2.2 MATERIALS AND METHODS

2.2.1 Sample collection and isolation of polyhydroxybutyrate (PHB) producing bacteria

The bacteria used in this study was collected and isolated from University of Agricultural sciences (U.A.S), Dharwad, Karnataka state India. Crop, Nursery, Wheat, Mulberry, Maize, Rice, Cotton, Fodder field and Botanical garden soil soils were screening for high PHB producing bacteria and the nutrient media was used as a nutrient source. One gram of soil sample is dispensed in 10 ml of sterile distilled water. This is mixed vigorously and 1ml from this is taken and added to another tube with 9 ml sterile distilled water to get a dilution of $10^{-1}$. This serial dilution is repeated to get dilutions of $10^{-2}$, $10^{-3}$, $10^{-4}$, $10^{-5}$, $10^{-6}$, $10^{-7}$, $10^{-8}$ and $10^{-9}$. For the isolation of organisms, 0.1ml of each dilution was plated onto a nutrient rich medium (g/L), with Peptone 2, Beef extract 2, NaCl 1, Agar 4, Distilled water 1lt, by spread plate method for the propagation of microbial growth. The plates were incubated at 30 °C for 48 hr. Colonies with different characteristic features were maintained as pure cultures on nutrient agar slants and stored at 4 °C.

2.2.2 Maintenance of culture and screening of PHB producing bacteria

The bacteria were streaked on to nutrient agar slants, incubated at 30 °C overnight and then stored at 4 °C for further use.

The isolates were tested for PHB production following the viable colony screening method based on the intensity of staining. All the six isolates gave the positive result for PHB accumulation through Sudan blank staining methods. All the six Sudan Black B positive isolates were subjected to quantitative estimation of PHB production. The synthesis of PHB was noticed from the log phase of growth and it continued until late exponential phase as the carbon source was utilized for both growth and PHB production.

2.2.2.1 Sudan black -B staining
All the bacteria isolates were tested for PHB positive or PHB negative based on the using Sudan black -B staining of the cells taken from slants and liquid cultures. Flame the loop and allow it to cool. Remove the cap from the culture bottle, flame the neck, remove a loop full of broth, flame the neck again and replace the cap. Spread the culture on a clean, grease-free slide, using the loop. The smear should cover an area about 10 mm x 30 mm. Flame the loop. Allow the smear to dry in the air. Fix the smear by holding the slide with forceps and passing it horizontally through a small Bunsen flame 2 to 3 times. Do not overheat the slide. Fixing kills the bacteria by coagulating the cytoplasm. It also sticks them to the slide. Place a few drops of Sudan Black solution on the fixed preparation. After 5 to 10 minutes the ethanol in the stain should have evaporated. Any excess liquid can be carefully drawn off using the edge of a piece of filter paper. Immerse the slide in xylene until it is completely decolorized (this takes about 10 seconds). Allow the slide to dry. Flood the slide with the counter stain, saffranin solution. After 10 seconds, gently rinse the slide with running water and allow it to dry again. When the slide is completely dry add a drop of immersion oil directly to the slide (no cover slip is needed). Examine with an oil immersion lens. The PHB can be seen as very dark granules inside pink cells. (Byrom, et al., 1991).

2. 2.2.2 Fluorescence staining (Acridine orange)

10 μL of 48 hr old culture of *Lysinibacillus sphaericus* BBKGBS6 was taken from the production medium and transferred to the eppendorf tube, containing 50 μl of acridine orange and incubated for 30 min at 30 °C. After the incubation period, the culture was centrifuged at 4000 rpm for 5 min. The pallet was collected and resuspended in distilled water. Smear prepared on a clean microscope slide observed under the fluorescent microscope at 460 nm (Senthilkumar and Prabhakaran, 2006). The appearance of yellow coloured granules inside the cell indicates
PHB production. The bacteria positive for PHA production was selected by observing the granules under fluorescence microscope (Carl Zeiss Fluorescence microscope under 100X), Bacterial culture showing substantial fluorescence were selected for further study.

