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Isolation and characterization of polyhydroxybutyrate (PHB) producing *Bacillus species* from agricultural soil

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ABSTRACT

Polyhydroxyalkanoates (PHAs), which is produced by several bacteria, is a biodegradable polymer that has many industrial and medical applications. The present study deals with the isolation, and characterization of biopolymer (PHB) producing bacteria from the agricultural soil. The biopolymer producing bacteria was isolated from soil samples like crop, nursery, and fodder fields. The bacteria were screened by morphological and biochemical characteristics. The bacteria were further identified by Sudan black- B staining and molecular characterization. High and efficient PHB accumulation bacteria was selected by quantification and extraction method. Further bacteria were confirmed by FTIR analysis to know the functional groups present in the bacteria. A total of six bacillus species were isolated, the strain BBKGBS12 and BBKGBS6 are gram positive and other isolated bacteria were gram negative. Black colour granulesof bacteria BBKGBS6 were seen by Sudan Black-B staining. Also granules of isolated bacteria were fluoroscenced by acridine orange staining . BBKGBS6 identified to be a strain of Lysinibacillus sphaericus BBKGBS6 by 16S r- RNA sequencing and high PHB accumulation. FTIR analysis gave prominent functional groups like CH_3, CH_2, C=O, C-O, CH, and OH were identified and the results revealed that the poly-3-hydroxybutyrate compound was produced by Lysinibacillus sphaericus BBKGBS6 (Accession number KP403811). However the nursery field soil has showed high amount of PHB accumulation bacteria.

Keywords: Polyhydroxybutyrate (PHB), Polyhydroxyalkanoates (PHAs), Fourier Transform Infra-Red Spectroscopy (FTIR)

INTRODUCTION

Polyhydroxybutyrate (PHB) is a biodegradable and biocompatible thermoplastic, there are a class of bacterial polyesters collectively called polyhydroxyalkanoates (PHAs), accumulated intracellular as reserve granules by many bacteria in harsh environmental conditions (1). PHB was first isolated and characterized in 1925 (2). PHB is primarily a product of carbon assimilation and is used by micro-organisms as a form of energy storage molecule (3). It can be made into many forms and shapes. PHB & PHV (Polyhydroxy valeric acid) are being developed for a variety of applications (4). PHB differentiates itself from other biodegradable plastics it has unique properties like insoluble in water, highly resistant to hydrolytic degradation, oxygen permeability, UV resistant, other biodegradable plastics are moisture sensitive and water soluble. PHB is poor resistance to acids and bases, soluble in chloroform and other chlorinated hydrocarbons and biocompatible and hence it is suitable for medical applications. Poly-β-hydroxybutyrate (PHB) is synthesized as an intracellular storage material and accumulates as distinct black granules during unbalanced growth in the cell; these are clearly visible in the cytoplasm of the cell. During the

adverse conditions PHB is used by the cell as an internal reserve of carbon and energy. Many bacteria, including those in the soil, are capable of PHB production and breakdown (5). A series of enzymes, synthetases or depolymerases, are implied in the biosynthesis and biodegradation of poly-β-hydroxybutyrates and also of other polyhydroxyalkanoates (6). These biodegradable polyesters display a special interest due to their possible use as substitutes of common plastics because they are completely degraded by the microorganisms present in the environment and they can be produced from regenerable carbon sources (7, 8). *Alicaligens eutrophs H16* is a facultative autotroph and can grow rapidly in simple media, for PHB production it requires anaerobic conditions with Co_2 and N source (9). Molecular structure of PHB does not depend on the features of the strain and conditions of carbon nutrition of microorganisms producing PHB (10). Most of the bacteria which produce PHB are nitrogen-fixing microorganisms. The *Azotobacter species* fix the molecular nitrogen and have the capacity to accumulate poly-β-hydroxybutyrates when they are grown on different carbon sources, including sucrose media (11). Therefore the study is focused on the producing of polyhydroxybutyrate (PHB) granules by strains isolated from different soil samples in University of Agricultural Sciences Dharwad. There were Screening and the isolation of the bacteria by using standard techniques. It was noticed that most of the soil had the PHB producing strains. The maximum density of the granules was recorded from the Nursery soil.

