

## SCREENING FOR NOVEL BACTERIAL STRAINS CAPABLE OF ACCUMULATING INTRACELLULAR PHB UNDER PHENOL STRESS

Indu C Nair

Assistant Professor, P.G Department of Biotechnology, SASSNDP Yogam College Konni, Pathanamthitta, K689691

[Inducnair73@gmail.com](mailto:Inducnair73@gmail.com)

**ABSTRACT:** Poly Hydroxy alkanooates production by different organisms is generally complex due to the diversity of PHAs, production organisms, substrates, and growth conditions used by different laboratories. An attempt was made to isolate PHB accumulating bacteria under phenol stress. Phenol degrading bacterial isolates were collected from five locations and the isolates were screened for PHB accumulation. The isolates were identified by conducting various biochemical tests and also by using rapid identification kit. The PHB accumulation in the selected isolate was confirmed by staining with Sudan Black and Nile Blue Sulphate followed by fluorescent microscopy. The intracellular accumulation of the PHB was traced and the optimum conditions were optimised. The optimum incubation period was 24 hours, optimum pH was 7 and the ideal temperature for incubation was 30<sup>0</sup>C. The PHB accumulated was extracted, purified and was subjected to FT/IR spectroscopy. The various bands observed at 3300 cm<sup>-1</sup>, 2900 cm<sup>-1</sup>, 2400 cm<sup>-1</sup>, 2200 cm<sup>-1</sup> and 1700 cm<sup>-1</sup> confirmed the presence of intracellular PHB. The selected organism could also produce 70 units of polyphenol oxidase enzyme after 16 hours of incubation in PHB producing medium. The organism used in the present study can be considered as a good source of PHB and further work is to be done to evaluate the possibility of scaling up the process.

**Key words:** PHB, screening, FT/IR spectroscopy

### INTRODUCTION

Planet earth is a unique piece of the universe. But this planet is constantly threatened by human activities. A dangerous situation is provided by the plastic wastes. The health of the biosphere is seriously affected by the accumulation of plastic wastes. Plastics are strongly recalcitrant, as they have long polymeric chains and specialized groups associated with them. Many microorganisms are unable to secrete suitable enzymes required for the decomposition of such compounds. Plastic wastes harm the aquatic and wild animals. As it is very difficult to subject the plastic wastes to degradation, alternative sources are always a better choice. The bacterial polymer PHA is a promising alternative for synthetic plastics. Poly hydroxy alkanooates (PHAs) are homo or hetero polyesters that are synthesized by different prokaryotes under specific conditions. They are biodegradable and can be moulded in different ways. They are completely biodegradable too. PHA can serve as a carbon or energy source for microorganisms during starvation periods. When the supply of the limiting nutrient is restored, PHA can be degraded by intracellular depolymerase and subsequently metabolized as carbon and energy source [1]. They are important polymers for at least three reasons. The primary reason, which stimulated interest in PHAs, is their synthesis from renewable sources and biodegradation to water and carbon dioxide [2]. PHA depolymerases have the property of being water-soluble and the ability to bind specifically to polyester surfaces [3]. Biodegradation may be enzymatically catalyzed hydrolysis or simple chemical hydrolysis of the polymer. Enzymatic degradation may be further *via* either intracellular [4] or extracellular PHA degradation [2]. The composition and extensive molecular size of plastics appears to provide resistance to microbial degradation. The mammoth scale use of plastics and their improper disposal has polluted natural environment the world over. Thus the need of the hour is to find alternative materials, having the physical and industrial properties similar to petrochemically derived plastics, which are biodegradable [5]. Among biodegradable plastics, Poly hydroxy alkanooates (PHAs) have been drawing much attention because of their similar material properties to conventional plastics and complete biodegradability. PHAs like PHB and PHBV are moldable thermoplastic polymers which can be obtained from bacteria.

