6. Summary

Samples were collected aseptically from the sludge of municipal wastewater treatment plant (K and C valley), Bangalore. They were processed and the bacterial strains were isolated according to the standard protocol. Primary isolation of bacteria was done by serial dilution technique in nitrogen limited mineral salt agar medium. Morphologically ten different colonies were isolated and transferred to the media several times until a pure culture was obtained.

The isolated bacterial colonies were maintained in nitrogen limited mineral salt agar slants and stored in refrigerator for further analysis. The pure culture of PHB producing *Alcaligenes eutrophus* (MTCC 1285), *A. latus* (MTCC 2309) and *A. latus* (MTCC 2311) strains obtained from IMTECH, Chandigarh were used as reference strains.

The ten bacterial isolates and commercial strains were screened for PHB production in shaken flask cultures. Culture grown in Nitrogen Limited Mineral Salt Broth (NLMSB) appeared as dirty white. The Sudan Black B stained cells were observed under microscope. The PHB granules appeared as a blue black color droplet and the cytoplasm appeared pink in color. Out of ten isolates, five strains which produce maximum PHB were selected .the five bacterial isolates and three commercial strains that produce grey to blue colored colonies in the Nitrogen Limited Mineral Salt Agar (NLMSA) plates were further chosen for the detailed study.

The five strains were characterized as per the guidelines of Bergey’s manual of bacteriology. Based on the cultural, morphological and biochemical characters, the isolates were identified as B1, B2, B3, B4 and B5. 16S rRNA gene sequence of the isolates (B1, B2, B3, B4 and B5) were compared to previously published sequences on the European Molecular Biology Laboratory (EMBL) database showed high
homology (99%) with *Pseudomonas* sp, *Pseudomonas* sp., *Arthrobacter* sp., *Aeromonas* sp. and *Bacillus* sp., respectively.

The production of PHB was carried out for the isolates and commercial strains in NLMSB and industrial waste based medium. The PHB produced was extracted with chloroform and was used for further estimation. According to the procedure of Lee *et al.* (1995), the PHB content was measured at 230 nm using UV spectrophotometer.

Seven sets of initial shake flask fermentation were carried out and higher level of PHB production was observed in NLMSB followed by sesame oil waste (40%) (w/v) without salt concentration. *A. eutrophus* and *Bacillus* sp. produced relatively higher amount of PHB i.e., 3.76 and 3.28 g/L, respectively. The maximum amounts of PHB produced in sesame oil waste (without salt) were about 3.36 and 1.28 g/L by *A. eutrophus* and *Bacillus* sp., respectively. Substantial decreases in the production of PHB were observed in 40% (w/v) sesame oil waste (with salts) and were about 3.76 and 1.96 g/L, respectively. Lower amount of PHB production was observed in *Pseudomonas* sp. in all cases of shake flask fermentation.

The PHB was extracted in pure form from the isolated strains and pure cultures. The samples were subjected to the qualitative analysis of PHB. Thus in order to find cheaper and better substrate different industrial waste was used in this study. Also different concentrations of nitrogen and phosphorous were tested to improve the productivity as and at the same time reduce the production cost of PHB.

Concentrations of nitrogen ranging from 50 to 200 mg/L were incorporated in standard production media (NLMSB) and then inoculated separately into each flask with isolated bacterial strains (*Pseudomonas* sp., *Pseudomonas* sp., *Arthrobacter* sp., *Aeromonas* sp., and *Bacillus* sp.) along with the standard MTCC cultures and allowed
to grow for 24, 48 and 72 h. Among the five isolated strains and the MTCC commercial strains, *Bacillus* sp. showed highest PHB production of 1.96 g/L at 72 hours of incubation in NLMSB amended with 150 mg/L of nitrogen source under aerobic condition, followed by *Bacillus* sp. which produced 1.72 g/L PHB at same conditions. Among the commercial strains tested, *Alcaligenes eutrophus* (MTCC 1285) showed highest production of PHB in the same condition with 3.64 g/L.

