Biological Forum – An International Journal **13(4): 923-927(2021)**

ISSN No. (Print): 0975-1130
ISSN No. (Online): 2249-3239

Isolation, Screening and Characterization of Bioplastic (Poly Hydroxy Butyrate) producing Bacteria from Termite Mound Soil

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(Received 22 September 2021, Accepted 17 November, 2021)
(Published by Research Trend, Website: www.researchtrend.net)

ABSTRACT: Polyhydroxybutyrate (PHB) is considered as a bio-plastic produced by a broad group of bacteria. This study was aimed to explore potential PHB producing bacteria from termite mound soil. Out of twenty isolates obtained from the termite ecosystem, six bacterial isolates namely PHB 01, PHB 02, PHB 03, PHB 04, PHB 05 and PHB 06 were positively stained for PHB production with the help of Sudan B Black and Nile blue staining. In addition to that, morphological and biochemical characterization of bacteria were revealed. PHB-producing strains were identified by molecular analysis (16S rRNA) and named *Bacillus siamensis* (PHB 01), *Bacillus subtilis* (PHB 02), *Azotobacter chroococcum* (PHB 03), *Bacillus safensis* (PHB 04), *Pseudomonas sp* (PHB 05) and *Bacillus megaterium* (PHB 06). All the selected PHB producers will be further utilized for the production of PHB using various nutrient sources to ensure sustainable production of bio plastics.

Keywords: *Bacillus* spp., Bio-plastic, PHB, 16S rRNA, termite mound soil.

INTRODUCTION

Plastics are a necessary part of daily life with ample applications in agriculture, household, medicine and packaging. Excessive use of petroleum-derived plastic resulted in environmental pollution because they remained in the soil for years which poses a serious threat to terrestrial and aquatic ecosystems. There is an urgent need to safeguard our environment from plastic-dumped world in an eco-friendly way. In recent days, the use of biodegradable plastic seeks attention globally. Bio-plastics such as starch derivates, polylactic acid (PLA), polymeric cellulose, poly hydroxyl alkanoates (PHA) plays a wide role with the added advantage of being obtained from plants as well as microbial sources. There are numerous sorts of bioplastics with various levels of degradability. But, polyhydroxybutyrate (PHB) is claimed to be 100% biodegradable. PHB is a biodegradable thermoplastic extracted from a wide range of micro-organisms. PHB and its copolymers are members of the PHA family commonly known as the best bacterial polyesters produced by a microbial process on an enriched sugar medium. Bio-degradable polymers were found to be a promising tool for reducing global environmental issues. In recent times, PHB is reported as biofuel also (Gao *et al*., 2011).

Microbial PHB shares the common property with petroleum-derived plastics (Chen *et al*., 2001). A broad range of microbes was producing PHB, which includes marine bacteria (Mohanrasu *et al*., 2020), algae (Selvaraj, 2021), actinomycetes (Trakunjae *et al*., 2021) some molds and yeast. Concerning prokaryotes, both Gram-positive and negative bacteria accumulated PHB as storage material. Of which, only a few were reported to accumulate a significant amount.

Termite soils provide a very particular biological climate that harbors and promotes very specialized cellulolytic and hemicellulolytic microorganisms which include PHB producers. Termite mound soil has been discovered to be a bacterial “gold mine” by researchers. Additionally, bacteria isolated from that soil could be used in an environmentally acceptable manner as a possible material for antibiotic production and ensuring environmental sustainability (Enagbonma and Babalola 2019).

Termite mound soil could be promoted as a possible source for bio-plastic production. Advancement in microbial synthesis leads to industrial-scale production of PHB, but the extraction and application are still far. Commercialization prospects of bacterial-based biopolymers are less because of the expensive cost of production. In this regard, an appropriate assortment of microbes for bio-plastic production is needed.

Bacillus spp are considered super microbial factories for commercial production of PHB *Bacilli* strains have their potential to accumulate PHAs which are biodegradable and biocompatible. These strains are appealing because of their faster production time and low endotoxicity (Kumar *et al*., 2009). Hence, the present work was aimed to obtain potent PHB producers from termite mound soil.

MATERIALS AND METHOD

A. Sampling and Isolation

Termite mound soil samples were collected from Western Ghats of Tamil Nadu, Coimbatore, located in 10°6″15″N and 79°44′20″E Latitude and Longitude respectively and at an altitude of 610m. Samples were stored in an ice pack and kept under -40°C for future use. Isolation was done by pour plate method using Nutrient Agar (NA) media (Beef Extract- 3 g; Glucose- 5g; Peptone-5g; Sodium Chloride- 5g; Agar- 20 g for 1000ml). Then the plates were incubated at 37°C for 24 h. Distinguished bacterial colonies were picked and grown in modified medium (Beef extract-3g; Glucose - 10g; Peptone-5g; Sodium Chloride-8; Agar-20g for 1000 ml) (Singh *et al*., 2011).

