Journal of Environmental Chemical Engineering 5 (2017) 1–9

Contents lists available at ScienceDirect

Journal of Environmental Chemical Engineering

journal homepage: www.elsevier.com/locate/jece

Bioconversion of glycerol to poly(HB-co-HV) copolymer in an inexpensive medium by a *Bacillus megaterium* strain isolated from marine sediments

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ARTICLE INFO

Article history:
Received 1 August 2016
Received in revised form 8 November 2016
Accepted 9 November 2016
Available online 10 November 2016

Keywords:
Poly(hydroxybutyrate-co-hydroxyvalerate)
Biocompatible polymers
Inexpensive media
Glycerol
Urea
Bacillus megaterium

ABSTRACT

A *Bacillus megaterium* strain, as an alternative to produce biopolymers from an industrial waste in a low cost medium, is studied. A culture medium composed by salt solution, trace elements, glycerol and urea was used. The growth of *B. megaterium* BBST4 and the production of PHA achieved by different concentrations of glycerol and urea, were analyzed at various C/N ratios. The effect of temperature, the incubation time and the addition of yeast extract, on the growth, PHA production and sporulation, were determined. The strain showed better growth and PHA production (30% w/w) after 48 h of incubation at 30 °C in a SWG medium with 3% of glycerol, 0.08% of urea, and 0.005% of yeast extract. Under these conditions no sporulating cells were observed. The obtained PHA was characterized by FTIR, NMR, DSC, SEC and GC–MS. It was determined that the biopolymer produced by *B. megaterium* BBST4 is a copolymer, composed of 3-hydroxybutyrate (92.2 mol%) and 3-hydroxyvalerate (7.8 mol%). This copolymer offer industrial and biomedical applications.

1. Introduction

Poly(hydroxyalkanoate)s (PHAs) are biodegradable polymers which are accumulated as carbon/energy source in several bacteria [1]. This family of biopolymers exhibit similar properties to non-biodegradable petrochemical-based polymers such as polyethylene or polypropylene. PHAs are synthesized and accumulated as intracytoplasmic granules. These polyesters can be employed either for packaging and coating materials, or as biodegradable carriers for controlled delivery of drugs, agrochemicals, etc. [2]. They can also have applications in both conventional medical devices and tissue engineering [3].

PHAs show in most cases excellent biodegradability, biocompatibility and mechanical properties directly related to the chemistry of the monomer units [4]. Incorporation of hydroxyvalerate units improves processability, impact strength and flexibility, while resulting in loss of stiffness as well as lower thermal stability and reduced barrier properties [5].

Despite PHAs friendly environmental properties, and that they have been recognized as good candidates to replace conventional petrochemical plastics, their high production costs have limited their use in a wide range of applications. A significant part of these costs is ascribed to the raw materials, in which the carbon source could sum up to 50% of the total expenses. The search of a more favorable economic equation enhance the interest in developing production processes based on cheap carbon sources, preferably from industrial by-products and wastes [6,7].

Several Gram negative bacteria have been employed for the efficient production of PHA [8]. However these bacteria can release endotoxins (pyrogen) which are present in lipopolysaccharides, a component of the outer cell wall. The endotoxins induce fever if they get into the bloodstream of humans or other animals [9–11]. Therefore, in order to use PHAs produced for Gram negative bacteria in biomedical applications, an extra separation stage should be carried out for removing the endotoxins [11]. This problem can be avoided if PHAs are obtained from Gram positive bacteria. It is well known their capacity to produce PHAs, especially

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http://dx.doi.org/10.1016/j.jece.2016.11.012
2213-3437/

those belonging to the genus *Bacillus* [12–15]. It has been reported that PHA contents in several *Bacillus* spp. strains were less than 41% in biomass (based on dry cell weights) [16]. Despite this, microorganisms from genus *Bacillus* possess many favorable characteristics which justify their use. They tolerate adverse environmental conditions, several *Bacillus* spp. strains exhibit low nutritional requirements, and can also produce numerous exoenzymes as proteases, amylases, xylanases, and surfactants. All these give them the possibility to utilize a wide range of substrates as carbon sources [17–19], among which glycerol has proved to be one of the most promising for PHA production in *Bacillus* spp. and other bacterial strains [1,20–32]. Glycerol is the major by-product generated in biodiesel industry, along with a few contaminants in minor quantities [20]. It is widely used in different products and industries, directly and without chemical modifications in most cases. However, these markets only consume a limited amount of glycerol. To support the sustained growth of the biodiesel industry new markets for the use of glycerol should be developed [33]. The present studies contribute to this aim by using glycerol as the sole carbon and energy source, as a part of low cost fermentation media developed for PHA copolymer production by a *Bacillus megaterium* strain. The Gram positive bacteria used in this work was isolated from coastal sediments of Bahía Blanca estuary (Argentine). Finally, characterization studies of the obtained PHA were performed.

2. Materials and methods

2.1. Microorganism and culture media

The bacterial strain used in the present work to produce PHA, was isolated from sediments of the Bahía Blanca Estuary (located at the Atlantic coast of Argentine, between 38° 42′ and 39° 26′ S and 62° 28′ and 61° 40′ W), in a previous work performed in our laboratory [34]. According to biochemical tests and the 16S rDNA analysis, the strain was a *Bacillus megaterium* (GenBank: accession number HM119600.1), and it was named *Bacillus megaterium* BBST4. The strain was stored at −72 °C in Trypticase Soy Broth (Britania B0210206, Argentine) with 20% glycerol.

Culture media used in this study were: a Winogradsky's mineral salt solution (WSG) containing NaCl, 2.5 g/L; K_2HPO_4, 2.5 g/L; KH_2PO_4, 2.5 g/L; $MgSO_4{\cdot}7H_2O$, 2.5 g/L; $MnSO_4{\cdot}4H_2O$, 0.05 g/L; $FeSO_4{\cdot}7H_2O$, 0.5 g/L [35] amended with 1 mL of trace element solution, containing $ZnSO_4{\cdot}7H_2O$, 2.25 g/L; $CuSO_4{\cdot}5H_2O$, 1 g/L; $CaCl_2{\cdot}2H_2O$, 2 g/L; H_3BO_4, 0.20 g/L, with a final pH of 8 adjusted with NaOH 1 N solution. The salt solution was supplemented with glycerol (Anedra, Argentine) and urea (Anedra, Argentine). WSG amended with ultrapure agar–agar (Merck, Germany), 12 g/L (**AWSG**) and Plate Count Agar, **PCA**, (Britania, Argentine), were used as solid mediums. All media and solutions were prepared with bi-distilled water. Winogradsky's salt solution and glycerol were autoclaved at 1 atm for 15 min. Trace element and urea solutions were sterilized by filtration through a membrane with 0.22 μm of pore (Merck-Millipore, Brazil) and added aseptically to the medium. The incubations in liquid medium were performed in a Gerhardt SW 20 (Denmark) shaking thermostatic bath.

