5.4 DISCUSSION

5.4.1 Bacterium and Media

In the present study *Lysinibacillus sphaericus* BBKGBS6 bacteria was produced PHB and it was isolated from nursery field soil (Kaliwal *et al.*., 2015). *Lysinibacillus sphaericus* BBKGBS6 was grown and maintained in a PHB production medium. The bacterium was grown under submerged fermentation. PHB for characterization was obtained by solvent extraction in a soxhlet apparatus. PHB was collected by decanting the hexane chloroform mixture and then air dried.

5.4.2 Characterization of Polyhydroxybutyrate (PHB) produced from *Lysinibacillus sphaericus* BBKGBS6

Polyhydroxybutyrate (PHB) was characterized by analytical methods (chemical properties), thermal properties and physical properties.

5.4.2.1 Analytical methods (chemical properties)

PHB can be converted quantitatively to crotonic acid by heating in concentrated sulfuric acid and the U-V absorption maximum of crotonic acid is shifted to 235 nm when concentrated sulfuric acid is the solvent (Law and Slepecky, 1961) carbonyl compounds absorb light below the ultra violet range and hence, are difficult to detect by spectrophotometer. Therefore PHB is converted to crotonic acid on dehydration or heating. Dehydration is done using concentrated sulfuric acid. The principle of U-V absorption lies in the fact that the ultra violet absorption maximum of α, β unsaturated acids undergoes a strong bathochromic shift or a shift to the lower frequency in sulfuric acid and can be recorded in the U-V range. The absorption maximum shifts to 235 nm (Williamson and Wilkinson, 1958; Slepecky and Law, 1960). In the present study
PHB can be detected even at 5 µg level. The purity of PHB obtained was observed by correlating the absorption of the compound and corresponding purity levels (µg) of standard PHB (Sigma). The purity of extracted sample was 79%. The results indicated the presence and levels of crotonic acid and in directly, indirectly confirmed the presence of polyhydroxybutyrate in the sample.

Characterization of PHB by FTIR is a routine chemical technique used to study the molecular structure. It can be both qualitative as well as quantitative. Basically FTIR is used for determining the presence or absence of specific functional groups in a reaction mixture. Any compound with covalent bonds will absorb frequencies of electromagnetic radiation in the infrared region. In FTIR, as the temperature of a substance is raised it begins to emit radiant energy. The amount of emitted radiation forms a curve as a functional wavelength or frequency depending on the temperature of the substance and its emissivity. Organic substances exhibit characteristic group frequencies in infrared region. The absorption spectrum of a given mixture is generally additive i.e., the sum of the individual spectra of the components. The intensity of the absorption bands is related to the concentration of the substance that absorbs the incident radiation. By substituting a wide variety of materials of organic origin in the beam of the radiating material, it is readily observed that certain wavelengths are found to be associated with changes in the structures of the absorbing molecules. The resulting curves can be distinguished from one another by the presence or the absence of particular bands of energy. Thus the absorption bands in an infrared spectrum are at frequencies of corresponding to the frequencies of vibration of the molecule concerned. These frequencies are also dependent upon the special interrelationship of the atoms contained in the molecular unit. Infrared spectra can thus provide a total simultaneous chemical analysis (Kaniz et al., 2000). Polyhydroxybutyrate as well as medium chain lengths PHAs have also been rapidly detected by FTIR (Hong et al., 1999).
In the present study IR spectrum obtained for PHB sample from *Lysinibacillus sphaericus* BBKGBS6. The IR spectra obtained showed characteristic absorption bonds for esters and the presence of C=O and C-O were obtained at 1724 cm\(^{-1}\) and 1281 cm\(^{-1}\) respectively (Misra *et al.*, 2000). Apart from this a peak at 1377 cm\(^{-1}\) was seen which is due to the CH\(_3\) or methyl bending. Peaks due to methyl stretching were also observed at 2975 cm\(^{-1}\) and 2926 cm\(^{-1}\). CH\(_2\) or methylene group was observed at 1450 cm\(^{-1}\) and methine or CH peak was at 3434 cm\(^{-1}\).

