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ORIGINAL PAPER

Production and characterization of PHB from two novel strains of *Bacillus* spp. isolated from soil and activated sludge

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Abstract The present study reports two bacteria, designated 87I and 112A, which were isolated from soil and activated sludge samples from Hyderabad, India, and that are capable of producing poly-3-hydroxybutyrate (PHB). Based on phenotypical features and genotypic investigations, these microorganisms were identified as *Bacillus* spp. Their optimal growth occurred between 28°C and 30°C and pH 7. *Bacillus* sp. 87I yielded a maximum of 70.04% dry cell weight (DCW) PHB in medium containing glucose as carbon source, followed by 55.5% DCW PHB in lactose-containing medium, whereas *Bacillus* sp. 112A produced a maximum of 67.73% PHB from glucose, 58.5% PHB from sucrose, followed by 50.5% PHB from starch as carbon substrates. The viscosity average molecular mass (M_v) of the polymers from *Bacillus* sp. 87I was 513 kDa and from *Bacillus* sp. 112A was 521 kDa. All the properties of the biopolymers produced by the two strains 87I and 112A were characterized.

Keywords *Bacillus* · Characterization · PHB · Production

Introduction

Poly-3-hydroxybutyrate (PHB), the most common representative of the polyhydroxyalkanoates (PHA), is widespread in different taxonomic groups of prokaryotes as an intracellular storage compound, acting as a carbon reserve and reducing equivalent that facilitates cell survival during stressful conditions [1, 2]. In general, PHB is synthesized by cells under growth-limiting conditions, when the carbon source is in excess and nitrogen, phosphorus, magnesium, oxygen or sulfur is present in a limiting concentration.

PHA are attracting considerable attention because of their potential as renewable and biodegradable plastics in the areas of tissue engineering, environmental friendly packaging materials, and as a chiral hydroxyalkanoate (HA) pool [3]. Poly-3-hydroxybutyrate (PHB), a representative compound of the family of PHA, has many potential applications in medicine, veterinary practice, and agriculture due to its biodegradability and biocompatibility [4].

Gram-positive bacteria, notably *Bacillus* and *Streptomyces*, have been used extensively in industry. However, these organisms have not yet been exploited for the production of PHA biodegradable polymers. Gram-negative bacteria, currently the only commercial source of PHA, have lipopolysaccharides (LPS) which copurify with the PHA and cause immunogenic reactions. On the other hand, Gram-positive bacteria lack LPS, a positive feature which justifies intensive investigation into their PHA production [5]. The genus *Bacillus* seems to be a potential candidate for production of PHB due to its better polymer yields and less stringent fermentation conditions. The novel PHA synthase discovered from *Bacillus* genus has the ability to incorporate both short chain length (scl) and medium chain length (mcl) PHA, indicating that the genus can be a potential producer of novel and known PHA with different ranges of monomeric compositions [6]. Hence, the accumulation of PHB by the *Bacillus* genera has distinct features which need extensive investigation.

At present, about 40 PHA synthase genes are known from various microorganisms [7]. The acquisition of a novel PHA synthase gene can facilitate the synthesis of a new type of PHA.

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One of the most important factors for the popularization of the use of PHB polymers as conventional plastics is their production cost [8]. Therefore, less expensive substrates, improved cultivation strategies, and easier downstream processing methods are required for cost reduction [9]. It is also essential to identify microorganisms that utilize cheap carbon sources efficiently to produce PHB [10], as the carbon substrate alone accounts for 50% of the total expense of PHB production.

In the present study, production and characterization of PHB from two novel strains of *Bacillus* spp., which were isolated from soil and activated sludge samples in an earlier investigation, are reported.

Materials and methods

Microorganisms

Bacillus sp. 87I and *Bacillus* sp. 112A isolated from soil and an activated sludge sample, respectively, collected at Hyderabad, India, have been described elsewhere [11]. These bacterial strains were isolated on Luria–Bertani (LB) medium (Hi media, India).