2.2. Assay of culture conditions

For all the experiments, *B. megaterium* BBST4 from −72 °C stock was recovered in Trypticase Soy Broth and incubated at 30 °C in an orbital shaker for 24 h. Then, an aliquot was taken with sterile loop and was transferred to AWSG (3% glycerol and 0.1% urea), and the plates were incubated for 48 h at 30 °C. One colony was picked and transferred to a 100 mL flask with 25 mL of WSG (with 3% glycerol and 0.1% urea). The flask was incubated at 30 °C and 150 rpm for 48 h. The culture was harvested by centrifugation (ROLCO CM 4080, Argentine) for 15 min at 2000 g, washed twice with saline solution (NaCl 0.85% w/v), and finally suspended in the same solution. Optical density of suspension was adjusted to an absorbance of 0.20 at 550 nm (OD550) [10^8 colony forming units/mL (CFU/mL)] using a spectrophotometer (Thermo Spectronic Genesys 20, Thermo Electron Corporation, MA, USA). The resulting cell suspension was used as inoculum for the experiments.

Sterile Erlenmeyer flasks of 250 mL were prepared with 100 mL of WSG and different concentrations of glycerol and urea. Different media assayed were the following: I- WSG amended with 0.1% (w/v) urea and three different concentrations of glycerol (v/v) (2%, 3%, and 4%). II- WSG amended with 3% glycerol and different concentrations of urea (0.05%, 0.08%, 0.1%, and 0.15%). Each media was assayed in triplicate. Each flask was inoculated with 1 mL of the cell suspension prepared from the pre-culture described above, to obtain a final bacterial density of 10^6 CFU/mL. These experiments were carried out in a shaking incubator at 30 °C and 150 rpm for 30 h. Growth was monitored by absorbance (OD_{550}) and viable count (in PCA) from broth samples over time. The specific growth rate (μ) was calculated as:

$$\boldsymbol{\mu} = \frac{\ln N - \ln \boldsymbol{N}_0}{\boldsymbol{t} - \boldsymbol{t}_0}$$

Where N and N_0 are the viable count at t and t_0, where t and t_0 are the final and initial times of exponential growth phase, respectively.

Furthermore, 20 μL of the culture were employed to determine the percentage of cells with PHA granules, by means of light microscopy (Zeiss, Primo Star Iled, Germany), after a Sudan Black staining [36,37]. The percentage of sporulating cells was determined by the Schaeffer-Fulton method for staining the endospores [38]. The stains were observed using a 100× oil-immersion objective. At the end of the experiments the remaining volume from each flask was centrifuged at 2000 g for 15 min to harvest the cells. The pellet was washed twice with distilled water and lyophilized (RIFICOR L-A-B3-C, with a WELCH 1402 vacuum pump). Subsequently dry cell weight (DCW) was determined in an analytical balance (Mettler AE 163, Mettler-Toledo Ltd, Leicester, UK). The resulting lyophilized biomass was used for PHA extraction as it is described in 2.5.

2.3. Effects of temperature and incubation time on PHA production

Bacillus megaterium BBST4 growth and PHA production were tested at 20 °C, 30 °C and 37 °C in WSG (3% glycerol and 0.08% urea) in a shaking incubator at 150 rpm. The inoculum was obtained as it was described before. At 18, 30, 40, 48 and 60 h of incubation, three flasks per each temperature assayed were removed from the incubator. The entire volume from each flask was centrifuged at 2000 *g* for 15 min. The pellet was washed twice with distilled water and lyophilized. Dry cell weight (DCW) and PHA content were determined (as described in 2.2 and 2.5, respectively). In addition, 20 μL of each culture was collected at each time, to determine the percentage of cells with PHA granules and the percentage of sporulating cells after Sudan Black and Schaeffer-Fulton staining, respectively.

2.4. Effect of addition of yeast extract

The effect of adding yeast extract in low concentrations to the WSG formulation that gave better results in the experiment described in 2.2, was tested. Two concentrations of yeast extract

Table 1
Bacillus megaterium BBST4 cultured in a minimal salt medium supplemented with different concentrations of glycerol and urea (WSG).

Culture medium	C:N	Biomass (DWC) ($g L^{-1} \pm SD$)	PHA ($g/L^{-1} \pm SD$)	PHA (% ± SD)
WSG (2% glycerol)*	17.0	1.33 ± 0.10^a	0.17 ± 0.037^a	12.9 ± 2.8^a
WSG (3% glycerol)*	25.4	2.20 ± 0.20^b	0.29 ± 0.036^b	13.2 ± 1.7^a
WSG (4% glycerol)*	34.0	1.40 ± 0.10^a	0.12 ± 0.020^a	8.6 ± 1.4^b
WSG (0.05% urea)**	50.2	2.32 ± 0.10^b	0.30 ± 0.046^b	13.1 ± 2.0^a
WSG (0.08% urea)**	31.6	2.60 ± 0.19^b	0.53 ± 0.049^c	20.4 ± 1.9^c
WSG (0.15% urea)**	16.9	2.30 ± 0.10^b	0.25 ± 0.021^b	11.0 ± 0.9^a

Culture conditions: 30 h at 30 °C and 150 rpm.
* 0.1% urea.
** 3% glycerol.

were assayed: 0.01% and 0.005% (w/v), using as a control the original WSG medium without yeast extract. Experiments, in triplicate, were carried out at the same conditions described in 2.2, in a shaking incubator at 150 rpm at 30 °C, for 48 h. Finally, the contents of DCW and PHA were determined.

2.5. Determination of PHA production

Polymer extraction was performed using a modification of the technique described by Manna et al. [39] and Valappil et al. [40]. Lyophilized cells were lysed in sodium hypochlorite (5.5% v/v in water) at 37 °C for 1 h. Then, the suspension was centrifuged at 6000 *g* (ALC-Multispeed Centrifuge PK131, Thermo Electron Corporation, France) and the obtained pellet was washed twice with water, acetone, and ethanol successively. Finally, pellets were subjected to extraction with boiling chloroform for 10 min and filtered with Whatman filter paper GF/C (47 mm, 1820047). The obtained PHA was recovered by solvent evaporation, and purified by precipitation with hexane [4]. The PHA content was defined based on the concentration of the PHA in the DCW (w/v).

