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ORIGINAL PAPER

A novel *Bacillus* sp. accumulating poly (3-hydroxybutyrate-co-3-hydroxyvalerate) from a single carbon substrate

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Abstract The objective of this paper was to report a bacterium designated as 88D, capable of producing poly (3-hydroxybutyrate-co-3-hydroxyvalerate) [P (3HB-co-3HV)] copolymer from a single carbon source, which was isolated from a municipal sewage treatment plant in Hyderabad, India. This microorganism, based on the phenotypical features and genotypic investigations, was identified as *Bacillus* sp. The optimal growth of *Bacillus* sp. 88D occurred between 28 and 30°C and at pH 7. The strain yielded a maximum of 64.62% dry cell weight (DCW) polymer in the medium containing glucose as carbon source, which was followed by 60.46% DCW polymer in glycerol containing medium. *Bacillus* sp. 88D produced P (3HB-co-3HV) from glucose or glycerol, when they were used as a single carbon substrate. This bacterium produced polyhydrxybutyrate (PHB) when sodium acetate was used as sole carbon substrate. The viscosity average molecular mass (Mv) of the copolymers ranged from 523 to 627 kDa. The physical, chemical and mechanical properties of the biopolymers were characterized.

Keywords *Bacillus* sp. · Biopolymer · Glucose · Glycerol · PHA · P (3HB-co-3HV) · Polyesters · Production

Introduction

Polyhydroxyalkanoates (PHAs) comprise a large class of polyesters that are accumulated in a wide variety of bacteria as carbon and energy storage material. The polymers are deposited as intracellular granules and sometimes amount to up to 90% of the dry cell weight [1, 2] when the bacteria are cultivated in the presence of excess carbon and limitation of any one of the growth nutrients. Owing to their thermoplastic properties and biodegradability, PHAs have attracted industrial interest and have been extensively studied recently. In addition to being potential substitutes for petrochemical plastics, PHAs are synthesized from renewable carbon sources and represent a new way of utilization of cheap carbon substrates from industrial wastes. PHAs can be divided into three groups depending on the number of carbon atoms in the monomeric units: short chain length PHA (3–5 carbon atoms, scl-PHA), medium chain length (6–15 carbon atoms, mcl-PHA) and long chain length (more than 15 atoms, lcl-PHA). The chemical composition of PHAs depends both on the bacterial strain and carbon source applied [2]. Hence, investigation for new bacteria producing PHAs could yield new polymers with different monomeric units. Bacterial PHAs have similar properties to those of petrochemical plastics, including material properties that resemble polypropylene. Polyhydroxybutyrate (PHB), a short chain length PHA, is found in most of the prokaryotes as storage material. However, PHB is rigid and fragile [3]; therefore, it is not a suitable polymer material. In contrast, the physical properties of poly (3-hydroxybutyrate-co-3-hydroxyvalerate) [P (3HB-co-3HV)] copolymer are superior to those of PHB [3, 4], and these polymers are commercially available. It has been shown that the monomer compositions of copolymers are affected by genes that encode proteins supplying the monomer units and by the substrate specifications of the PHA synthases from various microorganisms [5, 6]. Most bacteria that accumulate PHA can also produce different types of polyhydroxybutyrate copolymers [7, 8].

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Materials and methods

Bacterial strain

Different samples from the municipal sewage treatment plant at Hyderabad, India, were screened for PHA-producing bacteria and have been described elsewhere [9]. Bacterial strain 88D was isolated on Luria Bertani (LB) medium (Hi media, India).

Characterization of the strain 88D

Morphological, growth and biochemical studies were performed using standard methods [10, 11]. Nutrient agar was used for growth, maintenance of the strain and the determination of the phenotypic characteristics. The isolate 88D was characterized by its growth at various temperatures (5, 30, 45 and 60°C), tolerance at different salt levels (2, 4 and 10 g NaCl/100 ml) and reduction of nitrate. In addition, esculin, hippurate, casein and starch hydrolysis were examined. The production of acid from glucose, galactose, mannose, lactose, raffinose, xylose, cellobiose, fructose and salicin was tested. Along with lecithinase, lipase, gelatinase, urease, oxidase, VP test, utilization of citrate, succinate and anaerobic growth of the isolate were also performed. Also, the antibiotic sensitivity of the isolate 88D was tested. For phylogenetic characterization, the 16S rRNA gene was amplified [12], and the PCR product was purified using the QIA quick PCR purification kit (Qiagen). Sequencing was performed by using ABI PRISM model 3700 automatic DNA sequencer and the Big Dye Terminator cycle sequencing kit (both from Applied Biosystems). The 16S rRNA gene sequence (1,282 bp) was submitted to the RDP website, aligned and used to build a phylogenetic tree of the isolate 88D by neighbor-joining method using the tree builder tool.