MATERIALS AND METHODS

2.1 Sample collection and Isolation of PHB producing bacteria:

The bacteria used in this study was collected from University of Agricultural sciences, Dharwad crop, Nursery and Fodder field soils for screening of high PHB producing bacteria and the below mentioned media is used as a nutrient source. Various samples which are–collected were serially diluted and 10^{-5} dilution was plated on nutrient media (g/l), with Peptone- 2, Beef extract-2, NaCl-1, Agar-4, Distilled water- 1lt, the media was then autoclaved. These plates containing soil sample were incubated overnight.

2.2 Screening for PHB producing bacteria:

All the bacteria isolates were tested for PHB production using Sudan black –B stain (12) and also the PHB produces bacterial granules were detected by fluorescent staining methods using acridine orange as suggested by Senthilkumar and Prabhakaran. (13). The bacterial granules were observed by Carl Zeiss and fluroscent microscope under 100X.

2.3 Morphological characterizations of isolated bacteria:

Colony and cell morphology based on their colour, shape, margin, elevation, surface and arrangement of bacteria were studied.

2.4 Biochemical characterizations of isolated bacteria:

Gram staining, Nitrate reduction test, MR, VP test, indole test, citrate utilization test, oxidase test, gelatin liquifaction test, catalase activity, H_2S production, starch hydrolysis, D- fructose, sodium malonate, sodium acetate, D-fucose, D-sorbitol, D- glucose, L-alanine, salicin, rhamnose, propinoic acid, valeric acid, trisodium citrate, L-proline, L-rhamnose, D-ribose, inositol, glycogen, capric acid, L-histidine, L-arabinose, hydroxyl butyric acid tests were carried out using standard protocols proposed by Cappuccino and Sherman, (14), for the biochemical characterization of isolates.

3. Identification of isolated (BBKGBS6) bacteria by 16S r RNA gene sequencing:

The strain was further subjected for molecular identification to confirm by analyzing 16S r-RNA sequencing was done. The bacterial DNA was isolated using a standard protocol by Rohini *et al.,* (20) further confirmation of DNA was done using agarose gel electrophoresis, subsequently PCR was carried out for further amplification using Applied Biosystem Verti thermal cycler.

Universal primer (Lane, 1991)

>141121-28_A01_GBS6_518 Forward.ab1 1089
GGGGGTACGTTGATCGGCATATTGAGGCGTAAGCGCGCGCAGGTGGTTTC

>141121-28_C01_GBS6_800Reverse.ab1 847
GGGCGTCTTTCGCGCATCAGTGTCAGTTACAGACCAGATAGTCGCCTTCG

The presence of PCR products was determined by 2.5% agarose gel electrophoresis and to analyze the size of amplified PCR product DNA markers of 100bp was used which was provided by the Pure gene. The amplified product was further sequencing ABI 3500 XL Genetic Analyzer.

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3.1 Construction of phylogenetic tree of isolated bacteria:
By using the sequence the bacteria were identified and constructed a phylogenetic tree by using NCBI(http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&BLAST_PROGRAMS=megaBlast&PAGE_TYPE=BlastSearch&SHOW_DEFAULTS=on&LINK_LOC=blasthome)

4. Biopolymer production by isolated bacteria:
PHA production was carried out in LB medium plus 20 g/L glucose in a 500-mL shaking flask containing 100-mL medium at 30^0 C for 72 hrs.

4.1 Extraction and quantification of PHB and selection of isolates:
PHB was extracted from the BBK*GBS6* isolate by using the sodium hypochlorite method (15). All the Sudan black-B positives isolate were subjected to quantification of PHB production as per the method of John and Ralf method **(**16**).** Quantification was done by using the following formula.