2.6. Polymer characterization

2.6.1. Fourier transform infrared spectroscopy (FTIR)

The obtained PHA was dissolved in chloroform, and then a thin film of the biopolymer was obtained, by solvent evaporation, on a NaCl window. Spectra were obtained on FTIR equipment (Nicolet Nexus, Thermo Scientific, USA) in the range of 400–4000 cm^{-1} with a resolution of 4 cm^{-1}. The data was processed via *OMNIC* software.

2.6.2. Size exclusion chromatography (SEC)

The molar mass distribution and the average molar masses were obtained by Size Exclusion Chromatography (SEC). A system conformed by a Waters 515 pump with a refractive index detector Waters 410, and three 5 μm Phenogel Linear columns and a 5 μm pre-column, was used. Chloroform at 30 °C with a flow rate of 1 mL/min was used as solvent. The calibration was performed with polystyrene (PS) standards, and the following Mark-Houwink constants were used: $K_{PS} = 4.9\ 10^{-3}$ mL/g and $\alpha_{PS} = 0.794$ for PS and $K_{PHB} = 11.8\ 10^{-3}$ mL/g and $\alpha_{PHB} = 0.78$ for PHB. The data was processed via *Empower* software.

2.6.3. Differential scanning calorimetry (DSC)

Thermal properties were measured by Differential Scanning Calorimetry (DSC) using a Perkin Elmer Pyris 1 calorimeter. One heating-cooling-heating cycle was performed in the temperatures range of 30–200 °C at a rate of 10 °C/min. The properties on cooling (crystallization) and second heating (melting) were calculated. Temperatures of melting and crystallization were calculated with *Pyris* software (PerkinElmer, Inc.)

2.6.4. Gas chromatography–mass spectroscopy (GC–MS)

The monomeric composition of the purified PHA was determined with Gas Chromatography-Mass Spectroscopy (Perkin Elmer Clarus 500 Gas Chromatograph/Mass Spectrometer) with PE-5 (fused silica with 5% phenylpolydimethylsiloxane) column (60 m × 0.25 mm ID × 0.25 μm). Hydrogen was used as carrier gas. The oven temperature was maintained at 80 °C for 2 min then programed to 250 °C at 20 °C/min; with isothermal split ratio of 100:1 (at 250 °C), column flow of 1 mL/min, sampling time of 0.5 min with a sample volume of 1.0 μL. For MS, the mass range was 45–600 Da, the ionization mode was electron impact and the ionization energy operation was 70 eV. The data was processed via *TurboMass* software and *NIST* library spectra.

2.6.5. Nuclear magnetic resonance (MNR)

Nuclear magnetic resonance (NMR) spectra were used to confirm the monomeric composition of the purified PHA. The spectra were recorded at 300 MHz on a Bruker Avance III-300 (Top Spin 3.2) spectrophotometer, using CDCl3 Aldrich (99.8 atom% D, containing 0.003% v/v TMS) as solvent. Shifts (δ) are reported in ppm. ^1H and ^{13}C NMR (one-dimension NMR) spectra, and HSQC (heteronuclear single quantum coherence) two-dimensional NMR spectra were determined, with the following parameters for ^1H NMR: 11.25 μs 90° pulse length, 6009.615 Hz spectral width, 8 scans, and 1 s relaxation delay. ^1H NMR was calibrated on the solvent signal at 7.26 ppm, and tetramethylsilane (TMS) was used as internal reference (0.0 ppm). For ^{13}C NMR, chloroform ($CDCl_3$) was used as internal reference (77.0 ppm). The scans used for ^{13}C NMR and HSQC, were 4096 and 128 respectively. The composition of PHA monomers was calculated from the area ratio of absorption

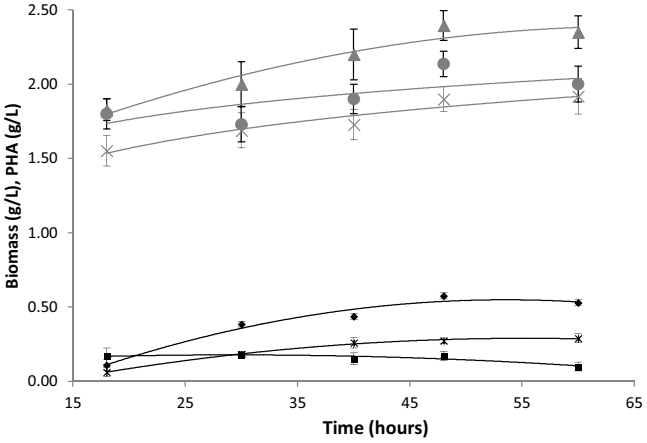

Fig. 1. Influence of temperature and culture time on PHA production (mean ± SD). Symbols: (*) PHA at T = 20 °C, (◆) PHA at T = 30 °C, (■) PHA at T = 37 °C, (×) Biomass at T = 20 °C, (▲) Biomass at T = 30 °C, and (●) Biomass at T = 37 °C, the curves represent the trend lines.

Table 2
Effect of yeast extract on cell density and PHA accumulation.

Culture medium*	Biomass ($g L^{-1} \pm SD$)	PHA ($g/L^{-1} \pm SD$)	PHA ($\% \pm SD$)
0.005% of yeast extract	3.80 ± 0.30^a	1.14 ± 0.057^a	30.0 ± 1.5^a
0.01% of yeast extract	2.90 ± 0.80^a	0.52 ± 0.058^b	18.0 ± 2.0^b
Control (without yeast extract)	2.55 ± 0.08^b	0.59 ± 0.026^b	23.0 ± 1.0^c

Culture conditions: 48 h at 30 °C and 150 rev min^{-1}.
* WSG (3% glycerol, 0.08% urea).

peaks of methyl groups corresponding to the HB and HV hydrogen signals (1.28 and 0.9 ppm from ^1H NMR, respectively) [41]. The data was processed via *TopSpin* software.

2.7. Statistical analysis

Significant differences between assays were performed using one-way ANOVA followed by LSD multiple comparison. A p-value < 0.05 was considered to be significant. The analyses were performed using R, a language and environment for statistical computing. [42]

3. Results and discussion

3.1. Effect of glycerol and urea in PHA production

The batch culture experiments carried out using *B. megaterium* BBST4 strain, were run in a mineral salt base medium with different concentrations of glycerol as the sole source of carbon and energy, and urea as the nitrogen source (Table 1). The agitation and the pH of the culture were determined in previous studies in our laboratory (personal communication). The *Bacillus megaterium* BBST4 strain showed to be capable of growing in a minimal salt

Table 3
Summary of literature describing PHAs production from glycerol by bacteria.

