Accumulation of poly(3-hydroxybutyrate) by *Microbacterium barkeri* DSM 20145

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**Abstract:** A gram-positive bacterium that accumulated poly(3-hydroxybutyric) acid (PHB) was isolated from petrol bunk soil. From its morphological and physiological properties and the nucleotide sequence (about 1.5 kb) of its 16S rDNA, it was identified as *Microbacterium barkeri* DSM 20145. Based on the available literature, this is the first report of PHB accumulation by *Microbacterium* sp. The isolate accumulated 0.6 g/L, or 29.7%, of PHB under growth conditions in a nitrogen-deficient medium containing glucose and yeast extract (4:1 ratio), with maximum production by 48 h. The capacity of the isolate to produce PHB under different carbon and nitrogen sources was also investigated. The strain produced appreciable levels of PHB by utilizing hemicellulosic sugars such as glucose, xylose, and arabinose, as well as disaccharides such as lactose and sucrose, alternative carbon sources like wheat bran and carboxymethyl cellulose, and organic acids such as propionic acid, with maximum production achieved using xylose (1.7 g/L, 68%). Among the nitrogen sources tested, peptone supported maximum production (1.4 g/L, 16%), followed by casein (0.9 g/L, 22.6%) and sodium nitrate (0.4 g/L, 26.4%), although the PHB yield was not appreciably high. The addition of xylose to the nitrogen-deficient medium increased the values of PHB yield (g/L) by 2.5 and the PHB accumulation (% dry cell weight) by 2.3; the replacement of yeast extract in the medium with peptone increased the PHB yield (g/L) by 2.05. The isolated polymer was highly soluble in chloroform and peaked sharply at 235 nm upon digestion with concentrated H2SO4. This study adds a new species to the family of PHB producers.

**Key words:** *Microbacterium barkeri* DSM 20145, poly(3-hydroxybutyrate) formation, carbon and nitrogen sources

**Introduction**

Poly(3-hydroxybutyric acid) (PHB), the best-known member of the polyhydroxyalkanoate (PHA) group of polymers, is produced as an intracellular carbon and energy reserve by a wide variety of bacteria when grown under conditions that limit nutrients other than carbon (1,2). Due to their biodegradability and biocompatibility, poly(3-hydroxybutyrate) and copolymers of 3-hydroxybutyrate (3HB) and 3-hydroxyvalerate (3HV), P(3HB-co-3HV), have attracted attention as possible alternatives to nonbiodegradable thermoplastics. At least 75 different genera of bacteria have been known to accumulate PHB as intracellular granules (1). PHB production has most commonly been studied in microorganisms belonging to the genera *Alcaligenes*, *Azotobacter*, *Bacillus*, and *Pseudomonas* (1,3-9). This polymer is synthesized under limited culture conditions and is used as a carbon and energy reserve. *Microbacterium* species are widely distributed in various environments (10-23).

The accumulation of PHB by members of the genus *Microbacterium* has thus far not been reported in the literature. In the present study, a
Microbacterium species capable of producing PHB was identified during screening for PHB-producing bacteria. Recent research has focused on the use of alternative substrates, novel extraction methods, genetically enhanced species, and mixed cultures in order to make PHB more commercially attractive. The aim of the present research was to determine PHB production by Microbacterium sp. and to test for further PHB production in different nitrogen and carbon sources.

**Materials and methods**

**Isolation of PHB-producing microorganisms**

Soil samples from different places in and around Bangalore were screened for PHB-positive colonies by plating them on nutrient agar and then flooding the colonies with Sudan Black (SB). Briefly, a 0.02% SB solution in 95% ethanol was spread over the colonies for 30-60 min, discarded, and washed with 95% ethanol (24). Bluish-black colonies were selected for PHB production studies. The isolated colonies were streaked on nutritive agar until pure cultures could be obtained for use in the identification of each separate morphotype from the colonies. Gram staining was used to determine the form, size, and type of aggregation as well as the presence of characteristic structures such as reserve granules, endospores, and cysts.

*M. barkeri* DSM 20145, isolated from a petrol bunk soil sample and identified by the established method, was used in this study. Stock cultures were grown and maintained at 4 °C on nutrient agar slants.