Experiment was carried out using four different concentrations of phosphorous (0.33 to 1.33 mg/L) to find the optimal phosphorus content for the production of PHB. The results showed that the bacterial strain *Aeromonas* sp. showed maximum production of PHB with 1.96 g/L followed by *Bacillus* sp. with 1.84 g/L at 72 h of incubation in NLMSB (0.99 mg) under aerobic condition.

Based on the experiment, with the above said concentration of nitrogen and phosphorous, a study was carried out by growing all the cultures independently in conical flasks with different substrates acquired from industrial waste. It was thought that this would also help in accessing the cheap substrate utilization capacity of the culture. In this connection, four different carbon sources (substrates) viz. sago waste, sesame oil waste, molasses and paper waste in different concentrations were taken into account.

For the environmental factor, oxygen content was also taken into an account and it was also found from the study that the aerobic condition paved the way for highest percentage of PHB at 72 hours of incubation, whereas anaerobic or semi-aerobic could not produce highest PHB accumulation at 72 h of incubation , in NLMSB. Preliminary experimental data were taken for further studies on the optimization of carbon sources.
Among the four different carbon sources tested, a maximum PHB content of 3.76 g/L was obtained with *A. eutrophus* (MTCC 1285) using seasame oil waste (40% w/v) followed by 3.56 g/L with the same organism using sago waste (30% w/v) in nitrogen limited mineral salt medium for 72 h of incubation in aerobic fermentation. The least PHB production of 1.12 g/L was observed in NLMS medium using *Pseudomonas* sp. in 50% (v/v) concentration of molasses.

The results obtained from the cultivated organisms were compared with the commercially available PHB producers, which showed highest production of PHB. *Alcaligenes eutrophus* (MTCC 1285) showed potential PHB production in seasame oil waste with 3.76 g/L in optimized conditions, whereas only 3.36 g/L PHB could be produced by *Aeromonas* sp. in seasame oil waste. The difference in the yields of a cultivated strain and commercial strain is negligible.

The effect of the concentration of salt in the medium on PHB production was evaluated. The experimental set up was carried out to evaluate the effect of carbon source alone without nitrogen and phosphorous. The study was carried out in different substrates amended in double distilled water.

In shake flask cultures, the PHB production by the isolated strains were tested and compared with the commercially available strains. The PHB production by *Pseudomonas* sp., *Pseudomonas* sp., Arthrobacter sp., Aeromonas sp. and *Bacillus* sp were 1.68, 1.76, 1.84, 1.92 and 1.84 g/L, respectively. Whereas *Alcaligenes eutrophus* (MTCC 1285), *A. latus* (MTCC 2309) and *A. latus* (MTCC 2311) was 2, 2.48 and 2.92, respectively in 40% (w/v) sago waste substrate.

The maximum PHB production was achieved for *Aeromonas* sp. (3.28 g/L) followed by *Arthrobacter* sp. (3.20 g/L), *Pseudomonas* sp. (3.05 g/L), *Pseudomonas* sp. (2.89 g/L) and *Bacillus* sp. (2.48 g/L) when grown in seasame oil waste at 40%
(w/v) concentration. Whereas, PHB production by *Alcaligenes eutrophus* (MTCC 1285), *A. latus* (MTCC 2309) and *A. latus* (MTCC 2311) was 3.36, 3.23 and 3.10 g/L, respectively.

*Aeromonas* sp. showed the highest PHB production (1.47 g/L) among all the selected bacterial cultures when grown in molasses at 40% (v/v). PHB production of *Pseudomonas* sp., *Pseudomonas* sp., *Arthrobacter* sp., and *Bacillus* sp. ranged between 1.24 and 1.39 g/L, which were significantly less than that of *Alcaligenes eutrophus* (MTCC 1285), *A. latus* (MTCC 2309) and *A. latus* (MTCC 2311) which showed highest PHB production viz. 1.53, 1.36 and 3.10 g/L, respectively at 40% (w/v) substrate except *A. latus* (MTCC 2311) which showed maximum production potential at 10% (w/v) substrate concentration.

