3.2 MATERIALS AND METHODS

3.2.1 Bacterium

*Lysinibacillus sphaericus* BBKGBS6 was used in the experiments. The culture was maintained on nutrient agar slants and regularly sub cultured and stored at 4 °C.

3.2.2 Media used for the growth of *Lysinibacillus sphaericus* BBKGBS6

Initially culture was grown using nutrient agar plates at 37 which was transferred to modified PHB production medium (pH 7) containing (g/L) Glucose 6.0, Urea 0.70, Dipotassium phosphate K$_2$HPO$_4$ 0.39, Magnesium sulphate heptahydrate MgSO$_4$7H$_2$O 0.2, Yeast extract 0.5 (Nath et al., 2005). To make solid medium, 1.5% Agar was added to the broth.

3.2.3 Growth kinetics with respect to biomass and PHB yield of *Lysinibacillus sphaericus* BBKGBS6

Growth study was carried out in PHB production media was containing (g/L), Glucose 55.0, Urea 0.65 and K$_2$HPO$_4$ 0.3 Final pH 7.0 at 30 °C and media was taken in 500 ml Erlenmeyer flasks and sterilized (121 °C, 20 min).The flasks were inoculated (in duplicate) with 0.1 ml of 18 hr old inoculums of *Lysinibacillus sphaericus* BBKGBS6. Thereafter, the flasks were incubated at 30 °C, pH 7.0 and 250 rpm up to 120 hr. Growth curve analyses was done at regular intervals of time 4 ml of the inoculums was taken and the optical density was measured at 600 nm using U-V spectrophotometer (Hitachi- U- 2900) against an un inoculated blank.

3.2.4 Cell cultivation of *Lysinibacillus sphaericus* BBKGBS6

For large-scale growth, inoculums was prepared in nutrient broth medium and transferred to 500 ml of nutrient broth in a wide necked 1 liter culture flask, incubated at 37 °C for 72 hrs with continuous gentle shaking.
3.2.5 Harvesting of *Lysinibacillus sphaericus* BBKGBS6

After incubation, cells were harvested by centrifugation (model 5804 R effendrop) at 8000 rpm for 12 min, washed in sterile water and recentrifuged. Pellets were collected aseptically, dried at 60 °C.

3.2.6 Cell disruption of *Lysinibacillus sphaericus* BBKGBS6 by ultra sonication for isolation of cell protein

The microbial cells were disrupted by ultra Sonication (Sonics vibra cell, U.S.A) and to determine the amount of protein released and rate constant for cell disruption. Ultra sound waves of frequencies greater than 20 k Hz rupture the cell walls by a phenomenon known as cavitation. The passage of ultrasound waves in a liquid medium creates alternating areas of compression and rarefaction which change rapidly. The cavities formed in the areas of rarefaction rapidly collapse as the area changes to one of compression.

The bubbles produced in the cavities are compressed to several thousand atmospheres. The collapse of bubbles creates shock waves which disrupt the cell walls in the surrounding region. The efficiency of the method depends on various factors such as the biological condition of the cells, pH, temperature, ionic strength and time of exposure. Ultrasonication leads to a rapid increase in the temperature and to avoid heat denaturation of the product it is necessary to cool the medium and also to limit the time of exposure. 2 ml of bacterial culture was taken in 5 eppendorf tubes and centrifuged at 10000 rpm for 5 min. The supernatant was discarded and dissolved the pellet in 1ml of distilled water. The eppendorf tubes were kept in ultrasonic wave generator tip for different time intervals (30 to150 seconds). Set the controller power 50 % and frequency of 25 kHz. After sonication, the eppendorf tubes were centrifuged at 10000 rpm for 5
min. 0.2 ml of supernatant was taken and determined the amount of protein in the sample by Lowry’s method.