Residual biomass (g/L) = DCW (g/L) – Dry weight of extracting PHB (g/L).

$$\text{PHB accumulation (\%)} = \frac{\text{Dry weight of extracting PHB+ (g/L)} \times 100\%}{\text{DCW (g/L)}}$$

4.2 FTIR analysis of isolated bacteria:
The biopolymer extracted was analyzed qualitatively in the MID IR region by FTIR using the Thermo Nicolet FTIR spectrometer, model 5700 range from 4000cm $^{-1}$ to 400cm $^{-1}$, using single bounce ATR accessory with Zinc selenide crystal. 64 scans were averaged to get the spectra. IR spectra were recorded with 4 cm $^{-1}$ resolution.

RESULTS AND DISCUSSION

5.1 Isolation of PHB producing bacteria by Sudan Black -B staining
Black color granules were taken as a positive result. A number of *Bacillus* sp. has been reported to accumulate 9–44.5% DCW PHB (17). By comparison, *Bacillus* sp.BBK*GBS6* produced 70% PHB from glucose. A relatively high yield of PHB was obtained in these strains, hence these strains were considered as potent organisms for industrial application. Black color colonies were taken as a positive result. Out of 50 soil samples three samples were shown positive results. The appearance of black colored granules in the cell indicates PHB production. The bacteria positive for PHB production were selected by observing the granules under a fluorescence microscope, Bacterial culture, showing substantial fluorescence were selected for further study.

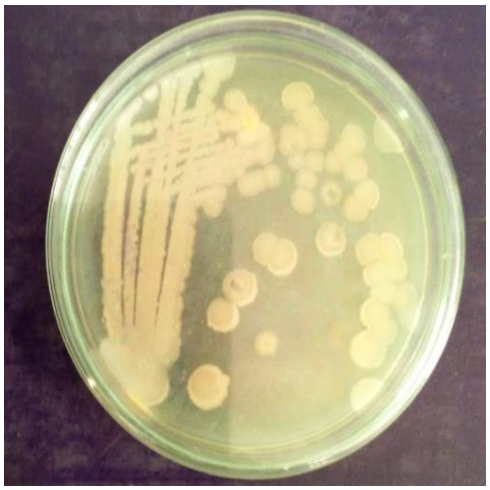

Fig. 1 Pure culture of isolatated bacteria BBKGBS6

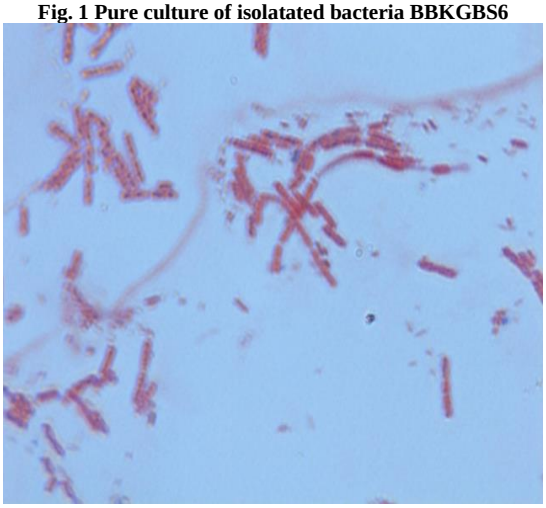

Fig. 2 Black color granules of isolated bacterial were seen by sudan black staining

