4. Results

4.1. Collection, isolation, cultivation and screening of PHB producing microorganisms

Samples which were collected aseptically from the sludge of municipal waste water treatment plant (K and C valley, Bengaluru) were processed and ten bacterial strains were isolated according to the standard protocol. These strains along with the pure cultures such as *Alkaligenes eutrophus* (MTCC 1285), *A. latus* (MTCC 2309) and *A. latus* (MTCC 2311) were cultivated on NLMS medium. After incubation (72 h at 37 °C), the colonies appeared as purpular, opaque and white in colour. These strains were further screened for PHB production by flooding with Sudan Black B. All the colonies appeared grey to blue colour confirming PHB producers. Further, these stained cells of colonies when observed under microscope showed PHB granules as bluish black coloured droplets against a pink coloured cytoplasm. Out of ten isolated strains, five were selected based on the yields of the PHB production and named them as B1, B2, B3, B4 and B5 (Plate 1-3).

4.2. Identification of PHB producing bacteria

4.2.1. Biochemical methods

Among the five PHB producing bacterial strains, all the isolates testing positive for the presence of lipophilic inclusions (Positive Sudan Black B Staining – slide method and plate method) were further chosen for characterization and study.

The five strains were B1, B2, B3, B4 and B5 were characterized as per the guidelines of Bergey’s manual of bacteriology (Table 4.1.)
Table 4.1. Morphological and biochemical characteristics of the isolates.

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<thead>
<tr>
<th>Characters</th>
<th>Interpretation</th>
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<tr>
<td></td>
<td>B1</td>
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<tr>
<td>Gram’s staining</td>
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<td>Shape</td>
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<td>Voges - Proskauer test</td>
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<td>Motility test</td>
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<td>Oxidase test</td>
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<td>Catalase test</td>
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<td>Starch utilization</td>
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<td>Gelatin liquefaction</td>
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<td>Urease test</td>
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<tr>
<td>Carbohydrate fermentation</td>
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<tr>
<td>D – Fructose</td>
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<tr>
<td>D – Mannitol</td>
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</table>

Based on the macroscopic, microscopic and biochemical characters, the five isolates were identified as *Pseudomonas* sp., *Pseudomonas* sp., *Arthrobacter* sp., *Aeromonas* sp., *Bacillus* sp., (we designated them as strain B1, B2, B3, B4 and B5 respectively) (Plate 2). The sequences of the partial 16S rRNA of the bacterial strains (950 bp, 891 bp, 954 bp, 941 bp and 971 bp) were compared against those available in the public databases. They were closely related to those of *Pseudomonas* sp. (95% homology), *Pseudomonas* sp. (95% homology), *Arthrobacter* sp. (95% homology), *Aeromonas* sp. (95% homology), *Bacillus* sp. (95% homology). The bacteria were
thus identified as *Pseudomonas* sp., *Pseudomonas* sp., *Arthrobacter* sp., *Aeromonas* sp., *Bacillus* sp. These nucleotide sequences of the five isolates (B1, B2, B3, B4 and B5) have been deposited in the GenBank database under accession number GQ505367, GQ505368, GQ505369, GQ505370 and KC508107 respectively.

**4.2.2. Identification of PHB producers by 16s rRNA sequencing**

When the 16S rRNA gene sequence of the isolates B1, B2, B3, B4 and B5 were compared to previously published sequences on the EMBL database, the highest homology i.e. 99% homology was found with *Pseudomonas* sp., *Pseudomonas* sp., *Arthrobacter* sp., *Aeromonas* sp., and *Bacillus* sp., respectively.