Poly Hydroxy Butyrate (PHB) or other polyhydroxyalkanoates (PHAs) have attracted commercial and academic interest as new biodegradable materials. PHB is nontoxic and water resistant. It is 100% biodegradable and can be processed like thermoplastics. Poly hydroxy butyric acids (PHB) are a group of storage compounds of carbon and energy that are accumulated during unbalanced growth by many bacteria. PHB is deposited intracellularly in the form of inclusion bodies and may account for up to 90% of the cellular dry weight. It is used in the manufacture of packaging films, bags, containers, biodegradable carriers for long-term storage of drugs, and ion conducting polymers. It is used as a raw material for chiral compounds that are enantiomerically pure chemicals and also for blood vessel replacements [6]. Depending upon the utilized sources of carbon and nitrogen, PHB may be selectively induced in bacterial species. A tremendous biochemical diversity is yet to be discovered in the world of microbiology. Evolved nearly 4 billion years ago microorganisms possess many a number of metabolic pathways which are not seen in advanced organisms. These pathways enable them to produce many useful compounds. Attempts to determine microbial diversity in natural environments are limited by the inability of the microbiologists to culture all microbes present in a particular environmental sample. A thorough screening always end in promising results to make use of their metabolic abilities. Though the industrial scale production is achieved in some countries we are yet to develop a suitable technology for this. A vast screening of microorganisms and designing of production process are necessary steps in this aim. In the present study an attempt is made to screen PHB producing bacteria from soil samples of different locations in Kerala. The present work is only a first step towards large scale production of PHB.

## MATERIALS AND METHODS

**Screening:** The soil bacteria capable of accumulating PHB are objectives of screening. The soil samples were collected from different detergent contaminated locations. 10 g of soil from each sample was weighed and mixed with 100 ml of sterile distilled water. This was shaken well and allowed to sediment for a while. The supernatant was used as inocula for serial dilution. From each sample serial dilution was done upto  $10^{10}$  dilutions. The plates were incubated overnight at  $37^{\circ}\text{C}$ .

### Sudan Black Staining

The cultured colonies were isolated and checked for PHB granules by sudan black staining [7] Identification of microorganisms. Identification of the selected PHB producing bacteria was done by conducting various morphological and biochemical tests based on Bergy's manual of systematic bacteriology [8] and also by using Rapid Identification kit (Hi Media).

### Standard Strain

Standard strain used was *Alcaligenes* sp d<sub>2</sub> [9]. The organism was available in the culture collection of School Of Biosciences, Mahatma Gandhi University. The culture was maintained in both nutrient agar and phenol contained media. Sub culturing was done once in two weeks.

### Phenol assay

Phenol estimation was done by standard method for phenol estimations [10]. From the phenol standards of known concentrations different aliquots were taken in clean test tubes. The aliquots were made into 5 ml using distilled water. To each of the standards 50 $\mu\text{l}$  2% 4-amino phenazone, 50 $\mu\text{l}$ , 25N ammonia solution and 25 $\mu\text{l}$  8% potassium ferricyanide were added. The tubes were vortexed and incubated for 15 minutes at room temperature ( $30 \pm 2^{\circ}\text{C}$ ). Optical density was recorded at 500 nm.

### Inoculum Preparation for PHB production

The strains which produced PHB granules were selected for the production of PHB. One loopful of the culture was inoculated to 50 ml of peptone broth containing 200ppm phenol and incubated overnight ( $30 \pm 2^{\circ}\text{C}$ , 150 rpm). The pellets were collected and diluted using physiological saline (0.85% NaCl) till the OD becomes 1. This was used as the inoculum for all studies.

The medium used in the study was mineral salt medium (MSPM) and the composition was as follows:  $\text{KH}_2\text{PO}_4$ -100 mg,  $(\text{NH}_4)_2\text{SO}_4$ -100 mg,  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ -50 mg,  $\text{CaCl}_2$ -1 mg and phenol-10 mg in 100ml of the medium. The pH was adjusted to 7.0. The solution was made up to 100 ml and was autoclaved at  $121^\circ\text{C}$  for 20 minutes. 10 microgram phenol was added after autoclaving.

### Staining of PHB granules using Sudan Black

The bacterial cells in MSPM medium were subjected to centrifugation at 7000 rpm for 15 minutes at  $4^\circ\text{C}$ . The supernatant was discarded and the cell pellets were resuspended in 1 ml of sterile saline. Fixed smear of the bacteria was stained with 3% aqueous solution of Sudan black for 10 minutes. The xylene decolourised slides were counterstained by saffranine for 10seconds. The slide was washed with tap water and blot dried, observed under an optical microscope.

