5. Discussion

5.1 Collection of samples from wastewater sludge, isolation and cultivation of PHB producing microorganisms

A variety of bacteria produces and accumulates PHAs. However, their use in industrial PHA production is not popular. Because of their high cost associated with the production of PHAs (Choi and Lee, 1999: Chan et al., 2001; Reddy et al., 2003). As a result, there is little incentive and thus, little progress in identifying and isolating an efficient PHA producer. Isolation of diverse PHAs producing bacteria by enrichment techniques can help to identify novel and more efficient PHA producer. This can lead to better PHA yields in a short period of time, thus cutting down production cost. The marine environment provides a virtually untapped resources for novel bacteria which have already been exploited for the industrial production of biopolymer (Exopolysaccharides, Melanins as well as polyhydroxyalkanoates) (Weiner, 1997; Lee et al., 2001).

Despite the common practice of exploiting the diversity of bacteria in the environment for the industrial production of novel compounds, few reports few reports available in the literature on industrial production of PHAs by the potential bacterial strains (Chan et al., 2001; Reddy et al., 2003). In view of these we have made attempt to isolate a diverse range of PHA producing bacteria from sludge of municipal wastewater treatment plant with the hope of identifying different classes of PHB produced.

5.2 Screening, identification and PHB producing capability of the isolates

Optical microscopic observations upon pollutant degrading microorganisms (Pseudomonas sp., Pseudomonas sp., Arthrobacter sp., Aeromonas sp. and Bacillus sp.) after SB staining indicated that more significant black blue granules accumulated
in all the strains revealing that those were likely feasible candidate microorganisms for PHA production which correlates the report of Chen et al. (2012). In contrast to the report of Chen et al. (2012), high level of significant accumulation of PHA granules was observed in the strains isolated from the wastewater sludge in this study. Intracellular compartments of five isolates of two *Pseudomonas* sps., (two spices) *Arthrobacter* sp., *Aeromonas* sp., *Bacillus* sp., and MTCC cultures viz *Alcaligenes eutrophus* (MTCC 1285), *A. latus* (MTCC 2309) and *A. latus* (MTCC 2311) in Nitrogen Limited Mineral Salt medium used in this study showed higher level of PHB accumulation which suggest that these strains are highly potent for the PHA production.

5.2.1. UV Spectrophotometry

The method for assay of poly-β-hydroxybutyric acid has been used to estimate polymer extracted from various organisms and under a variety of conditions (Slepeeky and Law, 1960). It has proved reliable and convenient in all instances. Certain materials, notably carbohydrates, cause some interference with this assay (Sleepecky and Law, 1960). In addition with some cells which do not contain polymer, an insoluble material is obtained after hypochlorite treatment which contains an interfering substance. This material gives a spectrum in sulfuric acid quite different from the typical crotonate spectrum. The nature of this material is not known. However, it is important to check carefully the entire spectrum from 220 to 260 mn when using this assay method with an unknown sample. The comparison of the gravimetric assay method with the spectrophotometric method for determination of poly-β-hydroxybutyric acid in crude samples reveals that the former gives values about 10% higher than the latter. The results of the present work indicate the peak of
the spectrum obtained by the pure form of PHB and the isolates were around 236 nm. This is probably due to chloroform-soluble impurities in the crude polymer which were not removed by acetone and alcohol washing of the native lipid particles. Reprecipitated polymer, on the other hand gives the theoretical amount of crotonic acid on sulfuric acid treatment.

5.2.2. FTIR Spectrum

Fourier-transform infrared (FT-IR) spectroscopy is used as a whole organism fingerprinting technique since it reflects the biochemical composition of intact cells. Although infrared spectroscopy was introduced already in the 1950s (Riddle et al., 1956; Stevenson and Bolduan, 1952), FT-IR spectroscopy required the advent of computer based data processing and multivariate statistical analysis to become suited for the accurate identification of prokaryotic and eukaryotic cells (Naumann et al., 1991). The resolving power of the method relies on the high specificity of FT-IR spectra, which represent the overall molecular composition of cells. These spectra can be compared with entries in spectral reference databases. Since even the molecular composition of different strains of the same species is often distinct, spectral analysis is capable of discriminating and identifying microorganisms down to the strain or serotype level (Rebuffo-Scheer et al., 2007). So far FT-IR spectroscopy has been used to identify pathogenic bacteria (Kuhm et al., 2009; Rebuffo-Scheer, et al., 2007; Samuels et al., 2009), food-borne yeasts (Büchl et al., 2008; Toubas et al., 2007), and filamentous fungi (Fischer et al., 2006). Additionally, FT-IR spectroscopy has been used to follow compositional changes in bacterial cells (Helm and Naumann, 1995; Marcotte et al., 2007; Naumann et al., 1996).
The extracted polymer from the isolates and pure cultures obtained from MTCC had characteristic bands with large absorption peak at 3395 – 3452 cm\(^{-1}\) was OH stretching and C-H was between 2924 – 2994 cm\(^{-1}\). The absorption band at 1723 -1728 cm\(^{-1}\) attributed to the stretching vibration of the carbonal bond (C=O). Hong et al., (1999) reported the absorption bands at 1728 cm\(^{-1}\), corresponding to the ester carbonyl group and at 1282 cm\(^{-1}\) corresponding to the–CH group which is the characteristic of PHB isolated from B. cereus. The band at 2321- 2359 cm\(^{-1}\) was assigned to the C≡C stretching of alkynes. Absorption peaks between 1537 cm\(^{-1}\) and 1655 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 – 1283 cm\(^{-1}\) were assigned to C-N vibrations of amine group and the above observations were identical to PHB.