**Characterization of the isolated bacteria**

The morphological and physiological properties of the isolate were investigated according to *Bergey’s Manual of Determinative Bacteriology* (25). The sequencing of 16S rDNA and the taxonomic studies of strain DSM 20145 were performed at Chromous Biotech Pvt. Ltd. in Bangalore, India. A partial 16S rDNA fragment of approximately 1.5 kb was amplified using high-fidelity polymerase chain reaction (PCR) polymerase. The PCR product was sequenced bidirectionally using the forward, reverse, and internal primers. The sequence data were then aligned and analyzed to identify the bacterium and find the most closely related strains.

**Inoculum preparation**

For all of the experiments, the inoculum was prepared in 250-mL Erlenmeyer flasks containing 50 mL of sterile nutrient broth. The flasks were incubated at 37 °C for 24 h on a rotary shaker at 120 rpm. The culture broth was centrifuged at 8000 × g for 15 min, washed twice, and suspended in sterile distilled water. The bacterial cell suspensions were inoculated into the nitrogen-deficient media for PHB production studies.

**PHB production studies in nitrogen-deficient media**

Unless otherwise stated, the nitrogen-deficient medium used for cell growth and PHB accumulation studies contained (in g/L): glucose, 10; MgSO₄, 0.2; NaCl, 0.1; KH₂PO₄, 0.5; and yeast extract, 2.5. PHB production studies were carried out in 250-mL flasks containing 50 mL of the culture medium and incubated at 37 °C on a rotary shaker at 120 rpm for 48 h (with the exception of the time-course study).

**Time-course study of PHB formation**

The inoculum was prepared as described above and PHB production as a function of time (0, 24, 48, 72, and 96 h) was determined during growth in the nitrogen-deficient media.

**Effect of different carbon and nitrogen sources on PHB production**

Glucose in the nitrogen-deficient medium was replaced by 10 g/L (w/v) of hemicellulosic sugars such as glucose, arabinose, and xylose in pure forms, as well as disaccharides such as sucrose and lactose, organic acids like propionic acid, and the sugar alcohol mannitol. Additionally, wheat bran and carboxymethyl cellulose (CMC) were used as carbon sources, while yeast extract was variously replaced by 0.25% (w/v) peptone, tryptone, casein, NH₄(SO₄)₂, NH₄Cl, and NaNO₃ as nitrogen sources. The experiments were conducted in duplicates and the averages of the readings were calculated for dry cell weight (DCW) and PHB content measurements.

**Dry cell weight**

Growth of the organisms was determined by measuring the dry weight of the bacterial pellet. The bacterial pellet was separated by centrifugation at 10,000 × g for 10 min, washed twice with distilled water, and dried to a constant weight at 80 °C.
**PHB extraction and estimation**

For the extraction of poly(3-hydroxybutyrate), the bacterial pellet was lysed in sodium hypochlorite at 37 °C for 2 h and centrifuged at 10,000 × g, and the residue was washed twice with each of the following: water, acetone, ethanol, and diethyl ether. Finally, the residue was extracted with boiling chloroform and filtered through Whatman No. 1 filter paper. The chloroform extract was evaporated to dryness, and the amount of PHB was quantified following the method of Law and Slepecky (26) with an ELICO SL150 UV-VIS spectrophotometer. For dry weight estimation, the pellet after extraction was dried to constant weight. The production of PHB was expressed as PHB yield (g/L) and PHB accumulation (% DCW).

**Statistical analysis**

The mean and standard deviation values of the DCW, PHB yield, and PHB content were calculated from at least 2 independent experiments run in duplicates. The correlation between bacterial DCW (g/L) and the PHB production (g/L) of the bacteria was determined according to Spearman’s correlation.

**Results and discussion**

Of the 30 total bacteria screened from different soil samples, the isolate *M. barkeri* DSM 20145, isolated from petrol bunk soil, was identified as a PHB-producing bacterium on the basis of the bluish-black colored colonies present upon flooding with Sudan Black B. The strain accumulated 0.25 g/L PHB with an 18.3% yield in nutrient broth by 48 h and was selected for further studies. The morphological and taxonomic features of the isolate were examined (Table). Based on the biochemical characteristics and an analysis of the partial nucleotide sequence of the 16S rDNA (approximately 1.5 kb), the isolate