### In vivo detection of PHB by fluorescent microscopy

The bacterial cells in MSPM medium were subjected to centrifugation at 10,000 g for 30 minutes at  $4^\circ\text{C}$ . The supernatant was discarded and the cell pellets were resuspended in 1 ml of sterile distilled water. Heat fixed smear of the bacteria was stained with 1% aqueous solution of Nile blue A. This solution was heated for 10 min. at  $55^\circ\text{C}$  in a coplin-staining jar. The slide was washed with tap water and was placed in 8% aqueous acetic acid solution for 1min.[11]. After washing the slide was observed under an epifluorescence microscope, using an exciter filter that provided an excitation wavelength of approximately 460 nm. (Plate 2)

### Optimization of the conditions for the production of PHB

**Incubation Period:** 100ml aliquots of the mineral salt phenol medium (pH-7) taken were inoculated with the selected isolate (5% in physiological saline) and incubated on a shaker ( $30\pm 2^\circ\text{C}$ , 150 rpm). Cells were separated from these samples over a time period of 8, 16,24,32,40 and 48 hrs respectively by centrifugation (10000 rpm for 30 min) in a centrifuge and the pellet was assayed for PHB production.

**pH:** Optimization of pH was done by conducting the PHB production as before at a medium pH of 3, 4, 5, 6, 7, 8 and 9 followed by the assay as mentioned earlier.

**Temperature:** Optimization of temperature was done by conducting the PHB production as before at temperatures of 30, 35, 40, 45, 50, 55, and  $60^\circ\text{C}$  followed by the assay as mentioned earlier.

### Recovery, purification and assay of poly- $\beta$ -hydroxy butyrate

Poly - $\beta$ -hydroxy butyrate was extracted, purified and assayed as per the method suggested by Law and Slepecky, 1961 [12]. PHB obtained from Sigma Aldrich was used as the standard. The two extracts prepared under similar conditions were subjected to FT/IR analysis at STIC, Cochin University, Cochin, Kerala, India.

## RESULT AND DISCUSSION

Petroleum based plastics have been produced by chemical industries since 1930s. They constitute an important group of materials in our modern society and are used in all manufacturing industries. But they act as sources of serious environmental contamination. Extensive molecular size of plastics make them insusceptible to the action of microbes. Most plastics remain at the site of disposal for long years. Incineration again is dangerous as it liberates poisonous gases thus adding to global warming. To avoid the environmental hazards created by plastics, an alternative is definitely needed. One of the best candidates for this is PHB. PHB has similar material properties to conventional plastics. They can be produced from renewable resources and they are readily biodegradable. Commercial and academic interest are being drawn by this compound as it solves a great environmental problem. The most important property of PHBs is their complete biodegradability and they are comparable to other plastics. PHAs like PHB and PHBV are moldable thermoplastic polymers. PHB has a melting temperature of  $175^\circ\text{C}$  and a glass transition temperature ( $T_g$ ) of  $4^\circ\text{C}$  [13,14,15]. Some of the applications of PHA are in the manufacture of packaging films, biodegradable carrier for long-term storage of drugs, medicines, disposable hygiene products like razors, diapers, water resistant layers, osteosynthetic material surgical pins, sutures, staples and swabs, blood vessel replacements, [6]. PHB has many physiological roles.

PHA accumulation has been implicated both as an energy source and a regulator for controlling the viability of reducing power for the operations of nitrogenase in symbiotic nitrogen fixing bacteria [16]. It is in association with inorganic phosphate, with highest concentrations in mitochondria and microsomes[17] (and possibly plays a role in regulation of intracellular calcium ion concentrations and in calcium signaling in eukaryotes). PHA synthases have been isolated which are responsible for the synthesis of PHB and are classified into three different Classes [18]. It was reported that Ammonium sulfate could induce the production of PHB [19]. Accumulation of PHB was observed under stress conditions as in the presence of phenol [19]. For separation of PHB, solvent extraction is used. This method is a widely used because it is applicable to many PHA producing microorganisms. The enzymatic digestion method was developed by ICI. [20]. An advanced stage of development in this field was the cloning and characterization of genes involved in PHB biosynthesis. To date 40 PHA synthase genes from more than 35 different bacteria have been cloned [21].