Strain identification

Both of the selected bacilli were identified by biochemical as well as *16S* rRNA sequence analysis and phylogenetic studies. Universal primers 8F (5′-AGAGTTTGATCCT GGCTCAG-3′) and 1492R (5′-ACGGCTACCTTGTTAC GACTT-3′) [12] were used for amplification of *16S* rRNA of both isolates. For quantitative analysis of the cellular fatty-acid compositions of the two bacilli, a loop of cell mass from each bacilli isolate was harvested, and fatty-acid methyl esters were prepared and identified following the instructions of the Microbial Identification system (MIDI).

Media and growth conditions

Isolates were grown aerobically (150 rpm) at 30°C in a chemically defined nitrogen-deficient E2 medium [13]. Sugars and mineral salts solutions were autoclaved separately at 110°C for 10 min.

Conditions of PHB production

For the production of PHB, the *Bacillus* isolates were cultivated in E2 mineral broth with 2% (w/v) glucose as carbon source. Each of these *Bacillus* isolates was grown in 250-ml Erlenmeyer flask containing 50 ml E2 mineral broth. After inoculation with overnight-grown 4% (v/v) inocula, the flasks were incubated at 30°C for 48 h on an orbital shaker at 150 rpm.

Extraction and quantitative assay of PHB

PHB was extracted from the *Bacillus* isolates by using the hypochlorite method [14]. Quantitative estimation of PHB extracted from the isolates was done by ultraviolet (UV) spectrophotometer method [15].

Growth pattern

The growth response of *Bacillus* spp. was studied using 500-ml Erlenmeyer flask containing 100 ml E2 broth with 2% 24-h-aged culture inoculum. Initial cell density was adjusted to A_{600} of 0.1, and incubated for 48 h at 30°C and 150 rpm. After inoculation, cell density was recorded for an interval from 4 h up to 48 h.

Effect of time on PHB production

Inoculated E2 broth was incubated for up to 48 h to determine the optimum incubation period. Ten milliliters of culture was drawn at 2-h intervals and centrifuged at 8,000 × *g* for 15 min to obtain the cell pellet. Quantitative assay was performed using the assay suggested by Aslim et al. [14].

Effect of temperature and pH on PHB production

PHB was accumulated by the two isolates, *Bacillus* sp. 87I and *Bacillus* sp. 112A, at various temperatures (25°C, 30°C, 35°C, 40°C, and 45°C) and at different pH values (6.8, 7.2, and 7.5) using E2 media and other standard conditions as described earlier.

PHB production from various carbon substrates

The capacity of these two isolates, *Bacillus* sp. 87I and *Bacillus* sp. 112A, to utilize various carbohydrates (lactose, galactose, fructose, glucose, mannitol, sucrose, maltose, and starch) was determined in the biochemical tests. For this, *Bacillus* sp. 87I and *Bacillus* sp. 112A were incubated separately in nitrogen-free E2 medium which had the above different carbon sources substituted for glucose. PHB content in the cells was extracted according to the method described earlier and quantified by UV spectrophotometer.

Polymer analysis

The fermentation broth was used for determination of dry cell weight (DCW). The cell concentration was determined by measuring DCW: 5 ml culture broth was centrifuged,

washed, and dried at 105°C until the weight did not decrease further. The residual mass was defined as total DCW–PHB weight; PHB (%) was defined as the percentage of the ratio of PHB to DCW. The polyesters content of the cell and the composition of polyesters were determined by using gas chromatography-mass spectrometry (GC–MS) analysis. The structure and mole fractions of HB units in the polymer samples were investigated by 400-MHz H^1 nuclear magnetic resonance (NMR) spectra recorded at 27°C in $CDCl_3$ solution of polyester. The average molecular mass of the polymers were estimated by the inherent viscosity method. Infrared (IR) spectra and differential scanning calorimeter (DSC) scans were also recorded for the polymers [16].

Results

Isolation and identification of strains

In an initial study [11], spore-forming bacteria were selectively isolated by heat or ethanol treatment from various environmental samples. A total of 240 colonies, representing many macroscopic differences in colony morphologies, were observed and picked up randomly from plates containing different samples for further analysis. Based on UV spectrophotometer quantifications, 25 *Bacillus* isolates with maximum PHB accumulation were selected from the total 240 *Bacillus* isolates as optimal PHB-accumulating *Bacillus* spp.