3.2.7 Cell Protein analysis of *Lysinibacillus sphaericus* BBKGS6

The amount of protein was analyzed by Lowry’s method. 0.2 ml of BSA was taken working standard in 5 test tubes and made up to 1ml using distilled water. The test tube with 1 ml distilled water serves as blank. 4.5 ml of reagent I was added and incubated for 10 min. After incubation 0.5 ml of reagent II was added and incubated for 30 minutes. The absorbance was measured at 660 nm and the standard graph was plotted. The amount of protein was estimated present in the given sample from the standard graph.

3.2.8 Effect of physical parameters on biomass and Polyhydroxybutyrate (PHB) production from *Lysinibacillus sphaericus* BBKGS6

The culture conditions favoring PHB accumulation in the organism. In bacteria the synthesis of large amounts of PHB is triggered by different environmental stimuli including limitation of oxygen, nitrogen, phosphorus, and potassium individually or simultaneously depending on the organisms. Apparently, all these stimuli act through the accumulation of reducing power and acetyl coenzyme A in the cytoplasm. Therefore, the present study deals with the effects of some of these factors, like; temperature, agitation, media pH, and inoculum concentration.

3.2.8.1 Temperature

*Lysinibacillus sphaericus* BBKGS6 was inoculated into sterile 500 ml Erlenmeyer flasks containing PHB production medium (100 ml). The flasks were incubated at 30, 35, 40, 45, and 50 °C at 150 rpm. Biomass and PHB production was monitored and recorded for 72 hr.
3.2.8.2 Agitation

PHB production medium was sterilized in 500 ml Erlenmeyer flasks and flasks were inoculated with *Lysinibacillus sphaericus* BBKGBS6. The flasks were incubated at 35 °C and at 100, 150, 200, 250, 300 rpm. Biomass and PHB production was monitored and recorded for 72 hr.

3.2.8.3 Media pH

PHB production media (100 ml) was prepared with different pH 4, 5, 6, 7 and 8, and sterilized at 121 °C under 15 lbs for 15 min. 0.1 ml of test culture was inoculated and incubated at 35 °C. Biomass and PHB production was monitored and recorded for 72 hr.

3.2.8.4 Inoculum

PHB production media (100 ml) was taken in 500 ml Erlenmeyer flasks. Different inoculums concentrations were taken as (ml) 0.1, 0.2, 0.3, 0.4, 0.5 and 0.6 and the flasks were sterilized (121 °C, 20 min). *Lysinibacillus sphaericus* BBKGBS6 was inoculated and the culture was grown at 30 °C (250 rpm). Biomass and PHB were analyzed after 72hr.

3.2.9 Effect of nutritional parameters on biomass and PHB Production from *Lysinibacillus sphaericus* BBKGBS6

Nutritional parameters like, nutrient media, carbon sources, fruits peel, nitrogen sources, urea, yeast extract and synergetic effect of *Lysinibacillus sphaericus* BBKGBS6 were, studied.

3.2.9.1 Nutrient media

There were four different media are used. Glucose yeast extract medium containing (g/L), Peptic digest of animal tissue 5.0, Yeast extract 5.0, Glucose 2.0, Monopotassium phosphate 0.5, Dipotassium phosphate 0.5, Magnesium sulphate 0.3, Sodium chloride 0.01,
Manganese sulphate 0.01, Zinc sulphate 0.0016, Copper sulphate 0.0016, Cobalt sulphate 0.0016 and Agar 15.0. Yeast mannitol agar medium containing (g/L), Yeast extract 1.0, Mannitol 10.0, Dipotassium phosphate 0.50, Magnesium sulphate 0.20, Sodium chloride 0.10, Calcium carbonate 1.0 and agar 15.0 final pH 6.8 ± 0.2 at 25 °C. PHB production medium containing (g/L), Glucose 55.0, Urea 0.65 and K$_2$HPO$_4$ 0.3 Final pH 7 at 30 °C and Luria Bertani medium containing (pH 7.0) containing, (g/L) Tryptone 10.0, Yeast extract 5.0, Sodium chloride 5.0 and Agar 10.0 Final pH 7.2 at 37 °C media were used for the highest growth and PHB production from Lysinibacillus sphaericus BBKGBS6.

