3.4 DISCUSSION

3.4.1 Growth kinetics of Lysinibacillus sphaericus BBKGBS6 with respect to biomass and Polyhydroxybutyrate (PHB) production

Growth as commonly applied to bacteria and other microorganisms refers to changes in the total population rather than an increase in the size or mass of an individual organism, growth denotes the increase in number beyond that present in the original inoculum.

In the present study the isolated strain Lysinibacillus sphaericus BBKGBS6, grew significantly faster under these conditions compared to the other selected strains. Its optical density (OD at 600 nm) increased from 0.2 to 10.1 after 48 hrs of incubation (growth rate 0.21 per hr). Consequently, strain BBKGBS6 was chosen for studies of polymer production. The synthesis of PHB was noticed from the log phase of growth and it was continued until late exponential phase as the carbon source was utilized for both growth and PHB production.

Economic and technological barriers are the main concerns regarding large scale microbial production of PHAs and poly-3-hydroxybutyrate (PHB). The large scale production of poly-β-hydroxybutyrate (PHB) by bacteria has become a subject of increasing interest (Byrom, 1897). It is well known that natural isolates of Actinobacillus, Azotobacter, Agrbacterium, hodobacter and Sphaerotilis have been focused on converting organic waste to bacterial PHB. The bacterium Alcaligens latus (Yamane et al., 1996) and Alcaligens eutrophus (Jian, 2001) are well known for their ability to produce PHA. Numerous factors affect the growth of R.sphaeroides in culture medium; these are concentration of substrates, pH, temperature, carbon to nitrogen ratio, and agitation (Grothe et al., 1999 and Du et al., 2001). Optimization of fermentation conditions has been used to enhance productivity. Ultra sound waves of frequencies
greater than 20 kHz 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 (Bio separation technology lab RSM University). The protein concentration in the medium was estimated using Lowry’s method (Lowry et al., 1951), which did not show any organized format. At the initial hour of incubation, the protein concentration was very low, after that the concentration increased. As the incubation time progressed, various enzymes might be synthesized in order to facilitate the cell growth and for other metabolic needs. After 16th hrs, protein concentration increased and at the same time, there was a decrease in the fructose concentration in the medium. This confirmed that this was the phase in which active metabolism was taking place (Lowry et al., 1951).

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

Different temperatures were tested for the optimization of biomass and PHB production from Lysinibacillus sphaericus BBKGS6. Present results indicated that maximum significant biomass and PHB yield was achieved at 35 °C incubation. Higher or lower temperatures showed inferior results. This temperature range is in accordance with the other reports. It has been
reported that the optimal temperature for growth and PHB synthesis appeared to be at 33 °C; however, over the 25 to 37 °C range, the effect of temperature was negligible (Grothe et al., 1999). According to Aslim et al., (2002), optimum incubation temperature for PHB production by Bacillus thuringiensis, Bacillus subtilis, and Bacillus pumilis was at 37 °C. Tamodgan & Sidal (2011) reported that temperature higher or lower than 30 °C lead to decrease in PHB synthesis by Bacillus subtilis ATCC 6633, as well as cell mass probably due to the low enzymes activity.

Zoogloea and Plasticicumulans acidivorans dominated the SBRs operated at 20 °C and 30 °C, respectively. Both enrichments accumulated PHB more than 75 % of cell dry weight. Short-term temperature change experiments revealed that P. acidivorans was more temperature sensitive as compared with Zoogloea. This is particularly true for the PHB degradation, resulting in incomplete PHB degradation in P. acidivorans at 20 °C. Incomplete PHB degradation limited biomass growth and allowed Zoogloea to outcompete P. acidivorans. The PHB content at the end of the feast phase correlated well with the cycle length at a constant solid retention time (SRT). These results suggest that to establish enrichment with the capacity to store a high fraction of PHB, the number of cycles per SRT should be minimized independent of the temperature (Yang Jiang, et al., 2011).

Agitation at 200 rpm was favorable for PHB production as reported (Luengo et al., 2003) and the similar results were found in the present study. In the present study different agitation speed rotations per minute (rpm) were tested for the biomass and PHB production by Lysinibacillus sphaericus BBKGBS6. Agitation of 200 rpm showed highest significant biomass and PHB production and less biomass at 350 rpm.