In the present attempt to screen PHB producing bacterial isolates 46 samples were obtained in the primary screening. All the soil samples were stressed by detergent/disinfectant contamination. The disinfectant generally carry phenol as the active component and therefore all the collected samples of soil were previously exposed to varying concentration of phenol. Phenol stress have been observed as one of the driving forces behind PHB accumulation [19]. From the 21 isolates obtained four were selected as PHB accumulating. These cultures were purified and was subjected to various biochemical tests. The methodology used in this study was rapid identification scheme making use of the Hi media Rapid kit. This ready made kit is extremely useful as it gives room for quick bacterial identification within two to three days of incubation.. The kit offers provision for conducting 23 biochemical tests at a stretch. The results obtained was compared with the standard and the values given. The isolates were found to be S<sub>1a</sub>- *Alcaligenes* sp, S<sub>1b</sub> *Neisseria* sp S<sub>2a</sub> *Bacillus* sp, S<sub>2b</sub> *Pseudomonas* sp( Plate 1). The standard strain used for comparison in this study was *Alcaligenes* sp d<sub>2</sub> [9]. The organism was early reported as an efficient strain capable of degrading phenol and accumulating PHB [19]. The organism was available in the culture collection of School Of Biosciences, Mahatma Gandhi University.Kottayam.

The S<sub>2a</sub> isolate, identified as *Bacillus* sp was selected for further studies. The intracellular accumulation of PHB was microscopically observed after sudan black staining followed by observation through Fluorescence microscope( Plate 2).

The presence of sudanophilic lipid like inclusions soluble in chloroform was initially observed in many *Bacillus* species and also in *Azotobacter chroococcum*. It was [22], who while studying *Bacillus megaterium* established the chemical nature of these inclusion bodies as poly-3-hydroxy-butyric acid P (3HB). Due to studies on several *Bacillus* strains [23] and phototropic bacteria [24] P HB became more widely known. They were first known to be associated with sporulation of bacteria[25]. PHB inclusions can be viewed with a phase-contrast microscope[26]. Native PHB inclusions can be stained using Sudan black B and oxazine dyes like Nile blue A exhibiting a strong fluorescence at an excitation wave length of 460 nm[11]. The production of any metabolite through microbial fermentation is mediated through enzymes and are generally influenced by various external factors. In the present work an attempt was also made to optimize some of the important factors affecting PHB production. The optimum incubation period was found to be 24 hour. Even though there was production from 16 hours onwards significant production was observed after 24 hour. The same trend was continued upto 32 hour. However the production got declined progressively upto 48 hour. (Fig.1) The optimum pH was found to be 7. The production was very less at pH 4 but consistently got increased from 5 to 7. As 7 is also the neutral pH it was selected as the optimum pH. On increasing the pH from 7, the PHB production got decreased to an insignificant amount. (Fig.2).

The optimum temperature was found to be 30 °C. However the PHB production remained high upto 50 °C. Any increase in the incubation temperature from 50 °C resulted in the decrease of PHB accumulation. Eventhough at 50 °C there was maximum PHB production, 30 °C was selected as the optimum temperature as it was also the ambient room temperature. (Fig.3)

The accumulated PHB was extracted and the purified PHB was submitted to FT/IR analysis. The spectrum gave all the characteristic bands indicative of PHB. The FT/IR analysis gave characteristic bands at 3300 cm<sup>-1</sup>, 2900 cm<sup>-1</sup>, 2400 cm<sup>-1</sup>, 2200 cm<sup>-1</sup> and 1700 cm<sup>-1</sup> confirming the presence of intracellular PHB. ( Fig.4). All these bands were respectively indicating polymerized ring structure, C-H bend, C-H stretch, ester linkage and carbonyl group.



