African Journal of Biotechnology Vol. 9(21), pp. 3144-3150, 24 May, 2010
Available online at http://www.academicjournals.org/AJB
ISSN 1684–5315 © 2010 Academic Journals

Full Length Research Paper

Microbial production of polyhydroxy alkanotes (PHA) from *Alcaligens* spp. and *Pseudomonas oleovorans* using different carbon sources

Amutha Santhanam* and Sreenivasan Sasidharan

Institute for Research in Molecular Medicine (INFORMM), Universiti Sains Malaysia, 11800, Pulau Pinang, Malaysia.

Accepted 6 April, 2010

Plastics have resistance to biological breakdown that result in its accumulation in the environment. This emphasizes the need to search for biodegradable polymer, which is easily disposable and degradable. Bacteria synthesize and accumulate polyhydroxy alkanote (PHA) as carbon source under limiting conditions of nutrients. The effect of different nutrient conditions on production of PHA by *Alcaligens eutrophus, Alcaligens latus* and *Pseudomonas oleovorans* was screened and characterized in this study. The influence of different carbon sources on PHA production showed that, medium with glucose as carbon source produced the maximum PHA content of 4.14 g/l from *A. eutrophus*. *P. oleovorans* produced 2.06 g/l from n-octane as carbon source. The functional groups of the extracted PHA granules were identified as C=O group by fourier transform infrared (FTIR)-spectroscopy analysis. Biodegrability studies showed that, the PHA produced is degradable by a number of soil microbes making it an ideal environmentally friendly material for regular human use.

Key words: Polyhydroxy alkanotes, polymer, polyhydroxy alkanote granules, biodegradable.

INTRODUCTION

In response to problems associated with plastic waste and its effect on the environment, there has been considerable interest in the development and production of biodegradable plastics. Polyhydroxy alkanoates (PHA) are polyesters that accumulate as inclusions in a wide variety of bacteria. These bacterial polymers have properties ranging from stiff and brittle plastics to rubber-like materials. Because of their inherent biodegradability, PHA are considered to be good candidates for biodegradable plastics and elastomers, since they possess material properties similar to those of synthetic polymers currently in use and are completely biodegradable after disposal (Holmes, 1988). Generally, seven classes of biopolymers are distinguished, namely, polynucleotides, polyamides, polysaccharides, polyisoprenes, lignin, polypohosphate, and PHA. PHA are produced by a variety of bacterial species (Shamala et al., 2003) under nutrient limiting conditions with excess carbon. These water insoluble storage polymers are biodegradable, exhibit thermoplastic properties and can be produced from renewable carbon sources (Brandl et al., 1990). The composition of the polymer synthesized is governed by two main factors, that is, the bacterial strain being used and the carbon source being used to grow the bacteria.

Polyhydroxyl butyrate (PHB) is a biodegradable thermoplastic polymer that has many advantages similar to that of many conventional petrochemical derived plastics (Oliveira et al., 2004). The main advantage is that, the biodegradable polymers are completely degraded to water, carbon dioxide and methane by anaerobic microorganisms in various environments such as soil, sea, lake water and sewage and hence, is easily disposable without harm to the environment. Poly 3-hydroxy butyrate (PHB) belongs to the PHA and is used widely as a storage compound produced by the bacteria. This is observed as hydrophobic inclusions in the cytoplasm of B*acillus megaterium* and in many gram negative and gram-positive bacteria (Brandl et al., 1988). Degradability can be categorized as either photodegradable or biode-

*Corresponding author Email: amutha_santhanam@yahoo.com. Fax: +604-6534803.

Abbreviations: PHA, Polyhydroxy alkanotes; **FTIR,** fourier transform infrared; PHB, polyhydroxyl butyrate.

gradable. Photo degradation leads to breakdown of the polymers into non-degradable smaller fragments leading to loss of structural integrity of the material.

In contrast, biodegradable polymers are either partly or fully decomposed. PHAs are high molecular mass polymers with properties similar to conventional plastics such as polypropylene (Reddy et al., 2003). Therefore, they have a wide range of applications, such as in the manufacture of bottles, packaging materials, films for agriculture and also in medical applications (Oliveira et al., 2004). The production cost of PHA is quite high compared with that of synthetic non biodegradable, and so great effort has been recently devoted to making this process economically more feasible, for instance, by changing the substrate from glucose to renewable resources.