Based on their high PHB productivity, the two best strains were selected for further studies. Morphologically, *Bacillus* sp. 87I (deposited as MTCC 9593) appeared as off-white, circular, smooth, large colonies and *Bacillus* sp. 112A (deposited as MTCC 9719) formed white, round-shaped, large colonies in nutrient agar. Microscopically, both isolates appeared as rod-shaped Gram-positive bacilli.

Genetic, biochemical, and fatty-acid analysis

16S rRNA sequencing followed by basic local alignment search tool (BLAST) analysis showed that the isolates had close *16S* rRNA database similarity with known *Bacillus* spp. The *16S* rRNA analysis revealed that *Bacillus* sp. 87I (1,428 bp; deposited in the EMBL database under accession number AM903377) had 95% nucleotide base homology with *B. mycoides*, and *Bacillus* sp. 112A (1,512 bp; deposited in the EMBL database under accession number AM900681) had only 94% homology with *B. flexus*.

A phylogenetic tree (Fig. 1) demonstrated that the isolated strains were members of the genus *Bacillus* and formed a monophyletic lineage. Sequence-similarity calculations after neighbor-joining analysis indicated that the closest relatives of strain 87I were *B. mycoides* (98.1%), *B. thurengenesis* (98.4%), and *B. weihenstephanensis* (98.1%) and those of strain 112A were *B. flexus* (95.4%), *B. megaterium* (94.8%), *B. simplex* (94.8%), and *B. nealsonii* (94.4%). Low *16S* rDNA gene sequence similarity values (<95%) were obtained between the novel strain 112A and all species with valid published names from the genus *Bacillus*. From the phylogenetic analysis, it was clear that, based on good *16S* rDNA (1,512 bp) sequences, the isolate belonged to the genus *Bacillus* and represented a distinct lineage that could be equated with a separate genomic species [17].

Results of biochemical analysis based on mannitol utilization and acid formation [18, 19] were also in agreement with *16S* rRNA analysis that *Bacillus* sp. 87I and *Bacillus* sp. 112A were close to *B. mycoides* and *B. flexus*, respectively. Apart from this, these were Gram-positive, endospore-forming, and catalase-positive organisms (results not shown).

The fatty-acid profile of *Bacillus* sp. 87I was characterized by predominance of C15:0 iso and C16:0, accounting for 23.12% and 19.67% of total acids, respectively, along with C13:0 iso (9.89%). Other fatty acids were present at lower concentrations, i.e., C15:0 anteiso (5.98%), C14:0 (4.21%), C13:0 anteiso (2.0%), C12:0 (1.44%), C12:0 iso (0.86%), C13:1 (0.58%), C16:1 w9c (0.55%), and C 10:0 (0.52%). The fatty-acid profile of *Bacillus* sp. 112A showed predominance of C 15:0 iso and C 15:0 anteiso, accounting for 32.57% and 29.91% of total acids, respectively, along with C16:0 (10.02%). Other fatty acids were present at lower concentrations.

Since the *Bacillus* sp. 87I fatty-acid profile showed variation from its close neighbor (Table 1), it could be a potent new strain or species of the genera *Bacillus*; further studies are needed to ascertain its position. On the other hand, the results of *Bacillus* sp. 112A *16S* rDNA sequence and also the variability in its fatty-acid profile (Table 1) make the isolate a unique strain of *Bacillus* sp., which also needs further investigation to ascertain its position.

Growth pattern of the selected isolates

Results showed that there was a long lag phase up to 4 and 8 h for 112A and 87I, respectively, whereas log phase was up to 28 h, followed by stationary phase (Fig. 2).