It has been reported that pH directly affects the PHB synthesis (Ali Hassan et al., 1997). In the present study, different media pH was tested at 24 hr to 72 hr on the biomass and PHB
production by *Lysinibacillus sphaericus* BBKGBS6. Maximum significant biomass and PHB production was obtained at pH 7. The pH 4 and 5 had less effect on the growth and PHB yield in 72 hrs of incubation. Less production of biomass and PHB were observed at pH 4 and 5 after 72 hr of incubation. It has been reported that in slightly acidic culture, wild type of *R.sphaeroides* was found to produce more by products such as methane or hydrogen and less PHB (Luengo *et al.*, 2003). Reports suggests that an initial pH value of 6.5 gave the best results; pH values that differed even slightly from the optimum reduced the culture performance (Flora *et al.*, 2010) present results recommended that, pH range of 6.0 to 7.5 for microbial growth. Therefore, pH 7 was selected for further experimentation. Higher or lower pH values showed inferior results. Metabolic processes are highly susceptible to even slight changes in pH (Wei *et al.*, 2011) and drastic changes in PHB production seems to be due to the effect of initial pH on the bioavailability of trace elements (Ramadas *et al.*, 2009 and Flora *et al.*, 2010) revealed that the maximum PHB production (25%) by *Bacillus sphaericus* was at pH range from 6.5 to 7.5, and the reduction of polymer accumulation at higher pH values is due to the effect on the degradative enzymes of polymer breakdown, so that PHB is utilized at a rate almost equal to the rate of its synthesis. Change in initial pH of the medium showed a strong influence on the production of PHB.

Inoculum concentrations are to affect biomass and PHB production. The pre inoculum prepared in the PHB production medium was rich in nitrogen concentration could involve in protein synthesis in the bacterium and thus increased the biomass. When these cells were put in the production medium, easy assimable carbon source facilitated the growth of microorganism. In the present report maximum significant PHB production was obtained with 0.5 ml of
Lysinibacillus sphaericus BBKGBS6 inoculum of 16 hr ages, which gave significant production of PHB. The inoculum size of the culture determined was $2 \times 10^8$ CFU/ml.

3.4.3 Effect of nutritional parameters on production of biomass and Polyhydroxybutyrate (PHB) from Lysinibacillus sphaericus BBKGBS6

Present study reveals that, PHB production media gave maximum significant biomass and PHB production by Lysinibacillus sphaericus BBKGBS6 compared with Yeast mannitol Agar medium, Glucose yeast extract medium, Luria Bertani medium and nutrient broth medium.