Strain	Culture[a]	X ($g L^{-1}$)	P (%)	PP ($g L h^{-1}$)	Yields[b]		Molar mass[c]			Thermal properties[d]				PHA	Work
					X/S	P/S	M_n	M_w	PI	T_m (°C)	T_c (°C)	ΔH_f (J/g)	X_c (%)		
Bacillus megaterium BBST4	D (SWG + 0.0005 g YE L^{-1}). 250 mL F (100 mL)	3.8	30	0.024	0.13	0.038	139	262	1.9	164	68	62	41.3	copolymer P (3HB-co-3HV) (8% HV)	this work
Bacillus megaterium OU303A	C (+0.004 g YE L^{-1}). 250 mL F (50 mL)		52				510 (M_v)			175			50.0	copolymer. P (3HB-co-3HV) (5% HV)	[20]
Bacillus megaterium MTCC10086	C (Fru + Gly). 250 mL F (50 mL)	1.5	39				610 (M_v)			171			44.1	PHB	[1]
Bacillus thuringiensis EGU45	C. CG. 500 mL F (125 mL)	2.1–3.5	46-54	0.023–0.064				385	2.1					copolymer. P (3HB-co-3HV) (~2%HV)	[25]
Burkholderia cepacia ATCC 17759	D. 500 mL F (100 mL)	5.8	82				173	304	1.8	182				PHB	[24]
Cupriavidus necator DSM 545	C (PG). FB. 2 L B (1.5 L)	82.5	62	1.520			304	957	3.2					PHB	[26]
Cupriavidus necator DSM 545	C (WG). FB. 2 L B (1.8 L) 2% DOC	23.8–27.3	15–18	0.06–0.15				550–1370	2.6–4					copolymer	[27]
Cupriavidus necator JMP 134	D (CGs). FB. 2 L B		70					620	3.7	173	97			PHB	[21]
Haloferax mediterranei DSM 1411	C (PG). FB. 10 L B		69	0.120		0.370		150	2.1	141	67			copolymer. P (3HB-co-3HV) (10-12% HV)	[23]
Halomonas sp. KM-1	C (Glu + Gly). F (20 mL)	2.8–2.4	41–45	0.040–0.049	0.14–0.048	0.095–0.046	32	140	4.2					PHB	[28]
Not identified (osmophilic microorganisms)	C (WG). FB (42 L B)			0.140		0.230		253	2.7	139	65			copolymer. P (3HB-co-3HV) (8-10% HV)	[22]
Paracoccus denitrificans	D (CGs + 0.2 g YE/L). FB. 2 L B		65					750	2.6	169	90			PHB	[21]
Pseudomonas oleovorans NRRL B-14682	C (Medium E). 1 L F (500 mL)	1.3	13–27	0.002–0.005		0.017–0.035	656							PHB	[29]
Pseudomonas corrugata 388	C (Medium E). 1 L F (500 mL)	1.8	42	0.012	0.21	0.088	107							copolymer	[29]
Mixed culture of *P. oleovorans* NRRL B-14682 and *P. corrugata* 388	C (Medium E). 1 L F (500 mL)	1.9–3.4	40–20		0.19–0.068	0.097–0.013								s/mcl-PHA blends	[30]
Pseudomonas oleovorans NRRL B-14682	C (Medium E). 2 L F (1 L)	2.2	36	0.011	0.23	0.090	322		1.9	172–173	95–84	80–91		PHB	[31]
Recombinant *Escherichia coli*	C (BMGL). Baffled F (100 mL)		60				107	285	2.6	173		85	58.22	PHB	[32]

X: DWC, P: PHA, PP: PHA productivity.
[a] D: defined media, C: complex media with glycerol, PG: pure glycerol. CG: crude glycerol. WG: waste glycerol. Gly: glycerol. Glu: glucose. Fru: fructose. F: flask, FB: fed-batch, B: bioreactor. DOC: dissolved oxygen concentration.
[b] X/S: biomass yield coefficient; P/S: PHA yield coefficient.
[c] M_n: Number-average molar mass ($\times 10^3$ g mol^{-1}), M_w: Weight-average molar mass ($\times 10^3$ g mol^{-1}), PI: Polydispersity index (M_w/M_n).
[d] T_m: Melting temperature, T_c: Crystallization temperature, ΔH_f: enthalpy of fusion, X_c: percent crystalinity.

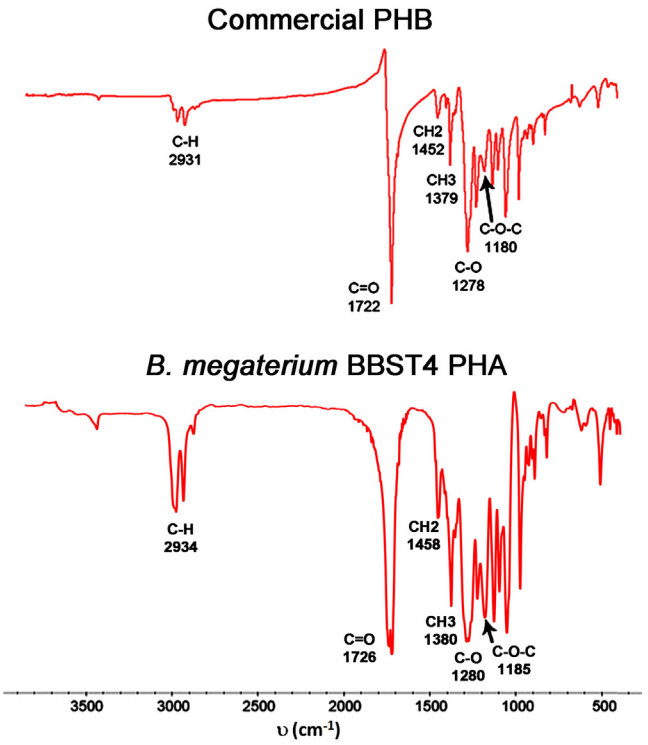

Fig. 2. FTIR spectrum of PHA produced by *Bacillus megaterium* BBST4 at 48 h of culture using glycerol as carbon source and urea as nitrogen source compared to a commercial PHB spectrum.