<table>
<thead>
<tr>
<th>Test</th>
<th>Observation</th>
<th>Genus <em>Microbacterium</em></th>
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<tbody>
<tr>
<td>Gram staining</td>
<td>Gram-positive</td>
<td>+</td>
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<tr>
<td>Endospores</td>
<td>_</td>
<td>_</td>
</tr>
<tr>
<td>Cell shape</td>
<td>Rods</td>
<td>+</td>
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<tr>
<td>Rod-coccus style</td>
<td>_</td>
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<tr>
<td>Arranged at angle to give V-formation</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Colony color</td>
<td>Yellow</td>
<td>+</td>
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<tr>
<td>Motility</td>
<td>Nonmotile</td>
<td>+/-</td>
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<tr>
<td>Catalase</td>
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<td>Voges-Proskauer</td>
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<td>Pectin hydrolysis</td>
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<tr>
<td>Tween 20 &amp; 80 hydrolysis</td>
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<td>Acid-fast</td>
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<td>+/-</td>
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<tr>
<td>Acid from glucose</td>
<td>+</td>
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</table>
was determined to be *Microbacterium barkeri* DSM 20145, with a close homology to *M. arborescens* DSM 20754 (Figure 1).

Time-course PHB formation by *M. barkeri* DSM 20145 was investigated in production media. There was a gradual increase in cell biomass until 24 h, after which it remained constant until 72 h; meanwhile, PHB formation and accumulation increased significantly and showed a maximal value by 48 h (from 0.18 g/L and 11.96% to 0.683 g/L and 29.69%), followed by a gradual decrease by 72 h (Figure 2). The decrease in PHB accumulation observed after the cessation of cell growth could be attributed to the intracellular consumption of PHB as a source of energy and carbon (27). The time profile of growth and PHB accumulation by this strain indicated that maximum PHB formation occurred during the stationary phase, which was followed by a decline in the polymer content. The existence of a relationship between the DCW and PHB production was also investigated, but statistical analysis showed that there was no correlation between DCW (g/L) and the PHB (g/L) content of the cultures (q = 0.099).

When tested on the various carbon and nitrogen sources, the strain exhibited nutritional versatility in terms of varied growth and PHB formation. The highest level of PHB accumulation was observed in the N₂-deficient medium with xylose (DCW 1.7 g/L, PHB accumulation 68%), followed by the media featuring sucrose (0.8 g/L, 57%), propionic acid (0.8 g/L, 57%), and arabinose (1.0 g/L, 40%). Wheat bran, CMC, mannitol, and lactose were also found to support PHB formation by this strain (Figure 3). Of the various nitrogen sources tested, peptone supported maximum PHB formation (1.4 g/L, 16%), followed by casein (0.95 g/L, 22.61%) and NaNO₃ (0.45 g/L, 26.4%), although the PHB yield was not appreciably high (Figure 4).

The purified polymer was highly soluble in chloroform and 1 N NaOH and moderately soluble in dioxane, pyridine, and toluene, but insoluble in water, sodium hypochlorite, acetone, ethanol, methanol, and diethyl ether. Digestion of the polymer with concentrated H₂SO₄ gave a sharp peak at 235 nm, characteristic of crotonic acid (Figure 5).

![Phylogenetic tree depicting close homologs of *M. barkeri* DSM 20145. The tree was constructed by neighbor-joining on a p distance matrix. PJ.txt - strain investigated.](image)}
Species from more than 75 genera are known to be capable of synthesizing PHB (28). The thermoplastic properties of the polymer and its biodegradability determine its importance as a substitute for petrochemical plastics (11).

According to the description of the genus *Microbacterium* (13,18,19), it has the following determinative phenotypic characteristics: irregular rods, but no marked rod-coccus cycle as exhibited by some genera of coryneform bacteria (14); the genus is also gram-positive and catalase-positive, not acid-fast, and not spore-forming. As shown in the Table, the taxonomic properties of the strain were consistent with those distinguishing characteristics of *Microbacterium* (18,19) and the strain should therefore be assigned to that genus. The sequence comparison results of the 16S rRNA gene of the strain with that of the related genera also supported the view that the strain may be a member of the genus *Microbacterium*. Comparisons of the 16S rRNA gene
sequences between the strain and the close relatives retrieved from the databases showed a clear affiliation with the genus Microbacterium. A phylogenetic tree of the strain, based on the 16S rRNA gene sequences, was constructed using the neighbor-joining method. These data, presented in Figure 1, strongly suggest that strain DSM 20145 should belong to the genus Microbacterium.

The occurrence of PHB has been reported from a wide variety of bacteria (1,2,29), but strains of Microbacterium spp. have not been investigated for this ability until now. A review of the available literature indicates that this is the first report on PHB production by Microbacterium sp.