Plate 1. Biochemical tests conducted for the newly isolated phenol degrading and PHB accumulating bacterial strains using RAPID Identification kit

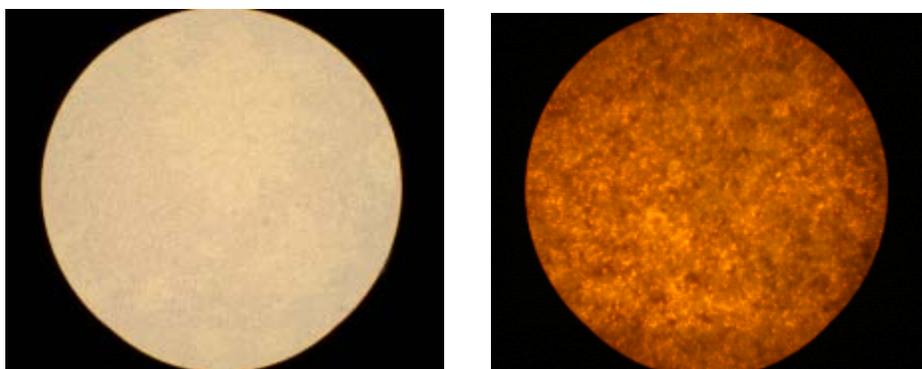


Plate 2. *In vivo* detection of PHB in selected isolate after staining with Nile Blue A and observed through fluorescent microscope at I: Visible light Plate II: Fluorescent light

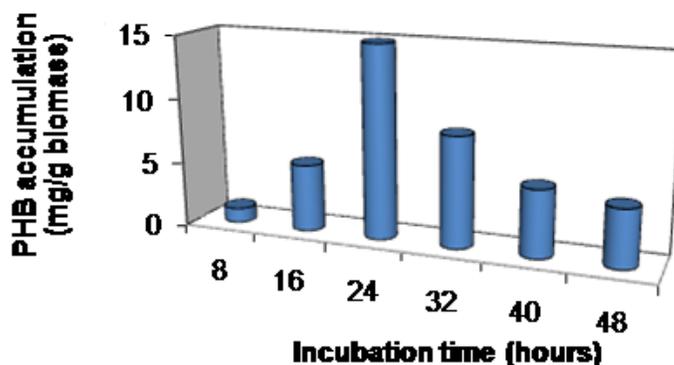


Fig. 1. Optimisation of incubation period of intracellular PHB accumulation in the selected isolate

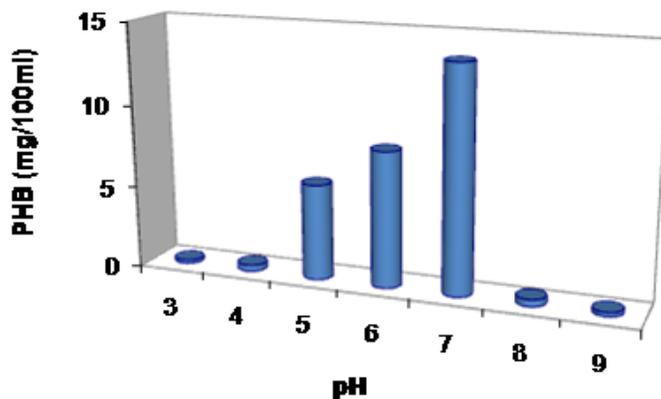


Fig.2.Optimization of pH for the production of Intracellular poly hydroxy butyrate in the selected isolate

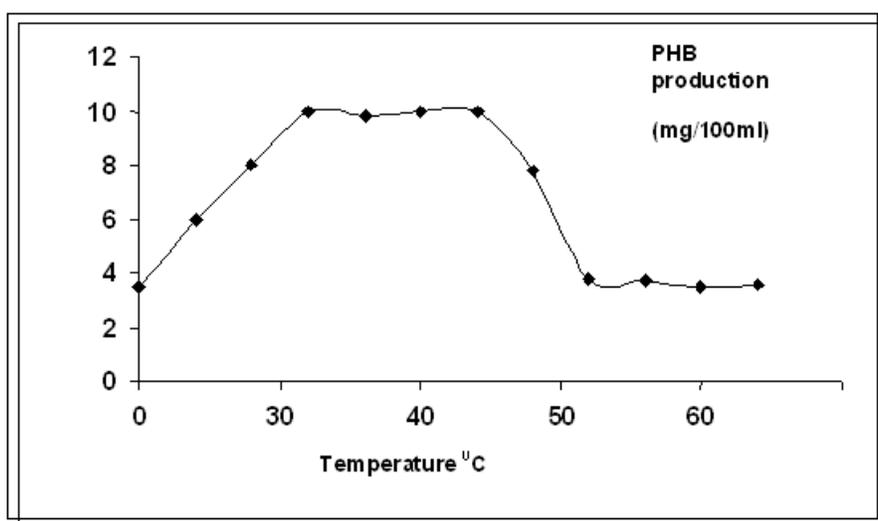


Fig.3.Optimization of temperature for the production of Intracellular poly hydroxy butyrate in the selected isolate