The yield of PHB from *Arthrobacter* sp. (2.24 g/L) in paper waste substrate in the concentration of 30% (v/v) was higher than that obtained from *Aeromonas* sp. (2.16 g/L) and *Bacillus* sp. (2.16 g/L), *Pseudomonas* sp. (2.08 g/L) and *Pseudomonas* sp. (2 g/L). Comparatively *A. latus* (MTCC 2309) showed the highest production of PHB with 3.01 g/L at 40% (v/v) substrate concentration.

There was a significant decrease in cationic and anionic concentration in the NLMSB used for the study. The feed components were modified and the initial chemical oxygen demand values were calculated and found to be 690 mg/L. The isolated bacterial strains were able to reduce the COD concentration in the range between 315 and 690 mg/L. Aerobic condition very well led to the decrease in the COD concentration in all the isolated and pure cultures used in the study. *Pseudomonas* sp. showed better results in decreasing the COD level to 315 mg/L while *Aeromonas* sp. was unable to reduce the COD level from 690 mg/L. All the organisms effectively reduced the COD content in the paper waste substrate in aerobic
condition. Anaerobic and semi aerobic conditions favored the low degradation potential of substrate by the microorganisms which had less decrease in the COD content of the industrial waste medium. Whereas, the COD level of commercial bacterial strains used such as Alcaligenes eutrophus (MTCC 1285), A. latus (MTCC 2309), A. latus (MTCC 2311) where 365, 435 and 366 mg/L, respectively. These strains where less effective in the reduction of COD when compared with the isolates Pseudomonas sp., Pseudomonas sp., Arthrobacter sp., Aeromonas sp. and Bacillus sp. in the range of 315, 410, 322, 350 and 365 mg/L respectively.

The experiments were carried out using standard protocol to find the influence of anionic concentration for the production of PHB. Aerobic conditions influenced a lot by decreasing the content of anions. The results showed that among the five isolated strains, Pseudomonas sp. was successful in decreasing the content of anion and increasing the PHB production. Nitrate, phosphate, ammonium and sulphate was noted to be less with 0.03, 0.19, 0.02, 0.05 mg/L, respectively when Pseudomonas sp. was inoculated in the NLMSB. Arthrobacter sp. and Pseudomonas sp. were effective in accumulating nitrite with 0.11 mg/L, Arthrobacter sp. in calcium with 40 mg/L, Arthrobacter sp. in potassium with 0.02 mg/L, and Bacillus sp. was effective in magnesium with 0.05 mg/L.

Among the cultures tested, the pure cultures showed higher PHB production with minimizing the content of cations. A. latus (MTCC 2309) showed effective reduction of phosphate (0.08 mg/L), ammonium (0.22 mg/L), sulphate (1 mg/L) and calcium (32 mg/L) followed by Alcaligenes eutrophus (MTCC 1285) which had reduced the potassium (0.07 mg/L), nitrate (0.01 mg/L) and nitrite (0.01 mg/L). A. latus (MTCC 2311) couldn’t make an effective attempt in reducing the cationic
content but had reduced magnesium (1 mg/L) effectively when compared with the other pure cultures.

PHB production in the NLMSB was studied under different optimized conditions. The media along with the substrate limitations were carried out in these experimental trials. PHB production by all the five isolated bacteria and the commercial strains were tested in the media alone. Bacterial isolates showed significantly less PHB production than that shown by the commercially available bacteria. *Aeromonas* sp. showed highest PHB production when compared with the isolated strains of bacterial PHB producers with 3.58 g/L. *Bacillus* sp. showed second highest PHB production with 3.48 g/L in aerobic condition and *Pseudomonas* sp. with the least production potential of 2.11 g/L in anaerobic condition.

*Aeromonas* sp. in NLMSB with reduced nitrogen source resulted in 3.89 g/L and B3 was able to produce 3.58 g/L of PHB while in commercially available PHB producer, *A. eutrophus* (MTCC 1285) could produce only 3.4 g/L in aerobic conditions. The effect of anaerobic condition influenced the PHB production with least value of 2.21 g/L in case of *Pseudomonas* sp.