5.2 Fluorescence staining of bacterial granules by acridine orange.

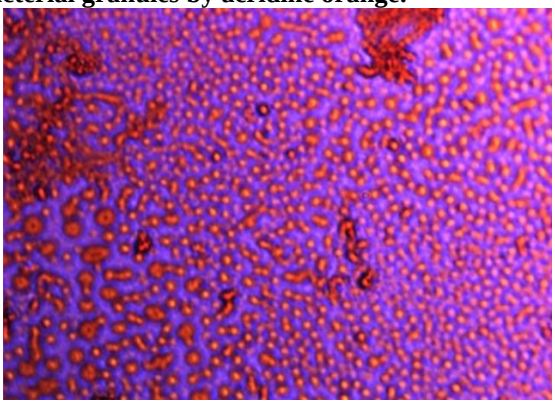

Fig. 3 The isolated bacterial granules were fluorescence by acridine orange stain

5.3 Characterization of PHB producing isolates

Six PHB producing bacterial strains were further characterized by Gram staining, morphological and biochemical tests as shown in Table 1 and 2. BBKGBS12 and BBKGBS6 isolates were Gram positive, rod shaped, spore formers. Growth was observed over a wide range of temperatures (15˚C to 45˚C). The *Lysinibacillus sphaericus BBKGBS6* utilized a wide range of sugars when tested for sugar fermentation and exhibited good growth over a wide range of pH (5.0 to 10.0). However, sporulation was observed when the bacteria were grown at extremes of pH, i.e., pH 5.0 and pH 10.0 all isolates are facultative anaerobic.

Table 1. Morphological characteristics of the isolates

Isolate no.	Whole colony appearance	Margin	Elevation	Opacity	Colour	Gram nature	No. of colonies	Surface of the colony
BBKGBS6	Irregular to flat	Undulate	Raised	Opaque	White	Positive rods with endospores	12	Smooth
BBKGBS9	Circular	Entire	Convex	Opaque	Cream	Negative rod in chain	7	Smooth
BBKGBS12	Irregular	Lobate	Umbonate	Translucent	Cream	Negative rod in chain	10	Rough
BBKGB27	Irregular	Undulate	Flat	Opaque	Cream	Positive rod in chain	5	Smooth
BBKGBS36	Irregular	Undulate	Flat	Opaque	Cream	Negative rod in chain with endospores	6	Smooth
BBKGBS45	Circular	Filamentous	Convex	Opaque	Cream	Negative rod in single	13	Smooth

Table 2. Biochemical characterizations of isolated *Lysinibacillus sphaericus BBKGBS6*

Characteristics	Results	Characteristics	Results
Gram staining	+	L-alanine	+
Anaerobic growth	-	Salicin	+
Gelatin hydrolysis	+	Rhamnose	-
Vogs proskauers	-	Propinoic acid	+
Indole production	-	Valeric acid	+
Oxidase activity	+	Trisodium citrate	+
Catalase activity	+	L-proline	+
H_2S production	-	L-rhamnose	-
Starch hydrolysis	-	D-ribose	-
Gelatin hydrolysis	-	Inositol	-
Nitrate reduction	-	Itaconic acid	-
D-frutose	-	Glycogen	-
Sodium malonate	+	Capric acid	-
Sodium acetate	+	L- histidine	-
D- fucose	-	L-arabinose	-
D-sorbitol	+	L-arabinose	-
D-glucose	+	Hydroxybutyric acid	+

5.4 16S rRNA gene amplification and sequence analysis of isolated bacteria

To confirm the conventional identification results, 16S rRNA gene investigation was performed. The PCR product of the 16S rRNA gene was amplified and comparison of the sequence to the database in Gen Bank by BLAST program, the alignment of 16S rRNA gene sequence of sample it is indicated that BBKGBS6 strain was identical to revealed a close relatedness to *Lysinibacillus sphaericus* with 74% similarity. Morphological results and phylogenetic analyses clearly demonstrated that strains BBK*GBS6, BBKGBS9, BBKGBS12, BBKGBS27, BBKGBS36* and BBK*GBS45* are members of the genus *Bacillus*. The optimal temperature for growth was between 28°C and 30°C, and the optimal pH was 7.0. Phylogenetic analysis of *16S* r-RNA demonstrated that BBK*GBS6* bacteria, grouped with *Lysinibacillus sp.1-2* and *Lysinibacilllus sphaericus S2*, are well-defined taxa. Further characterizations of these bacteria are necessary before proposing them as novel species.