An increase or shift to left or higher frequency of 1739 cm\(^{-1}\) was seen in some samples. This peak has been interpreted as to the presence of higher alkanoates. PHAMCL at 1740 cm\(^{-1}\), (HB + mcl HA) at 1732 cm\(^{-1}\) have been reported (Hong *et al.*, 1999). But soil bacteria are known to accumulate only short chain length PHB such. Hence the shift could be because of a possible conjugation with the ester single-bonded oxygen. Such conjugation interferes with possible resonance with carbonyl group leading to an increase in absorption frequency for the C=O bond. This was seen along with decrease in the frequency of the C-O stretch to 1215 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 shift to higher frequency was interpreted as said above, since further studies confirmed only the presence of PHB in the sample. Strong absorption was observed at 1731 cm\(^{-1}\) due to C=O stretching vibration 1215 cm\(^{-1}\) characteristic of C-O stretch at 3000 cm\(^{-1}\) and 1460 cm\(^{-1}\) C-H stretching and bending respectively. A bond at 1380 cm\(^{-1}\) showed the presence of methyl group. Intensity of absorption at 3000 cm\(^{-1}\) in relation to intensity at 1731 cm\(^{-1}\) was longer. Absorption at 3000 cm\(^{-1}\) indicates longer aliphatic chains. IR spectroscopy revealed the possible presence of a copolymer such as Polyhydroxy butyrate in the sample.
Gas chromatography is a very efficient method for quantitative estimation as well as characterization of PHB. GCMS is a useful technique to determine and understand the molecular structure of any compound. In the present results the methyl esters obtained after methanolysis of the sample showed fragmentation patterns in GCMS that enabled to define the structure of PHB obtained from *Lysinibacillus sphaericus* BBKGBS6. The major molecular fragmentation were m/z 115 (C$_5$H$_9$O$_3$)$^+$, m/z 105 (C$_4$H$_7$O$_3$)$^+$, m/z 85 (C$_4$H$_7$O$_2$)$^+$, m/z 78 (C$_3$H$_6$O$_2$)$^+$, m/z 63 (C$_2$H$_3$O$_2$)$^+$, m/z 43 (C$_2$H$_3$O), m/z 57 (C$_2$H$_5$O$_2$) (C$_3$H$_7$O$^+$), m/z 71 (C$_3$H$_7$O$_2^+$) obtained. The α-cleavage reaction resulted in the loss of the alkoxy group from the ester to corresponding acylium ion and was observed at m/z 43, m/z 71, m/z 85, a second useful peak observed from the loss of the alkyl group from the acyl portion of the ester molecule which appeared as m/z 59 and m/z 87, m/z 117 m/z 105 (Findlay and White, 1983; Eversloh *et al.*, 2001). The other fragment ions seen as a β-cleavage reaction to methyl esters (Mc Lafferty rearrangement) was m/z 74. Other rearrangements of the alkyl positions of the molecule in which a hydrogen atom from the alkyl portion is transferred to the carbonyl oxygen of the acyl portion of the ester results in fragments of m/z 61, m/z 75. The present results confirmed that the PHB extracted from the sample contains 3-hydroxy functional group and the presence of methyl esters of hydroxy butyrate. The peaks at m/z 105 and m/z 74 define the 3-hydroxy functional structure (Lee *et al.*, 1995). The molecular fragments of 3-hydroxy functional group are m/z 103, m/z 74, m/z 71, m/z 61, and m/z 43. The molecular ion related peaks were rather weak because of high energy electron impart.

NMR spectroscopy is a valuable non-destructive method for monitoring polymer formation and degradation and has the advantages of accuracy, speed and sensitivity (Yan *et al.*, 2000). NMR has been used to investigate various aspects of PHB like monomer composition,
cellular content, conformational analysis, monomer linkage sequence, copolymer analysis and PHA metabolic pathway studies (Jacob et al., 1986). NMR has been used effectively to characterize the structure of polyhydroxyalkanoates (Choi and Yoon, 1994; Hori et al., 1994; Rodrigues et al., 1995; Matsusaki et al., 2000; Labuzek and Radecka 2001 and Lee et al., 2001).