medium at all glycerol and urea concentrations assayed, without need of the addition of any complex nutritional source. An average specific growth rate of $0.39 \pm 0.09\ h^{-1}$, was defined for all tested media formulations. No significant differences with this value were detected in any of the assays ($p > 0.05$). The onset of the stationary phase was observed at about 20 h of culture. The studies through light microscopy suggested that PHA inclusion bodies increased throughout the culture process, reaching their maximum size in the stationary phase. No sporulating cells were observed in any tested formulation during these experiments. The effect of C/N molar ratio in the media, using a fixed glycerol concentration of 30 g/L, and varying urea concentration from 0.5 to 1 g/L, did not show differences in DCW. However, PHA accumulation showed to be greater at a ratio of C/N = 31.6, than at any higher or lower ratio. That "optimal" ratio, which can be considered as moderately unbalanced, was in agreement with the results obtained by Kulpreecha et al. [43], using *Bacillus* spp. However, as may be seen in Table 1, in the medium where urea concentration was fixed at 1 g/L, with varying glycerol concentrations, the most unbalanced C/N ratio (C/N: 34) gave lower production of DCW and PHA, although this ratio was similar to the "optimal" ratio (i.e. C/N:31.6). These results are in concordance with those observed by Zafar et al. [44], who worked with *Bacillus* sp. RER002. Within a glycerol concentration range of 10–40 g/L, they found that the maximum production of PHAs was observed at 30 g/L. Despite that the negative effect of higher concentrations of glycerol on PHA production is not analyzed in the above mentioned work, a glycerol excess could in principle, induce an elevation of the osmolarity in the growth medium.

Therefore, according to the above considerations, 3% of glycerol and 0.08% of urea were used in the subsequent experiments.

3.2. Effect of temperature in PHA production

Bacillus megaterium strains have been reported as mesophiles, with an optimum growth temperature located between 30 and 37 °C [45–47]. Using the above-mentioned medium, the effect of incubation temperature on the PHA production (Fig. 1) was studied by means of experiences carried along 60 h at 30, 37 °C, and at 20 °C as a control temperature. Fig. 1 shows the behavior of the strain from 18 h of culture, because approximately at this moment occurs the onset of the stationary phase, and the PHA accumulation begins to be significant. Although the strain studied in this work showed good growth at 20 °C and at 37 °C (Fig. 1), both DCW and PHA productions were significantly higher at 30 °C, in coincidence with the results obtained by Thirumala et al. [48]. These results were also in concordance with Prabhu et al. [49], who found that the optimum temperature for the PHA synthase activity is 30 °C. The highest accumulation of PHA at 30 °C was found after 48 h of culture, and no significant differences were found after 60 h. Therefore, 48 h culture could be proposed as the optimal harvest time. Another advantage of the culture at 30 °C was the low rate of sporulation (<10% after 60 h of culture). This sporulation rate was similar to the observed at 20 °C. Contrarily, at 37 °C the percentage of sporulating cells began to gradually increase since 30 h of incubation. In this case, at the end of the experiments the percentage of sporulating cells was in the range of 30–40%. This result could be important because it has been reported that the maximal PHA accumulation occurs prior to the formation of spores, and that also the PHA is degraded during the sporulation process [50,51].

3.3. Effect of yeast extract in PHA production

Employing glycerol as carbon source, Reddy et al. [20] obtained P(3HB-co-3HV) copolymer with *Bacillus megaterium* OU303A, adding yeast extract at a low concentration (0.004 g/L). On the other hand, Mothes et al. [21], employing a higher concentration of yeast extract (0.2 g/L) and the same carbon source, obtain a PHB homopolymer with *Cupriavidus necator* JMP 134. Yeast extract is a mixture of amino acids, peptides, water soluble vitamins and carbohydrates, which act as growth factors. In this work, yeast extract was used to provide trace nutrients aiming to improve the production of PHA in the *Bacillus megaterium* strain, without affecting the C:N:P ratios in the selected medium. Otherwise, the yeast extract could result in an unexpected alternative source of nitrogen and/or carbon. Table 2 shows the results obtained when the SWG medium (3% glycerol, 0.08% urea), was amended by addition of low concentrations of yeast extract. It was found that the addition of yeast extract at 0.005% improved the PHA production ($p < 0.01$), and that 0.01% of yeast extract produced a negative effect on the process. Although *Bacillus megaterium* has no extra nutritional requirements for cultivation, the presence of the factors contained in the yeast extract, probably implies a saving of energy for the biosynthetic processes. However, yeast extract at higher concentrations (0.01%) probably implies an extra supply of nitrogen, which alters the C/N ratio, provoking a less favorable condition for PHA production. Additionally, the lower yeast extract concentration employed is about 10 fold lower to that used by Reddy et al. [20] and would not significantly change the final cost of the culture medium.

3.4. Polymer characterization

In an ulterior procedure, the PHA obtained from *B. megaterium* BBST4 in the best culture conditions explored (3% glycerol, 0.08% urea, 0.005% yeast extract, 30 °C, 150 rpm, pH 8) was characterized. A summary of the results obtained is shown in Table 3, compared