2. 2.3.3 Nile red staining

The bacteria were grown in 1.0% (w/v) nutrient broth glucose medium for inoculums development. PHB production in shake flasks was studied using the modified basal mineral salt medium (Greenspan et al., 1985) with appropriate carbon source. For shake flask experiments, quantities of 50 ml medium in 250 ml capacity Erlenmeyer flasks sterilized by autoclaving at (15 lb, 20 min) and cooled was used. They were inoculated with 1.0% (v/v) inoculums of overnight culture and incubated at 37 °C at 120 rpm/min for 48 hr. The bacteria were screened for PHB granule accumulation from 16 hrs onwards using Nile red (Ostle and Holt, 1982) staining methods. The bacteria positive for PHB production were selected by observing the granules under fluorescence microscope, with fluorescence illuminator, fitted with image analyzer. Nile red stain was prepared from stock solution in acetone (Greenspan, 1985). The Nile red stained preparations were observed at an excitation wavelength of 540 nm and emission at 590 nm respectively. Bacterial cultures showing substantial fluorescence were selected for further study.

2. 2.2.4 Nile blue staining

Nile blue is a basic oxazine dye which is soluble in water and ethyl alcohol (Lillie, 1977). The oxazone form of the dye (Nile pink) is formed by the spontaneous oxidation of Nile blue in aqueous solution or by refluxing Nile blue with dilute sulfuric acid. Nile pink is soluble in neutral lipids which are liquid at the staining temperature (Thompson, 1966). The Nile blue and Nile pink mixture has been used as a histological fat stain in prepared tissue sections (Smith,
In this chapter followed a method for selectively staining PHB granules with Nile blue. 1% aqueous solution of Nile blue (Matheson, Coleman & Bell stock NX0395) was prepared and filtered before use. Mild heating may be necessary to fully dissolve the stain, but acid reflux to produce the oxazine form was not required. Azotobacter chroococcum and Bacillus megaterium KM cells were grown under conditions favorable for PHB production (Greibel and Merrick, 1971). 2 drops of 0.2% Nile blue solution were added to 300 µl sample of each culture, vortexes and incubated in a water bath for 10 minutes at 55 °C. After 1 min of mild sonication, inclusion bodies were visualized at 1000x magnification under fluorescent light (excitation wavelength 550 nm, observation at 580 nm) as bright orange intracellular granules. PHB extraction heat fixed smears of bacterial cells were stained with the Nile blue solution at 55 °C for 10 min in a coplin staining jar. After being stained, the slides were washed with tap water to remove excess stain and with 8% aqueous acetic acid for 1 min. The stained smear was washed and blotted dry with bibulous paper, remoistened with tap water, and covered with glass cover slip. The cover slip is necessary, as standard immersion oil will extract some of the fluorescent dye and obscure the field with a general yellow fluorescence. The cover slip thus protects the stained cells from immersion oil. The preparation was examined with a Nikon Labphot microscope with an episcopic fluorescence attachment. The Nikon blue excitation method, which provides an excitation wavelength of approximately 460 nm, was used.

2.2.3 Morphological characterization of polyhydroxybutyrate (PHB) producing bacteria from soil

Isolation of bacteria from different sources and sites, the best producer of PHB was selected and identified. The tested bacterial isolate was grown on nutrient agar plates and the
cellular morphology was examined with light and scanning electron microscope. Morphological characterizations like Colony, cell morphology based on their colour, shape, margin, elevation, surface and arrangement of bacteria were studied under bright field compound microscope were carried out according to morphological and biochemical characters (Williams et al., 1994).

### 2.2.3.1 Cell size

Cell size was measured using ocular micrometer (one scale =1/10 mm (100 µ) and stage micrometer (1 scale division =1/100 mm) in the microscope. The ocular micrometer was calibrated by using the stage micrometer. Under 10X lens each division corresponds to 11.2µ, with 25X, 4.6µ, with 40X, 2.8µ and with 100X, 1.1µ per division of ocular micrometer.