The major restriction in the commercialization of bioplastics is their high production cost. The use of readily available cheap agro industrial residues as the carbon sources may reduce the higher cost. Several studies have shown the utilization of various carbon sources for different bacterial strains. The glycerol utilization as a carbon source was reported by Taidi et al., (1994) for Ralstonia Eutroph (Taidi et al., 1994). The culture showed no difference in the PHB production in media containing sucrose and glycerol as carbon source. Fatty acids from the fermented fruit and vegetable residues also can be supplemented as a carbon source for the microorganisms, to reduce the production cost (Halami, 1999 and Nonato et al., 2001). There are reports describing 25 g/L of PHB when soluble starch was utilized as a carbon source with Azetobacter chroococcum in fed batch mode (Kim, 2007). PHB production by 11 different Bacillus sp. was studied by Chen et al., (1991). Present results showed a maximum of 50% (w/v) PHB of dry cell weight of the bacteria. Twenty nine Bacillus strains were assessed for PHB production and found that B. megaterium showed maximum production of 0.207 g/L and productivity percentage of 48.13%. Lowest PHB was 6.53% in B. subtilis K1 (Aslim et al., 2002 and Van Thuoc et al., 2007), using wheat bran hydrolyzate as the carbon source, reported the PHB concentration as 1.08 g/L, which was almost similar to the result of this study (1.06 g/L)
but the PHB content was 6.8% in this study in comparison to the reported value of 33.8 % by Van Thuoc et al., (2007). The Wheat bran hydrolysate is rich in protein concentration (Van Thuoc et al., 2007). Although the production medium contained the yeast extract (0.2 g/L), the protein in the hydrolysate might have contributed to the cell growth (CDW 15.5 g/L). The cassava bagasse hydrolysate also resulted good growth (CDW 2.5 g/L) but the amount of PHB was low several agro industrial residues such as potato starch, soy cake (Oliveira et al., 2004) cane molasses, whey (Ahn et al., 2001) have been reported for PHB production. Fukui and Doi, (1998) reported that the plant oils such as olive oil, corn oil and palm were good carbon substrates for R. eutropha for PHB production. Thakor et al., (2005) found the coconut oil as one of the best carbon source for Comamonas testosteroni. Rusendi and John, (1995) used waste potato starch hydrolysate for the production of PHB and reported a yield of 77% of the biomass dry weight. For sugar estimation an alternative to Nelson-Somogyi method is the Dinitrosalicylic acid method simple, sensitive and adoptable during handling of a large number of samples at a time and present sample showed moderate amount of reducing sugars. In the present study, ten different carbon sources of Glucose, Sucrose, Mannitol, Maltose, Rhaminose, Fructose, Arabinose, Adonitol and Cellobiose were tested. In the present study for biomass and PHB production from Lysinibacillus sphaericus BBKGBS6 isolate have showed maximum significant production of biomass and PHB in media with Glucose at 72 hr of incubation. However with Maltose, yield was not significant. Fructose served as the second best carbon source in terms of PHB production, among the ten carbon sources tested. However, as compared to glucose the PHB content was less. Minimum biomass and PHB were obtained with arabinose, guava biomass and PHB content and also mannose showed less biomass and PHB content at 72 hrs. This was due to inherent inhibitory effect of high concentration of Arabinose on the bacterial
growth. Glucose being monosaccharide were readily utilizes by bacteria, hence their growth and subsequent production of PHB was higher. In case of Sucrose and Maltose complexity of the carbon increased and hence PHA yields were low. Similar conclusion was made by Chandrashekharaih et al., (2008). Composition of the carbon substrate used for fermentation and utilization of appropriate bacterial strain, controls the copolymer production where substrate cost represents nearly 40% of total cost. Thus, it is essential to explore an alternate substrate for bacterial growth and polymer production. Cheaper raw materials, such as whey, wastewater from olive mills, molasses, corn steep liquor, starchy wastewater, palm oil mill effluent, have been used as nutrient supplements for bacterial PHB production (Page 1989; Hassan et al., 1997; Gouda et al. 2001; Yu 2001; Lapointe et al., 2002; Marangoni et al., 2002 and Pozo et al., 2002).

Therefore, the identification of alternative cost effective substrates for the production of PHB has become an important objective for the commercialization of bioplastics. Microorganisms which can produce and store PHB under nutrient limited conditions can normally degrade and metabolize it when the carbon or energy source is limited (Williams et al., 1996). It has been reported that, production of PHB by the strains of SPY and Ralstonia eutropha was done in Mineral Salt Media with Jambul seed as a carbon source the biomass of the organism was 4.2 g/L and PHB was 0.2 g/L (Preethi et al., 2012). Less reducing sugar were reported from the Lysinibacillus sphaericus BBKGBS6.

Three fruits peel were used as carbon source and tested at various time intervals, these substrates were selected based on easy availability and cheaper cost, the biomass and PHB content obtained with the different substrates tested. In the present study Sapota fruit feel showed maximum biomass (6.63 g/L) and PHB content (0.265 g/L) at 4% after 48 hrs incubation time.
Nitrogen source play an important role in synthesis of PHB. In recent years, PHB and other PHAs have been considered commercially important because of their possible use as biodegradable thermoplastics (Lee, 1996).

In the present study, different nitrogen sources were tested for the highest biomass and PHB production. Urea was showed maximum significant biomass and PHB yield. Further the different concentrations of urea (g/L) were tested for the production of PHB. 3 g/L of Urea showed highest biomass and PHB production respectively. Peptone showed less biomass and PHB production. However, addition of sodium nitrite leads to accumulation of PHB. Sodium nitrite the only nitrogen of the sodium was available for use, and enzymes for assimilation of nitrite were not synthesized, thus leading to a low PHB content.

