5.2 MATERIALS AND METHODS

5.2.1 Bacterium

In the present study *Lysinibacillus sphaericus* BBKGBS6 soil bacteria from chapter 2 was used and produced Polyhydroxybutyrate (PHB) and it was isolated from nursery field soil (Kaliwal *et al.*, 2015).

5.2.2 Medium

*Lysinibacillus sphaericus* BBKGBS6 was grown and maintained in a PHB production medium. The bacterium was grown under submerged fermentation. The culture medium was consisted of (g/L), glucose 2.0, (NH$_4$)$_2$SO$_4$ 4.0, KH$_2$PO$_4$ 13.3, MgSO$_4$·7H$_2$O 1.2, citric acid 1.7 and trace element 10ml. The glucose and MgSO$_4$·7H$_2$O were added after autoclave and mention pH of 6.8 with NaOH at 35 °C. When the pH was higher than 6.9 because of glucose depletion, feeding nutrient solution corresponding to 20 g of glucose (28.6 ml of feeding solution) was added at a rate of 5.5 ml/min for a definite time.

5.2.3 Production and storage of Polyhydroxybutyrate (PHB) containing biomass

PHB contents used in this study were 137 g/L, 85 g/L, and 62%, respectively. After fermentation, the cell broth was concentrated by centrifugation at 4,000 rpm for 15 min at 25 °C, washed twice with distilled water, and then freeze dried. The resulting cell powders were stored at 48 °C until they were needed.

5.2.4 PHB recovery by using dispersions of sodium hypochlorite and chloroform

1 gm portion of cell powder was treated with a dispersion containing 50 ml of chloroform and 50 ml of a diluted sodium hypochlorite solution. The hypochlorite concentrations in the aqueous solutions used were 3, 5, 10, and 20% (vol/ vol). After the cell powder was treated at 30
193 °C for 1 hr, the mixture was centrifuged at 4,000 rpm for 10 min, which resulted in three separate phases. The upper phase was a hypochlorite solution, the middle phase contained non PHB cell material and undisrupted cells, and the bottom phase was chloroform containing PHB. The upper phase was removed first with a pipette, and the middle phase was separated by filtration from the chloroform phase. Finally, PHB was recovered from the chloroform phase by non solvent precipitation and filtration. The non solvent used was a mixture of methanol and water (7:3, vol/vol).

5.2.5 Purification of Polyhydroxybutyrate (PHB) produced from Lysinibacillus sphaericus BBKGB6

PHB for characterization was obtained by solvent extraction in a soxhlet apparatus. Cells were collected by centrifugation from a 72 hr old culture. They were washed thoroughly with distilled water and dried with acetone. Acetone dried cells were refluxed in chloroform for 6 hr in the soxhlet apparatus. PHB in the cells dissolved in chloroform and a highly viscous solution was obtained after 6 hr of refluxing. Insoluble matter that was found floating in chloroform was removed by filtering using glass wool followed by centrifugation. PHB was recovered from chloroform solution by precipitation with hexane five volumes of the polymer. PHB precipitated as a white cottony mass. PHB was collected by decanting the hexane chloroform mixture and then air dried.

5.2.6 Characterization of Polyhydroxybutyrate (PHB) produced from Lysinibacillus sphaericus BBKGB6

Polyhydroxybutyrate (PHB) produced from Lysinibacillus sphaericus BBKGB6 were characterized by analytical method, thermal properties and physical properties.
5.2.6.1 Analytical method

Chemical properties of Polyhydroxybutyrate (PHB) produced by *Lysinibacillus sphaericus* BBKGBS6 like, Crotonate assay of Polyhydroxybutyrate (PHB) by using U-V Spectroscopy, Fourier Transform Infrared spectroscopy (FTIR), Gas Chromatography Mass spectroscopy, (GCMS), Nuclear magnetic resonance (NMR), X-ray powder crystallography were analyzed.