**4.3. Production of PHB in shake flask cultures**

The production of PHB was carried out by growing B1, B2, B3, B4, B5 and *Alcaligenes eutrophus* (MTCC 1285), *A. latus* (MTCC 2309) and *A. latus* (MTCC 2311) in NLMSB and industrial waste based medium (sago waste, sesame oil waste, molasses waste, paper waste – with and without salt concentration). All the strains produced, high level of PHB in NLMSB followed by sesame oil waste (40% w/v) without salt concentration. Out of seven strains selected for the study, *A. eutrophus* and B5 produced relatively high amount of PHB viz. 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 B5, respectively. Substantial decreases in the production of PHB were observed in sesame oil waste (40 % w/v with salts) and were about 3.76 and 1.96 g / L respectively. Lower amount of PHB production was observed in B2 in all cases of shake flask fermentation (Table 4.2.a – 4.2.f).
4.4. Influence of nitrogen and phosphorous on PHB production

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 to study the PHB production (Table 4.3.a – 4.3.c; Plate 4).

4.4.1. NLMSB medium only

All the five isolated bacterial strains and the commercial strains were tested for PHB production in the medium alone (i.e. NLMSB). Bacterial isolates showed significantly less PHB production than that shown by the commercially available bacteria. B4 showed highest PHB production when compared to other isolated strains of bacteria with 3.58 g / L. B5 showed second highest PHB production with 3.48 g / L in aerobic condition and B1 with the least production potential of 2.11 g / L in anaerobic condition.

4.4.2. NLMSB with reduced nitrogen concentration

B4 in the nitrogen minimized NLMSB 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 B1.

4.4.3. NLMSB with reduced phosphorous concentration

The change in salt concentration in the media leads to the increase in the PHB production between 1.70 and 3.70 g / L with the least in anaerobic condition. Phosphorous minimization to B4 was more effective than the other supplements increasing the PHB to 3.7 g / L on the 3rd day 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).

4.4.4. NLMSB with reduced nitrogen and phosphorous concentration

Minimized nitrogen and phosphorous content were more effective than their individual minimization as they induced a higher PHB production in B4 with 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 lesser than the isolated strains.

4.4.5. NLMSB without nitrogen and phosphorous

Nitrogen and phosphorous content in the NLMSB was removed to check the production of PHB using the isolated bacterial strains and commercially available PHB producers. The isolated strain B4 increased the PHB production in the medium lacking nitrogen and phosphorous 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 of the media lacking both the supplements was less when compared to all the other parameters studied.

4.5. Optimization studies
4.5.1. Media optimization
4.5.1.1. Optimization of nitrogen concentration

The major obstacle in large scale production of PHB is the high cost of production. 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 so 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 batched in standard production media and then inoculated with B1, B2, B3, B4 and B5 along with the standard MTCC cultures and allowed to grow for 24, 48 and 72 h. Among the five isolated strains (B1, B2, B3, B4, and B5) and the MTCC commercial strains, B5 showed highest PHB production at 72 hours incubation in NLMSB amended with 150 mg of nitrogen source with 1.96 g / L in aerobic condition. Following B5, second highest PHB production was observed in B4 with 1.72 g / L at same conditions. *Alcaligenes eutrophus* (MTCC 1285) showed highest production of PHB in the same condition with 3.64 g / L (Table 4.4.a – 4.4.h; Plate 5 - 8).

### 4.5.1.2. Optimization of phosphorous concentration

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 B4 showed maximum production of PHB at 72 h of incubation in NLMSB (0.99 mg of phosphorous) with 1.96 g / L in aerobic condition followed by B5 with 1.84 g / L in same concentration and incubation time (Table 4.5.a – 4.5.h; Plate 9 - 12).

Based on the experiment, with the above said concentration of nitrogen and phosphorous, a study was carried out by growing all the cultures in the above said batch cultures in conical flask with different substrates acquired from industrial waste. It was thought that this would not only help in finding cheaper substrate but also the optimized carbon, nitrogen and phosphorous concentration and would also help in accessing the cheap substrate utilization capacity of the culture. Four different carbon sources (substrates) viz. sago waste, seasame oil waste, molasses and paper waste in different concentrations were taken into account.
4.5.2. Optimization of carbon source

4.5.2.1. NLMSB with reduced nitrogen and phosphorous concentration

The effect of the concentration of nitrogen and phosphorous in the medium for PHB production by the strains were evaluated. PHB accumulation was found to be maximum at higher dosage of nitrogen (150 mg / L) and phosphorous (0.99 mg / L). For the environmental factor, oxygen content was taken into account and it was found from the study that the aerobic condition paved the way for highest percentage of PHB production at 72 h of incubation, whereas in anaerobic or semiaerobic couldn’t produce higher PHB in NLMSB. Preliminary experimental data’s were taken for further studies on the optimization of carbon sources.

