International Journal of Pharmacy and Biological Sciences
ISSN: 2321-3272 (Print), ISSN: 2230-7605 (Online)
IJPBS | Volume 9 | Special Issue 2- ICESMT| 2019 | 179-185
International Conference on "Empowering Society with Microbial Technology"
Held at Tuljaram Chaturchand College of Arts, Science and Commerce, Baramati - 413 102,
Dist. Pune, Maharashtra, India, 7-9 February 2019
| Research Article | Biological Sciences |Open Access |MCI Approved|
|UGC Approved Journal|

# Studies on Polyhydroxy alkanoates Synthesized by *Geobacillus thermoglucosidasius* and *Geobacillus toebiiw*

**Seema P. Rodge, Snehal A. Patil, Rutuja V. Bhalekar, Neelima M. Deshpande and Niranjan P. Patil***
**Department of Microbiology, MES Abasaheb Garware College, Karve road, Pune-411004, Maharashtra, India.**

Received: 30 Jan 2019 / Accepted: 20 Feb 2019 / Published online: 01 Apr 2019
**Corresponding Author Email:** niranjan75@gmail.com

## Abstract
Polyhydroxybutyrate (PHB) is bio-based, biodegradable and biocompatible polymer. It is an attractive member for an environment friendly replacement of non-biodegradable synthetic plastics. In this study, 21 microorganisms were isolated from hot spring situated in Kasol, Himachal Pradesh, India. Bacterial isolates were selected for the studies on Polyhydroxyalkanoates based on the Sudan Black B staining as a primary screening test. The isolates were screened for their ability for PHB production in Davis Minimal Medium containing 2% glucose. Extraction of PHB was carried out using sodium hypochlorite digestion method. The concentration of PHB in extracted sample was quantified by UV-Visible spectrophotometer based Crotonic acid assay, which resulted in selection of two potential isolates for further studies. FTIR (Fourier Transform Infrared Spectroscopy) was employed to know functional groups in extracted crude PHB preparation. Morphological & biochemical characterisation and 16S rRNA gene sequencing of PHB producing bacteria was performed to know their taxonomic positions. The two isolates were identified as *Geobacillus thermoglucosidasius* and *Geobacillus toebii.* It can be concluded that thermophiles *Geobacillus thermoglucosidasius* and *Geobacillus toebii* may be potential candidate for the PHB production.

**Keywords**
Crotonic acid assay, FTIR, *Geobacillus thermoglucosidasius, Geobacillus toebii,* Polyhydroxyalkanoates.

*****

## INTRODUCTION:
We can look at plastic invention as one of the milestones in the history of civilization. Birth of plastic was responsible for expansion of various industries as well as it had made drastic changes in our day to day lives. Alexander Parkes is remembered as father of plastic. Plastic manufacturing requires the major raw material such as oil and natural gas. These are typically organic polymers of high molecular mass, and they are commonly derived from petrochemicals. Plastic is

resistant to chemicals, thermal and electrical insulators, light in weight. Due to these properties, plastic contributes to the health and safety of consumers in food and water packaging applications. Plastics can also be used to improve the performance and reduce the costs of building materials; examples of this include lightweight fixings, window and door frames, fixtures and insulation materials. [1]

However, there is dark side of use of plastic manufacturing of plastic require tons of petrochemicals, plastic materials are non-biodegradable, non-biocompatible. Plastic materials are not degraded by action of solar UV radiations, but it gets fragmented into smaller plastics called microplastics. It can cause injury and death to birds, fishes and other creatures. Plastic hampers the growth of plants and trees by blocking absorption of minerals, nutrients and water [1]. Thus, such a large negative impact of synthetic plastic leads to development of bioplastic which is going to eliminate all problems related with use of synthetic plastics. Nowadays plastic is being one of the major environmental pollution. In order to avoid all these circumstances there is need of strong substitute of bioplastic i.e., Poly-3-hydroxybutyrate (PHB).