5.2.6.2 Crotonate assay of PHB by U-V Spectroscopy

The polymer obtained by extraction method described above was used for crotonic assay. About 10 mg of the extracted polymer was taken in a test tube and was dissolved in 5 ml of concentrated sulfuric acid. This was digested in a water bath at 100 °C for 10 min to hydrolyze the product to crotonic acid. The absorbency at 235 nm of the solution was measured in a U-V spectrophotometer (Hitachi-U-3310 spectrophotometer Japan) against sulfuric acid blank (Law and Slepecky, 1961). PHB was quantified based on this assay by comparing with the purity of the standard PHB. A standard graph was drawn with standard PHB obtained from Sigma. Standard PHB was taken in 0.5, 1, 1.5, 2.0 and 10 mg and was treated as mentioned above. The absorbance was measured at 235 nm. Concentration of test sample was calculated from standard graph and purity was estimated based on concentration of PHB in known weight of sample was used.

5.2.6.3 Preparation of PHB sample for the analysis by Fourier transform infrared spectroscopy (FTIR)

Extracted PHB was dissolved in chloroform. A drop of the chloroform solution was placed on the kbr windows and analyzed. Analysis was done by Fourier transform infrared spectroscopy (model no.4700, Nicolet). The scanning conditions were a spectra range of 4000 -
400 cm\(^{-1}\).

### 5.2.6.4 Preparation of PHB sample for the analysis of Gas chromatography

GC analysis of PHB was carried out by the method described by Brandl et al., (1988). 10 mg of extracted PHA was taken in a glass test tube. 1ml chloroform, 0.85 ml of methanol and 0.15 ml of sulfuric acid were added. All the chemicals used were of AR grade. The tube was sealed and kept for hydrolysis in an oil bath at 100 °C for 160 min. After hydrolysis contents were allowed to cool and were mixed with 5 ml of water. After phase separation the bottom chloroform phase was taken and used for GC analysis. PHB from Sigma were also prepared similarly as standards. The methyl esters obtained were analyzed in a Fisons gas chromatograph (GC Fisons, 8000, CE instruments, Italy) with a DB 1 (Durabond) capillary column (DB series, Shimadzu, Japan; Mfd by JW Scientific USA) and flame ionization detector. Nitrogen 1 ml/min was used as a carrier gas. The temperatures of the injector and detector were 220 °C and 230 °C, respectively. Temperature program used was 55 °C for 7 min; temp ramp of 4 °C per min up to 100 °C; 10 °C rise up to 200 °C followed by 10 min hold.

### 5.2.6.5 PHB sample preparation for the analysis of Gas chromatography mass spectroscopy (GCMS)

Samples were esterifies as mentioned and were analyzed in a Gas chromatography Mass Spectrophotometer (GCMS QP-2010S M Shimadzu Japan). Column used was DB 1 (Durabond) capillary column (DB series, Shimadzu, Japan) and flame ionization detector. Nitrogen (1 ml/min) was used as a carrier gas. The temperatures of the injector and detector were 220 °C and 230 °C respectively. Temperature program used was 45 °C for 7 min; temp ramp of 4 °C per min up to 100 °C; 10 °C rise up to 200 °C followed by 10 min hold. Standard PHB (sigma) was used for comparison.
5.2.6.6 PHB sample analysis by Nuclear magnetic resonance (NMR) spectroscopy

Nuclear magnetic resonance (NMR) analysis of PHB sample was done at sophisticated instruments facility, Indian institute of Science, Bangalore, India. $^1$H and C NMR spectra were recorded using purified samples. PHB was extracted by chloroform as mentioned under purification of PHB. 5 mg of sample were dissolved in deuterated chloroform (CDCl$_3$) and the solution was analyzed at 400 MHz on an AMX 400 (Bruker) spectrophotometer. The spectrum was recorded at 25 °C with a pulse repetition of 3 seconds. The enhancement of proton resonance in $^1$H NMR was determined from a comparison with the spectrum of standard P (3HB-co-HV) obtained from sigma. Copolymer concentration was expressed as mol%. This was calculated from ratios of peak areas due to HV methyl resonance and the sum of HB and HV methyl resonances in $^1$H NMR. The carbon resonance obtained in the $^{13}$C NMR was compared with the peaks from a standard PHB resonance graph.