5.2.3. \(^1\)H NMR Spectral analysis

The extracted polymer (40 mg) was dissolved in 1 ml CDCl\(_3\) followed by \(^1\)H NMR analysis. Three groups of signals characteristic of polymer PHB were found in the spectrum. A doublet at 1.26 δ represented the methyl group (CH\(_3\)) coupled to one proton while a doublet of quadruplet at 2.5 δ resulted from methylene group (CH\(_2\)) adjacent to an asymmetric carbon atom bearing a single proton. The third signal was a multiplet at 5.2 δ, which was attributed to a methyne group (CH) which correlates the report of Arun et al. (2009) and Rohini et al. (2006). From the contribution of various groups to the NMR spectra, it was concluded that the waste activated sludge could serve as an inexpensive source of biodegradable polymer and that the bacterial biomass in sludge produced PHA exclusively in the form of PHB.
5.3. Extraction and estimation of PHB

After the successful scaling up of PHB production, the efficiency of polymer extraction using hypochlorite and organic solvents were evaluated. Three different techniques namely chloroform extraction (Ramsay et al., 1994), chloroform–hypochlorite dispersion extraction (Hahn et al., 1993, 1994 and 1995) and soxhlet extraction (Ramsay et al., 1994; Manna et al., 1999) were tested for the PHB extraction. The extraction involves the dissolution of the polymer into chloroform. The resulting solution is centrifuged to remove debris concentrated and the polymer was precipitated on of the polymer, and the polymer precipitated using methanol or ethanol, leaving low molecular weight lipids in solution. In chloroform hypochlorite dispersion techniques, the anionic surfactant hypochlorite is used to remove cellular debris before the intracellular polymer is dissolved in chloroform. Hypochlorite extraction is known to cause severe degradation of PHB leading to a significant reduction of the polymer chain length (Berger et al., 1989).

The PHB extracted from five isolated bacterial strains and commercial strains in synthetic and industrial waste based medium (sago waste, sesame oil waste, molasses waste, paper waste – with and without salt concentration) was estimated in seven sets of initial shake flask fermentation in which higher level of PHB production was observed in NLMSB followed by sesame oil waste (40% w/v) without salt concentration. The present result corroborates well with the results of Khanna and Srivastava (2005 and 2006), Patwardhan and Srivastava (2008), in that formation of PHB is favored by an ample amount of carbon and shortage of nitrogen. Under nitrogen starved condition slow and less microbial growth was observed, which may be due to the utilization of PHB as a nitrogen source. However, the extent of this degradation varies considerably between organisms. Within the organisms tested, the
amount of PHB degraded to a lower molecular weight compound in *A. eutrophus* (75% reduction in the number average molecular weight) during the recovery process. The chloroform extraction method relies solely on the solubility of the PHB in chloroform and does not involve any treatment with sodium hypochlorite. This method is widely used to recover PHB as it results in less polymer degradation.

**5.4. Optimization studies**

A satisfying process to produce PHA has not yet been achieved. The choice of a suitable carbon source is an important factor in the optimization of the PHA production. The nature of the carbon source not only determines the PHA content, but also the composition of monomers, which subsequently affects the final properties of the polymer. 80% of the final cost corresponds to the carbon source used. Therefore the price of PHA can easily be reduced by using cheap substrates (Oliveira *et al.*, 2007). Consequently, simple sources of carbon, such as glucose or fructose, were used to produce PHA (as PHB), in addition to organic volatile acids such as acetic, propionic and butyric acids. Other sources of carbon which have been used were molasses, whey and vegetable oil (Oliveira *et al.*, 2007). The production of biopolymers with vegetable oil has shown an increase in monomeric units with 6–14 carbons atoms. For instance, Akiyama *et al.* (2003) reported an yield of 0.8 g and 0.3 g of PHA per g of plant oil and glucose used, respectively. Vegetable oils are desirable inexpensive feedstocks for the production of various bioproducts including PHA (Kim *et al.*, 2008). The yields from vegetable oils are high compared with those from other carbon sources, because they consist of a much higher number of carbon atoms per weight (Kahar *et al.*, 2004, Akiyama *et al.*, 2003).
Although the use of molecular biology techniques and recombinant DNA are of great utility to direct the production of PHA to very specific products, that type of methodologies increase the cost of PHA production. Fed batch culture fermentation process is typically used in industries to reach high cell density in the bioreactor and avoid problems of inhibition by substrate, which limits the amount of the final product in the culture. It has been reported that large amounts of carbohydrates (>30 g L\(^{-1}\)) inhibit biomass production and final product (Shang et al., 2004). Srivastava and Khanna (2005) compared the effect of fructose, glucose, molasses, glycerol and sucrose on residual biomass and PHB production. They obtained similar biomass production, but less PHB production when glucose was used compared to fructose, presumably these because of the inability of the culture to utilize these sugars that contain ions and minerals, which have a deleterious effect on microbial growth and product synthesis.