B. Screening of the PHB accumulating isolates

Primary Screening. For the primary screening of PHB producers, Sudan Black B staining was performed, using the protocol described by (Burdon *et al*., 1942). Dye solution (0.3 %) was prepared using 70% ethanol. Ten to twenty microlitre of bacterial culture was heat fixed on glass slide. Then the dye solution was added to the slide and left undisturbed. After 15 minutes, immerse the slide in xylene followed by 5% safranin as counterstain. PHB positive isolate was determined by the observation of blue-black colored cells.

Secondary Screening (Ostle and Holt, 1982). Primarily screened isolates were subjected to secondary screening using Nile blue dye. Twenty four hours old bacterial culture was spread onto the modified nutrient agar plates incubated at 37°C for one day. Nile blue (0.5%) dye in acetone solution was spread onto the plates. After 15 minutes, the plates were observed under UV-Transilluminator. Positive cultures showed orange fluorescence at 460nm wavelength.

Morphological Characterization. Morphological characterization of isolates was done based on the distinctive shape, color and size. Gerhardt *et al*. (1981) method was adopted to study the cell morphology.

Biochemical Characterization (John *et al.*, 2009). Standard protocols were used for the biochemical characterization of positively screened bacteria (sugar utilization test, IMViC test, catalase test, nitrate test, urease test).

Molecular Identification of PHB isolates. Genomic DNA from bacterial isolates were extracted using the standard protocol. 16s rRNA sequencing was done. Then the sequence was deposited in GenBank to obtain accession number. Finally, phylogenetic tree was constructed and the isolates were listed in the Table 4.

RESULTS AND DISCUSSION

Petroleum based plastics play major role in our daily lives due to their availability and ease accessability. Besides, it creates environmental pollution. Scientists were creating an alternative, which ends in biodegradable plastic. There has been an increased demand in public as well as scientific research for the development of biodegradable polymers. Microorganisms provide a source of bioplastic (PHB) as cytoplasmic inclusions which are similar to polypropylene. PHB is accumulated during stress conditions. Based on the properties like biodegradable and nontoxic, it can be used for medical applications also. But the drawback in PHB stretches out in its cost of production, which is preferably high than the petroleum plastics with the current production rate it can't compete with petroleum derivatives.

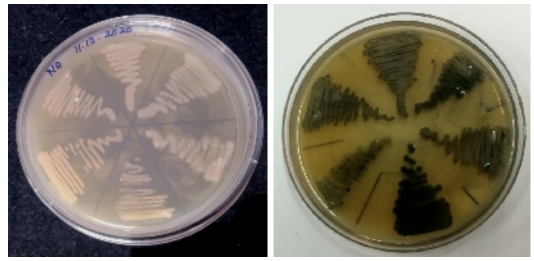
Different genera (*Pseudomonas*, *Ralstonia, Acinetobacter*, *Bacillus*, *Rhizobium*) of bacteria synthesize PHA and *Bacillus* strains were considered as hyper accumulator (Lee *et al*., 1996). Other genus like *Serratia* and *Proteus* also revelaed the PHB producing ability that was isolated from agricultural soil at southern region of Morocco (Aragosa *et al*., 2021). In this present work, attempts were made to isolate potent PHB producers from termite mound soil. In nature, termites solely depend on plant litter as the main source of food, it confirms the rich source of organic matter present around the termite mound. Hence the termite soil from the mound is used for the isolation of PHB producers. PHB-producing microbes were isolated from different environments such as municipal sewage sludge (Reddy *et al*., 2009), fish gut (Kaynar and Beyatli, 2009), marine environments (Arun *et al*., 2009), palm oil mill effluent (Alias and Tan, 2005).

During the isolation of PHB producing microorganisms, it is essential to screen the bacteria with the help of lipophilic stain Sudan Black-B. It reveals the intracellular lipid matter present in the cytoplasm (Burdon, 1946). Bluish black colonies were regarded as PHB producers and white coloured colonies were recognized as non PHB producers. Based on colour change, the bacterial isolates can be selected as positive for the production of PHB in plate assay. The microbial isolates were positive for the two stains Stain I (Sudan black B) and Stain II (Nile Blue A) were quantitatively screened for PHB production. Among the twenty isolates, only 6 isolates having the capacity to accumulate PHB was screened using Sudan B Black and Nile Blue staining respectively (Table 1). Appearance of bluish-black color and the orange fluorescence under UV-Transilluminator confirms the PHB production (Fig. 1A, B). In this study, six positive bacterial isolates (PHB 01, PHB 02, PHB 03, PHB 04, PHB 05, and PHB 06) are screened and purified by streaking and refrigerated (-20°C) for futher experiments. Liu *et al.,* (1998) isolated PHB-producing *Rhizobium meliloti* strains using the Sudan Black B stain as a viable colony screening method.