Lemoigne in 1925 discovered a linear polyester of D (-)-3-hydroxybutyric named PHB. PHB is accumulated in the form of intracellular granules by a variety of Gram-positive and Gram-negative bacteria under conditions of a limiting phosphorous and nitrogen elements and abundant carbon source. The general properties of PHB include biodegradability, biocompatibility, and resistance to ultraviolet light, solubility in chloroform, water insolublility and non-toxicity [2]. PHB is a best and effective biodegradable thermoplastic. Very less work is done on plant sources as promising source of PHB production [3]. Significant amount of PHA accumulation is reported for many representatives of the microbial domains growing under high salinity or extreme pH or extreme temperature conditions of both Archaea and Bacteria. PHA biosynthesis, from thermophilic bacterium *Thermus thermophilus* was studied by Pantazaki et al. [4]. PHB synthesis by the thermophilic bacterium *Geobacillus* sp. AY 946034 was studied by Giedraityte et al., this bacterium is able to grow at the temperatures up to 60 °C [5]. *Haloferax mediterannei* can grow at high salinity and according to Koller et al. [6] is currently considered to be the most promising candidate for whey based PHA production on industrial scale.

The present work deals with isolation of different types of PHB producing bacteria, extraction of PHB and its characterisation by FTIR.

**MATERIALS AND METHODS:**

**Collection of samples**: For the isolation of PHB producing bacteria, the water samples were collected from hot spring of Kasol, Himachal Pradesh, India.

**Isolation of bacteria**: The water samples were appropriately diluted, and isolation was carried out by pour plate method using sterile nutrient agar medium. The plates were incubated at 37° C & 55 ° C for 24 hrs. Isolated colonies were selected and subcultured on nutrient agar plate till further use.

**Screening of PHB producers**: All the isolates were screened for their PHB producing ability. For this purpose, these isolates were grown on PHA detection agar (Glucose, 20 g/l; $KH_2PO_4$ , 13.3 g/l; $MgSO_4$, 1.3 g/l; $(NH_4)$ $SO_4$, 2 g/l; Citric acid, 1.7 g/l; Trace elemental solution, 10 ml; Agar, 15 g/l; pH, 7).

**Screening by Sudan Black B staining:** The thin smear of cell suspension was prepared on slide and air dried. The smear was stained with Sudan Black B solution and was allowed to stand for 10-15 min. The slide was air dred.. Then the slide was flooded with xylene and air dried. The slide was immersed in counter stain safranine for 5 minutes and then washed gently under tap water and air dried. The slide was observed under oil immersion microscope for detection of PHB granules [7]. Bacteria showing positive in bluish-black and negative in yellow orange (Figure 2).

**Characterization of PHB producing isolates**: Isolates were grown on nutrient agar plate and incubated for 24 hours at 37˚C and 55˚C. These morphological characteristics of the colonies were identified by growing the bacteria on the nutrient agar medium and Gram character was identified using Gram Staining. Biochemical characterization was carried out using Bergey's manual. The molecular characterization was done by 16S rRNA sequencing [8].

**Media Ingredients and culture conditions:** The PHB production was carried out using Davis minimal broth and 2 gm % dextrose. Sterile media were inoculated with one loopful inoculum and incubated on shaker at room temperature at 110 rpm. The themophilic

cultures were incubated on shaker at $60^0$ C at 110 rpm. The bacterial isolates were incubated for seven days.

**Extraction of intracellular PHB**: After 120 hrs of incubation at $37^0$ C & $55^0$ C, the cultures were collected in the sterile Falcon tubes. Then the samples were centrifuged at 4000 rpm for 20 minutes at $4^0$ C. After centrifugation, supernatant was discarded, and the cell pellet was washed with acetone and ethanol (1:1). The cell pellet was dried overnight. Then, the cell pellet was digested by adding 10 ml 4% sodium hypochlorite for 30 minutes. After centrifugation at 4000 rpm for 20 minutes at $4^0$ C, the supernatant was discarded, and the precipitate was washed with acetone and ethanol (1:1). The precipitate was dissolved in chloroform and then the chloroform was allowed to evaporate [9].

**Quantification of extracted crude PHB:**

**Cell dry weight:** After 120 hrs of incubation $37^0$ C & $55^0$ C, the cells were collected and centrifuged at 4000 rpm for 20 minutes at $4^0$C. Supernatant was discarded, and cell pellet was dried to estimate the dry cell weight (DCW) [10].