In the present investigation, among the four different carbon sources tested, a maximum PHB content of 3.76 g / L was obtained with sesame oil waste followed by 3.56 g / L with sago waste in nitrogen limited media. These results obtained from the isolated organisms were compared with the commercially available PHB producers, viz *Alcaligenes eutrophus* (MTCC 1285) showed high PHB production in sesame oil waste with 3.76 g/L in optimized conditions, whereas only 3.36 g/L of PHB could be produced by B4 in sesame oil waste. This was 11% decrease in the PHB production when compared with the commercially available PHB producers. The theoretical yield coefficients of PHA production from vegetable oils are as high as 1.0 g of PHA/g vegetable oils used, while the yield from glucose is only 0.32–0.48 g per of PHA/g glucose used were reported (Yamane, 1992). Shilpi Khanna and Srivastava (2005) reported the utilization of twelve different carbon sources for the
optimized production of PHB. In their results, maximum PHB content of 1.4 g / L was obtained with fructose after 60 h of incubation. Kahar et al.(2004) reported on the production of P(3HB) and P(3HB-co-3HHx) by using the wild type strain H16 of A. eutropha and its recombinant strain, respectively, with soybean oil as a sole carbon source, producing a high PHA yield ranging from 0.72 to 0.76 g of PHA/g soybean oil used. From these its clear that yields of PHAs produced by isolated strains of the present study was found to be higher than isolated strain.

In order to find a better substrate, different carbon sources, nitrogen sources, salt concentrations and pH levels were tested so as to increase the productivity of PHB. The activated sludge was inhabited by PHB producing bacteria and also by other non PHB producing organisms such as ciliates, rotifers, nematodes and oligochaetes (Rastak et al., 1993). The relative abundance and occurrence of the different organisms varied with the ratio of food (F) (Chemical Oxygen Demand-COD/Biochemical Oxygen Demand-BOD) to microorganisms (M) and high F:M ratios favoured the increase in bacterial biomass in comparison to other organisms (Mishoe, 1999; Chua et al., 2000). This selectively enriched sludge could serve as a low cost source of PHB producing biomass. In general, the PHB yield is significantly affected by the nutritional conditions (Johnson et al., 2009, Chua et al., 2003). It has been reported that high yield PHB accumulation could be achieved by limiting the nitrogen and/or phosphorous sources (Johnson et al., 2009, Bernat et al., 2008, Md Din et al., 2006). Variation in the PHB concentration over time in the presence/absence of nitrogen and/or phosphorous sources was investigated by Liu et al. (2011) under continuous aeration with sodium acetate as carbon source.
Valappil et al. (2007) reported that in shaken flask cultures, the cell mass increased steadily, leading to a maximum cell density within 24 h of cultivation after which there was a gradual decrease. The pH of the culture medium decreased during the growth, from its initial value of 7 to a minimum of 4.5. Cessation of logarithmic growth coincided with the approach of the pH minimum and rapid consumption of glucose. PHB accumulated rapidly during the stationary phase and reached a maximum concentration of 38% of dry cell weight (dcw) at 60 h of growth in modified Kannan and Rehacek medium which is the best for PHB production using the newly characterised B. cereus as compared to other reported minimal media such as potassium deficient production medium (Wakisaka et al., 1982) and phosphate deficient production medium (Lopez et al., 1986).

Most bacterial strains that are known to accumulate PHA under conditions of nutrient limitation. Nitrogen or phosphate limitation has been successfully applied to the fedbatch culture of A. eutropha for the production of P(3HB) and P(3HB-co-3HV) with high productivity (Kim et al., 1994a, Kim et al., 1994b; Ryu et al., 1997). In addition, some bacteria such as A. latus and recombinant Escherichia coli do not require nutrient limitation for PHA biosynthesis and can accumulate PHA during growth (Hanggi 1990, Kim et al., 1992).

