4.2 MATERIALS AND METHODS

4.2.1 Use of solvents and chemicals for extraction and purification of Polyhydroxybutyrate (PHB) produced from Lysinibacillus sphaericus BBKGBS6

AR grade of Chloroform, Dichloromethane, Acetone, Diethyl ether, absolute Ethanol, Sodium hypochlorite (5% chlorine), Sodium hydroxide and Ammonium hydroxide were used. Surfactant, Triton X 100 and Chelating agent, Ethylene diamine tetra acetic acid (EDTA) were used obtained from Himedia chemicals.

4.2.2 Bacterium growth and medium

The medium used for inoculum contained the following components (g/L), Na$_2$HPO$_4$ 2H$_2$O 4.4, KH$_2$PO$_4$ 1.5, (NH$_4$)$_2$SO$_4$ 1.0, MgSO$_4$ 7H$_2$O 0.2, Sucrose 10, Yeast extract 0.5 and pH 7. 10% inoculum was prepared at 30 °C and 250 rpm for 24 hr. The production medium was prepared similar to the inoculum medium but without yeast extract. The concentration of sucrose was increased to 20 g/L. PHA production was carried out in 100 ml of the above medium taken in 500 ml Erlenmeyer flasks. Incubation was performed at 250 rpm and 30 °C for 72 hr.

4.2.3 Methods of extraction and purification of Polyhydroxybutyrate (PHB) produced from Lysinibacillus sphaericus BBKGBS6

4.2.3.3 Analysis of molecular weight of Polyhydroxybutyrate (PHB)

Molecular weight analysis was carried out. The molecular weight of PHB was estimated based on viscosity measurement to give a viscosity average molecular wt. 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 (Lundgren et al., 1965 and Quagliano et al., 2001).

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

Were, T1 and T2 are time in seconds.

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

\(M_V\) was calculated according to Mark – Houwink – Sakurada equation

\[\eta = K_1 (M_V)^{a}\]

\[K_1 = 7.7 \times 10^{-5} \text{ dl/g and } a = 0.82 \text{ for PHB}\]

4.2.3.4 Extraction by Sodium hypochlorite

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 °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).

4.2.3.5 Extraction by Chloroform

Cells were collected by centrifugation (6000 rpm for 20 min at room temperature) and
were washed with sufficient acetone for 10 min. Acetone dried cells were held in 5 volumes of chloroform and left overnight at room temperature. Clear chloroform layer was obtained by filtering with glass wool. This was again centrifuged at 4000 rpm at 10 °C to obtain a clear solution of PHB in chloroform. PHB was then precipitated with hexane (5 vol) (Choi and Lee, 1999). Solvent extraction of PHB from cells was also done using soxhlet apparatus PHB for characterization was obtained by solvent extraction in a soxhlet apparatus. Cells were collected by centrifugation form 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 hrs in the soxhlet apparatus. PHB in the cells dissolved in chloroform and a highly viscous solution was obtained after 6 hrs 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 hexane were used against one volume of chloroform solution for precipitation of the polymer. PHB precipitated as a white cottony mass. PHB was collected by decanting the hexane chloroform mixture and then air dried. Chloroform was used as a solvent.

4.2.3.6 Extraction of PHB by dispersions of hypochlorite solution and chloroform

PHB containing cells were collected by centrifugation (6000 rpm for 20 min at room temperature). These cells were treated with dispersion of hypochlorite and chloroform in the ratio of 1:1 and kept for digestion for 1 hr at 37 °C. The digested cell material was then recovered by centrifugation at 6000 rpm for 10 min. Centrifugation resulted in three separate phases. The upper phase was that of hypochlorite solution, the middle phase contained non PHB cell material and undisrupted cells, and the bottom phase was chloroform containing PHB (Hahn et al., 1994). The bottom phase was carefully removed by pipetting or by using a separating funnel. PHB was
recovered by precipitation with hexane.

4.2.3.7 Extraction by surfactant and chelating agents

Biomass containing PHB was collected by centrifugation (6000 rpm for 20 min at room temperature). Cells were suspended in known quantity of water. 0.6% of triton X 100 and 0.06% of EDTA were added to the cells and pH was adjusted to 13 with 1 N NaOH solution and they were kept at 50 °C for 10 min. They were then centrifuged at 6000 rpm for 10 min. Digested cell material was washed with acetone and dried. The precipitate containing PHB was further purified by dissolving in chloroform and precipitated with hexane (Chen et al., 2001). Alkaline extraction Biomass containing PHB was collected by centrifugation (6000 rpm for 20 min at room temperature). The precipitated cells obtained were resuspended in a known quantity of water. pH was adjusted to 11 with ammonium hydroxide or sodium hydroxide and kept at 50 °C for 10 min. The digested cell material obtained was then washed thoroughly with water and then with acetone. The precipitate containing PHB was further purified by dissolving in chloroform and precipitated with hexane (Choi and Lee, 1999).

4.2.3.8 Extraction by enzyme

PHB containing cells PHB was collected by centrifugation (6000 rpm for 20 min at room temperature). The cells were suspended in a known quantity of water and thermally treated at 80 °C for 10 min and cooled. A proteolytic enzyme such as protease (Novozyme) was used for extraction of PHB. Protease (22500 units / ml) was added in the range of 0.5 ml of enzyme to 10 ml of substrate (biomass) containing 1% suspension of biomass (on dry weight basis) and was incubated at 40 °C, for 2 ½ hrs.
4.2.3.8.2 Cell lytic activity of Lysinibacillus sphaericus BBKGBS6

*Lysinibacillus sphaericus* BBKGBS6 biomass was thermally inactivated at 80 °C for 10 min and suspended in phosphate buffer. Optimization of different pH (at 50 °C), different temperature (at pH 8), enzyme concentration and concentration of biomass for lysis and time required were tested using the lytic culture filtrate. Lytic activity was determined by hydrolyzing *Lysinibacillus sphaericus* BBKGBS6 cells of known dry weight (3 mg/ 5 ml) with 48 to 72 hr old clarified culture filtrate (0.5 ml) of cell lytic culture at 50 °C, pH 6 to 7 for 1 hr. O.D was measured at 620 nm and activity was calculated as 1000 X (Triveni and Shamala, 1999).

4.2.3.8.3 Crotonoate assay of PHB extracted by enzymatic method

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 (Shimadzu U-V 160, Japan) against sulfuric acid blank (Law and Slepecky 1961). PHA 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 used.

4.2.3.8.4 Characterization of PHB extracted by using lytic enzyme

Characterization of the extracted polymer was carried out using Infrared spectroscopy, gas chromatography and differential scanning calorimeter. Scanning electron microscopy (SEM)
of enzyme treated biomass scanning electron microscopic analysis of the lytic enzyme treated biomass was studied.