In the present study the $^1$H NMR spectrum of PHB showed three groups of signals characteristic of PHB a doublet at 1.29 ppm which is characteristic of methyl group, a doublet of a quadruplet at 2.5 ppm which is attributed to methylene group and a multiplet at 5.28 ppm characteristic of a methyne group. A triplet at 0.9 ppm and a methylene resonance at 1.59 and methyne resonance at 5.5 indicated the presence of polyhydroxybutyrate in the polymer. This was comparable to reported data (Tombolini and Nuti, 1989; Tan et al., 1997). The intensity of carbonyl carbon peak resonance, methine carbon resonance, methyl carbon resonance and methylene carbon resonance are given in the spectrum. The absorbance in ppm is 169.143, 67.656, 40.864 and 19.787 for carbonyl, methine, methylene and methyl resonance respectively. The present data was comparable with reported values (Doi et al., 1986; Eversloh et al., 2001; Kamiya et al., 1989). The $^1$H NMR results confirmed the presence of polyhydroxybutyrate in the polymer. The molecular weight of PHB depends on the organism, source of carbon and also the downstream processing.

The details structure analysis of PHB to point out that access to available model (Cornibert et al., 1972; Yokouchi et al., 1973; Bruckner et al., 1986 and Bruckner et al., 1987) and cell parameters and space group were determined without “a priori” information through a trial and error procedure while packing and conformational energy calculations were very helpful in defining the starting point for the refinement procedure. The optically active thermoplastic aliphatic polyester is produced and accumulated as a source of energy and carbon by a number of
microorganisms (Lundergren et al., 1965). Many years after the first physicochemical studied by Stokdale et al., 1968. PHB is again attracting attention (Marchessault et al., 1970) because of it is commercial interest. Furthermore, it has been showed that its natural production is more a rule than an exception in the microbial world and those other stereo regular polyesters with longer aliphatic side chains are also synthesized.

Among the many nonpathogenic microorganisms accumulating PHB, *Rhizobium* was chosen (Morikawa et al., 1981) as source because of its agricultural importance and for the extensive knowledge of its biochemistry, physiology, and ecology. The production, extraction and characterization procedures are described in the Experimental Section. In the present study the outstanding stereo regularity and crystallinity of this polymer giving rise to X-ray powder diffraction spectra very well detailed up to high 2θ values and the present compound was optically.

Two structural studies were carried out on PHB, both of them based on X-ray diffraction data from oriented fibers. In the present study showed that sample PHB was optically active compound and the similar results were reported (Cornibert and Marchessault, 1972; Tadokoro and co workers and Briickner et al., 1987). It is worth while pointing out that these two studies were carried out following different criteria. Model II is the result of a least squares procedure that optimizes the agreement between observed and calculated intensities by adjusting all internal coordinates of the chain, while model I was obtained, together with other possible conformers, by a preliminary conformational analysis. Agreement with diffracted intensities for model I was optimized by allowing only rigid body refinement; it assumes therefore the meaning of a figure of merit to discriminate, among many conformers, the most likely to be present in the crystalline state (Briickner et al., 1987). Traditional methods for evaluating integrated intensities in X-ray diffraction patterns from oriented samples involve also some uncertainty. The measurement of
local intensity, its correction through proper spot shape factors, and the background estimate are subject to errors due to the approximations involved. The resulting uncertainties are well testified by simply comparing observed data reported (Conibert et al., 1972 and Yokou hi et al., 1973). A disagreement factor of ~ 11% on equatorial peaks and of ~ 21% on layer peaks. Such discrepancies are really hardly detectable among powder diagrams unless marked texturing is present.

The recent introduction of two dimensional scanning microdensitometers has indeed made it feasible to utilize far more of the information contained in the pattern directly, thus reducing the need for approximations and subjective decisions (French and Gardner, 1984). This is achieved, however, at the cost of heavy computational procedures handling a high number of data points (on the order of 10^6).

The comparisons carried out in this work are useful, in the present study point out that a well detailed powder profile can be highly discriminatory toward structural models that show only modest conformational differences; this means that geometrical parameters resulting from a riveted analysis carried out on such experimental data are well defined within rather narrow limits. Moreover, an indication that this minimum is unique comes from having obtained the same refined structure starting from different models and the present result was match with the earlier reports.