Some of the A. eutrophus strains used for commercial PHB production have a PHB concentration that is approximately 80% (w/w) of the DCW (29). Chen et al. (30) studied PHA in 11 different Bacillus spp. and found PHB at up to 50% (w/v) of the DCW of the bacteria. Microbacterium barkeri DSM 20145 accumulated 29.69% PHB by 40 h. When compared to related studies in the literature, these results show a lower PHB production. The disparity may result from differences in the strains, types of media, or the cultivation methods used in the individual studies.

Bacteria able to synthesize PHA can be divided into 2 groups (29). The first group, accumulating PHA during the stationary phase, requires the limitation of N, P, Mg, and oxygen, for example, and an excess of the carbon sources. The most important microorganism for industrial PHA production, Ralstonia eutropha, belongs to this group. The second group, which accumulates PHA during the growth phase, includes Alcaligenes latus, a mutant strain of Azotobacter vinelandii, A. beijerinckii (27), and recombinant strains of Escherichia coli bearing the PHA operon of R. eutropha. Our strain, M. barkeri DSM 20145, belongs to the first group, because PHB accumulates during the stationary phase. The physiological characteristics of the organism are important for the bioprocess to produce polymer.

Several factors influence the economics of biodegradable polymer production. These factors include substrate cost and the ability to produce biodegradable polymers from inexpensive or renewable substrates (14). The type of carbon source used has a huge influence on PHB productivity. The carbon source must always be provided in excess to allow for maximum PHB accumulation in the biomass. M. barkeri DSM 20145 exhibited high nutritional versatility by showing a capacity to utilize hemicellulose sugars, pentoses, hexoses, disaccharides, sugar alcohol, wheat bran, CMC, and organic acid to form PHB. The addition of xylose to the N$_2$-deficient medium increased the values of PHB yield (g/L) 2.5-fold and PHB accumulation (% DCW) 2.3-fold. Replacing the yeast in the medium with peptone increased the PHB yield (g/L) 2.05-fold. This observation indicates the strain's potential for use in the industrial production of PHB using cheaper alternative carbon sources such as hemicellulosic waste. M. barkeri DSM 12045 showed varied levels of PHB production under different nitrogen sources with maximum values obtained in the presence of peptone. The enhancement of PHB accumulation may result from the presence of amino acids and peptides in peptone, tryptone, and casein. Such stimulation of biosynthesis might be due to the channeling of the carbon source into PHB accumulation. Similar enhancement of PHB accumulation was reported in A. vinelandii, A. chroococcum, A. beijerinckii, and E. coli when the organisms were grown in media containing organic nitrogen sources (31-36). Several renewable materials, including casein hydrolysates and gluten, are known to be available from agriculture as nitrogen sources that can be used as cheap substrates (37). The addition of inorganic nitrogen sources also supported PHB production. In this study, the ratio of carbon and nitrogen was always maintained at 4:1 to allow for maximum PHB accumulation in the biomass. Further optimization of the C:N ratio may encourage increased PHB formation by this strain. Xi et al. (20) determined that PHB synthesis was highly dependent on the nitrogen source. While PHB synthesis by Pseudomonas oleovorans was not significantly stimulated by nitrogen limitation, P. resinovorans responded to nitrogen limitation by greatly increasing the PHB production rate (16). These observations corroborate our results. The identity and purity of the PHB obtained from M. barkeri DSM 20145 were confirmed by solubility properties and UV absorption spectra. The Law and Slepecky method detects only PHB and no other polyhydroxyalkanoates. Future studies will focus on
investigating the ability of this strain to accumulate various other polyhydroxyalkanoates.

The present study highlights the accumulation and partial characterization of PHB by a new strain, *M. barkeri* DSM 20145, and examines the influence of a variety of carbon and nitrogen sources on the growth and PHB production of this strain. PHB accumulation by this strain is comparable to most other PHB-producing gram-positive *Bacillus* spp. and much higher than the results of *Streptomyces*, *Clostridium*, and *Nocardia* sp. previously reported (38). *M. barkeri* DSM 12045 may be an attractive candidate for the production of biodegradable plastics, but further research is needed to characterize the type of PHA produced by this strain on glucose and different carbon feeds in order to verify its potential use in the commercial production of biodegradable polymers. The addition of a new species to the existing list of PHB-producing microorganisms will certainly provide new opportunities for the production of cost-effective biodegradable plastics.

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