5.2.6.7 X-ray Diffraction analysis of Polyhydroxybutyrate (PHB)

Extracted PHB, 150 mg, was used to fill the sample holder of a D-500 Siemens Goniometer equipped with an Anton Paar temperature controller. Annealing was directly carried out in situ by raising the temperature of the sample holder to 170 °C for 15 min under vacuum conditions (~ 0.001 mmHg). After spontaneous cooling (~30 min) to room temperature a quantitative recording of the powder X-ray diffraction profile started under conditions.

5.2.7 Thermal properties

Thermal properties of Polyhydroxybutyrate (PHB) produced from *Lysinibacillus sphaericus* BBKGBS6 were, Differential Scanning Calorimeter (DSC) and Thermo gravimetric analysis (TGA) analyzed.

5.2.7.1 Differential Scanning Calorimeter (DSC) analysis

Differential scanning calorimetric (DSC) experiments was performed using
Universal V4.5A TA Instruments, (m.p.156.61 °C; ΔH = 28.54 J/g) USIC Dharwad. The data was collected by heat and cool method. Samples of cast films weighed 5-10 mg were packed in aluminum pan and then heated from 20 °C to 300 °C at a scanning rate of 10 °C per minute under nitrogen atmosphere. The melting temperature (Tm) and melting enthalpy (ΔH_f) were determined from DSC endothermal peaks. After one minute annealing, the sample was cooled to 20 °C.

The crystallinity (X_c) of PHB in the blends is calculated as per equation given below.

\[
X_c = \frac{\Delta H_f}{\Delta H_0} \times W
\]

Where,

\(\Delta H_f\) = melting enthalpy of the sample (J/g).

\(\Delta H_0\) = 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.

5.2.7.2 Thermo gravimetric analysis (TGA)

TGA is commonly used to determine selected characteristics of materials that exhibit either mass loss or gain due to decomposition, oxidation, or loss of volatiles (such as moisture). Common applications of TGA are materials characterization through analysis of characteristic decomposition patterns, studies of degradation mechanisms and reaction kinetics, determination of organic content in a sample, and determination of inorganic (e.g. ash) content in a sample, which may be useful for corroborating predicted material structures or simply used as a chemical analysis.

Thermo gravimetric analysis (TGA) was performed on a Shimadzu instrument (Japan). The temperature was ramped at a heating rate of 10 °C / min under nitrogen, to a temperature well above the degradation temperature of the polymers (500 °C).
5.2.8 Physical properties

Mechanical properties of Polyhydroxybutyrate (PHB) produced by *Lysinibacillus sphaericus* BBKGBS6, Viscosity, solvent casting method for PHB film preparation, tensile strength, water vapor transmission rate (WVTR) and Oxygen transmission rate (OTR) were analyzed.

5.2.8.1 Estimation of molecular weight of PHB by Viscometry

The molecular weight of PHB was estimated based on viscosity measurement to give a viscosity average molecular weight. Predetermined amounts of the sample at a concentration of 0.1, 0.2, 0.3, 0.4 and 0.5% were analyzed for their reduced viscosity. The viscosity of PHB chloroform solution was determined at 20 °C using Ostwald’s viscometer. Time for the flow of the solvent and solution was recorded in triplicates. Density of solvent and solution were determined using a specific gravity bottle. Intrinsic viscosity was determined by plotting a graph with reduced viscosity versus concentration on y and x-axes respectively (Quagliano *et al.*, 2001).

\[
\text{Viscosity (}\eta\text{)} = \frac{\text{Density of solution}}{\text{Density of solvent}} \times \frac{T1 \text{ solution}}{T2 \text{ solvent}}
\]

\[
\text{Specific viscosity (}\eta_{Sp}\text{)} = \eta - 1
\]

\[M_v\] was calculated according to Mark – Houwink – Sakurada equation

\[
\eta = K1 \ (M_v)^a
\]

\[K1 = 7.7 \times 10^{-5} \text{ dl/g and } a = 0.82 \text{ for PHB}\]

5.2.8.2 Preparation of PHB film by solvent casting method
Films were prepared by as per the solvent casting method (Savenkova et al., 2000). 2% solution of PHA in chloroform was prepared. Uniform, flat and scratch less glass plates were selected for casting. These plates were placed on a flat and leveled 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 hr at room temperature. Care was taken not to disturb the plates during drying. After drying the films were peeled out of the plates.