Table 1: Screening of PHB producing bacteria from termite ecosystem.

Sr. No.	Isolates	Stain I	Stain II
1.	PHB 01	+	+
2.	PHB 02	+	+
3.	PHB 03	+	+
4.	PHB 04	+	+
5.	PHB 05	+	+
6.	PHB 06	+	+
7.	PHB 07	-	-
8.	PHB 08	-	-
9.	PHB 09	-	-
10.	PHB 10	-	-
11.	PHB 11	-	-
12.	PHB 12	-	-
13.	PHB 13	-	-
14.	PHB 14	-	-
15.	PHB 15	-	-
16.	PHB 16	-	-
17.	PHB 17	-	-
18.	PHB 18	-	-
19.	PHB 19	-	-
20.	PHB 20	-	-

+ Positive; - Negative; Stain I:Sudan Black B; Stain II: Nile Blue



(A) Before (B) After

Fig. 1. Sudan Black Staining.

Morphological characteristics of selected PHB producing isolates were listed in Table 2. The positive isolates from Stain I and stain II were subjected to morphological characterization. Most of the isolates appeared as white colored smooth surfaced with translucent opacity. PHB03 showed brown colored colonies, drop like elevation with translucent opacity. The shape of the isolates was observed as rod and cocci. In the microscopic observation, Gram positive and Gram negative bacteria was identified. SEM (Scanning Electron Microscope) observation confirms the morphology (rod shaped) and increased size of the bacteria after three days of incubation. Isolate PHB 01 was observed under SEM after 36 h fermentation to observe the growth of the rod-shaped bacteria.

Table 2: Morphological Characteristics of selected PHB producing isolates.

Isolates	Color	Shape	Gram Staining	Elevation	Opacity
PHB 01	White	Rod	+	Flat	Translucent
PHB 02	White	Rod	+	Elevated	Opaque
PHB 03	Brown	Rod	_	Drop Like	Translucent
PHB 04	White	Rod	+	Convex	Opaque
PHB 05	Yellow	Cocci	_	Convex	Opaque
PHB 06	Pale Yelow	Rod	+	Drop like	Opaque

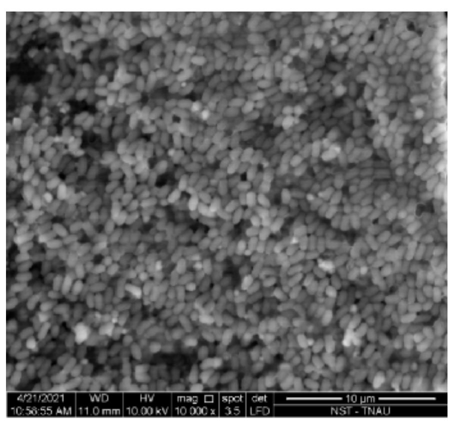


Fig. 2. Growth of Cells under Scanning Electron Microscope.

The majority of cells (Fig. 2) appeared to be rod and grouped. All the cells appeared to have large quantities of PHA and suspected as *Bacillus* species was appeared in bluish-black color. On the basis of morphological and biochemical characters, the isolated bacteria was classified using Bergey's Manual (upto genus level). This methodology was followed to identify *Bacillus, Micrococcus, Pseudomonas* from compost sample (Karim *et al*., 2018). Similarly, biochemical characterization helped to identify several genus (*Bacillus, Micrococcus, Arthrobacter*) isolated from animal manure and industrial sites were reported by Thapa *et al*., (2019). Likewise our results also grouped and classified under the genus level of *Bacillus, Azotobacter and Pseudomonas.* Then the isolates were confirmed molecularly using 16S rRNA sequencing and listed in Table 4. Most of the scientist reported *Bacillus* species have the ability to produce PHA using industrial waste water, industrial effluents (Full *et al.*, 2006; Otari and Ghosh 2009). Present investigation also coherent with the earlier studies, because out of 6 PHB producers 4 were *Bacillus* sp. (Table 4, Plate 1).

Table 3: Biochemical characterization of selected PHB producing isolates.

Isolates	Sugar Utilization Test				IMViC Test				Nitrate Reduction Test	Catalase Test	Urease Test
	Glucose	Lactose	Maltose	Sucrose	Indole	MR	VP	Citrate			
PHB 01	+	+	+	+	-	-	-	+	+	+	+
PHB 02	+	+	+	+	-	-	-	-	+	+	-
PHB 03	+	+	+	+	+	-	-	+	-	-	-
PHB 04	+	+	+	+	+	-	+	+	+	-	+
PHB 05	+	+	+	+	+	+	+	+	-	+	-
PHB 06	+	+	+	+	+	-	+	+	-	-	+

+ Positive, - Negative, MR: Methyl Red, VP: Voges- Proskauer

Table 4: Molecular identification of selected PHB producing bacterial isolates.