Effect of time on PHB production

In the time course of PHB production, maximum PHB accumulation was recorded at around 32 and 36 h for 87I and 112A, respectively (Fig. 3). Accumulation of the polymer begins in the late log phase of growth and becomes maximum during the late stationary phase of growth; it is

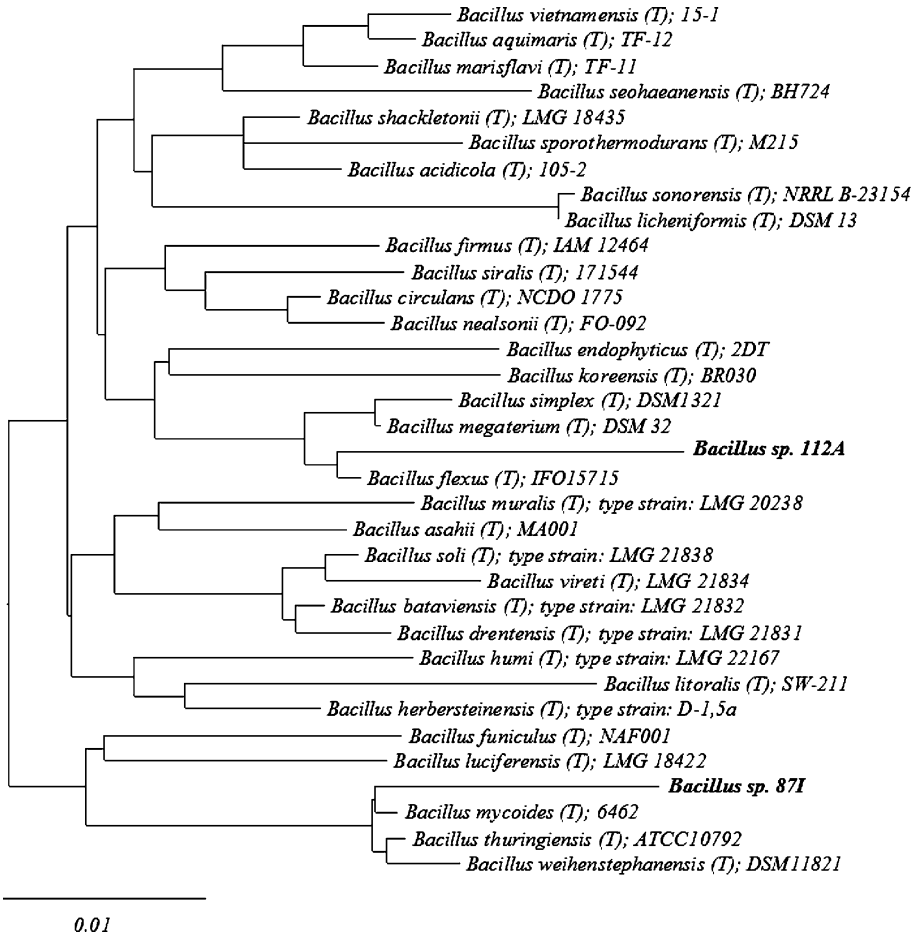

Fig. 1 Unrooted phylogram obtained by neighbor-joining of the *16S* rDNA sequence from the *Bacillus* spp. type strains obtained from the GenBank with *Bacillus* sp. 87I and *Bacillus* sp. 112A

therefore important to harvest cells at the optimum time (late stationary phase) to obtain a maximum yield of PHB.

Effect of temperature and pH on PHB production

PHB was accumulated in *Bacillus* sp. 87I and *Bacillus* sp. 112A at various temperatures to estimate PHA productivity (Table 2). To compare PHA productivities, *Bacillus megaterium* MTCC 453 and *Ralstonia eutropha* MTCC 1472 were used; these are the most common PHB-producing bacteria and have been studied in detail. The PHB productivities of *Bacillus* sp. 87I and *Bacillus* sp. 112A were higher than those of *B. megaterium* at all temperatures. Although *B. megaterium* and *R. eutropha* could not accumulate PHB at temperatures above 40°C, strain 112A accumulated PHB even at 45°C. The PHB synthesis-related enzymes of 112A could have moderate thermostability (due to its ability to grow even at 45°C), which is an important feature, useful for industrial application of such strain. Isolate 87I could not grow at 45°C (Table 2).