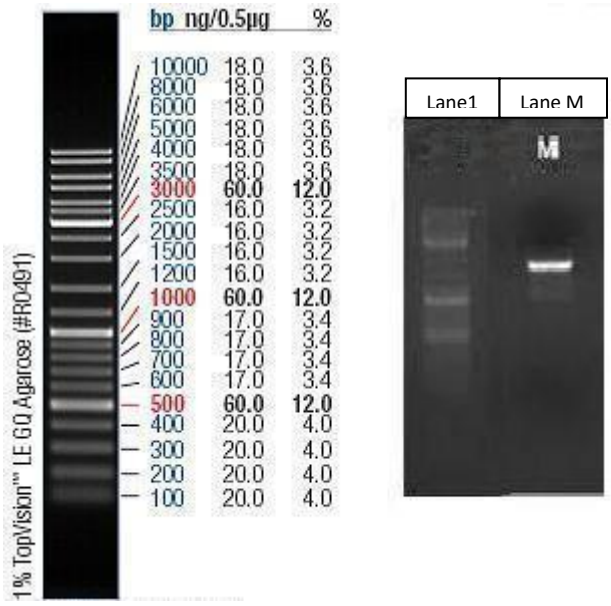

Fig. 4 An agarose gel showing the PCR amplification product of *Lysinibacillus sphaericus BBKGBS6* (lane 1: 200 bp of DNA step ladder, lane M: amplified region of the *Lysinibacillus sphaericus BBKGBS6)*

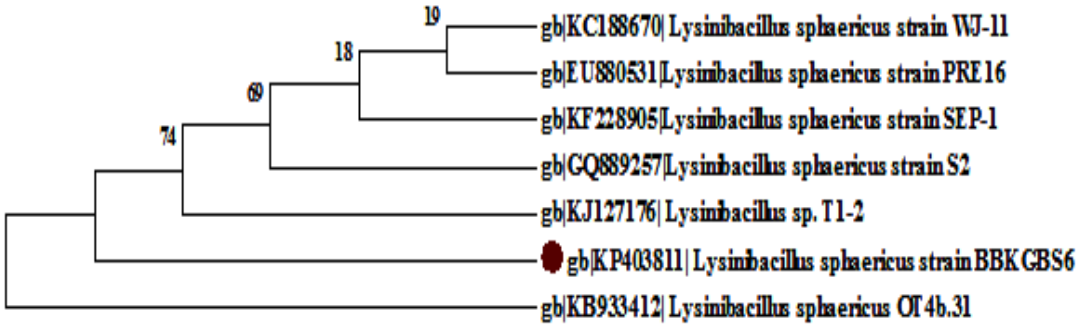

Fig.5 Phylogenetic tree analysis of PHB producing bacteria (*Lysinibacillus sphaericus* BBKGBS6), constructed through neighbor joining method with bootstrap values as percentage at the nodes