Recently, much effort (Chakravarty et al., 2010) has been given to produce PHA in pilot scale of continuous mode from waste water systems to make this production feasible. One of the major stumbling blocks in the large scale synthesis is the high production cost. Studies have shown that, the raw material costs (mainly carbon source) contribute most significantly to the overall production cost (Choi and Lee, 1997). By changing the carbon source and bacterial strains used in the fermentation process, it is possible to produce related biopolymers having properties ranging from stiff and brittle plastics to rubbery polymers (Anderson and Dawes, 1990).

The bacterium *Alcaligens eutrophus* (Jian, 2001), and *Alcaligens latus (*Yamane et al., 1996*)* are well known for their ability to produce PHA. *Pseudomonas oleovorans* (Prieto et al., 1999) which accumulates PHA only when alkanes are provided as carbon source was included in this study for PHA production under different carbon sources. Hence, this study is aimed at the microbial production of PHA from *A. eutrophus*, A. *latus*, and *P. oleovorans,* towards the optimization of inoculums and production of PHA utilizing different carbon sources of glucose, fructose and sucrose.

MATERIALS AND METHODS

Microorganisms

A. eutrophus (MTCC 1285) *A. latus* (MTCC 2309) and *P. oleovorans* (MTCC 617) strains were obtained from the Institute of Microbial Technology, India. *Alcaligens* spp were grown in ABM medium (Yeast extract- 2 g/l, Peptone- 5 g/l, Agar- 20 g /l Soil extract- 50 ml/l) and *P. oleovorans* was in nutrient agar medium. The soil extract was prepared by boiling ground soil in water followed by filtration.

The original cultures were maintained as glycerol stock at –20°C. For experimental purpose the strains were maintained as slant cultures in their respective medium and stored in the refrigerator.

PHA production

For PHA production, the stock cultures were initially inoculated in a nutrient rich (with nitrogen source) medium. After growth for 24 h in the above medium, the cells were harvested and again re-inoculated into nitrogen free medium containing the carbon source. The organisms were allowed to grow for 48 h under aeration at 30°C. The seed cultures of *A. eutrophus, A. latus* and *P. oleovorans* were centrifuged at 5000 rpm for 10 min and the cell pellet was used as initial inoculums. Polymer production was carried out at 30°C for 48 h, and then, the samples were withdrawn and analyzed for PHA production. For all the three strains, the effect of different initial inoculums (1, 2 and 5%, v/v) and different carbon sources (2%, w/v) as substrates of fructose, lactose, glucose, commercial sugar (sucrose) and n-octane were studied in triplicates. In this study, commercial sugar was used instead of sucrose. This commercial sugar was purchased from the grocery shop.

Extraction of PHA in large volume

The PHA was directly extracted using the solvent chloroform. First, the bacterial cultures were harvested by centrifugation at 5000 rpm for 10 min. The lipids were removed from the cell pellet-using methanol (40 times the volume of cell pellets) and the cells were incubated at 95°C for 1 h. Then it was filtered to remove the methanol completely and the sediment granules were incubated in an oven at 65°C till dry. Chloroform was added to the dried granules and was incubated at 95°C for 10 min and after cooling, the mixture was gently mixed overnight. The solution was then filtered to get the debris. Finally, the PHA was precipitated from the debris with 7:3 (v/v) mixtures of methanol and water. The precipitated PHA was then washed with acetone and dried.

Staining procedure

The presence of PHA as intracellular granules was confirmed by staining the cells with Sudan black-B. After the complete production of PHA under suitable growth conditions, thin smear of strains were made on a clean glass slide and was heat fixed. This slide was immersed in a filtered solution of 0.3% (w/v) Sudan black-B (in ethylene glycol) for 15 - 20 min. Then, the slide was immersed in xylem and blot dried with absorbent paper. Finally, the microscopic slide was counter-stained for 10 s with (0.5% w/v) aqueous safranin. The slide was then rinsed with tap water and blot dried and examined under a microscope.