**Residual Biomass:** The residual biomass was estimated by using the following formula-

Residual Biomass (gm/ml) = DCW (gm/ml) - Dry weight of extracted PHB (gm/ml).

**PHB accumulation**: This is the percentage of intracellular accumulation of PHB is estimated by using the following formula-

PHB accumulation (%) = Dry weight of extracted PHB (gm/ml)/ DCW (gm/ml) × 100

**Estimation of Concentration of PHB:** Estimation of concentration of PHB was done by crotonic acid assay [11].

**Qualitative Analysis by Thin Layer Chromatography:** The sample was prepared by dissolving the 4µg extracted polymer sample in 60µl of hexane. The prepared sample was loaded on the TLC plates, pre-coated with silica and it was placed in solvent system consisting of Ethyl acetate and Hexane (1:1). It was allowed to run for 40 minutes, after sufficient run the plate were removed and dried. And after drying, iodine vapours were used to stain PHB present in the sample. Presence of PHB was confirmed by appearance of brown spot on the TLC plate and Rf value was calculated.

**Characterization of extracted PHB:** FTIR analysis was carried out to detect the different functional groups in PHB. This infra-red spectroscopy detects the characteristic vibration of chemical functional groups in the sample. FTIR spectrum was recorded using FTIR 4100 typeA spectrophotometer [12].

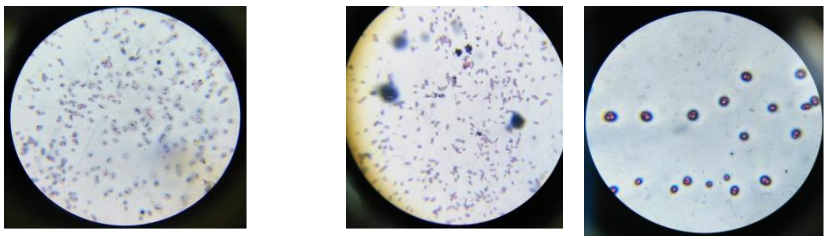
**RESULTS AND DISCUSSION:**

In the present study, morphologically distinct isolates were obtained from Kasol, Hot spring samples situated in Himachal Pradesh, India using routine microbiological isolation procedure (Figure 1). The isolated colonies were screened for PHB granule production ability by Sudan Black B staining. Out of 21 isolates screened, seven isolates were showed presence of intracellular PHB granules (Figure 2). In order to select isolates having maximum PHB production ability, seven isolates were investigated for PHB accumulation and percent PHB yield. Production of PHB was studied with Minimal Davis medium. Optimization of pH, temperature and incubation period was observed to be 7 , $55^o$ C for 8 days respectively. PHB was extracted using Sodium Hypochlorite digestion method (Figure 3). After extraction, % accumulation of PHB showed that the *Geobacillus thermoglucosidasius*, *Geobacillus toebii*, B1, CB, T47, Yeast sp. and X1B isolates produces maximum PHB accumulation within the cell (Table 1). In order to quantitate PHB produced by each isolate under stated conditions crotonic acid standard dose response curve (data not shown) was obtained and used for extrapolating unknown concentration of PHB produced by isolates. It was found that thermophilic isolate *Geobacillus toebii* exhibited highest yield followed by *Geobacillus thermoglucosidasius.* (Table 2). Thin layer chromatography (Figure 4) of extracted PHB showed the characteristic brown spots. The Rf values were calculated from the obtained spots found closer to the standard PHB. FTIR spectra of extracted PHB produced by isolate *Geobacillus toebii* and *Geobacillus thermoglucosidasius* shows all functional groups present in that of standard PHB i.e. C=O, C-O, $CH_2$, $CH_3$ (Figure 5).FTIR spectrum of extracted polymer from *Geobacillus thermoglucosidasius* shows peaks at 1734.66/cm corresponding to C=O functional group whereas peak at 3000.69/cm and 2879.20/cm corresponds to C-H (SP2) and C-H(SP3) groups. FTIR spectrum of PHB extracted from *Geobacillus toebii* shows a characteristic sharp peak at 1734.66/cm and 1217.83/cm which corresponds to C=O and C-O functional groups. The peaks obtained are nearly