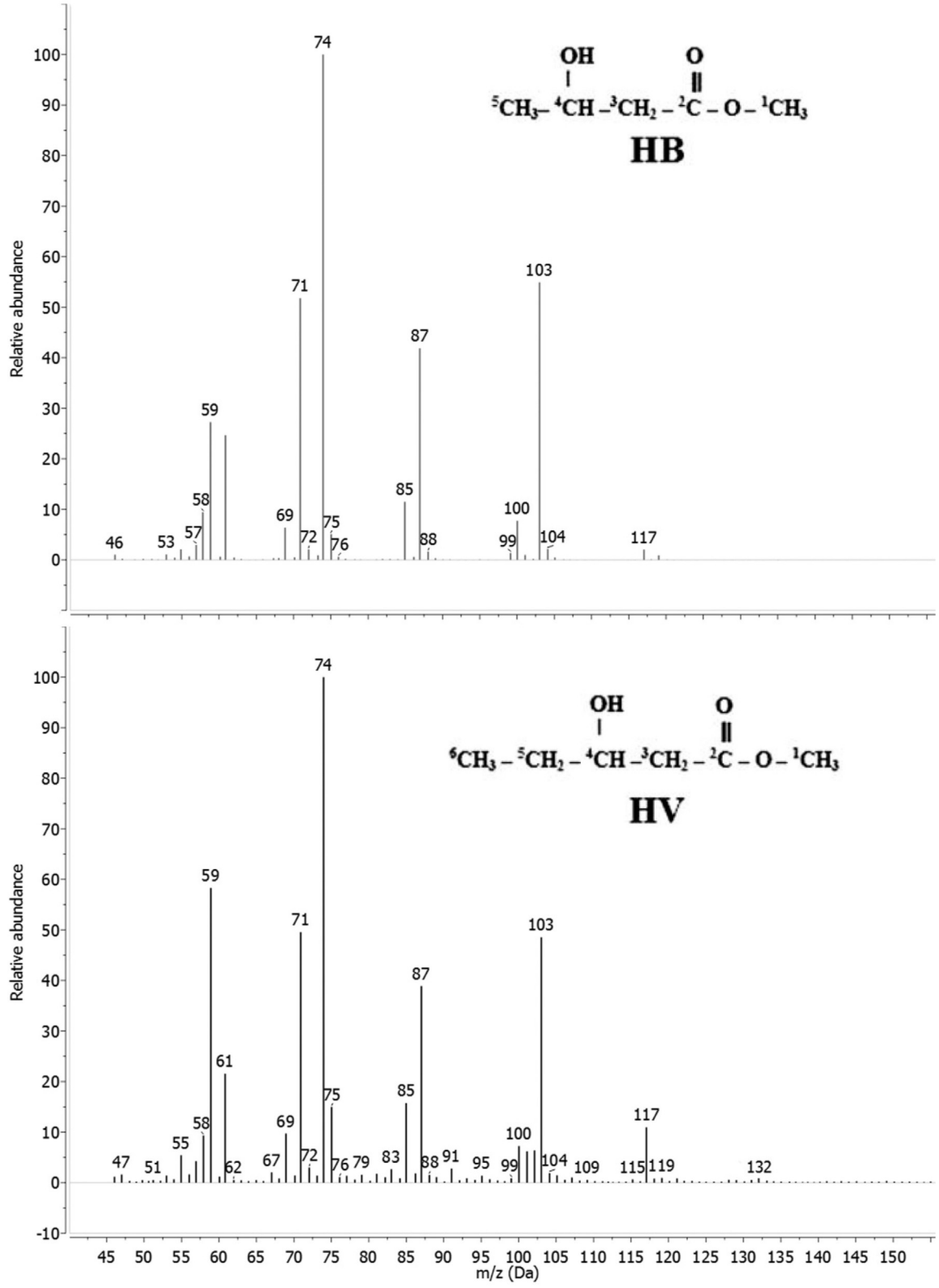

Fig. 3. Mass spectra of the two peaks of the PHA isolated from *B. megaterium* BBST4 at 48 h of culture.

with those found in the literature. The characterization results obtained are discussed below.

Fig. 2 shows the FTIR spectrum of the PHA produced by the *B. megaterium* BBST4 strain. Similar peaks to those of other PHAs produced using glycerol as a carbon source can be found [20,25,52,53], including two intense absorption bands around 1726 and 1280 cm^{-1} corresponding to C=O and C—O stretching groups, respectively [20]. Other absorption bands at 1185, 1381, 1458 and 2932 cm^{-1}, corresponding to C—O, —CH_3, —CH_2 and —CH groups, respectively, were observed. The differences between

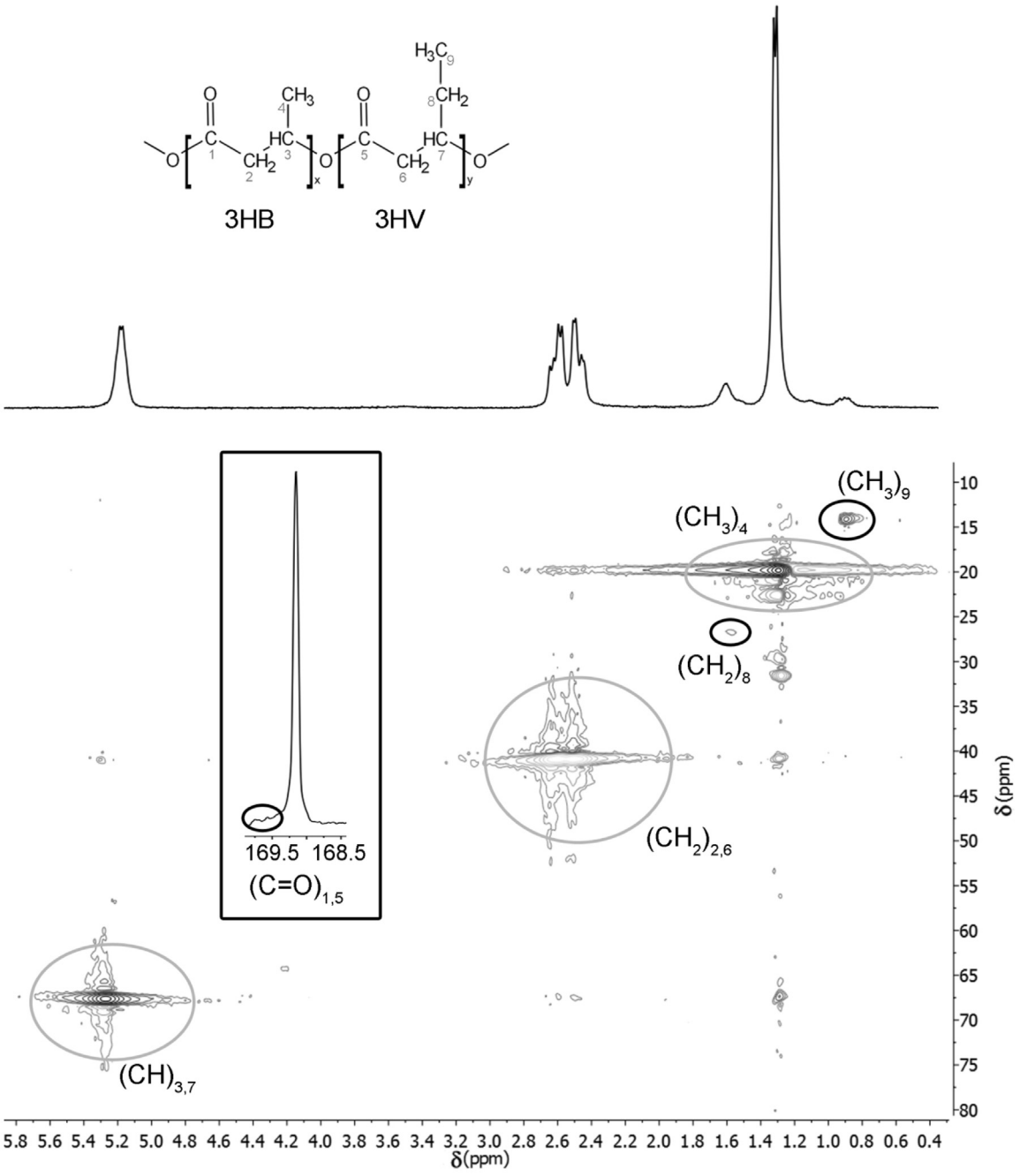

Fig. 4. 1D and 2D NMR spectra for the PHA isolated from *B. megaterium* BBST4 at 48 h of culture.

commercial PHB and PHA spectra were determined in previous studies with statistical techniques by means of the Principal Component Analysis [54].