The two selected isolates grew best at pH 7.2, and hence maximum accumulation of PHB was obtained at pH 7.2. Although the cultures grew at lower and higher pH values, a drastic fall in PHB yield was seen. Lower PHB contents were obtained at pH values of 6.8 and 7.5 (Table 2).

Under limited amounts of nitrogen, potassium, and phosphorous, PHB accumulation of *Bacillus cereus* SPV was reported to improve [22]. A similar and significant increase in the amount of PHB accumulation was seen in the present study in *Bacillus* sp. 87I and *Bacillus* sp. 112A by limiting the nitrogen content to 5 mM, which probably increases the activity of enzymes metabolizing PHB.

PHB production from various carbon substrates

The two isolates (*Bacillus* sp. 87I and *Bacillus* sp. 112A) produced maximum PHB with glucose but exhibited varying specificity in utilizing all other sugars studied. Galactose and starch were not included in the study involving *Bacillus* sp. 87I, whereas lactose was not tested with *Bacillus* sp. 112A. *Bacillus* sp. 87I produced a good PHB yield in lactose, followed by maltose, sucrose, fructose, and mannitol. In the case of *Bacillus* sp. 112A, the highest PHB productivity in glucose was followed by that in starch, sucrose, fructose, galactose, maltose, and mannitol (Table 3).

Table 1 Comparison of fatty-acid content (%) of *Bacillus* sp. 87I and *Bacillus* sp. 112A with their closely related *B. cereus* and *B. flexus*, respectively

Fatty-acid methyl ester	*Bacillus* sp. 87I	*B. cereus*[a]	*Bacillus* sp. 112A	*B. flexus*[a]
10:0	0.52	–	–	–
12:0	1.44	–	0.19	–
12:0 iso	0.86	1.9	–	–
13:0 iso	9.89	10.7	0.54	–
13:0 anteiso	2.00	2.9	0.24	–
13:1	0.58	–	–	–
14:0	4.21	4.8	2.53	–
14:0 iso	–	3.8	3.11	1.6
14:0 anteiso	–	–	0.05	–
15:1 iso G	0.48	19.3	–	–
15:1 iso F	–	5.1	0.08	–
15:0 iso	23.12	–	32.57	32.2
15:0 anteiso	5.98	–	29.91	35.4
16:1 w9c	0.55	1.7	–	–
16:1 w7c OH	–	2.9	0.34	6.4
16:0 iso	–	9.3	2.42	8.6
16:0 anteiso	–	–	0.08	–
16:1 w11c	–	2.9	1.64	–
16:0	19.67	8.1	10.02	1.2
15:0 iso 3OH	0.58	10.4	0.13	–
17:1 iso w10c	–	6.9	0.95	–
17:0 iso	–	5.6	6.00	2.1
17:0 anteiso	–	1.6	5.86	3.4
17:0 cyclo	–	–	0.20	–
17:0	–	–	0.17	–
16:0 3OH	–	–	0.21	–
18:0 iso	–	–	0.17	–
18:1 w9c	–	–	0.13	–

[a] Data from Matsumoto et al. [20] and Suresh et al. [21]