similar to that of standard PHB at 1728/cm and 1282/cm confirming that extracted polymer is PHB. The methane group gave strong band at 1366.32 The cumulative results of staining, crotonic acid assay, TLC and FTIR confirmed the extracted biological polymer as PHB. In order to investigate the economical production of PHB, utilising waste containing complex nutrients for the growth of selected isolates qualitative enzyme (amylase, protease, lipase) detection tests were performed. *Geobacillus thermoglucosidasius and Geobacillus toebii* were found to produce amylase and lipase.

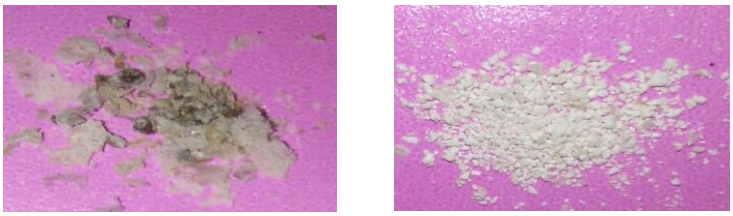
**(1) CB isolate** **(2) *Geobacillus toebii,*** ***(3) Geobacillus themoglucosidasius***

**Figure 1. Isolated colonies obtained by streak plate method on Nutrient agar medium incubated (1) 37$^0$C and (2 and 3) 55$^0$C temperature**



***Geobacillus themoglucosidasius*** ***Geobacillus toebii*** **CB**

**Figure 2. Sudan Black B staining of PHB granules, showing presence of black granules within pink coloured cells**.



*Geobacillus thermoglucosidasius* *Geobacillus toebii*

**Figure 3. Extracted and dried PHB obtained from selected isolates.**

**Qualitative Analysis of extracted PHB by Thin Layer Chromatography**

**Spot showing presence of PHB in crude extracted sample.**

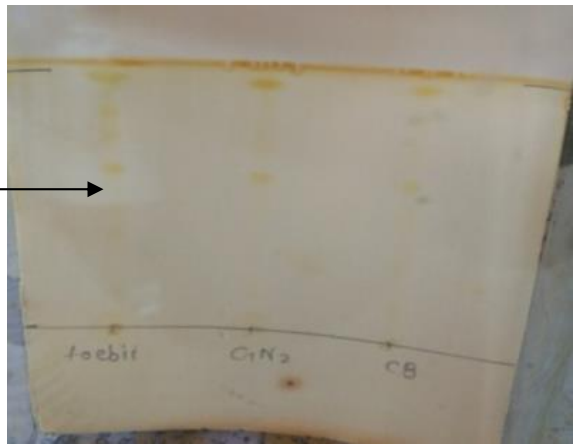
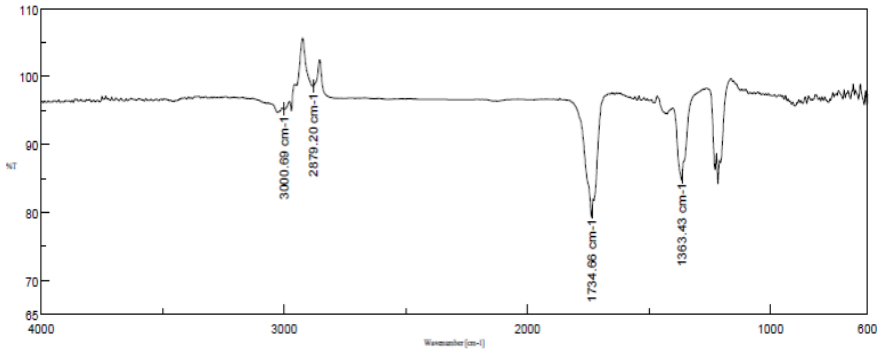