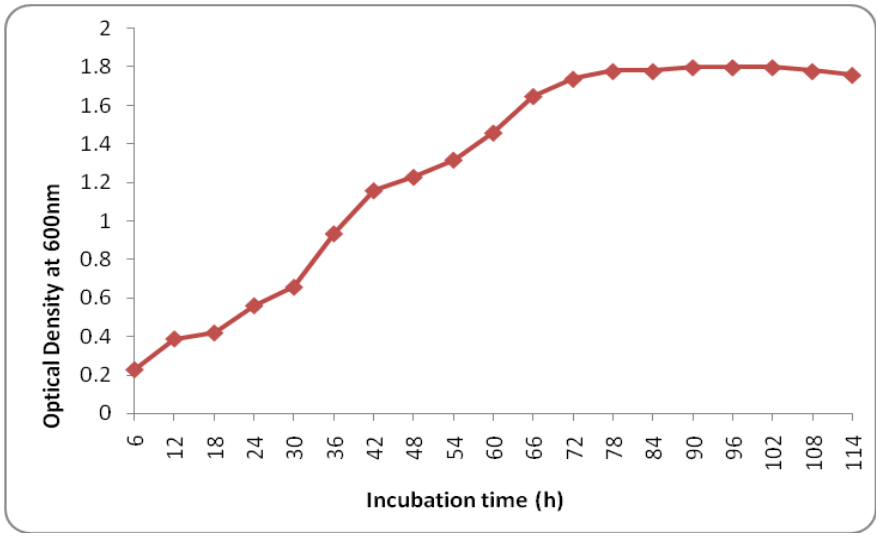

Fig. 6 Growth curve of *Lysinibacillus spaericus BBKGBS6*

5.5 Growth studies of isolated bacteria

0.1ml of the inoculums was transferred into 100 ml of nutrient broth for growth curve analyses. At regular intervals of time 4 ml of the inoculums was taken and the optical density was measured at 600 nm using UV-spectrophotometer against an un-inoculated blank. The synthesis of PHB was noticed from the log phase of growth and it continued until late exponential phase as the carbon source was utilized for both growth and PHB production.

5.6 Production of PHB using simplified media

In the present study, we have noticed that all the bacterial isolates were able to produce substantial amounts of PHB during growth using the simplified media mentioned above containing a single carbon and nitrogen source. The PHB accumulation was noticed as early as 16 hours of incubation in the bacterial cells. Carbon sources serve three different functions within the organisms: biomass synthesis, cell maintenance, and PHA polymerization. Among the carbon sources tested with the strains BBKGBS6 and BBKGBS27 glucose was found to be the preferred substrate for polymer accumulation. However, strain BBK*GBS6* also showed good polymer yield with glucose. The PHB extracted was calculated as a percentage yield of the cellular dry weight obtained. The maximum yield of PHB was observed in *Lysinibacillus spaericus BBKGBS6* amounting to 1.3 g/l from 3.2g/L of biomass resulting 70% yield at the end of 72hrs (Table 3). This strain was used for further studies. The other isolates, BBKGBS9, BBKGBS12, BBKGBS27, BBKGBS36, and BBKGBS45 are producing 60%, 50%, 33%, 14% and 66% respectively (Table 3).

Table. 3 Quantification of PHB production and selection of isolates

Sl.No	source	Isolates	Yield (g/L)	PHB accumulation (%)
1	Crop soil	BBKGBS36	0.2	14
2	Nursery siol	BBKGBS12	0.9	50
		BBKGBS45	0.12	66
		BBKGBS6	1.3	70
3	Fodder field soil	BBKGBS9	1.2	60
		BBKGBS27	1.3	33