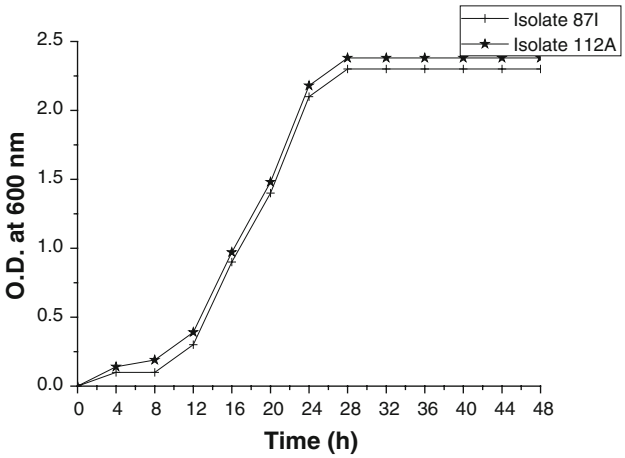

Fig. 2 Growth pattern of the isolates

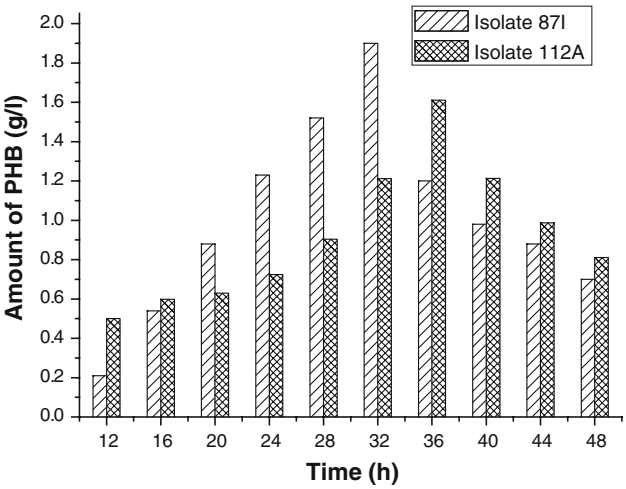

Fig. 3 Effect of time on PHB production

A *Bacillus* strain utilized soya molasses to produce 90% cell dry weight of the polymer without any nutrient limitation [23]. Rohini et al. [24] reported synthesis and accumulation of a higher amount of PHB (64.10%) by *Bacillus thuringiensis* (isolated from natural soils) when grown on glycerol, whereas *Bacillus* sp. 87I produced 70.04% PHB DCW when grown on glucose, higher than all earlier reports with *Bacillus* wild-type strains. *Bacillus* sp. 112A produced 67.63% PHB DCW when grown on glucose as sole carbon source.

Polymer analysis

To study its physical properties, PHB was dissolved in hot chloroform, and the film formed after evaporation of chloroform was used for analysis. PHB with molecular mass around 2×10^5 to 3×10^6 has been reported in the literature for cultures grown on long-chain hydrocarbons, while the molecular mass of the polymers also influences the crystallinity of PHA [25]. *Bacillus* sp. 87I produced polymer with average molecular mass of 513 kDa, whereas, the average molecular mass of the PHB polymer from *Bacillus* sp. 112A was of 521 kDa (Table 4).

The T_m values of the polymers produced by *Bacillus* sp. 87I and *Bacillus* sp. 112A were 152.9°C and 147.7°C, respectively (Table 4). Both polymers were found to be melt-stable; when heated above their T_m to 190°C, quenched to room temperature, and reheated to 190°C, the peak at their T_m was obtained again on second heating, suggesting their stability. However, when heated beyond T_m to 210°C and quenched to 50°C, the peak at T_m was lost on subsequent heating. The percentage crystallinity of the PHB films was studied by X-ray diffraction patterns.

Table 2 PHB produced by *Bacillus* sp. 87I and *Bacillus* sp. 112A, when grown at different incubation temperatures and different pH values

		Bacillus sp. 87I (g/l)	*Bacillus* sp. 112A (g/l)	*B. megaterium* (g/l)	*R. eutropha* (g/l)
Temperature (°C)	25	0.600	0.712	0.517	0.155
	30	1.890	1.579	1.467	1.569
	35	0.570	0.969	0.433	0.673
	40	0.420	0.765	0.324	0.666
	45	0.000	0.515	0.000	0.000
pH	6.8	1.816	1.302	–	–
	7.2	1.917	1.539	–	–
	7.5	1.419	1.246	–	–

Table 3 PHB production by *Bacillus* sp. 87I and *Bacillus* sp. 112A grown on different carbon sources

Carbon source	OD at 600 nm		Biomass content (g/l)		PHB content (g/l)		%PHB (DCW)	
	Bacillus sp. 87I	*Bacillus* sp. 112A	*Bacillus* sp. 87I	*Bacillus* sp. 112A	*Bacillus* sp. 87I	*Bacillus* sp. 112A	*Bacillus* sp. 87I	*Bacillus* sp. 112A
Lactose	1.285	–	1.90	–	1.755	–	55.5	–
Galactose	–	0.950	–	1.02	–	0.879	–	35.50
Fructose	0.665	1.005	0.88	1.25	0.550	0.950	28.1	30.3
Glucose	1.654	1.950	2.28	2.10	1.901	1.827	70.04	67.63
Mannitol	0.545	0.450	0.75	0.75	0.345	0.350	20.3	24.2
Sucrose	0.889	1.659	0.95	1.30	0.780	1.512	30	58.90
Maltose	1.025	0.550	1.67	0.82	1.256	0.390	45.5	25.7
Starch	–	1.725	–	1.85	–	1.689	–	50.04