Degradation of the extracted PHA

To assess the degrading capacity of the extracted PHA obtained from *A. eutrophus*, the chloroform solvent method was used. For this, media containing PHA as the sole carbon source was used for the isolation of microbes that degrade PHA from soil and water. Nitrogen free mineral agar medium was prepared to a portion of the medium that has been melted and cooled to 45 - 50°C; a sufficient amount of sterile concentrated PHA granules suspension was added to give a final concentration of 2.5% (w/v) in the medium. Granule-agar suspension was poured over the surface of the plates of solidified medium to form a thin layer. After the overlay solidified, serially diluted samples were spread over the medium. The plates were incubated for 3 - 5 days at 37°C

Fourier transform-infrared spectroscopy (FT-IR analysis)

The PHA extracted from the organism was analyzed by FT-IR spectroscopy (JASCO FT/IR). It was used under the following conditions: spectral range, 4000-400 cm^{-1} to confirm the functional groups of the extracted polymer.

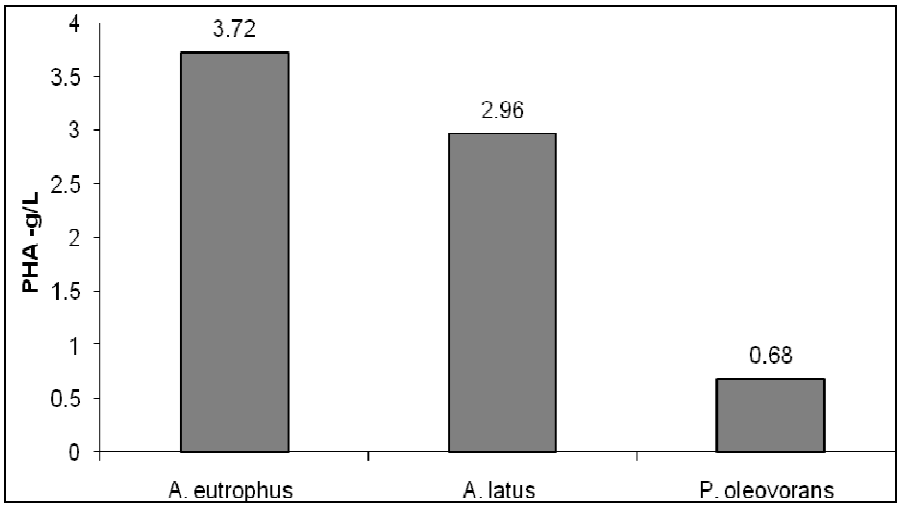

Figure 1. Comparison of poly hydroxyalkanoate (PHA) production of *A. eutrophus, A. latus*, and *P. oleovorans* ($P < 0.05$).

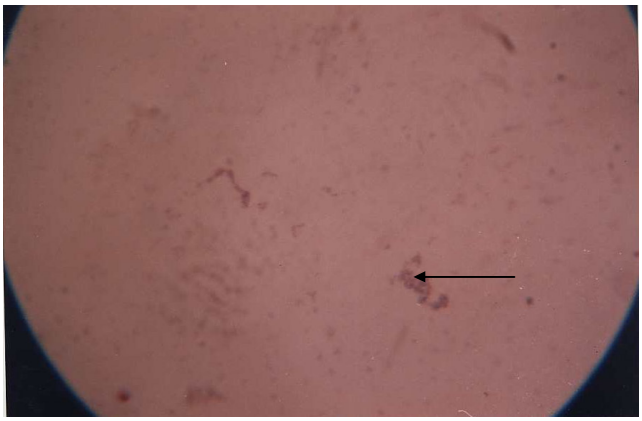

Figure 2. Photomicrograph of PHA granules in *Pseudomonas oleovorans* (cytoplasm as pink, PHA granules as black). Arrow shows poly hydroxyalkanoate granules.

Analytical methods

PHA production was estimated as previously described by Law and Slepecky (1961). Briefly, 3 ml of bacterial culture grown in N-free medium was transferred to glass centrifuge tubes (tubes were washed with acetone and methanol to remove plasticizers if plastic tubes were used) and centrifuged at 5000 rpm for 10 min. The cell pellet was suspended in 1 ml of standard alkaline hypochlorite solution and incubated at 37°C for 1 - 2 h for complete digestion of cell components except PHA. The mixture was centrifuged to collect PHA granules and the supernatant was discarded. The sediment was washed twice with 10 ml of distilled water and centrifuged. The PHA granules in the sediment were washed twice with three portions of acetone, methanol and diethyl ether, respectively. The polymer granule was dissolved with boiling chloroform and the chloroform was allowed to evaporate.