Various salts of ammonium were tested and provided at amounts equivalent to the amount of ammonium sulphate taken in the media initially used (Mulchandani et al., 1989 and Raje et al., 1998). It has been reported that the complex nitrogen sources increased the yield of PHB by Bacillus megaterium, Bacillus licheniformis, Anaerobiospirillum succiniproducens and Phafia rhodozyma in the presence of Yeast extract, and a combination of Yeast extract and peptone (Lee et al., 1999 and shah, 2012). In the present report, the isolated strain, Lysinibacillus sphaericus BBKGBS6 has showed significant PHB production on Urea and Yeast extract. Similar results were obtained from cultivation of PHB production in a variety of complex nitrogen sources has been tried and it has been found that addition of complex nitrogen sources increased the yield of PHB produced by Azotobacter vinelandii UWD strain (Pagew, 1992). Bonartseva et al., (1994) tested the capacity for PHB production in active and less active strains of Rhizobium phaseoli, R. meliloti and R. trifolii during growth on media with different carbon and nitrogen sources.
It was found that PHB synthesis can be selectively induced either in active or less active Rhizobium strains by sources of carbon and nitrogen (Tombolini et al., 1989). In the present study increasing concentration of Nitrogen and Carbon source (N: C) were assessed for the growth and PHB production in Lysinibacillus sphaericus BBKGBS6. 0.6:5.2 (N: C) ratio showed highest significant PHB production. 0.3:6 ratios were showed less biomass and PHB production. It has been reported that the less active strain of R. phaseoli 680 was a promising producer of PHB and the PHB content in cells of this strain was up to 65% of dry cell weight during growth on a medium with sucrose and nitrate; the PHB content was much lower when organic acids were used. Investigators has been reported the ability of biodegradable polymer production by various members of the genera Pseudomonas (Smet et al., 1983; Suzuki et al., 1986; Taylor et al., 1976 and Aremu et al., 2010). The type of nitrogen source and the carbon to nitrogen ratio affect biomass and PHB yields. Urea is a suitable nitrogen source; Ammonium nitrate is unsatisfactory and is not metabolized to a significant extent. When using ammonium nitrate and sucrose, the optimal C: N ratio is 28:3. The sensitivity of the biomass and PHB yields to changes in C: N ratio depends on the type of the nitrogen source used (Grothe et al., 1999).

3.4.3 Media optimization for the growth of Lysinibacillus sphaericus BBKGBS6 using Response Surface Methodology (RSM)

For the determination of the optimum operating concentration and interaction of factors on growth and PHB production, the response surfaces were studied in detail for all possible combinations by keeping two parameters constant at a time. The contour plots between various factors were analyzed and optimized concentrations of the media components were found. The predicted values obtained were confirmed by point prediction feature of the software. This is a feature of Design-Expert Software, which allows the study of responses by variation of one
parameter at a time. It was found by contour plots and point prediction that when KH$_2$PO$_4$ was varied from higher to lower limit, they were found to converge at a point. Above and below which, value of both response decreased. The converging value for KH$_2$PO$_4$ was found to be 1.6 g/L. The predicted values for glucose and KH$_2$PO$_4$ for the response was found to be increasing for their entire range given. The contours clearly show that if the upper limit of KH$_2$PO$_4$ would have been increased more, then the contours would have converged at a point. Thus, the optimum values of the media components obtained in this way are as follows: glucose, 42 g/L; KH$_2$PO$_4$, 8.7 g/L; urea, 8.7 g/L; inoculum, 0.55 ml/L. Shake flask studies were then carried out for experimental verification of the predicted media concentration. In the present study the *Lysinibacillus sphaericus* BBKGBS6 grown on 42 g/L glucose as carbon source and 1 g/L urea as nitrogen source. An optimized residual biomass of 7.10 g/L and PHB concentration of 5.16 g/L was predicted by the Design Expert Software. A maximum of 6.65 g/L residual biomass and 2.75 g/L PHB was obtained by using the concentrations specified by the design expert, representing 93 and 81% validity of the predicted models for residual biomass and PHB production, respectively.