Studies performed with GPC showed that the obtained PHA had relatively low *Mn* and *Mw* (Table 3), compared to those determined for PHB homopolymer (in the range of 1.0–3.0 10^6 g/mol [55,56]) and to others values determined for PHAs using glycerol as carbon source (see Table 3). The Mw is similar to that reported by the osmophilic microorganisms copolymer producers [22], using glycerol as carbon source. It has been reported that the polydispersity index (*Mw*/*Mn*) is variable for the PHAs produced by different strains using glycerol as carbon source. However, the polydispersity index obtained for the PHA copolymer produced by *Bacillus thuringiensis* EGU45 was similar to that obtained in the present work (see Table 3). This value is also consistent with those reported for the PHA synthesized through different *Bacillus* spp. using various carbon sources [40,57].

The thermal analysis of the PHA produced by *B. megaterium* BBST4 (Table 3) showed a low crystallization temperature, similar to that obtained for the P(HB-co-HV) produced with *Haloferax mediterranei* DSM 1411 [22] and osmophilic microorganisms [23] using complex media and glycerol as carbon source. Based on the melting enthalpy of a perfect PHB crystal (149.37 J/g, [58]), the percent crystallinity of the obtained biopolymer was calculated. The calculated value for the obtained PHA (Table 3) was lower than that of the PHB produced with the same strain using glucose as sole carbon source (X% = 61.5, [34]), and similar to the P(HB-co-HV) produced with *Bacillus* spp. strain using glycerol and propionic acid as carbon sources [20]. All these data become relevant because a diminution in the melting temperature increases the

Table 4
Cross-peak assignments for the PHA produced by *Bacillus megaterium* BBST4 at pH 8 and 48 h of incubation based on NMR data.

Group[a]	δ (ppm)	
	^{13}C NMR	^{1}H NMR
$(COO)_{1,5}$	169.8–169.0	–
$(CH)_{3,7}$	66.3–71.5	5.21–5.40
$(CH_2)_{2,6}$	43.8–40.6	2.35–2.73
$(CH_2)_8$	26.8	1.58
$(CH_3)_4$	18.4–21.7	1.38–1.20
$(CH_3)_9$	14.1	0.90

[a] The group numbers follow the assignments showed in Fig. 4.

processability without degradation of the biopolymer. In addition, a decrease in the degree of crystallinity increases the degradation rate, since amorphous regions are degraded more rapidly than crystalline regions [40]. The melting point value obtained (Tm = 164.1 °C) is lower than that found for the PHB homopolymer produced by the same strain using glucose as a sole carbon source (Tm = 170.6 °C, [34]), and similar to the P(HB-co-HV) produced with *Halomonas campisalis* (MCM B-1027) using maltose as carbon source [59]. Furthermore, a second melting peak is observed at lower temperatures (Tm = 148.5 °C). It can be attributed either to imperfect crystals of PHA, or to the presence of comonomers in the biopolymer. Indeed, based on the Gas chromatography spectrum for the PHA produced by the strain (Fig. 3), two peaks were observed. Both peaks coincide with those corresponding to the fragmentation of methylated 3HA monomers [20] with 5 and 6 carbons. The highest peak would correspond to the structure of 3HB, showing a molecular weight of 118. The lower peak would correspond to the structure of 3HV, showing a molecular weight of 132. This was confirmed by means of one-dimensional NMR and two-dimensional HSQC spectra (Fig. 4). The ^{1}H NMR spectrum of PHA exhibited the typical chemical shifts patterns which are found in the spectra for P(HB-co-HV) [25,59–61]. The expected resonances for HB were demonstrated by the methyl group around 1.28 ppm, the doublet for the methylene group (CH2) between 2.35 and 2.73 ppm and the multiplet signal for methine group (CH) among 5.21 and 5.40 ppm [24,48,59,60]. At the same time, the presence of minor peaks at 0.90 ppm and between 1.52 and 1.61 ppm can be observed, indicating the presence of HV in the biopolymer [2]. The cross-peak assignments observed in the HSQC spectrum (Fig. 4) are reported in Table 4. The cross-peaks between 0.90 ppm and 14.1 ppm, and between 1.58 ppm and 26.8 ppm, can be attributed to the signals produced by the CH3 and CH2 groups of the side chain of 3HV, respectively.

Based on FTIR characterization, the biopolymer produced by the *B. megaterium* BBST4 strain was defined as PHA. From thermal properties it was defined that this biopolymer would probably be a copolymer. That was confirmed through GC/MS and NMR data. The mole fractions of the monomeric units in the copolymer produced by *B. megaterium* BBST4 would be composed of 3HB (approximately 92.2 mol%), and 3HV (approximately 7.8 mol%).

Shahid et al. [62] demonstrated that using glycerol as the sole carbon source and an excess of nitrogen source (3 g/L of $(NH_4)_2SO_4$), *B. megaterium* DSM 509 produces a PHB homopolymer. Under the same conditions but with total limitation of available nitrogen, these authors found that this strain produces copolymers. In the present paper, it was used a strategy of partial limitations of the nitrogen source respect to the amount used by Shahid et al. [62] (a half and a sixth part of that amount). The studied strain responded generating a copolymer with small HV proportion.

4. Conclusions

Bacillus megaterium BBST4 strain was capable to produce P(HB-co-HV) copolymer using glycerol as sole carbon source. This copolymer type has advantageous properties as a material for tissue engineering, and is suitable for its use as substrate for implants and cell cultures [63]. The results obtained in this work showed that the copolymer can be produced by *B. megaterium* BBST4 when it grows in a minimal salt base medium with glycerol as sole carbon source. In synthesis, the most suitable conditions found for P(HB-co-HV) production were: incubation at 30 °C for 48 h with a composition of the culture medium of 3% w/v of glycerol, 0.08% w/v of urea, and 0.005% w/v of yeast extract. These fermentation conditions seem to prevent the sporulation process and the PHA degradation, once maximal production has been achieved. This is a relevant point to analyze the feasibility of the use of the *Bacillus* spp. as PHA producer. Based on these optimum conditions for biopolymer production, the yield coefficients found are in concordance with those obtained by other authors in flask culture employing glycerol as carbon source (Table 3).