The percentage crystallinity was calculated from diffraction intensity data according to Vonk's method. Polymer from *Bacillus* sp. 112A was more crystalline (50.17%) than polymer from *Bacillus* sp. 87I (45.8%) (Table 4).

Young's modulus, tensile strength, and elongation to breakage are three important mechanical properties of polymers. The Young's modulus of the polymers from *Bacillus* sp. 87I and *Bacillus* sp. 112A were 5.8 and 3.5 GPa, respectively. The tensile strength of PHB from *Bacillus* sp. 87I was 38 MPa, similar to polypropylene, suggesting the polymer to be as tough as polypropylene. However, the elongation to breakage was found to be very low for the two studied polymers when compared with polypropylene and polyethylene, suggesting their low elasticity (Table 4).

Table 4 Physical and mechanical properties of PHB extracted from *Bacillus* sp. 87I and *Bacillus* sp. 112A grown on glucose as carbon substrate

Properties	*Bacillus* sp. 87I	*Bacillus* sp. 112A
Viscosity average molecular mass (M_v) (kDa)	513	521
Glass-transition temperature (°C) (T_g)	0.8	1.8
Melting temperature (T_m) (°C)	152.9	147.7
Percent crystallinity (%)	45.8	50.17
Young's modulus (GPa)	5.8	3.5
Tensile strength (MPa)	38	15
Elongation to breakage (%)	8	5

IR spectra were recorded for PHB dissolved in chloroform. Spectra of the standard PHB showed two intense absorption bands at 1,724.3 and 1,280.3 cm^{-1}, corresponding to C=O and C–O stretching groups, respectively (Fig. 4). The polymer from *Bacillus* sp. 87I exhibited a sharp peak at 1,714 cm^{-1}, corresponding to C=O stretching. The peak at 1,190 cm^{-1} confirmed C–O stretching in the polymer as ester of HB. The polymer from *Bacillus* sp. 112A showed sharp peaks at 1,722.43 and 1,276.88 cm^{-1}, suggesting it also as PHB. Rohini et al. [24] 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 polymers from both *Bacillus* sp. 87I and *Bacillus* sp. 112A showed similar absorption bands, characteristic of the poly-3-hydroxybutyrate (PHB) polymer. The H^1 NMR scans of the polymers from *Bacillus* sp. 87I and *Bacillus* sp. 112A were recorded. A doublet was recorded at 2.57 ppm, corresponding to the methylene group (–CH_2–), while a multiplet signal at 5.27 ppm corresponded to the methyne group (–CH–) (Fig. 5). Another doublet signal at 1.30 ppm corresponded

