W. H. Lee, T. Tsuge, Y. Doi and K. Sudesh, Biosynthesis and characterization of poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) from palm oil products in a *Wautersia eutropha* mutant. *Biotechnol Lett,* **27**, 1405 (2005).
19. B. T. Bäckström, J. A. Brockelbank and B. H. Rehm, Recombinant *Escherichia coli* produces tailor-made biopolyester granules for applications in fluorescence activated cell sorting: functional display of the mouse interleukin-2 and myelin oligodendrocyte glycoprotein. *BMC Biotechnol,* **7**, 3 (2007).
20. M. Singh, S. K. Patel and V. C. Kalia, Bacillus subtilis as potential producer for polyhydroxyalkanoates. *Microb Cell Fact,* **8**, 38 (2009).
21. J. Tian, A. Sinskey, J. and Stubbe, Kinetic studies of polyhydroxybutyrate granule formation in *Wautersia eutropha* H16 by transmission electron microscopy. *J Bacteriol*, 3814 (2005a).
22. polyhydroxyalkanoate (PHA)-producing Bacillus spp. using the polymerase chain reaction (PCR). *J Appl Microbiol* **94**, 369 (2003).
23. S. Otari and J. Ghosh, Production and characterization of the polymer polyhydroxy butyrate-co-polyhydroxy valerate by *Bacillus megaterium* NCIM 2475. *Curr Res J Biol Sci,* **1**, 23 (2009).
24. M. R. Zakaria, S. Abd-Aziz, H. Ariffin, N. A. A. Rahman, P. L. Yee and M. A. Hassan, Comamonas sp. EB172 isolated from digester treating palm oil mill effluent as potential polyhydroxyalkanoate (PHA) producer. *Afr J Biotechnol,* **7,** 4118 (2008).
25. D. Jendrossek and R. Handrick, Microbial degradation of polyhydroxyalkanoates. *Annu Rev Microbiol,* **56**, 403 (2002).
26. G. G. Choi, M. W. Kim, J. Y. Kim and Y. H. Rhee, Production of poly (3-hydroxybutyrate-co-3-hydroxyvalerate) with high molar fractions of 3-hydroxyvalerate by a threonine-overproducing

mutant of *Alcaligenes* sp. SH-69. *Biotechnol Lett,* **9**, 665 (2003).

27. R. Sindhu, B. Ammu, P. Binod, S. K. Deepthi, K. Ramachandran, C. R. Soccol and A. Pandey, Production and characterization of poly-3-hydroxybutyrate from crude glycerol by Bacillus sphaericus NII 0838 and improving its thermal properties by blending with other polymers. *Braz Arch Of Biol Techn,* **54**, 783 (2011).
28. J. Lee and S. Y. Choi, In Polyhydroxyalkanoates: Biodegradable Polymer, 2nd ed. *Manual of industrial microbiology and biotechnology*, American Society for Microbiology,Washington, p. 616 (1999).
29. N. D. O'Leary, K. E. O'Connor, P. Ward, M. Goff and A. D. Dobson, Genetic characterization of accumulation of polyhydroxyalkanoate from styrene in *Pseudomonas putida* CA-3. *Appl Environ Microbiol,* **71**, 4380 (2005).
30. S. Labuzek and I. Radecka, Biosynthesis of PHB tercopolymer by Bacillus cereus UW85. *J Appl Microbiol,* **90**, 353 (2001).
31. A. M. Gumel, M. S. M. Annuar and T. Heidelberg, Biosynthesis and characterization of polyhydroxyalkanoates copolymers produced by Pseudomonas putida Bet001 isolated from palm oil mill effluent. *PLoS One*, *7*, p.e45214. (2012).
32. S. Randriamahefa, E. Renard, P. Gue´rin, and V. Langlois, Fourier transform infrared spectroscopy for screening and quantifying production of PHAs by Pseudomonas grown on sodium octanoate. *Biomacromolecules*, **4,** 1092 (2003).