5.2.8.3 Measurement of PHB tensile strength

Tensile strength of PHB film was carried out according to ASTMD 882 using universal testing machine (Model Lx 5, LYOD ISNT). The test was performed in triplicates at room temperature. PHB strips of 1cm breadth and 10 cm length were cut from the films. Thickness of the film was measured using micrometer, model 549E (Testing machines inc., New York, USA) and was expressed in terms of gauge. The test specimens were placed in the grips of the test machine. The grip of the machine was tightened evenly so as to hold the filmstrip firmly during testing. The speed was set at the rate of 50 mm/min. As the test strip elongates the resistance of the strip increases and was detected by a load cell. The elongation of the strip was calculated as % elongation.

\[
\text{Tensile strength} = \frac{\text{Maximum load in N}}{\text{cross section area in mm}^2}
\]

\[
\% \text{ of Elongation} = \frac{\text{Extension (mm)}}{\text{length of the sample in mm}} \times 100
\]
5.2.8.4 Water vapor transmission rate (WVTR) of PHB film

Water vapor transmission rate of PHB film was measured as per ASTM E 96-95 and carried out according to the desiccant method. Anhydrous calcium chloride was enclosed in an aluminum dish, closed and the assembly was placed in a humidity chamber. The chamber was maintained at 38 °C and 90% RH. The initial weight was noted. Then the dish was covered with the test film (12 cm diameter) and sealed using paraffin wax. This assembly was placed in the humidity chamber maintained at 38 °C and 90% RH. The aluminum dish was weighed at regular intervals of 60 minutes till a constant weight gain was achieved. Each time the assembly was cooled to room temperature before recording weight. Experiments were conducted in triplicates. The water vapour transmission rate was calculated using the following formula

\[
WVTR = \frac{\text{pick of moisture in grams per hr}}{\text{area of the specimen}}
\]

Unit: g / m² / 24 hr at 38 °C and 90% RH

5.2.8.5 Measurement of Oxygen transmission rate (OTR) of PHB film

Oxygen transmission rate of PHB film was measured as per ASTM D-1434-66. Experiments were conducted in triplicates. For oxygen transmission rate permeability cell consisting of two stainless steel discs were used. The discs formed a cylindrical cavity when superimposed. The test film (9 cm) was clamped between the two discs using 6 equally spaced bolts after placing 3 filter circles on the upper disc as support. A rubber gasket on it ensured a pressure tight fit. The cell was connected with a short plug of mercury contained in a capillary glass tube in a vertical position with an opening in the centre of the upper disc. Gas inlets and vent lines were provided on both sides of the cell. Oxygen was supplied at a constant rate, over atmospheric pressure to the bottom of the cell and the permeated gas was allowed to expand on
the opposite side against atmospheric pressure. Pressure of oxygen was maintained at 20 psi. The upward displacement of mercury due to permeation of the gas through the film gives the rate of oxygen transmission. An electromechanical vibration was used to avoid friction to the movement of mercury in the tube. The displacement was measured as a function of time and displacement of mercury versus time was plotted and the slope of straight line was obtained. OTR was calculated as follows.

\[
\text{OTR} = \frac{\text{VX6.566X1010}}{\text{A X P}} \text{ in cc/24 hr/ m2/ tam}
\]

A = area of the test film, cm\(^2\)

P = test gas pressure differential, cm Hg

V = volume of the gas transmitted through the material (slope X a)

Slope = rate of rise in the capillary plug, cm/s. a = C/S area of capillary, cm\(^{-2}\).