Finally, the granules were mixed with 10 ml of concentrated H_2SO_4 and the tube was capped and heated for 10 min at 100°C in a water bath. The concentration of PHA was determined from an established standard graph in which the absorbance was plotted against the concentration of crotonic acid as standard (235 nm). PHA granules extracted with the boiling chloroform method were dissolved in concentrated sulfuric acid and was taken for measurement at a UV spectrum between 220 and 225 nm. The presence of PHA was confirmed by the presence of a peak obtained between 230 - 240 nm.

Statistical analysis

Data were analyzed by using one way analysis of variance (ANOVA). P values of < 0.05 were considered significant. Statistical calculations were performed using the statistical package for the social sciences (SPSS) version 12.0 statistical software.

RESULTS

Estimation of efficiency of PHA production

In order to find out the efficiency of the organism to produce PHA, the three micro organisms were grown under nitrogen limiting condition and the sample was taken for estimation after 48 h. *A. eutrophus* produced a higher amount of PHA compared to *A. latus* and *P. oleovorans.* The concentrations of the polyhydroxy alkanote produced by these microorganisms are summarized in Figure 1. The photomicrograph of PHA granules in *P. oleovorans* is shown in Figure 2

Effect of initial inoculum

The concentration of PHA polymer produced with 2% v/v initial inoculum, by *A. eutrophus, A. latus* and *P. oleovorans* are as follows: 3.96 g/l, 2.81 g/l and 0.65 g/l. The production of PHA by different inoculum was compared as shown in Table 1.

Table 1. Comparison of PHA production by different types of microorganism with different concentration of initial inoculum.

Microorganism	Polyhydroxyalkanoate (g/l) with different inoculum		
	1% (v/v)	2% (v/v)	5% (v/v)
Alcaligens eutrophus	1.85	3.96	2.41
Alcaligens latus	1.04	2.81	1.97
Pseudomonas oleovorans	0.41	0.65	0.54