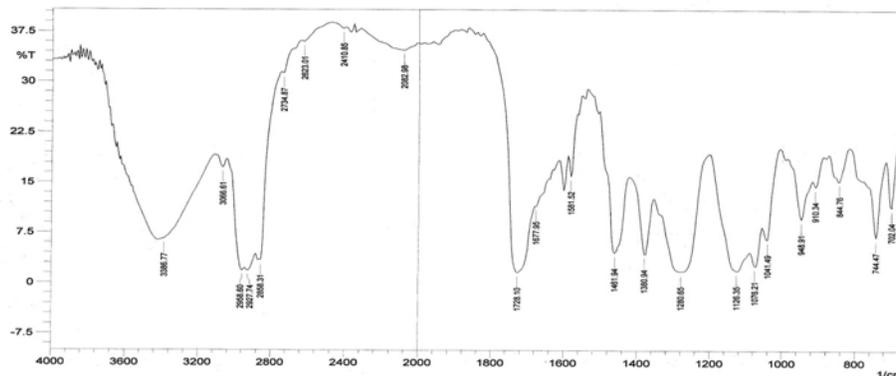


Fig.4. FT/IR of the PHB accumulated in selected isolate

The accumulation of PHB in the selected isolate was confirmed with Florescence microscope and the extracted PHB was structurally conformed with FT/IR analysis. The proposed results gives enough room for further investigation into the molecular mechanism taking place in the organism facilitating PHB accumulation PHB under phenol stress. PHB is an ideal alternative to plastics as it is biodegradable. But to meet the increasing demand it is essential to establish a large scale process for the production, extraction and purification of PHB. This can be materialized only by the convergence of the principles of all branches of applied Life Sciences. The present proposal is only a first step towards large scale production of PHB and it very much involves basic Microbiology and Biotechnology aspects. However to look into the mechanism of PHB accumulation in vivo and also to evaluate the possibility of metabolic regulation we have to analyze the biochemical aspects of PHB accumulation. An insight into the Molecular biological aspects of the process will definitely help in improving the efficiency of the process. Towards the end, it is highly essential to apply the principles of Biochemical Engineering for scaling up the process. Hence the proposed problem is very much interdisciplinary in its approach and holds ample opportunities for further development and research.