Isolates Code	Name of the bacteria	Similarity (%)	Accession No.
PHB 01	*Bacillus siamensis*	98	MW440618
PHB 02	*Bacillus subtilis*	97	MW440620
PHB 03	*Azotobacter chroococcum*	96	MZ542284
PHB 04	*Bacillus safensis*	97	MZ542355
PHB 05	*Pseudomonas sp*	95	MZ542455
PHB 06	*Bacillus megaterium*	94	MZ542333

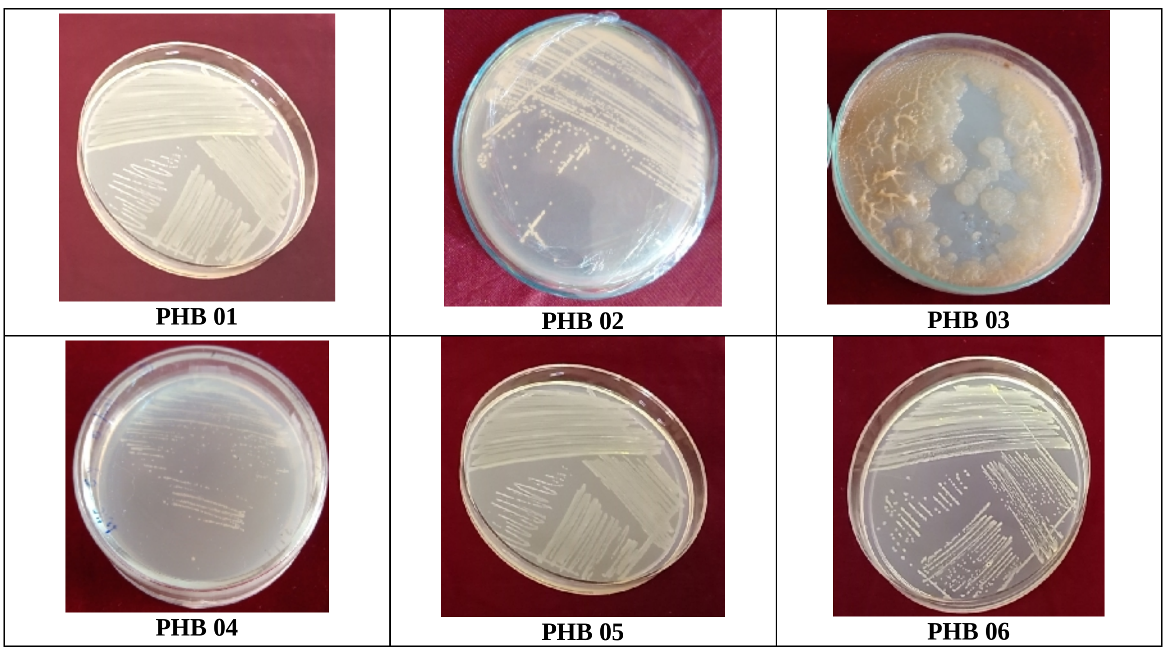


Plate 1. PHP producing isolates :- *Bacillus siamensis* (PHB 01), *Bacillus subtilis* (PHB 02), *Azotobacter chroococcum* (PHB 03), *Bacillus safensis* (PHB 04), *Pseudomonas sp* (PHB 05) and *Bacillus megaterium* (PHB 06).

CONCLUSION

Poly hydroxy butrate synthesized from microorganism is known for its plasticity and have similar physiochemical properties with petroleum derived plastics. Recently many studies investigate and explore potential PHB producers and try to increase the PHB yield by optimizing the growth influencing factors. In the present study out of 20 isolates obtained from termite ecosystem, 6 bacterial isolates were conformed for PHB production and they were identified molecularly. In subsequent studies, yield of PHB may be increased under optimized conditions. Cheap carbon substrates like agro residues, agro industrial wastes and food wastes will also used for PHB production with optimized growing condition in order to reduce the cost of production. Produced PHB will be further mixed with other bio based plastics *viz.*, Poly Lactic Acid and starch to develop biodegradable bio plastics. Present investigation is a first step towards sustainable production of bioplastic which ultimately result in sustainable environment with reduced use of petroleum derived plastics.

Acknowledgment. All the authors are grateful to the Department of Agricultural Microbiology, Tamil Nadu Agricultural University, Coimbatore-03.
Conflict of Interest. None.

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How to cite this article: Subasri, M.; V. Gomathi[2] and Mary, J. K. (2021). Isolation, Screening and characterization of Bioplastic (Poly Hydroxy Butyrate) producing Bacteria from Termite Mound Soil. *Biological Forum – An International Journal*, *13*(4): 923-927.