5.4.2.2 Thermal properties

In the present results of DSC showed higher crystalline degree for pure PHB (50%). The crystalline degrees of PHB were maintained between 41% and 46%. The lower crystalline degree and higher Tm resulted from better plasticizing effect which depresses the crystallization of PHB and pushes the already formed micro crystals or ordered chains to pack into more perfect structures. An identical result was earlier reported by Xu et al., (2006). The less compact
structure would enhance the molecular entanglement and the molecular penetrating in PHB. This interaction would suppress the crystallization of PHB which in turn decrease the degree of crystallinity. The intermolecular interactions in the PHB would lead to plasticizing or anti plasticizing effect depends on which is stronger.

In the present results melting points of the PHB film ranged between 260.70 to 191.31 °C. This indicates that there is no shift in the melting point of the polyesters suggesting that there is no interaction between the polymers. Identical results were earlier reported by Godbole et al., (2003).

The decomposition of PHB and enhance the thermal stability. Identical observations were reported by Choi et al, 2003 who prepared PHB blends. The blends showed a higher thermal stability. Parra et al., 2006 reported PHB blend preparation. It showed improved properties.

In the present study TGA reports that the thermal degradation temperature increase with increasing heating rate was caused by heat hysteresis. The thermal degradation temperature can be expressed more exactly as an equilibrium degradation temperature $T (0)$ when the heating rate approaches zero: $T_0 (0) = 259 \degree C$, $T_p (0) = 273 \degree C$, and $T_f (0) = 280 \degree C$. The peak width was $T_f - T_0 = 21 + 0.13B$ and it increased with the heating rate. Thermal degradation lost that corresponds to $T_p$ and $T_f$, respectively. The $C_p$ is the thermal degradation loss at $T = T_p$, and $C_p = 100\% \times$ the weight percent of residue at $T_p$. The $C_f$ is the thermal degradation loss at $T = T_f$ and $C_f = 100\% \times$ the weight percent of residue at $T_f$. The values of $C_p$ and $C_f$ were not significantly affected by the heating rate. The $C_p$ and $C_f$ were $69 \pm 1$ and $96 \pm 1\%$, respectively. The thermal degradation of PHB proceeded to completion.

5.4.2.3 Physical properties
In the present study molecular weight was measured by viscometry and the viscosity average molecular weight of the biopolymer PHB was 11310 k Da. Decrease of aeration level (series 2, shaking at 190 rpm) at the stationary phase of growth caused an increase in PHB molecular weight from 1480 to 1670 k Da. Culture growth was not observed under microaerophilic conditions, but young (12 hrs) cells intensely produced the polymer. Its molecular weight (1820 k Da) exceeded the molecular weight of the polymer produced under aerobic conditions by about 30%. The culture did not grow under anaerobic conditions. Young 12 hrs cells intensely (as under micro aerophilic conditions) produced PHB. Its molecular weight (2215 k Da) was twice as high as that of the aerobically produced polymer. A decrease in the molecular weight of the PHB produced by A. chroococcum 7B at higher aeration rates was also observed by other scientists in a semi batch process. At the air flow rate 0.5 vol/ (vol/min), the strain produced PHB of 1100 k Da, whereas flow rate elevation to 2.5 vol/ (vol/min) reduced molecular weight to 111 k Da. Obviously, the high intracellular NADH / NAD⁺ ratio at oxygen shortage inhibit enzymes involved in glucose catabolism and the tri carboxylic acid cycle; therefore, acetyl-CoA is channeled to PHB production. Thus, lower aeration at the second stage of A. chroococcum 7B growth yields PHB with high molecular weight, 1.5 to 2.2 M Da (Myshkina et al., 2007).

As a new member of the PHA family, PHB still has many properties that may be interesting to a wide range of applications. The solution of the polymer was casted on to clean glass plates. Clear and transparent films could be easily peeled off from the glass plate. In the present study experiments also showed that the polymer could be heat sealed. These films were used for tensile strength, water vapour permeability and oxygen transmission rate studies. PHB has much better mechanical properties over PHB and PHBV (Doi et al., 1995). However, no
study was done so far concerning this promising material for applications such as tissue engineering material. Viable cell growth on untreated PHB was better than that on films, indicating a promising property of PHB for use as biomaterial. This study may provide first hand data to compare the biocompatibility of PLA, PHB. The surface properties of a biomaterial, especially hydrophilicity, influence cell adhesion to the materials (Zhao and Geuskens, 1999). In general, the higher the hydrophilicity of a material surface, the stronger the cells attached to the material. In this study, lipases and NaOH were employed to improve the hydrophilicity of the polymer films. The improved hydrophilicity of the films allowed cells in its suspension to easily attach on the polymer films compared to that on the untreated ones. This is why we observed better cell growth on the treated PHB and PLA (Xianshuang et al., 2002).