## ACKNOWLEDGEMENT

The author acknowledges UGC, Government of India for the financial support

## REFERENCES

- [1] Thakor, NS, Patel MA, Trivedi UB, Patel KC. 2003. Production of poly  $\beta$ hydroxy butyrate by *Comamonas testosteroni* during growth on naphthalene. *World J. Microbiol. Biotechnol.* 19:185-189.
- [2] Jendrossek D, Handrick R. 2002. Microbial degradation of polyhydroxyalkanoates. *Ann. Rev. Microbiol.* 56: 403-432.
- [3] Briese BH, Jendrossek D, Schegel HG. 1994. Degradation of poly (3-hydroxy butyrate -co-3-hydroxyvalerate) by aerobic sewage sludge. *FEMS Microbiol. Lett.* 117: 107-112. .
- [4] Handrick R, Reinhardt S, Focarete ML, Scandola M, Adamus G, Kowalozuk M, Jendrossek D 2001. A new type of thermoalkalophilic hydrolase of *Paucimonas lemoignei* with high speciality for amorphous poly esters of short chain length hydroxy alkanolic acids. *J. Biol. Chem.* 276: 36215-24.
- [5] Sujatha K, Mahalakshmi A, Shenbagarathai R. 2005. Analysis of PCR products for PHB production in indigenous *Pseudomonas* sp. LDC-5 Indian *J. Biotechnology* 4: 323-335.
- [6] (Madison LL, Huisman GW. 1999. Metabolic engineering of poly (3-hydroxyalkanoates): From DNA to plastic. *Microbiol. Mol. Biol Rev.* 63: 21-53.
- [7] Fernandez-Castilla, R., V.F. Rodriguez, R.J. Gonzalez and B.F. Ruiz 1986. Accumulation of poly hydroxyl butyrate by *Halobacteria*. *Appl. Environ. Microbiol.* 51, 214-6.
- [8] Buchanan, R. G and Gibbons, N.E 1974. *Bergey's manual of determinative bacteriology*. 8th edition. The Williams and Williams Co., Baltimore.
- [9] Nair, C. Indu, Shashidhar S. 2004. Microbial degradation of phenol by an *Alcaligenes* sp. isolated from tropical soil. *J. Soil Science.* 3(4): 4-51.
- [10] Mordocco, A., Clern, K. and Roger, J 1999. Continuous degradation of phenol at low concentration using immobilized *Pseudomonas putida*. *Enzyme and Microbial Technology*, 25, 530-536.
- [11] Ostle AG, Holt JG. 1982. Nile blue as a fluorescent stain for PHB. *Appl. Environ. Microbiol.* 44: 238-241
- [12] Law JH, Slepecky RA. (1961) Assay of Poly- $\beta$ -hydroxybutyric acid. *J. Bacteriol.* 82: 33-36.
- [13] Byrom D. 1987. Polymer synthesis by microorganisms: technology and economics. *Trends Biotechnol.* 5: 246-250.
- [14] Marchessault RH, Monasterios CJ, Morin FG, Sundararajan PR. 1990. Chiral poly ( $\beta$ -hydroxyalkanoates): an adaptable helix influenced by the alkane side-chain. *Int. J. Biol. Macromol.* 12: 158-165
- [15] Holmes PA. 1998. Biologically produced PHA polymers and co-polymers. In: D. C. Bassett (ed.). *Developments in crystalline polymers*, Elsevier, London. 2: 1-65.
- [16] McDermott TR, Griffith SM, Vance CP, Graham PH. 1989. Carbon metabolism in *Bradyrhizobium japonicum* bacteroids. *FEMS Microbiol. Rev.* 63: 327-340.
- [17] Reusch RN, Huang R, Kosk-Kosicka D 1997. Novel components and enzymatic activities of human erythrocyte plasma membrane calcium pump. *FEBS Lett.* 412: 592-596.
- [18] Steinbüchel A, Schlegel HG. 1991. Physiology and molecular genetics of poly ( $\beta$ - hydroxyalkanoic acid) synthesis in *Alcaligenes eutrophus*. *Mol. Microbiol.* 5: 535-542.

- [19] Nair, C. Indu , Pradeep S, Ajayan M.S, Jayachandran K and Shankar Shashidhar 2009. Polyhydroxy butyrate production by a phenol degrading *Alcaligenes sp d2* Applied Biochemistry and Biotechnology, 159.2.2009.545-552 . Springer
- [20]Steinbüchel A, Hustede E, Liebergesell M, Piper U, Timm A and Valentin H. 1992. Molecular basis for biosynthesis and accumulation of polyhydroxyalkanoic acids in bacteria. FEMS Microbiol. Rev. 103: 217-230
- [21] Sudesh K, Abe H, Doi Y. 2000. Synthesis and properties of polyhydroxyalkanoates: biological polyesters. Prog. Polym. Sci. 25: 1503-1555.
- [22] Lemoigne M. 1927. Etudes sur l'autolyse microbienne origine de l'acide  $\beta$ -oxybutyrique forme par autolyse. Ann. Inst. Pasteur. 41: 148-65.
- [23] Macrae RM, Wilkinson JR. 1958. Poly- $\beta$ -hydroxybutyrate metabolism in washed suspensions of *Bacillus cereus* and *Bacillus megaterium*. J. Gen. Microbiol. 19: 210-222.
- [24] Doudorff M, Stainer RY. 1959. Role of Poly- $\beta$ -hydroxybutyric acid in the assimilation of organic carbon by bacteria. Nature 183:1440-1442.
- [25] Slepecky RA, Law JH. 1961. Synthesis and degradation of poly- $\beta$ -hydroxybutyric acid in connection with sporulation of *Bacillus megaterium*. J. Bacteriol. 86: 37-42.
- [26] Dawes EA, Senior PJ. 1973. The role and regulation of energy reserve polymers in microorganisms. Adv. Micro. Physiol. 10: 135-266.