Transmission electron microscopy (TEM)

The sample preparation for TEM was done as described by Quillaguam et al. [13]. The cells on attaining maximum PHB content were separated by centrifugation at 8,000*g* for 13 min and fixed at room temperature in a solution of 4% (v/v) glutaraldehyde in 0.1 M sodium cacodylate, pH 7.1 and 0.1% (w/v) Brij 35, followed by an overnight treatment in the same solution without Brij 35. The cells were then rinsed with 0.1 M sodium cacodylate, pH 7.1, transferred to 2% osmium tetroxide for 8 h at room temperature and subsequently to 2% uranyl acetate in 10% ethanol for 40 min. The cells were dehydrated through a graded series of ethanol–water solutions with a final treatment of propylene oxide and embedded in epon/araldite resin, which was then cut with a diamond knife. The fine sections of 50 nm were placed on Formvar-coated copper grids, contrasted with a 2% aqueous solution of uranyl acetate and examined under a transmission electron microscope, TEM (Hitachi).

Growth medium and culture conditions

E2 medium [14] was used for PHB production. The composition of the medium was (gl^{-1}): microcosmic salt ($NaNH_4HPO_4$) 3.5; $K_2HPO_4.3H_2O$ 7.5; KH_2PO_4 3.7; $MgSO_4.7H_2O$ (100 mM) 10 ml; mineral trace (MT)–microelement stock 1 ml; yeast extract 0.004; carbon source (w/v) 2% (final concentration); distilled water 989 ml; pH 7.2. The MT microelement stock solution contained (gl^{-1}): $FeSO_4.7H_2O$ 2.78; $MnCl_2.4H_2O$ 1.98; $CoSO_4.7H_2O$ 2.81; $CaCl_2.2H_2O$ 1.47; $CuCl_2.2H_2O$ 0.17; $ZnSO_4.7H_2O$ 0.29. Sugars and mineral salts solutions were autoclaved separately at 121°C for 15 min.

Extraction of PHA from the isolate

PHA was extracted from the isolate by using the hypochlorite method [15]. For this, the isolate was grown in 250 ml Erlenmeyer flasks containing 50 ml E2 mineral medium with different carbon sources. These flasks were incubated at 28°C for 48 h with an environmental shaker at 150*g*. After incubation, cell suspension (10 ml) was centrifuged at 6,000*g* for 10 min. The cell pellet was washed once with 10 ml saline and was recentrifuged to get the pellet. The cell pellet was then suspended in 5 ml sodium hypochlorite and incubated at 37°C for 10 min with stirring. This extract was centrifuged at 8,000*g* for 20 min, and the pellet of PHA was washed with cold diethyl ether. The pellet was again centrifuged at 8,000*g* to get purified PHA.

Analytical methods

The fermentation broth sample was used for the determination of the 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 it reached a constant weight. The residual mass was defined as total DCW minus PHB weight; PHB (%) was defined as the percentage of the ratio of PHB to DCW.

Characterization of the produced polymers

The polyester content of the cell and the composition of polyesters were determined by using the gas chromatography–mass spectrometry (GC–MS) analysis. The structure and mole fractions of PHA units in the polymer samples were investigated by the 400 MHz H^1 nuclear magnetic resonance (NMR) spectra recorded at 27°C in a $CDCl_3$ solution of polyester. The average molecular weights of the

polymers were estimated by the inherent viscosity method. The crystallinity of the polymers was determined by X-ray diffraction (XRD) studies. Infrared spectra (IR) and differential scanning calorimeter (DSC) scans were recorded for the polymers [16].

Results

Morphology

Strain 88D, deposited as MTCC 9592, was a motile, mesophilic, aerobic, gram-positive rod. Microscopic observation performed on cells during the accumulation of PHA clearly showed the existence of intracellular PHA granules (Fig. 1). The presence of endospores and the positive catalase activity of the bacterium indicate that the bacterium could be a *Bacillus* sp.