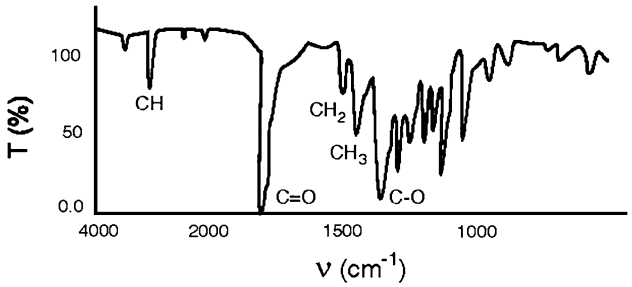

Fig. 4 IR spectrum of the PHB polymer produced by *Bacillus* sp. 87I

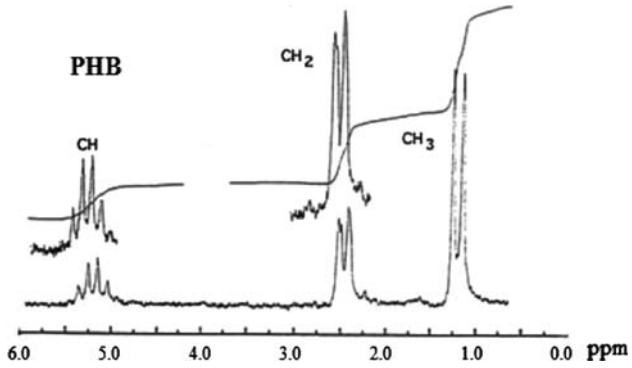

Fig. 5 NMR scan of polymer produced by *Bacillus* sp. 112A

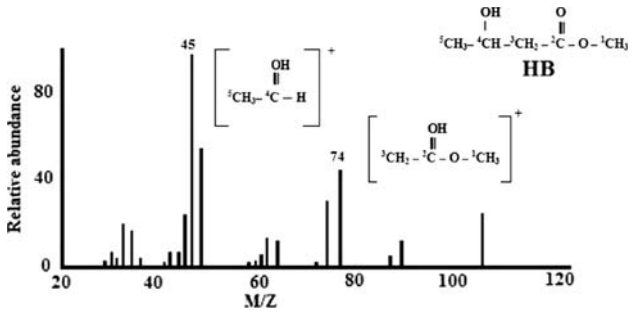

Fig. 6 Mass spectra of methyl ester of 3-hydroxybutyrate (HB) synthesized by *Bacillus* sp. 112A by GC–MS analysis. The numbering of carbon atoms in methyl-3-hydroxyalkanoates is indicated in *superscript*

to the methyl group (–CH_3–). The methyl esters showed a sharp signal around 3.50 ppm, corresponding to the CH_3–O group of the esters. These results suggest the polymers to be poly-3-hydroxybutyrate (PHB).

The methanolysis products of the polymer samples were analyzed using GC–MS. By analyzing the potential fragmentation patterns and the molecular mass of the fragments, the identities of specific peaks in the mass spectra were correlated to the carbonyl and hydroxyl ends of the representative hydroxyalkanoates. The polymer samples from *Bacillus* sp. 87I and *Bacillus* sp. 112A showed two fragments in mass spectrum at 45 and 74 *m/z* (Fig. 6). The peak at 45 *m/z* represented the hydroxyl end of the molecule, originating from cleavage of the bond between C_3 and C_4. The peak at 74 *m/z* represented the carbonyl end of the molecule, originating from McLafferty rearrangement [26] after cleavage of the bond between C_3 and C_4.

Discussion

A number of *Bacillus* spp. have been reported to accumulate 9–44.5% DCW PHB [27]. By comparison, *Bacillus* sp. 87I produced as little as 20% DCW PHB and as much as 70.4% DCW PHB from mannitol and glucose, respectively. Similarly, *Bacillus* sp. 112A produced a minimum of 24.2% DCW PHB from mannitol and a maximum of 67.63% DCW PHB from glucose. A relatively high yield of PHB was obtained in these wild strains, hence these strains were considered as potent organisms for industrial application.

Morphological results (not shown) and phylogenetic analyses clearly demonstrated that strains 87I and 112A 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* rDNA demonstrated that these bacteria, grouped with *B. mycoides* (*B. cereus*) and *B. flexus*, are well-defined taxa. Further characterizations of these bacteria are necessary before proposing them as novel species.

Carbon sources serve three different functions within the organisms: biomass synthesis, cell maintenance, and PHA polymerization. Among the carbon sources tested with the strains 87I and 112A, glucose was found to be the preferred substrate for polymer accumulation. However, strain 87I also showed good polymer yield (55.5% DCW PHB) with lactose. On the other hand, strain 112A yielded 58.9% DCW PHB with sucrose and also a significant amount (50% DCW PHB) with starch as the carbon source. This study was important, since it yielded two strains of *Bacillus* sp. that were capable of producing polymer not only from glucose but also from carbon substrates such as lactose, sucrose, and starch.

In further studies, these strains of *Bacillus* spp. could be effectively utilized to produce PHB from substrates such as whey and starch-containing material.

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