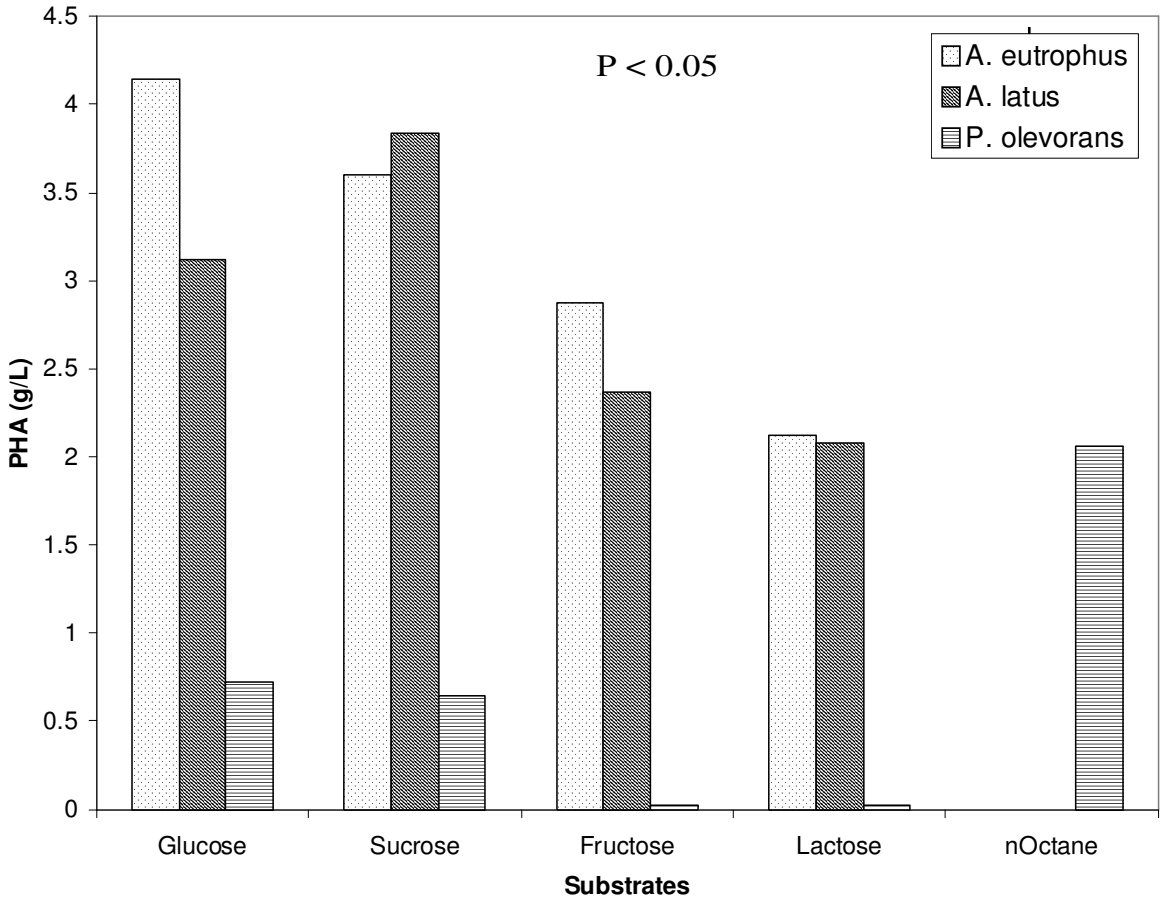

Figure 3. Comparative data of PHA production by *A. eutrophus, A. latus,* and *P. oleovorans* with various carbon sources.

Effect of different carbon sources

To increase the yields of polymer PHA, various carbon sources (2% w/v) such as sucrose, fructose and lactose and n-octane were added to the nitrogen free medium with the inoculum of 2% (v/v). In this study, *A. eutrophus* efficiently produced the maximum concentration of 4.14 g/l with glucose in comparison to other carbon sources. The PHA production of *A. eutrophus* from different carbon sources showed significant difference $p < 0.05$ (one way ANOVA). The effect of different carbon sources on production of PHA by different types of microorganisms are shown in Figure 3.

Biodegradability

To analyze the biodegradability, the polymer produced by *A. eutrophus* was supplied as carbon source instead of glucose in the medium for the isolation of microbes from soil. Many soil bacteria effectively degraded the extracted PHA granules. This was confirmed by the growth of soil born microbes in the plates with PHA as carbon source.