Metabolic properties

The morphological, cultural and biochemical characteristics of the strain 88D are presented in Table 1. The results obtained from these tests were compared with the standard taxonomic descriptions [17], and the isolate was identified.

Phylogenetic analysis

The sequence of the 16S rDNA gene of strain 88D was determined (1,282 bp) and deposited in the Genbank sequence database under accession no. EF688467. A phylogenetic tree (Fig. 2) demonstrated that the isolated strain was a member of the genus *Bacillus*, and it formed a monophyletic lineage. Sequence similarity calculations after a neighbor-joining analysis indicated that the closest relatives of strain 88D were

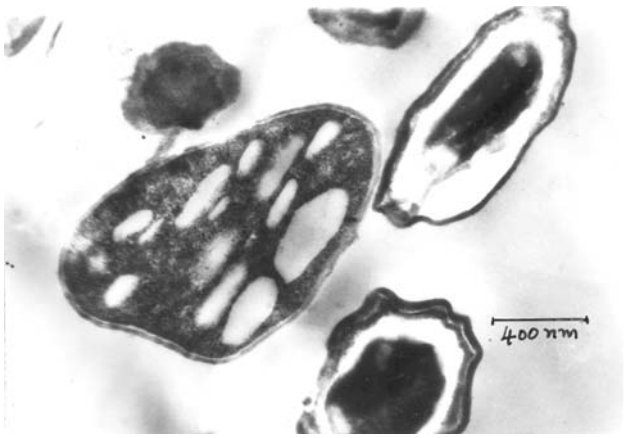

Fig. 1 TEM of *Bacillus* sp. 88D showing PHB accumulation as internal granules

Table 1 Morphological, cultural and biochemical properties of *Bacillus* sp. 88D

Characteristic	*Bacillus* sp. 88D
Morphology	
Cell shape	Rod
Cell size	1.0–1.2 × 3.0–5.0 μm
Motility	+
Endospore	+
Spore position	Terminal
Spore shape	Oval
Gram staining	+
Cultural characteristics	
Colony shape	Round
Optimum temperature	28–30°C
Optimum pH	7.0
Growth on nutrient agar	+
Growth in NaCl 2, 4 and 10%	−, −, −
Growth at 5, 30, 45 and 60°C	−, +, −, −
Biochemical properties	
Voges–Proskauer	−
Nitrate reduction	+
Casein hydrolysis	+
Catalase	+
Oxidase	+
Urease production	+
Hippurate hydrolysis	−
Esculin hydrolysis	−
Starch hydrolysis	+
Anaerobic growth	−
Citrate utilization	+
Succinate utilization	+
Lecithinase	−
Gelatinase	−
Lipase	−
Acid production from	
Glucose	+
Galactose	+
Fructose	+
Mannose	−
Lactose	+
Raffinose	+
Xylose	−
Cellobiose	−
Salicin	−
Antibiotic sensitivity	
Polymyxin	+
Chloramphenicol	+
Nalidixic acid	+
Streptomycin	+