The effect of the concentration of salt in the medium to PHB production was also evaluated in the present study which depicted the PHB accumulation 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 at 72 h of incubation, whereas in anaerobic or semi-aerobic couldn’t produce highest PHB in NLMSB.
5.4.1. Cationic and anionic concentrations

Pepaske and Repaske, (1976) reported that the depletion of ammonium, phosphate, sulphate and magnesium ions was accompanied by a sudden transition from the exponential to the stationary growth phase; 1 g cellular dry weight was obtained from 11.5 mM ammonium, 350 µM phosphate, 145 µM sulphate or 75 µM magnesium. These yields were slightly found higher than that of wild type strain. In contrast to these nutrients, the exhaustion of potassium did not completely cut off cell growth but retarded it significantly. With 20, 40 and 80 µM potassium in the medium the cell grew with a doubling time of 2 h until they reached a density of 0.16, 0.28 or 0.52 g of cells /L. After this initial phase the doubling time increased to 12 h. Therefore, a concentration of 140 µM potassium allowed maximum exponential growth of the cells to a density of 1 g dry weight /L. in another study, Novak (2001) showed that has indicated that lower biomass production was caused by the increase in ammonium ions concentration in wastewater.

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 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.
The results of the present work correlated with Dobrzyńska et al. (2004), which showed on average of 700 mg / L in organic compound sludge loading.

The commercial bacterial strains used in the study Alcaligenes eutrophus (MTCC 1285), A. latus (MTCC 2309), A. latus (MTCC 2311) with 365 mg / L, 435 mg / L and 366 mg / L, respectively were 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 mg / L, 410 mg / L, 322 mg / L, 350 mg / L and 365 mg / L, respectively which correlates with the work reported by Pepaske and Repaske,(1976).

5.4.2. Influence of nitrogen and phosphorous on PHB production

After the accumulation phase the concentration of nitrogen was gradually reduced in the medium for N₂ limitation. The weight ratio of C:N in the influent was adjusted to 10, 20, 30, 40 and 50. Samples were taken after 72 h of incubation with each nitrogen concentration to measure the PHB production.

The results showed that the % of PHB accumulation in the biomass increased from 10 – 30 later in 40 and 50 concentrations it decreased. The maximum% of PHA accumulation was observed with a C:N weight ratio 30. This correlates with the report of Wen et al. (2010) as they observed maximum of 59% PHA accumulation was achieved at C:N ratio of 25 with a PHA productivity equal to 1.61 mg PHA/mg COD consumed.

The accumulation of PHA in the activated sludge system was high under nitrogen limitation, the NLMS concentration in the system reduced sharply by the end of the experiment. This may be attributed due to the high C:N ratio applied in the medium, which is lower than the minimum C:N ratio required for the biomass to continue cell growth and anabolic metabolism.
For most cases of PHB production under reduced phosphorous condition, the maximum accumulation occurred either at the end of the anaerobic half hour after the commencement of aeration. This is probably due to less C:P ratio 0.33mg/ l, leading to a lag phase for PHB synthesis. When the C:P ratio was equal to 0.99 mg / L, PHB accumulation achieved maximum of cell dry weight at the end of the aerobic PHAse.

5.4.3. Influence of aeration on PHB production.

In the present study, the results showed that PHB was continuously synthesized and accumulated in the medium under aerobic condition by the strains isolated from the activated sludge. These results are in agreement with results Saito et al. (1995) how that the sludge accumulated more PHB under aerobic conditions than under anaerobic conditions. Investigated three types of operating systems for PHB production viz, aerobic, semi aerobic, anaerobic. It was found that the two stage bioprocess approach was the most successful strategy for PHA production and aerobic conditions were found better and less complex for PHA production.

For the typical anaerobic, aerobic activated sludge process, microorganisms consume energy sourced from polyphosphate or glycogen to absorb organic substrates such as short chain fatty acids under anaerobic conditions and they temporarily store the organic substrates, such as PHB, until the conditions become aerobic. Under aerobic conditions, they grow and regenerate polyphosphate and glycogen while aerobically utilizing the temporal carbon storage, PHB (Cech and Hartman, 1993).

The production of PHB in aerobic, semiaerobic and anaerobic conditions using activated sludge was carried out, concentrating on the biochemical mechanisms and on the trials to increase PHB content in activated sludge. The system selects microorganisms that have very interesting capabilities in all of the growth condition.
Although it is not easy to investigate what is carrying out these interesting metabolisms, it is worth trying to find new isolates with unique PHB production capabilities. The attempt to produce PHB by activated sludge indicates another way of PHB production. Most of the research on PHB production has been concentrated on pure culture of microorganisms or genetically engineered plants. Using activated sludge, or culture isolates enriched under adequate conditions, may also be a promising option for PHA production.