### 2.2.3.2 Scanning electron microscopy (SEM) analysis

To study the morphology, isolated bacteria was observed under the scanning electron microscope. Fresh culture (12 hr to 18 hr old) was centrifuged and washed twice in phosphate buffer 0.1M (pH 6.5). Centrifuged bacterial cells were fixed in 1 to 2% glutaraldehyde overnight. The cells were then separated by centrifugation at 8000 rpm for 5 min. In order to avoid initial osmotic damage, cells were successively suspended in gradient ethanol (10-100%) followed by methanol. The sample was then dried in desiccators. Dried cells were placed on aluminum stubs and sputter coated with gold using argon as the ionizing plasma. Cells were scanned by using scanning electron microscope.

### 2.2.3.3 Transmission electron microscopy (TEM) analysis

*Lysinibacillus sphaericus* BBKGBS6 was cultivated with aeration at 30 °C. Gentamicin was included in all growth media, except when PHB utilization was being measured. A single colony from a dextrose-free tryptic soy broth (TSB) (Becton Dickinson Microbiology Systems, Cockeysville, MD) plate was cultivated in 5 ml of TSB to saturation (~40 h), at which time 2 ml
was transferred into 100 ml of TSB in 500-ml baffled flasks and grown for 24 hr. The doubling time of *Lysinibacillus sphaericus* BBKGBS6 in TSB is between 3 and 4 hr. Cells harvested by centrifugation were washed and transferred into 200 ml of TSB or 200 ml of PHB (minimal medium supplemented with 1% fructose and 0.01% [wt/vol] ammonium chloride) in 1-liter baffled flasks to obtain cultures with an initial optical density at 600 nm of 0.5. For cells grown under TSB conditions, 5 ml of cells was removed at 4 and 24 hr for TEM analysis. For cells grown in PHB, 5 ml of cells was removed from the culture at 2.5, 5, 9, 24, and 73 hr. In all cases, cells were immediately fixed for TEM studies. For PHB utilization, 100 ml of cells grown in PHB for 73 hr was harvested, washed with 0.85% (wt/vol) saline, and transferred into 200 ml of PHB utilization medium (PHB_U) (minimal medium supplemented with 0.5% [wt/vol] ammonium chloride). Samples were harvested at 48 hr for TEM analysis.

### 2.2.4 Biochemical characterization of *Lysinibacillus sphaericus* BBKGBS6

The ability of the selected isolate *Lysinibacillus sphaericus* BBKGBS6 to hydrolyze starch, gelatin and casein was detected on the defined medium, for which the cultures were incubated at 30 °C. Other biochemical tests like Gram’s staining, Litmus milk test, oxidase test and Catalase test, Vogues Proskauer test, hydroxybutyric acid and citrate utilization were done in the defined medium using specific substrates.
2.2.4.1 Gram’s staining

Bacterium was heat fixed on the slide. Slide was flooded with crystal (or gentian) violet for 60 seconds and Gram's iodine for 180 seconds. Carefully decolorize with 95% ethanol until thinnest parts of the smear are colorless. (Wash with water). This third step is the most critical and also the one most affected by technical variations in timing and reagents. Saffranin was flooded with (10% Fuchsine) for 60 seconds. (Wash with water). Air dry, or blot with absorbent paper.

2.2.5.2 Litmus Milk test

Litmus milk was used for characterization of soil bacteria. It is prepared by adding a saturated alcoholic solution of litmus to fresh skimmed milk until a pale lavender colour is obtained. The prepared litmus milk was dispensed in 10 ml amounts in test tubes and autoclaved at 121 °C for 20 min. Milk turns pink if there is acid formation. Soil bacteria produce slow changes in litmus milk mainly towards slight alkalinity (Kersters, 1971).

2.2.4.3 Oxidase test

A sterile filter paper strip was moistened in 1% solution of N, N, N\textsuperscript{1}, N\textsuperscript{1} - tetramethyl-p-phenylenediamine-p-ndihydrochloride. The growth of the test culture from a slant was placed on the paper. A platinum loop was used (Kersters \textit{et al.}, 1971).