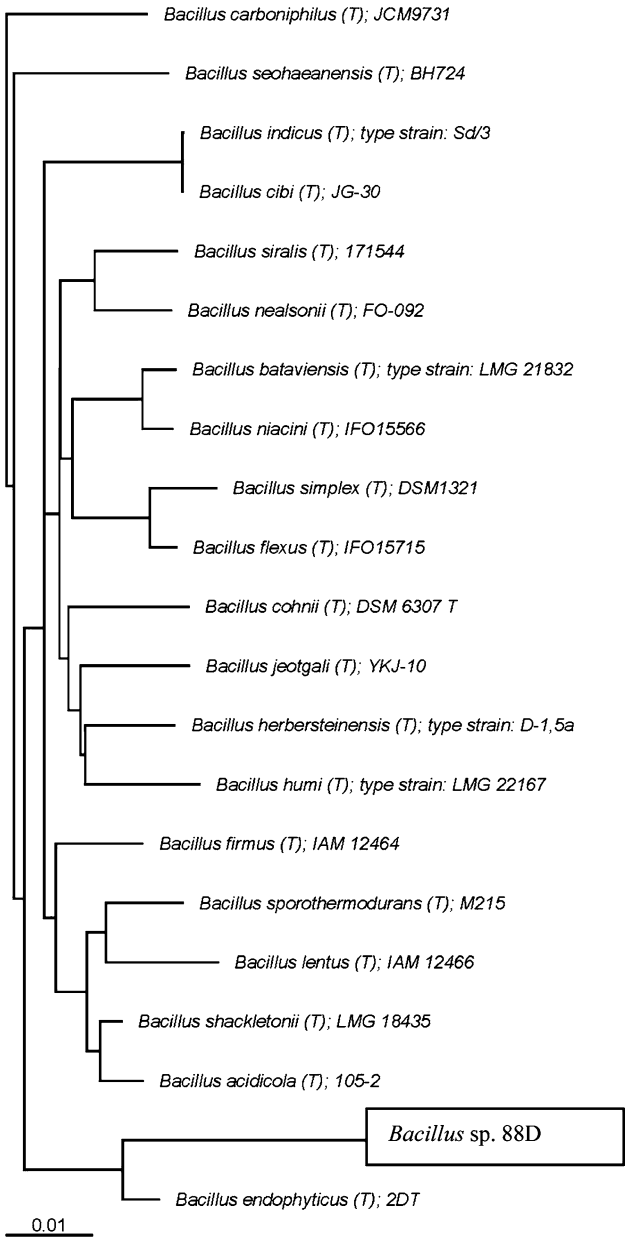

Fig. 2 Phylogenetic tree of *Bacillus* sp. 88D

B. endophyticus (95%), *B. nealsonii* (93%) and *B. flexus* (92%). Low 16S rDNA gene sequence similarity values (<95%) were obtained between the novel strain and all species with valid published names from the genus *Bacillus*. From the phylogenetic analysis, it was clear that based on a good 16S rDNA (1,282 bp) sequence, the isolate belonged to the genus *Bacillus* and represented a distinct lineage that could be equated with a separate genomic species [18].

Production of PHA

During the batch fermentation of strain 88D, the production of PHA began after 18 h of incubation in glucose, glycerol or sodium acetate enriched E2 medium and ended after 2 days. Under these conditions, the yield of PHA was 1.64, 1.51 and 1.01 g l^{-1}, respectively.

Characterization of PHA

The H^1 NMR spectra of polymers in $CDCl_3$ solution were obtained. The methyl protons ($-CH_3$) appear to have a double resonance at 1.274 ppm, methylene protons ($-CH_2$) appear to have a multiplet resonance at 2.520 ppm, and methine proton ($-CH$) of bacterial polyhydroxybutyrate also has a multiplet resonance at 5.260 ppm. The H^1 NMR spectra of polymer produced by the bacterium, when grown on glucose or glycerol as the sole source of carbon, implied that the polymers contained the two monomeric units, HB and HV (Fig. 3). The H^1 NMR spectrum resonances were determined for P (HB-co-HV) at 0.894, 1.250, 1.620, 2.650 and 5.250 ppm for $-CH_3$ (HV side group), $-CH_3$ (HB side group), $-CH_2$ (HV side group), $-CH_2$ (HV and HB bulk structure) and $-CH$ (HV and HB bulk structure) groups, respectively. The mole fractions of the monomeric units were determined from the intensity ratio of doublet CH_3-proton resonance 1 at 1.274 ppm to the triplet CH_3-proton resonance 5 at 0.894 ppm in the H^1 NMR spectra [19].

The monomeric compositions of the polymers produced by the strain when grown on different carbon sources are shown in Table 2. The PHA content and its composition are influenced mainly by the strain of microorganism, the type of substrate employed, its concentration and environmental growth conditions [2].

By analyzing the potential fragmentation patterns and the molecular weight of the fragments, the identities of specific peaks in the mass spectra were correlated to the carbonyl and hydroxyl ends of the representative hydroxyalkanoates. In the mass spectrum of methyl 3-hydroxybutyrate, the peak at *m/z* 45 represented the hydroxyl end of

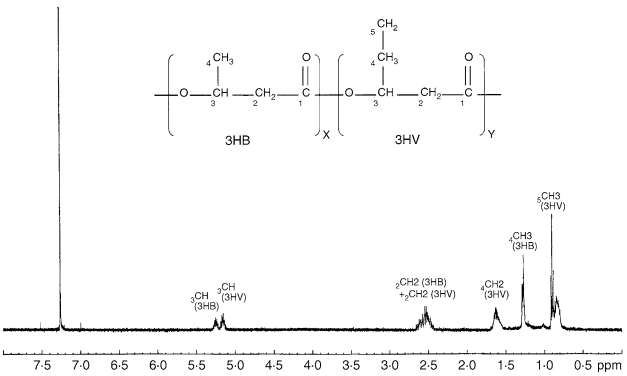

Fig. 3 H^1 NMR spectrum of P (3HB-co-3HV) polymer produced by *Bacillus* sp. 88D, when grown on glucose or glycerol