The change in salt concentration in the media leads to the increase in the PHB production between 1.70 g/L and 3.70 g/L with the least in anaerobic condition. *Aeromonas* sp. in NLMSB with reduced phosphorous source was found more effective in increasing the PHB to 3.7 g/L on 72 h of incubation. This also influenced the commercial bacterial strain in the production of PHB with 3.21 g/L in *Alcaligenes eutrophus* (MTCC 1285); 3.12 g/L in *A. latus* (MTCC 2309) and 3.05 g/L in *A. latus* (MTCC 2311).
NLMSB with reduced nitrogen and phosphorous content were found more effective than their individual minimization as they induced a higher PHB production in *Aeromonas* sp. With an yield of 4.06 g/L. When this was compared to the commercially available bacterial PHB producer, *A. eutrophus* (MTCC 1285) showed significantly highest production potential with 3.74 g/L which is significantly lesser than the isolated strains.

NLMSB without nitrogen and phosphorous was checked for the production of PHB using the isolated bacterial strains and commercially available PHB producers. The isolated strains *Aeromonas* sp. increased the PHB production with 2.53 g/L. Even the commercial PHB producer, *Alcaligenes eutrophus* (MTCC 1285) was able to accumulate only 2.50 g/L of PHB. The PHB content in media lacking both the supplements was significantly less when compared to all the other parameters studied.

The presence of PHB was monitored by UV Spectrophotometer analysis of samples of pure cultures and five bacterial PHB producers. The results indicate that the peak of the spectrum obtained by the pure form of PHB was around 236 nm. In all the cases of the strains used in the present study i.e isolated bacterial strains and commercially available bacterial strains, the peak was observed at 236 nm. From this, it was shown that the spectrum of the bacterial strains used in the study correlate well with the peak observed by the commercially available PHB. This clearly reveals the presence of PHB in the samples under investigation.

In the FT-IR Spectrum of the extracted polymer, characteristic bands for PHB were obtained for the samples of isolated cultures and pure cultures used in the study. The FT-IR spectra obtained were compared with the spectrum of commercially available PHB. The large absorption peak at 3452 cm\(^{-1}\) – 3395 cm\(^{-1}\) was OH stretching and C-H was between 2994 cm\(^{-1}\) – 2924 cm\(^{-1}\). The absorption band at 1728
cm$^{-1}$ attributed to the stretching vibration of the carbonyl bond (C=O). The band at 2359 cm$^{-1}$ - 2321 cm$^{-1}$ was assigned to the C≡C stretching of alkynes. Absorption peaks between 1537.95 cm$^{-1}$ and 1655.59 cm$^{-1}$ indicates the presence of nitro compounds. The bands between 1547 cm$^{-1}$ and 1597 cm$^{-1}$ arise from N-H vibration of amines. Intense bands centered at 1078 cm$^{-1}$ – 1283 cm$^{-1}$ were assigned to C-N vibrations of amine group. The obtained IR absorption peaks correlated with the literature value and with the spectrum of pure PHB. From the above details it is concluded that the compound should be PHB.

The obtained NMR spectrum for PHB of the selected isolates, pure cultures and commercially available PHB showed the following results. The NMR spectra identified the polymer as an isocratic homopolymer. The spectrum revealed the presence of three group of signals characteristic of PHB homopolymer. The doublet at 1.26 $\delta$ was attributed to the methyl group coupled to one proton; the doublet of the quadruplet around 2.5 $\delta$ to the methylene group adjacent to an asymmetric carbon atom bearing a single proton and the singlet at 5.2 $\delta$ to the methyne group. Chloroform-d gave a chemical shift signal at 7.26 $\delta$. Thus the spectrum was found to correlate with the results obtained by Rohini et al., (2006). From the above qualitative analysis, it is concluded that the compound should be PHB.