The total cfu obtained was $8X10^4$ ml when the polymer

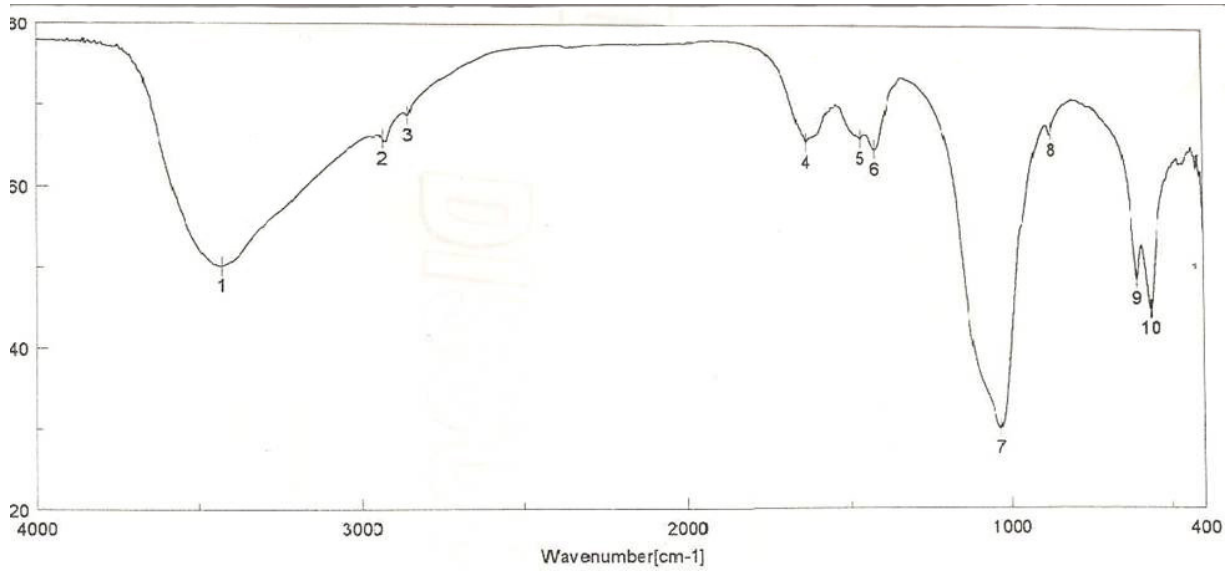

Figure 4. FT-IR spectrum of PHA produced by *P. oleovorans*. Accumulation = 16; Zerofilling = Off; Gain = 2; Resolution = 4cm^{-1}; Apodization = Cosine; Scanning speed = 2 mm/sec; **1.** 3428.81, 50.1299; **2.** 2933.20, 65.7954; **3.** 2854.13, 68.6438; **4.** 1631.48, 64.9650; **5.** 1465.63, 65.4094; **6.** 1419.35, 64.0721; **7.** 1031.73, 29.9060; **8.** 867.81, 66.2923; **9.** 603.61, 48.1949; **10.** 561.18, 44.5129.

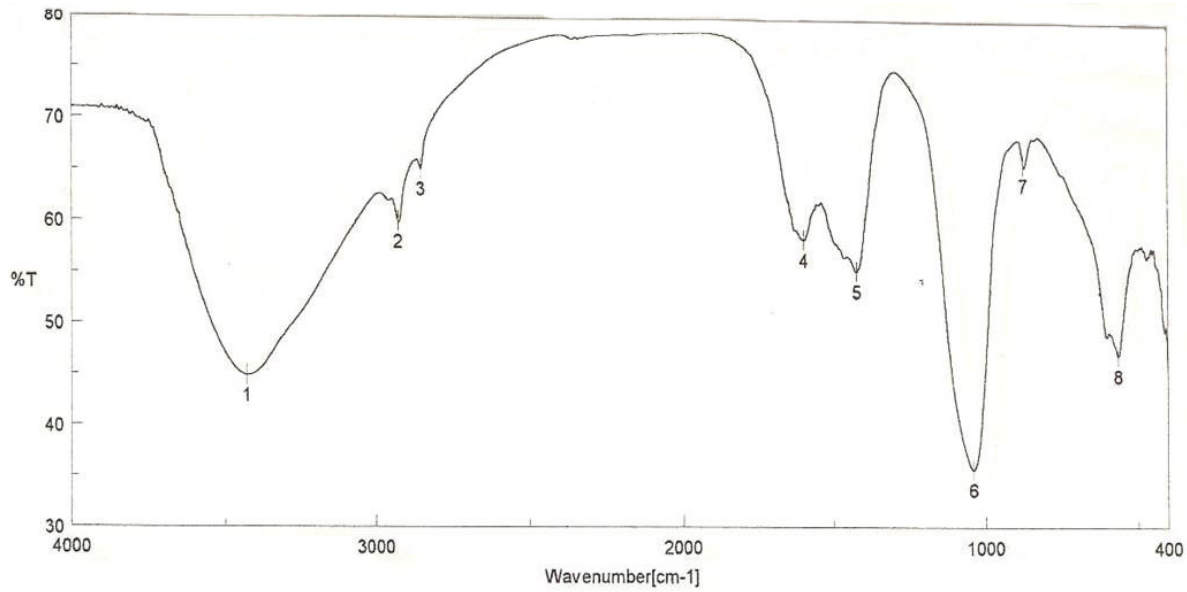

Figure 5. FT-IR Spectrum of PHB produced by *A. eutrophus*. Accumulation =16; Resolution = 4cm^{-1} Zerofilling = Off; Apodization = Cosine Gain = 2; Scanning speed = 2 mm/s; **1.** 3426.89, 44.9160; **2.** 2927.4, 59.9219; **3.** 2854.13, 65.0217; 4**.** 1598.70, 58.1044; **5.**1421.28, 55.0307; **6.**1039.44, 35.6863; **7.** 875.52, 65, 4236; **8.** 565.04, 46.8555.

produced by *A. eutrophus* was supplied as carbon source

FT-IR analysis

The functional groups of the extracted PHA granules were identified as C=O group by FT-IR spectroscopy. The results of FT-IR spectroscopy are shown in Figures 4 and 5.