2.2.4.4 Catalase test

LBA agar slants were inoculated with the culture and incubated at 30 °C. 3 ml of 3% \( \text{H}_2\text{O}_2 \) was trickled down the slant. The slant was tested for the evolution of bubbles, the presence of which indicated a positive test for catalase (Kersters \textit{et al.}, 1971).
2.2.5 Molecular characterization of *Lysinibacillus sphaericus* BBKGBS6 by 16S rRNA sequencing

The genomic DNA was extracted from the strain which proved its efficacy in the production of biopolymer and for identification.

2.2.6.1 DNA Extraction

Bacterial Genomic DNA was isolated using the Insta Gene TM Matrix Genomic DNA isolation kit as per the kit instruction the procedure was followed. Isolated bacterial colonies were picked and suspend in 1ml of sterile water in a microfuge tube and Centrifuge it for 1 minute at 10,000 - 12,000 rpm to remove the supernatant. 200 μl of Insta Gene matrix was added to the pellet and incubated at 56 °C for 15 min. Vortex at high speed for ten seconds and the tube was kept in a 100 °C in heat block or boiling water bath for 8 min. The contents were vertexes at high speeds for 10 seconds and spin at 10,000 to 12,000 rpm for 2 min. In result, 20 μl of the supernatant was used per 50 μl PCR reaction.

2.2.5.2 PCR Protocol

Using below 16S rRNA Universal primers gene fragment was amplified using MJ research Peltier thermal cycler.

2.2.5.2 Primer Details

\[27F \text{ AGAGTTTGATCMTGGCTCAG} \quad 20\]
\[1492R \text{ TACGGYTACCTTGTTACGACTT} \quad 22\]

1μL of template DNA was added in 20 μl of PCR reaction solution. Use 27F/1492R primers used for bacteria, and then PCR reaction performed with below conditions, initial denaturation at 94 °C for 2 min and then 35 amplification cycles at 94 °C for 45 sec, 55 °C for 60 sec, and 72 °C for 60 sec. Final Extension at 72 °C for 10 min. DNA fragments are amplified
about 1,400 bp in the case of bacteria. Include a positive control (\textit{E.coli} genomic DNA) and a negative control in the PCR.

\textbf{2.2.5.3 Purification of PCR products}

Removed unincorporated PCR primers and dNTPs from PCR products by using Montage PCR Clean up kit (Millipore). The PCR product was sequenced using the 518F/800R primers. Sequencing reactions were performed using a ABI PRISM® Big DyeTM terminator cycle Sequencing kits with AmpliTaq® DNA polymerase (FS enzyme) (Applied Biosystems).

\textbf{2.2.5.4 Sequencing protocol}

Single-pass sequencing was performed on each template using below 16S rRNA universal primers. The fluorescent-labeled fragments were purified from the unincorporated terminators with an ethanol precipitation protocol. The samples were resuspended in distilled water and subjected to electrophoresis in an ABI 3730x1 sequencer (Applied biosystems). Sequence data was aligned and analyzed for Identifying the Sample.

\textbf{2.2.5.5 Construction of phylogenetic tree}

The 16 S rRNA gene sequence was used to carry out BLAST with the non redundant database of NCBI gen bank database. The obtained isolated bacterial sequence was analyzed using the BLAST tool. The pylogenitic tree was constructed using the neighbor joining method apply general data clustering techniques to sequence analysis using genetic distance as a clustering metric using Mega 7 version software (Dereeper \textit{et al.}, 2008).