Among the four different carbon sources tested, a maximum PHB of 3.76 g / L was obtained with seasame oil waste followed by 3.56 g / L with sago waste. About 3.24 g / L of PHB was produced when the same organism was grown in nitrogen limited media. Minimum PHB production of 1 g / L was obtained at 50 mg nitrogen concentration for 72 h of incubation in anaerobic fermentation. When different concentrations of sago wastes were added to the medium, highest PHB production was achieved in 30% (w/v) concentration. The yield was achieved upto 3.24 g / L in B5. In contrast, the decreased PHB production was observed in 10% (w/v) sago waste concentration; anaerobic condition with optimal nitrogen and phosphorous concentration achieved PHB of 1.28 g / L by strain B1. A maximum PHB production by strain B4 was found to be 3.36 g / L with seasame oil waste at the concentration of 40% (w/v). This was followed by 1.28 g / L of PHB production by B2 strain with concentration of 10% (w/v) seasame oil waste in anaerobic condition. PHB accumulation was found to be maximum in B4 at 20% (v/v) concentration of molasses (3.26 g / L) followed by 3.21 g / L at 30% concentration. The least PHB
production was found to be present in 50% (v/v) of paper waste in B2 (1.2 g / L). About 3.20 g / L of PHB was produced when the organism B4 was grown in 40% (w/v) concentration of paper waste substrate. Minimum PHB production of 1.12 g / L was obtained for B2 strain at 20% (v/v) paper waste substrate (Table 4.6.a. – 4.6.c; Plate 13 - 28).

The results obtained from the cultivated organisms were compared with the MTCC cultures, which showed highest production of PHB. *Alcaligenes eutrophus* (MTCC 1285) showed potential PHB production in seasame oil waste with 3.76 g / L under optimum conditions, whereas only 3.36 g / L of PHB could be produced by B4 strain in seasame oil waste. This was 11% decrease in the PHB production when compared with the pure cultures of MTCC used in this study (Table 4.6.a. – 4.6.c; Plate 13 - 28).

4.5.2.2. NLMSB without nitrogen and phosphorous

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 selected strains such as B1, B2, B3, B4 and B5 were tested and compared with the MTCC cultures of *Alcaligenes eutrophus* (MTCC 1285), *A. latus* (MTCC 2309) and *A. latus* (MTCC 2311). The PHB production by B1, B2, B3, B4 and B5 were 1.68, 1.76, 1.84, 1.92 and 1.84 g / L, respectively in 40% (w/v) sago waste substrate. Whereas PHB production by *Alcaligenes eutrophus* (MTCC 1285), *A. latus* (MTCC 2309) and *A. latus* (MTCC 2311) was 2, 2.48 and 2.92 g / L, respectively in 40% (w/v) sago waste substrate (Table 4.6.a. – 4.6.c; Plate 13 - 28).
The efficiency of PHB production measured in seasame oil waste (without nitrogen and phosphorous) was the highest for B4 (3.28 g / L), B3 (3.20 g / L), B1 (3.05 g / L), B2 (2.89 g / L) and B5 (2.48 g / L) at 40% (w/v) concentration of substrate indicating that PHB production potential was proportionate with their concentration. PHB production in *Alcaligenes eutrophus* (MTCC 1285), *A. latus* (MTCC 2309) and *A. latus* (MTCC 2311) was 3.36, 3.23 and 3.10 g / L, respectively in 40% (w/v) seasame oil substrate (Table 4.6.a – 4.6.c; Plate 13 - 28).

B4 showed the highest PHB production (1.47 g / L) among all the selected bacterial strains at 40% (v/v) molasses substrate. PHB production of B1, B2, B3 and B5 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 in 40% (v/v) substrate except *A. latus* (MTCC 2311) which showed maximum production potential at 10% (v/v) substrate (Table 4.6.a – 4.6.c; Plate 13 - 28).