DISCUSSION

PHA polymers were accumulated in the cells in nitrogen-

free mineral media containing various carbon substrates, and the accumulated PHA polymers were subsequently degraded after the carbon sources were exhausted. In this study, three different types of microorganisms were studied to evaluate their PHA production under different types of carbon source. Optimization of initial microbial load and suitable carbon source are very important for high production of PHA. Polyhydroxy alkanote obtained in this study were found to be sudanophilic, as confirmed by Sudan black-B staining procedures. Previous reports (Forsyth et al., 1958) have confirmed the sudanophilic nature of PHA granules. A conventional method developed for the analysis of PHA by Kim et al. (1994) revealed that the polymer could be converted quantitatively to crotonic acid by heating in concentrated sulphuric acid and the ultra violet absorption was shifted to 235 nm. So crotonic acid was used as the standard in this study to quantify PHA. At high concentration (5% v/v) of inoculums, *A. eutrophus*, *A. latus* and *P. oleovorans* were not able to produce high amount of PHA. At low concentration of initial inoculum (1% v/v), *A. eutrophus* produced low amount of PHA as compared to 2% v/v initial inoculum. This showed that, the higher inoculum of bacterial cells rapidly utilized the already accumulated intra cellular PHA granules as carbon and energy source (Yamane et al., 1996). According to Valappil et al. (2007) *Bacillus* spp required 10% inoculum to produce PHA. However, under optimized substrate and suitable culture conditions, the *Alcaligens* spp and *P. oleovorans* produced PHA even at low inoculum.

A. eutrophus produced the maximum concentration of PHA using glucose as carbon substrate compared to sucrose, fructose and lactose. This proved that glucose was a better substrate for production of PHA by *A. eutrophus* when compared to other carbon sources. This might be due to the low efficiency utilization of these disaccharides. In addition to that, the maximum PHA production was 3.6 g/l when *A. eutrophus* was grown in fructose, this was good enough as Jung and Lee (2000) reported 3.2 g/l. A higher concentration of PHA polymer obtained with commercial sugar suggests the possibility of using cane molasses or other cheaper sucrose substrates in future. Biotechnological production of PHAs from different sugars via condensation of acetyl-CoA units stemming from hexose catabolism is well described in the literature (Sudesh et al., 2000) but only a limited number of micro-organisms directly convert lactose into PHAs. This explained the low production of PHA from lactose compared to glucose by *Alcaligens* spp in this study. In glucose medium, *P. oleovorans* produced low concentration of PHA polymer. In the present study, *P. oleovorans* produced low concentration of polymer PHA with glucose. According to Haywood et al. (1989) *P. oleovorans* ATCC 29347 does not synthesize PHA granules in a medium containing glucose and the organisms yield higher amounts of PHA with n-alkanes such as n-octane. Similarly, *P. oleovorans* produced higher amounts of PHA (2.06 g/l) with n-octane. The PHA degradation capacity of microbes was reported to be due to the presence of intracellular depolymerases. This enzyme converts the polymer to water and carbon dioxide aerobically and methane under anaerobic condition. The PHA produced by *A. eutrophus* was found to be easily degraded by a number of soil microbes showing its utility as a biodegradable agent. The presence of growth of soil microorganisms in the agar plate using PHA granules as sole carbon source had proven this. In this study, the functional groups of the polymer PHA was confirmed as C=O groups by FT-IR spectroscopy. The result obtained by this is exactly similar to that of other researchers (De Smet et al., 1983; Castillo et al., 1986).

In conclusion, this study has led to the preliminary finding of bacterial strains of *A. eutrophus*, *A. latus* and *P. oleovorans* capable of producing PHA from glucose, noctane and that commercial sugar was one of the better substrate for PHAs production by *A. eutrophus*. The optimized initial inoculum would help the microorganism to grow in different carbon source. In addition, it was also observed that, many soil bacteria effectively degraded the PHA granules obtained in this study. This preliminary comparative analysis of three types of bacteria has led to the novel invention of a biodegradable environment friendly polymer with a high potential for regular use.

REFERENCES

Anderson AJ, Dawes EA (1990). Occurrence, metabolism, metabolic role and industrial uses of bacterial Polyhydroxy alkanotes. Microbiol. Rev. 54: 450-472.

Brandl H, Gross RA, Lenz RW, Fuller RC (1990). Plastics from bacteria and for bacteria: Poly (β hydroxyalkanotes) as natural,biocompatible, and biodegradable polyesters. Adv. Biochem. Eng. Biotechnol. 41: 77-93.