Table 2 Monomer compositions of the polymers produced by *Bacillus* sp. 88D, grown on different substrates, as determined by H^1 NMR spectra

Substrate	Monomer composition		PHB (%)
	3HB (%)	3HV (%)	DCW
Glucose	96	4	64.62
Glucose + P	87	13	59.82
Glycerol	85	15	60.46
Glycerol + P	96	4	55.90
Na-acetate	100	0	48.11
Na-acetate + P	93.7	6.3	42.03

P propionic acid

the molecule. It originated from cleavage of the bond between C_3 and C_4. The peak at *m/z* 74 represented the carbonyl end of the molecule, which originated from McLafferty rearrangement [20] after cleavage of the bond between C_3 and C_4. In the mass spectrum of methyl 3-hydroxyvalerate, the peak at *m/z* 74 also originated from the carbonyl end of the molecule due to McLafferty rearrangement. The peak at *m/z* 59 represented the hydroxyl end released when the molecule was cleaved at the bond between C_3 and C_4 (Fig. 4).

IR spectra were recorded for the polymers dissolved in chloroform. Spectra 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. Other absorption bands at 1,380, 1,455, 2,930 and 3,750 cm^{-1} corresponding to CH_3, $-CH_2$, $-CH$ and O–H groups are shown in Fig. 5.

The average molecular weights of the polymer extracted from the isolate grown on different substrates are reported in Table 3. The average molecular weights for the PHA polymers from isolate 88D were in the range of 523–627 kDa.

The temperature at which the peak is obtained is considered as the temperature of melting (T_m); if two peaks were obtained, the higher temperature value was considered as the T_m. The T_m and glass transition temperature (T_g) values for the polymers are reported in the Table 3. The T_m values of the polymer ranged between 152.9 and 176.32°C. All the

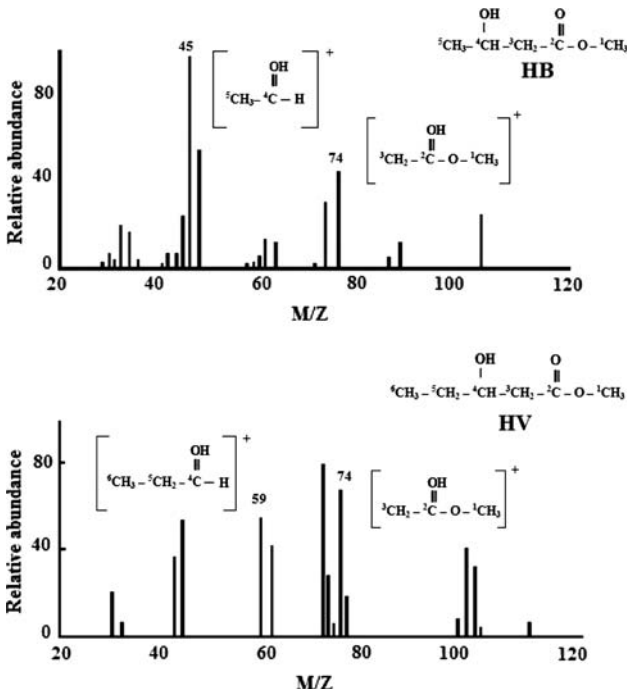

Fig. 4 Mass spectra of methyl ester of 3-hydroxybutyrate (HB) and methyl ester of 3-hydroxyvalerate in a GC-MS analysis of polymer synthesized by *Bacillus* sp. 88D. The numbering of carbon atoms in methyl 3-hydroxyalkanoates is in superscript