The yield of PHB from B4 (2.24 g / L) in 30% paper waste substrate was higher than that obtained from B3 and B5 (2.16 g / L each), B2 (2.08 g / L) and B1 (2 g / L). Comparatively *A. latus* (MTCC 2309) showed the highest production of PHB with 3.01 g / L at 40% (v/v) substrate (Table 4.6.a – 4.6.c; Plate 13 - 28).

**4.5.2.3 Optimization of cations 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. B1 showed better results in decreasing the COD level to 315 mg/L while B4 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 semiaerobic conditions favored the low degradation potential of substrate by the microorganisms which had less decrease in the COD content of the industrial waste medium. The commercial bacterial strains used in the study *Alcaligenes eutrophus* (MTCC 1285), *A. latus* (MTCC 2309), *A. latus* (MTCC 2311) with 365, 435 and 366 mg/L, respectively were less effective in the reduction of COD when compared with the isolated strains B1, B2, B3, B4 and B5 in the range of 315, 410, 322, 350 and 365 mg/L, respectively (Table 4.7.a – 4.7.c).

### 4.5.2.4 Optimization of anion concentration

In the experiments carried out using standard experimental protocol to find out the influence of anionic concentration, aerobic conditions influenced a lot by decreasing the content of anions. The results showed that among the five isolated strains, B2 was successful in decreasing the content of anion and increasing the PHB production. Nitrate, phosphate, ammonium, sulphate was noted to be less with 0.03, 0.19, 0.02 and 0.05 mg/L, respectively when B2 was inoculated in the NLMSB. While B3 and B1 were effective in accumulating nitrite with 0.11 mg/L, B3 in calcium with 40 mg/L, B4 in potassium with 0.02 mg/L, and B5 in magnesium with 0.05 mg/L (Table 4.8.a – 4.8.af).

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 cation content but had reduced magnesium (1 mg / L) effectively when compared to other pure cultures (Table 4.8.a. – 4.8.af).

### 4.6. UV Spectrophotometry

The presence of PHB was monitored by UV Spectrophotometer analysis and showed the following results in the samples of pure cultures and five bacterial PHB producers.

The results indicate that the absorption of the spectrum obtained by the pure form of PHB was around 236 nm. In all the cases of the cultures used in the present study – B1, B2, B3, B4, B5 and pure cultures of *Alcaligenes eutrophus* (MTCC 1285), *A. latus* (MTCC 2309), *A. latus* (MTCC 2311) the absorption were observed at 236 nm. From this, it was shown that the spectrum of the bacterial strains used in the study correlate well with the absorption observed by the commercially available PHB. This clearly reveals the presence of PHB in the samples under investigation (Fig. 4.1).

### 4.7. FT – IR spectrum analysis

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 spectrums obtained were compared with the spectrum of commercially available PHB. The large absorption peak at 3395.07 cm\(^{-1}\) – 3452.34 cm\(^{-1}\) was OH stretching and C-H was between 2924.25 cm\(^{-1}\) – 2994.59 cm\(^{-1}\). The absorption band at 1723.45 cm\(^{-1}\)- 1728.87 cm\(^{-1}\) attributed to the stretching vibration of the carboxyl bond (C=O). The band at 2321.87 cm\(^{-1}\)- 2359.02 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 1547.59 cm\(^{-1}\) and 1597.11 cm\(^{-1}\) arise from N-H vibration of amines. Intense bands centered at 1078.01 cm\(^{-1}\) – 1283.39 cm\(^{-1}\) were assigned to C-N vibrations of amine group (Fig. 4.2a - e).

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.

4.8. \(^1\text{H NMR Spectral Analysis}\)

The obtained spectrum for 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 ppm was attributed to the methyl group coupled to one proton; the doublet of the quadruplet around 2.5 ppm to the methylene group adjacent to an asymmetric carbon atom bearing a single proton and the singlet at 5.2 ppm to the methyne group. Chloroform-d gave a chemical shift signal at 7.26 ppm (Table 4.9; (Fig. 4.3a - e).

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.