Brandl H, Gross RA, Lenz RW, Fuller RC (1988). *Pseudomonas oleovorans* as a source of Poly (β hydroxyalkanotes) for potential applications as biodegradable polyesters. Appl. Environ. Microbiol. 54: 1977-1982.

Castillo RF, Valera FR, Ramos JG, Berraquero FR (1986). Accumulation of Poly (β hydroxybutyrate) by *Halobacteria*. Appl. Environ. Microbiol. 51: 214-216.

Chakravarty P, Mhaisalkar V, Chakrabarti T (2010). Study on Poly-hydroxy alkanote (PHA) production in pilot scale continuous mode waste water treatment system. Bioresour. Technol. 101: 2896-2899.

Choi JI, Lee SY (1997). Process analysis and economic evaluation for poly (3hydroxybutyrate) production by fermentation. Bioprocess Eng. 17: 335-342

De Smet MJ, Eggin G, Witholt KB, Kingma J, Wynberg H (1983). Characterization of intracellular inclusions formed by *Pseudomonas oleovorans* during growth on octane. J. Bacteriol. 154: 870-878.

Forsyth WGC, Haward AC, Roberts JB (1958). Occurrence of Poly- β hydroxybutyric acid in aerobic gram negative bacteria. Nature, 182: 800-801.

Haywood GW, Anderson AJ, Dawes EA (1989). A survey of the accumulation of novel poly hydroxyalkanotes by bacteria. Biotechnol. lett. 11: 471-476.

Holmes PA (1988). Biologically produced PHA polymers and copolymers. In: Bassett DC, editor; Bassett DC, editor. Developments in crystalline polymers. Vol. 2. London, England: Elsevier pp. 1-65.

Jian Yu (2001). Production of PHA from starchy waste water via organic acids. J. Biotechnol. 86: 105-112.

Jung YM, Lee YH (2000). Utilization of oxidative pressure for enhanced

production of Poly β hydroxybutyrate and Poly (3 hydroxybutyrate-3 hydroxyvalerate) in *Ralstonia eutropha*. J. Biosci. Bioeng. 90: 266-270.

Kim BS, Lee SC, Lee SY, Chang HN, Chang YK, Woo SI (1994). Production of Poly (3hydroxybutyric–co-hydroxyl valeric acid) by fed batch culture of *Alcaligens eutrophus* with substrate control using on line glucose analyzer. Enzyme Microb. Technol. 16: 556-561.

Law JH, Slepecky RA (1961). Assay of Poly β hydroxyl butyric acid. J. Bacteriol. 82: 33-36.

Oliveira FC, Freire DMG, Castilho LR (2004). Production of Poly (3-hydroxybutyrate) by solid state fermentation with *Ralstonia eutropha*. Biotechnol. Lett. 26: 1851-1855

Prieto MA, Buhler B, Jung K, Witholt B, Kessler B (1999). PhaF, a Polyhydroxyalkanote-granule asociated protein of *Pseudomonas oleovorans* GPo1 involved in the regulatory expression system for pha genes. J. Bacteriol. 181: 58-868.

Reddy CSK, Ghai R, RashmiKalia VC (2003). Study on poly-hydroxyalkanoate (PHA) production in pilot scale continuous mode wastewater treatment system. Bioresour. Technol. 87: 37-146

Shamala TR, Chandrashekar A, Vijayendra SV, Kshama L (2003). Identification of polyhydroxyalkanoate (PHA)-producing *Bacillus* spp. using the polymerase chain reaction (PCR). J. Appl. Microbiol. 94: 69-74.

Sudesh K, Abe H, Doi Y (2000). Synthesis, structure and properties of polyhydroxyalkanoates: Biological polyesters. Prog. Polym. Sci. 25: 1503-1555.

Valappil SP, Peiris D, Langley GJ, Herniman JM, Boccaccini AR, Bucke C, Roy I (2007). Polyhydroxyalkanote (PHA) biosynthesis from structurally unrelated carbon sources by a newly characterized *Bacillus spp*. J. Biotechnol. 127: 475-487.

Yamane T, Fukunage M, Lee YW (1996). Increased PHB productivity by high cell density fed batch culture of *Alcaligens latus* a growth associated PHB producer. Biotechnol. Bioeng. 50: 197-202.