**Figure 4. TLC plate showing presence of PHB in Extracted sample.**




**Characterization of extracted PHB by FTIR FTIR spectrum of *Geobacillus thermoglucosidasus***

**Figure 5.1 (Culture-*Geobacillus thermoglucosidasus*) - FTIR spectrum of extracted polymer shows peaks at 1734.66/cm corresponding to C=O functional group where as peak at 3000.69/cm and 2879.20/cm corresponds to C-H (SP2) and C-H(SP3) groups.**

**FTIR spectrum of *Geobacillus toebii.***

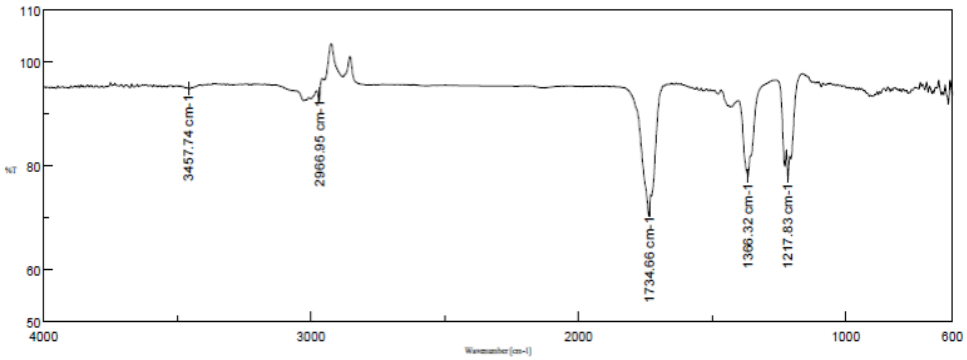



**Figure 5.2 (Culture-*Geobacillus toebii*) - FTIR spectrum of this particular sample shows a Characteristic sharp peaks at 1734.66/cm and 1217.83/cm which corresponds to C=O and C-O functional groups.The peaks obtained are nearly similar to that of standard PHB at 1728/cm and 1282/cm confirming that extracted polymer is PHB. The methane group gave strong band at 1366.32**

**Table.1 Percentage accumulation PHB in 9 isolates.**

| Name of the bacterial isolate | % accumulation of PHB |
|---|---|
| *Geobacillus thermoglucosidasus* | 82% |
| *Geobacillus toebii* | 81.08% |
| T47 | 80.5% |
| B1 | 78.12% |
| CB | 76.11% |
| Yeast sp. | 74.46% |
| X1b | 65.41% |

**Table. 2 Concentration of PHB obtained from each extracted sample from different isolates.**

| Name of the bacterial isolate | PHB yield (µg/80 ml) |
|---|---|
| *Geobacillus toebii* | 15.24 |
| *Geobacillus thermoglucosidasius* | 12.5 |
| T47 | 9.1 |
| CB | 8.78 |
| B1 | 2.15 |
| Yeast sp. | 2.43 |
| X1b | 1.04 |

The present study emphasizes isolation of microorganisms capable of accumulating PHB in limiting concentration of N, P, O but in the presence of excess carbon source. Further the study is directed in selecting thermophilic isolates having PHB production attribute. Giedraitytė et.al (2015) investigated PHB production ability thermophilic *Geobacillus* sp. AY 946034 and it was found to accumulate 68.9% of cell dry weight PHB [5]. Our isolates *Geobacillus toebii* (81.08 %) and *Geobacillus thermoglucosidasius* (82%) accumulated higher amount of PHB in the cells. To our best of knowledge, we are reporting production of PHB by *Geobacillus toebii* for the first time. According to Gomes et.al (2016) use of thermophilic isolate can result in reduction in the risk of contamination by mesophilic microorganisms in biotechnological processes, with reference to this thermophilic, *Geobacillus toebii* and *Geobacillus thermoglucosidasius* may have added advantage over the mesophilic PHB producing microorganism [13]. There is a need of low-cost substrate, for making the PHB synthesis process more economical, one of the ways of achieving this could be use of cheap carbon sources such as wastewater. Wastewater contains many complex substrates such as starch, lipids etc. As *Geobacillus toebii* and *Geobacillus thermoglucosidasius* have ability to utilize complex substrate such as starch and lipid, wastewater might be implemented for the production of PHB. According to the literature survey, production of PHB from *Geobacillus toebii* is studied for the first time as there were no earlier reports found for the same.