3.2.9.2 Carbon sources

Mannitol, Glucose, Sucrose, Arabinose, Adonitol, Fructose, Cellobiose and Mannose at (20 g/L) were tested as carbon substrates for biomass and PHB production. These carbon sources were incorporated into PHB production medium. Each medium was then sterilized (121 °C, 20 min). Lysinibacillus sphaericus BBKGBS6 was inoculated into 100 ml of each of the flasks and was grown at 30 °C and 250 rpm for 72 hr.

3.2.9.3 Reducing sugar analysis

Reducing sugar analysis was done by as per the method of Dinitrosalicylic acid (DNS). Sugars with reducing property (arising out of the presence of a potential aldehyde or keto group) are called reducing sugars. 100 mg of the sample was weighed and extract the sugars with hot 80% ethanol twice (5 ml each time). The supernatant was collected and evaporated it by keeping it on a water bath at 80 °C. 10 ml water was added and dissolved the sugars. Pipette out aliquots of 0.1 or 0.2 ml to separate test tubes and pipette out 0.2, 0.4, 0.6, 0.8 and 1 ml of the working standard solution (10 ml of stock diluted to 100 ml with distilled water, 100 μg/ml) into a series of test tubes. Make up the volume in both sample and standard tubes to 2 ml with distilled water.
Pipette out 2 ml distilled water in a separate tube to set a blank. 1 ml of alkaline copper tartrate reagent was added to each tube (A- 2.5 gm anhydrous sodium carbonate was dissolved in 2 gm of sodium bicarbonate, 2.5 gm of Potassium sodium tartrate and 20 gm of anhydrous sodium sulphate in 80 ml water and make up to 100 ml. B- 15 g Copper sulphate was dissolved in a small volume of distilled water. one drop of Sulphuric acid was added and make up to 100 ml. Mix 4 ml of B and 96 ml of solution A before use). The tubes were placed in boiling water for 10 minutes. Cool the tubes and 1 ml of arsenomolybolic acid reagent was added (2.5 gm ammonium molybdate was dissolved in 45 ml of water and 2.5 ml of Sulphuric acid and mix well). Then 0.3 gm disodium hydrogen arsenate was dissolved in 25 ml water then, mixed well and incubated at 37 °C for 24 to 48 hr) to all the tubes. The volume in each tube was made up to 10 ml with water. The absorbance was recorded of blue colour at 620 nm after 10 min. The amount of reducing sugars was calculated present in the sample from the graph (Somogyi et al., 1952 and Krishnaveni et al., 1984).

3.2.9.4 Fruits peel

Fruits peel of Guava, Banana and Sapota was collected and dried in an oven at 60 °C to reduce moisture content and was milled into fine particles and milled powder was directly added into the PHB production medium at the respective concentration of 2 g/L. The effect of fruits peel was determined in the PHB production medium was replaced with concentration of Guava, Banana and Sapota peel with optimum pH of 7 at 35 °C. The biomass and PHB production was measured at different time intervals every 24 hr up to 72 hr.

3.2.9.5 Nitrogen sources

Different nitrogen sources such as Peptone, Tryptone, Sodium Nitrate, Ammonium sulphate, Ammonium phosphate, Ammonium acetate, Ammonium chloride, Urea, Sodium
nitrate were tested as nitrogen substrates for PHB production these were incorporated at Nitrogen levels equivalent to that of total nitrogen present in 0.65 g/L of urea. These nitrogen sources were incorporated into medium (instead of urea into PHB production medium). The medium was then dispensed into 500 ml Erlenmeyer flasks and sterilized (121 °C, 20 min). The culture was grown at 35 °C and 150 rpm. Biomass and PHB were analyzed every 24 hr up to 72 hr.

3.2.9.6 Urea

Urea at different nitrogen levels were (g/l) 1.0, 1.5, 2.0, 2.5, 3.0, 3.5 and 4.0 examined for efficient PHB synthesis by *Lysinibacillus sphaericus* BBKGBS6. The medium was then dispensed into 500 ml Erlenmeyer flasks and sterilized (121 °C 20 min). The culture was grown at 35 °C and 150 rpm. Biomass and PHB were analyzed every 24 hr up to 72 hr.