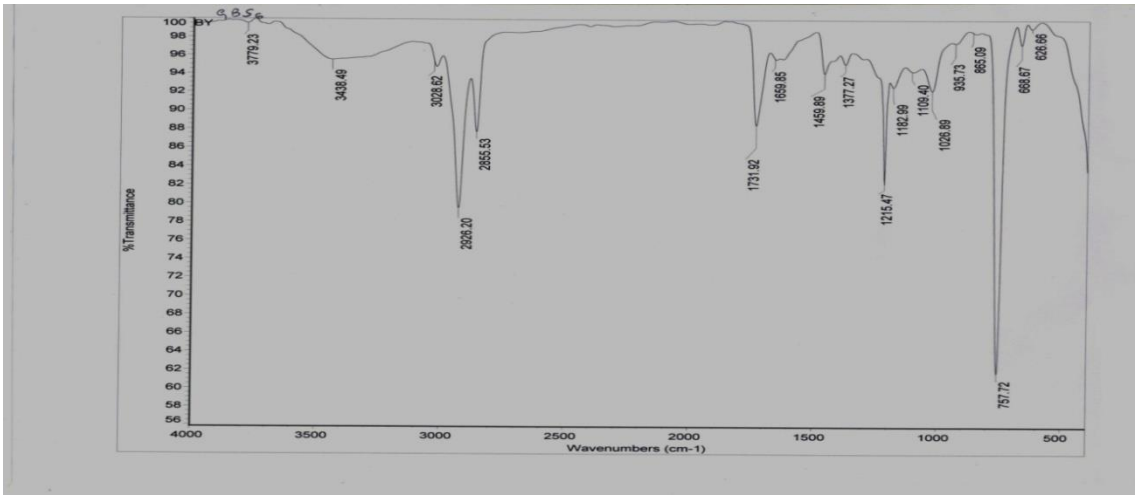

Fig. 7 FTIR spectroscopy of extracting biopolymer showing absorption at 1720 cm $^{-1}$for C=O group from *Lysinibacillus sphaericus BBKGBS6*)

5.7 FTIR analysis

FTIR analysis was done, in order to know the functional groups present in bacterial extract. About 1mg extracted sample of PHB was dissolved in 5 ml of chloroform. Chloroform was allowed to evaporate to get PHB polymer film and was subjected to FTIR analysis by using FTIR spectrophotometer. Spectra were recorded at 4000 cm^{-1} to 400 cm^{-1} range. To investigate about the functional group present in the PHB and for the conformation FTIR spectroscopy study was carried out with standard PHB (sigma) (18). FTIR spectra of the extracted polymer show peaks at 1731.92 cm^{-1} and 1215.47 cm^{-1} corresponds to specific rotations around carbon atoms specific to certain functional groups (Fig.7). The peak at 1731.92 cm^{-1} corresponds to C=O stretch of the ester group present in the molecular chain of highly ordered crystalline structure (19). The peak at 1215.47 cm^{-1} corresponds to –CH group. These peaks are corresponding to the peaks obtained for the standard PHB, at 1730 cm^{-1} and 1216 cm^{-1} exactly confirming that the extracted polymer is PHB (20) reported that the Fourier-transform infrared (FTIR) absorption band at about 1,730 cm^{-1} is a characteristic of the carbonyl group and that a band at about 1,280–1,053 cm^{-1} characterizes valance vibration of the carboxyl group. The extracted polymers from *Lysinibacillus sphaericus* BBKGBS6 showed similar absorption bands, characteristic of the poly-3-hydroxybutyrate polymer. These results suggest the polymers to be poly-3-hydroxybutyrate. Therefore, this study was important, *Bacillus spp.* BBK*GBS6* from soil that was capable of producing polymer glucose as carbon substrate. In further studies, these strains of *Bacillus* spp. could be effectively utilized to produce PHB from substrates such as lactose, sucrose, and starch.

CONCLUSION

In the present study showed that isolation of Biopolymer producing bacteria *Lysinibacillus sphaaericus BBKGBS6* which can utilize glucose in simple media containing only peptone as nitrogen source for PHB production has been identified and characterized from the soil. Among the three soil samples which were used Fodder field soil and nursery field soil gave the number of isolated single positive and high amount of PHB accumulation colonies respectively. Further bacterial biopolymer was confirmed by FTIR analysis gives prominent functional groups like CH_3, CH_2, C=O, C-O, CH, and OH were identified and the results suggest that the poly-3-hydroxybutyrate compound was produced by *Lysinibacillus sphaericus BBKGBS6* (Accession No. KP403811). The production of PHB was found to increase along with the increase in the biomass. Further studies are required to optimize the growth media

to improve the PHB yield and to reduce the cost of production media along with suitable PHB induction media components. However the nursery field soil has showed high amount of PHB accumulation bacteria.

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