**CONCLUSION:**

The problem of pollution of the environment due to use of non-biodegradable plastic can be solved by replacing the non- biodegradable plastic with the biodegradable plastic. Use of PHB can be considered as

a promising alternative for this purpose. *Geobacillus toebii* and *Geobacillus thermoglucosidasius* may be considered as useful in the process of making of bioplastic because of their ability to produce high amount of PHB. *Geobacillus toebii* and *Geobacillus thermoglucosidasius* being thermophile have an added advantage in the biotechnological process, as there might be less chances of contamination by mesophilic microorganisms.

**ACKNOWLEDGEMENT:**

We are thankful to Dr. P. B. Buchade, Principal of MES, Abasaheb Garware College, Pune-04. We express our sincere thanks to Dr. R. A. Walhe, Head, Department of Microbiology for granting us permission to work.

**REFERENCES:**

[1] Hammer J., Kraak M., Parsons J., Plastics in the marine environment: the dark side of a modern gift. Rev Environ Contam Toxicol, 220: 1-44, (2012).

[2] Bugnicourt E., Cinelli P., Lazzeri A., Alvarez V., Polyhydroxyalkanoate (PHA): Review of synthesis, characteristics, processing and potential applications in packaging. eXPRESS PolymerLetters, 8(11): 791-808, (2014).

[3] Wagale A., Dixit Y., Vakil B., Screening and Isolation of PHB Producers from Plant Sources. Int. J. Curr Microbiol. App. Sci, 5(4):413-423, (2016).

[4] Pantazaki A., Tambaka M., Langlois V., Guerin P., Kyriakidis, Polyhydroxyalkanoate (PHA) biosynthesis in *Thermus thermophilus:* purification and biochemical properties of PHA synthase. Mol Cell Biochem , 254(1): 173-183, (2003).

[5] Giedraitytė G., Kalėdienė L., Purification and characterization of polyhydroxybutyrate produced from thermophilic *Geobacillus* sp. AY 946034 strain. Chemija, 26(1): 38-45, (2015).

[6] Koller M., Hesse P., Bona R., Kutschera C., Atlić A et al., Potential of various archae-and eubacterial strains as industrial polyhydroxyalkanoate producers from whey. Macromol Biosci, 7(2): 218-226, (2007).

[7] Burdon, K. L., Fatty Material in Bacteria and Fungi Revealed by Staining Dried, Fixed Slide Preparations 1. Journal of Bacteriology, *52*(6):665–678, (1946).

[8] Packialakshmi N., Badhru N., screening and identification of efficient polyhydroxybutyrate producing bacteria from plastic wastes dumped soil samples. World Journal of Pharmaceutical Research, 4(10): 1785-1794, (2015).

[9] Ramsay J.A, Berger E., Voyer R., Chavarie C., Ramsay B.A., Extraction of poly-3-hydroxybutyrate using chlorinated solvents. Biotechnology Techniques, 8(8): 589-594, (1994).

[10] Mikkili I., Karlapudi A., T.C. Venkateswarulu., D. John Babu., Kodali V., Isolation, Screening and Extraction of Polyhydroxybutyrate (PHB) producing bacteria from Sewage sample. Int. J. Pharm Tech:850-857, (2014).

[11] Law J., Slepecky R. , Assay of poly-beta-hydroxybutyric acid. *J Bacteriol,* 82(1):33-6, (1961).

[12] Shah R., FTIR analysis of polyhydroxyalkanoates by novel *Bacillus sp.* AS 3-2 from soil of Kadi region, North Gujarat, India. India J Biochem Tech, 3(4): 380-383, (2012).

[13] Gomes E., de Souza A.R., Orjuela G.L., Da Silva R., de Oliveira T.B., Rodrigues A. Applications and Benefits of Thermophilic Microorganisms and Their Enzymes for Industrial Biotechnology. In: Schmoll M., Dattenböck C. (eds) Gene Expression Systems in Fungi: Advancements and Applications. Fungal Biology. Springer, Cham (2016)