3.2.9.7 Yeast extract

Yeast extract at different concentrations (g/L) were, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0 examined for efficient biomass and PHB production by *Lysinibacillus sphaericus* BBKGBS6 respectively. Yeast extract was incorporated into medium. The medium was then dispensed into 500 ml Erlenmeyer flasks and sterilized (121 °C, 20 min). The culture was grown at 35 °C and 150 rpm. Biomass and PHB were analyzed every 24 hr up to 72 hr.
3.2.9.8 Synergetic effect of Urea and Glucose

Urea and Glucose at different levels, 0.3: 6.0 to 0.9: 21.0 (g/L) were examined for efficient biomass and PHB production by *Lysinibacillus sphaericus* BBKGBS6 respectively. These nitrogen and carbon sources were incorporated into medium. The medium was then dispensed into 500 ml Erlenmeyer flasks and sterilized (121 °C, 20 min). The culture was grown at 35 °C and 150 rpm. Biomass and PHB were analyzed every 24 hr up to 72 hr.

3.2.9.9 Media optimization for the growth of *Lysinibacillus sphaericus* BBKGBS6 using response surface methodology (RSM)

The carbon, nitrogen and mineral sources, which had been screened earlier, were used along with other components for media optimization studies. A total of four factors were tested, which were, (A) Potassium dihydrogen phosphate, (B) Urea, (C) Glucose and (D) inoculum concentration. A design of 26 experiments was formulated for eight factors using the software. Each parameter was tested at two levels, high (+1) and low (−1). Concentration range for the variables was decided on the basis of literature reports for PHB production by *R. eutropha*. The experiments were done in Erlenmeyer flasks containing 50 ml media at 150 rpm for 60 hrs in duplicate. Response was measured in terms of residual biomass and PHB production. The residual biomass and PHB obtained in these 12 experiments was subjected to compatible analysis, which yielded *t*-values. The components giving higher positive *t*-value were taken up for further studies. Four factors (glucose, urea, KH$_2$PO$_4$ and inoculum concentration), screened from CCD design, were studied for determining their optimum concentration values for residual biomass and PHB production.
3.2.9.9.1 Central Composite Design (CCD)

Once the relevant factors having high $t$-values were selected, RSM was used to determine the optimum concentration of these factors affecting cell growth, rest of the factors being kept at a constant level. A $2^n$ factorial Central Composite Design (CCD) developed using design Expert (version 5.0.9) software (Stat-Ease Corporation, USA) was used to optimize the concentration of the factors selected. Factors yielding high positive $t$-value were selected and an experimental design of 30 experiments was formulated using the Design Expert software. The remaining parameters, which were found not to be influencing the residual biomass and PHB production ($\text{CaCl}_2$ (0.02 g/L), trace metal solution (10 ml/L)) were maintained at a constant level. Experiments were conducted in 250 ml Erlenmeyer flask containing 50 ml of media (pH 7) prepared according to the design. The flasks were kept in incubator shaker maintained at 30 °C and 150 rpm. Responses studied were residual biomass ($X'$) (g/L) and PHB (g/L) at the end of 60 hr. Contour plots (3D) were generated to understand the interaction of various factors and then used to find the optimized concentration of the media components majorly affecting the response. A special feature of the software, point-prediction was used to confirm the above obtained optimized values.

To check the validity of optimized media predicted by the Design Expert software, growth kinetics of the culture was studied in shake flask. Five percent inoculum (containing 10 g/L fructose) was added to 200 ml of media taken in 1 L Erlenmeyer flask. The experiment was carried out in duplicate for 60 hrs. Samples were withdrawn at regular intervals and were analyzed for biomass, nutrients and PHB content.
3.2.9.10 Statistical analysis

ANOVA test was performed for knowing the significance of Polyhydroxybutyrate (PHB) and biomass production from the *Lysinibacillus sphaericus* BBKGBS6 and the p value is $\leq 0.05$. 