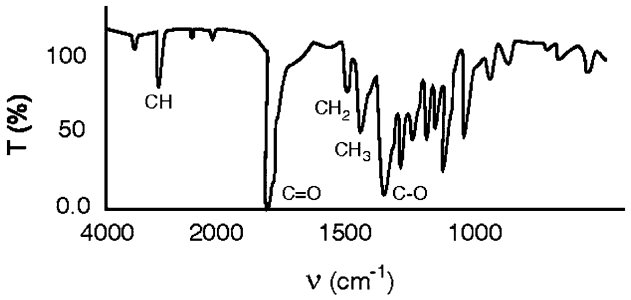

Fig. 5 IR spectrum of the PHB polymer synthesized by *Bacillus* sp. 88D, when grown on sodium acetate as carbon substrate

polymers were found to be melt stable when heated above their T_m till 180°C, quenched to room temperature and reheated till 180°C. The peak at their T_m was reobtained on

Table 3 Physical properties of the polymers extracted from *Bacillus* sp. 88D, when grown on different substrates

Carbon substrate	Viscosity average molecular mass (M_v) (kDa)	Glass transition temperature (°C) (T_g)	Temperature of melting (T_m) (°C)	Percent crystallinity (%)
Glucose	540	8.11	166.12	35.76
Glucose + P	627	8.70	176.32	40.96
Glycerol	610	8.97	170.9	44.09
Glycerol +P	546	8.61	162.36	42.11
Na-acetate	523	8.0	152.9	36.89
Na-acetate + P	560	8.19	158.9	36.01

P propionic acid

Table 4 Mechanical properties of the polymers determined using universal testing machine

Carbon substrate (isolate)	Young's modulus (GPa)	Tensile strength (MPa)	Elongation to break (%)
Glucose (88D)	1.3	35	45
Glycerol (88D)	1.7	37	69
Na-acetate (88D)	1.1	31	22
Polypropylene[a]	2	38	140
Polyethylene[a]	0.3	NA	700

NA not available

[a] Reported by [2, 21]

second heating, suggesting their stability. When heated beyond the T_m till 210°C and quenched to 50°C, the peak at T_m was lost on subsequent heating.

The percentage of crystallinity of the PHA films was studied by X-ray diffraction patterns. The percentage crystallinity calculated from diffracted intensity data according to Vonk's method is presented in Table 3. Polymer from *Bacillus* sp. 88D grown on glycerol as sole carbon substrate was the most crystalline with a value of 44.09% (Table 3).

Young's modulus, tensile strength and elongation to break were the three important mechanical properties tested for the polymers (Table 4). The tensile strength of the polymers from the isolate grown on glucose and glycerol, individually, were 35 and 37 MPa, respectively. These results suggest that the toughness of these polymers was very similar to that of polypropylene (38 MPa). However, the elongation to break was found to be very low for all the polymers studied as compared with polypropylene and polyethylene, suggesting their low elasticity.

Discussion

Morphological and phylogenetic analyses have clearly demonstrated that strain 88D is a member of the genus *Bacillus.* The optimal temperature for growth was between 28 and 30°C, and the optimal pH was 7.0. Phylogenetic analysis of 16S rDNA demonstrated that this bacterium is grouped with *B. endophyticus*, a well-defined taxon. Further characterizations of the bacterium are necessary before proposing it as a 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 strain 88D, glucose and glycerol were found to be the choice of substrates for the polymer accumulation. Also, these carbon substrates yielded hydroxyvalerate (HV) copolymer of PHB as confirmed by H^1 NMR and GC–mass spectra. This is a significant result because there have been only a few reports of P (3HB-co-3HV) synthesis from a single carbon source [22]. Composition of the carbon substrate used for fermentation and utilization of appropriate bacterial strain control the production of copolymers of PHB [23]. When tested in combination with propionic acid, the precursor for 3HV copolymer accumulation, at a concentration of 2 ml/l, *Bacillus* sp. 88D produced polymer containing 13% HV copolymer in glucose-containing medium. In a medium with glycerol and propionic acid, the isolate 88D produced a copolymer with meager HV (4%). Though the actual reason for this decrease in the HV content of the copolymer even in presence of the precursor propionic acid is not understood, one suggested reason could be the propionic acid itself, which has shown some toxic and inhibitory effect in different strains of bacteria [24]. However, the strain, *Bacillus* sp. 88D could be used effectively to produce P (3HB-co-3HV) (15% HV), with glycerol as sole carbon source. This study was important, since it yielded a new strain of *Bacillus* sp., capable of producing copolymer from glucose and glycerol, individually, when these were used as sole carbon substrates. Therefore, *Bacillus* sp. 88D could be an interesting candidate for industrial production of biopolymers with different monomeric units.

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