In the present study the solution cast films of PHB extracted from *Lysinibacillus spearicus* BBKGBS6 were examined for its tensile strength and elongation to break. Tensile strength of the film was 28.23 MPa and was comparable to polypropylene (38 MPa) when the film could bear a load of 16.9 N. and the maximum load taken was 18.5 N and the tensile strength at this load was 41.1 Mpa. The extension to break was 1.29 %. Research on biodegradable plastics based on starch began in the 1970s and continues today at various labs all over the world. Technologies have been developed for continuous production of extrusion blown films and injection-molded articles containing 50% or more of starch. Water sensitivity of such films has been reduced by lamination with poly (vinyl chloride) (Shogren et al., 1993). Combination of urea with certain polyols provides better plasticization of starch with good quality films (Doane, 1992). Melted or destructurized starch, obtained by disruption of the granular architecture resulting in loss of crystallinity, has emerged as a new type of thermoplastic material for commercial development. To increase the compatibility of hydrophilic starch with
the hydrophobic plastic matrix, starch granules have been surface treated, for example with silanes (Doane, 1992). Pro-oxidants can sometimes be added to enhance oxidative degradation of the synthetic polymer. Tensile strength is a measure to what extent the material stretches before breaking.

Plasticizers, for biopolymer-based films, can be divided into water soluble and water insoluble (Siepmann et al., 1998). The type and the amount of plasticizer strongly affect the film formation from polymeric aqueous dispersions (Johnson et al., 1991). Hydrophilic plasticizers dissolve in the aqueous medium when they are added to polymer dispersions and if added in high concentration they can lead to an increase in water diffusion in the polymer. In contrast, hydrophobic plasticizers may close the micro-voids in the film, leading to a decrease in water uptake. However, water insoluble plasticizers can cause phase separation leading to flexibility losses or yet to the formation of discontinuity zones during film drying. As a consequence, water vapor permeability rates are increased. Complete uptake of insoluble plasticizer by the polymer can be achieved by an optimum stirring rate of the polymeric dispersion with the plasticizer (Siepmann et al., 1998). In the present study the water vapour permeability of the biopolymer PHB film was 29 g/m²/day.

Water vapour and oxygen are two of the main parameters studied in packaging applications, because they may transfer from the internal or external environment through the polymer package wall, resulting in a continuous change in product quality and shelf-life (Germain, 1997). Carbon dioxide is now important for the packaging in modified atmosphere (MAP technology) because it can potentially reduce the problems associated with processed fresh product, leading a significantly longer shelf-life. For example, for fresh product respiration
rate is of great importance in MAP design so identify the best packaging is a crucial factor. The most important barrier properties of polymer films used in packaging application are described.

The oxygen barrier property of a food packaging container for fresh product (e.g. fruits, salad, ready-to-eat meals) plays an important role on its preservation. The oxygen barrier is quantified by the oxygen permeability coefficients (OPC) which indicate the amount of oxygen that permeates per unit of area and time in a packaging materials \( \text{[kg m}^{-2}\text{s}^{-1}\text{Pa}^{-1}] \). So, when a polymer film packaging has a low oxygen permeability coefficient, the oxygen pressure inside the container drops to the point where the oxidation is retarded, extending the shelf-life of the product. Generally the biodegradable polymers present a value one or more order of magnitude below the synthetic polymer used in the same field like PET and OPS. Several authors reported in literature the oxygen permeability coefficients of one of the most commercialized biodegradable polymer like the PLA (Auras et al., 2005, Lehermeier et al., 2001 and Oliveira et al., 2004).

In the present study the oxygen transmission rate was calculated as 472.36 (cc/m2/day/atm 65% Rh and 27 \(^{\circ}\)C). Relatively low oxygen diffusivity has been reported for PHB films and this property is considered important for the film to be used as a food packaging material, the same results were reported (Eggink et al., 1994). The low water permeability is significant for applications of this film as a packaging material.