\textbf{2.2.6 Extraction and quantitative analysis of polyhydroxybutyrate (PHB) production and selection of isolates}
The PHB production was observed in 250 ml Erlenmeyer flask containing 50 ml of treated cardboard industry waste water, as production medium under stationary conditions of growth. PHB was extracted from the bacterium BBKGBS6 by using the sodium hypochlorite method (Cappuccino et al., 1992). After 72 hr of incubation at 37 °C, culture broth was centrifuged at 8000 rpm for 15 min. The pellet along with 10 ml sodium hypochlorite was incubated at 50 °C for 1 hr for lyses of cells. The cell extract obtained was centrifuged at 12000 rpm for 30 min and then washed sequentially with distilled water, acetone, and absolute ethanol. After washing, the pellet was dissolved in 10 ml chloroform (Analytical grade) and incubated overnight at 50 °C and was evaporated at room temperature (Singh et al., 2011). After evaporation, 10 ml of Sulphuric acid was added to it and placed in water bath for 10 min at 100 °C. This converts polyhydroxyalkanoic acids (PHAs) into crotonic acid, which gives maximum absorbance at 235 nm by using U-V spectrophotometer (Hitachi- U-2900) (Law and Slepecky, 1961 and Lee et al., 1995). PHB (Sigma Aldrich) was used as standard for making standard curve. For quantitative analysis of PHA, cell culture was grown as described earlier and cell pellet was dried to estimate the dry cell weight (DCW) in units of g/l (Du et al., 2001). Residual biomass was estimated as the difference between dry cell weight and dry weight of extracted PHB (Zakaria et al., 2010). This was calculated to determine the cellular weight and accumulation other than PHB. The percentage of intracellular PHB accumulation is estimated as the percentage composition of PHB present in the dry cell weight. The bacterial cells containing All the Sudan black- B positives isolate were subjected to quantification of PHB production as per the method of John and Ralf.

\[
\text{Residual biomass (g/L)} = \text{DCW (g/L)} - \text{Dry weight of extracting PHB (g/L)}.
\]
\[
\text{PHB accumulation (%)} = \frac{\text{Dry weight of extracting PHB+ (g/L) × 100%}}{\text{Dry cell weight (g/L)}}
\]
2.2.7 Identification of polyhydroxybutyrate (PHB) producing bacteria by Fourier transform infrared (FTIR) spectroscopy

PHB producing bacteria were identified by Fourier Transform Infrared (FTIR) Spectroscopy (Nicolet - 6700 model). Attempts to apply IR technology to biology began as early as the 1910s, when the use of IR spectroscopy for the analysis of biological samples was first suggested. By the late 1940s, the technique was being successfully explored for the study of biological materials; in fact, IR spectroscopy has become an accepted tool for the characterization of biomolecules (Margaria and Quinterio, 2000). An advantage of Fourier transform infrared (FTIR) spectrometer FTIR spectroscopy is that this method can be applied to powdered, dehydrated, or aqueous samples. Also, the FTIR spectrometer can be modified in order to make the study of very small samples, such as tissues sections and single colonies, a possibility. The technique is exquisitely sensitive (Sacksteder and Barry, 2001). The composition of polymer materials can be readily determined by measuring their infrared spectra using a FTIR spectrometer and then comparing the results with a commercially available or specifically prepared spectral data base (Staurt, 2000). The FTIR spectrum of a cell will exhibit contributions from all cellular macromolecules, including protein, lipid, carbohydrates and DNA. Although the spectra of macromolecules are complex proteins, lipids and DNA provide characteristic, non overlapping spectral contributions permit the determination of macromolecular concentration from the band’s amplitude (Naumann, 1998; Sacksteder and Barry, 2001). IR spectroscopy and evaluation of data by using and absorption coefficient at selected wave number values have been developed by Zagreba et al., (1990) and have been used for PHB analysis (Savenkova et al., 1994). FTIR spectra of intact microbial cells are highly specific, finger prints like signature
which can be used discriminate between diverse microbial species and strains, detect in situ intracellular components or structures such as inclusion bodies, storage materials or endospores, detect and quantify metabolically released CO$_2$ in response to various different substrates and characterize growth dependent phenomena of cell-drug interactions. The characteristic information was extracted from the spectral contours by applying resolution enhancement techniques and pattern recognition methods such as factor, cluster, linear discriminant analysis, and artificial neural networks. Particularly interesting applications arise by means of a light microscope coupled to the spectrophotometer (Naumann, 1998).