The efficiency of *B. megaterium* BBST4 for PHA production in a low cost system using glycerol, and the characteristics of the biopolymer thus obtained would justify further work, aiming to production improvement.

Acknowledgments

Authors wish to thank CONICET (National Scientific and Technical Research Council) and UNS (Universidad Nacional del Sur) for financial support.

References

[1] L. Bora, R. Das, D. Gohain, A novel melt stable and high tensile strength biopolymer (polyhydroxyalkanoates) from *Bacillus megaterium* (MTCC10086) and its characterization, J. Basic Microbiol. 54 (9) (2013) 1012–1016.
[2] S.O. Kulkarni, P.P. Kanekar, S.S. Nilegaonkar, S.S. Sarnaik, J.P. Jog, Production and characterization of a biodegradable poly (hydroxybutyrate-co-hydroxyvalerate) (PHB-co-PHV) copolymer by moderately haloalkali tolerant *Halomonas campisalis* MCM B-1027 isolated from Lonar Lake, India, Bioresour. Technol. 101 (2010) 9765–9771.
[3] G.Q. Chen, Q. Wu, The application of polyhydroxyalkanoates as tissue engineering materials, Biomaterials 26 (2005) 6565–6578.
[4] J.A. Chuah, M. Yamada, S. Taguchi, K. Sudesh, Biosynthesis and characterization of polyhydroxyalkanoate containing 5-hydroxyvalerate units: effects of 5HV units on biodegradability, cytotoxicity, mechanical and thermal properties, Polym. Degrad. Stab. 98 (2013) 331–338.
[5] E. Chiellini, R. Solaro, Biodegradable polymeric materials, Adv. Mater. (Weinheim Ger.) 8 (1996) 305–313.
[6] J.-I. Choi, S.Y. Lee, Process analysis and economic evaluation for poly(3-hydroxybutyrate) production by fermentation, Bioprocess. Eng. 17 (1997) 335–342.
[7] A.A. Koutinas, R.-H. Wang, C. Webb, The biochemurgist-bioconversion of agricultural raw materials for chemical production, Biofuel. Bioprod. Biorefin. 1 (2006) 34–48.
[8] S. Khanna, A.K. Srivastava, Recent advances in microbial polyhydroxyalkanoates, Process Biochem. 40 (2005) 607–619.
[9] J. Levin, T.E. Poore, N.P. Zauber, R.S. Oser, Detection of endotoxin in the blood of patients with sepsis due to gram-negative bacteria, N. Engl. J. Med. 283 (1970) 1313–1316.
[10] J. Roth, J.L. McClellan, M.J. Kluger, E. Zeisberger, Attenuation of fever and release of cytokines after repeated injections of lipopolysaccharide in guinea-pigs, J. Physiol. 477 (1994) 177–185.
[11] G.Q. Chen, Q. Wu, The application of polyhydroxyalkanoates as tissue engineering materials, Biomaterials 26 (2005) 6565–6578.
[12] R.M. Macrae, J.F. Wilkinson, Poly-beta-hyroxybutyrate metabolism in washed suspensions of *Bacillus cereus* and *Bacillus megaterium*, J. Gen. Microbiol. 19 (1958) 210–222.
[13] G.Q. Chen, K.H. Konig, R.M. Lafferty, Occurrence of poly β-hydroxyalkanoates in the genus *Bacillus*, FEMS Microbiol. Lett. 84 (1991) 173–176.
[14] Q. Wu, H.H. Huang, G.H. Hu, J.C. Chen, K.P. Ho, G.Q. Chen, Production of poly-3-hydroxybutyrate by *Bacillus* sp. JMa5 cultivated in molasses media, Anton. Leeuw. Int. J. Gen. Mol. Microbiol. 80 (2001) 111–118.
[15] B. Ghate, P. Pandit, C. Kulkarni, D.D. Mungi, T.S. Patel, PHB production using novel agroindustrial sources from different *Bacillus* species, Int. J. Pharm. Bio. Sci. 2 (2011) 242–249.

[16] T.R. Shamala, A. Chandrashekar, S.V.N. Vijayendra, L. Kshama, Identification of polyhydroxyalkanoate (PHA)-producing *Bacillus* spp. using the polymerase chain reaction (PCR), J. Appl. Microbiol. 94 (2003) 369–374.

[17] P. Anuradha, K. Vijayalakshmi, N.D. Prasanna, K. Sridevi, Production and properties of alkaline xylanases from *Bacillus* sp. isolated from sugarcane fields, Curr. Sci. 90 (2007) 1283–1286.

[18] K.K. Kiran, T.S. Chandra, Production of surfactant and detergent-stable halophilic, and alkalitolerant alpha-amylase by a moderately halophilic *Bacillus* sp. strain TSCVKK, Appl. Microbiol. Biot. 77 (2008) 1023–1031.

[19] S.R.K. Pandian, V. Deepak, K. Kalishwaralal, N. Rameshkumar, M. Jeyaraja, S. Gurunathana, Optimization and fed-batch production of PHB utilizing dairy waste and sea water as nutrient sources by *Bacillus megaterium* SRKP-3, Bioresour. Technol. 101 (2010) 705–711.

[20] S.V. Reddy, M. Thirumala, S.K. Mahmood, Production of PHB and P(3HB-co-3HV) biopolymers by *Bacillus megaterium* strain OU303A isolated from municipal sewage sludge, World J. Microbiol. Biotechnol. 25 (3) (2009) 391–397.

[21] G. Mothes, C. Schnorpfeil, J.U. Ackermann, Production of PHB from crude glycerol, Eng. Life Sci. 7 (5) (2007) 475–479.

[22] M. Koller, R. Bona, G. Braunegg, C. Hermann, P. Horvat, M. Kroutil, P. Varila, Production of polyhydroxyalkanoates from agricultural waste and surplus materials, Biomacromolecules 6 (2) (2005) 561–565.

[23] C. Hermann-Krauss, M. Koller, A. Muhr, H. Fasl, F. Stelzer, G. Braunegg, Archaeal Production of Polyhydroxyalkanoate (PHA) Co- and Terpolyesters from Biodiesel Industry-derived By-products, Archaea, 2013.

[24] C. Zhu, C.T. Nomura, J.A. Perrotta, A.J. Stipanovic, J.P. Nakas, Production and characterization of poly-3-hydroxybutyrate from biodiesel-glycerol by *Burkholderia cepacia* ATCC 17759, Biotechnol. Progr. 26 (2) (2010) 424–430.

[25] P. Kumar, S. Ray, S.K. Patel, J.K. Lee, V.C. Kalia, Bioconversion of crude glycerol to polyhydroxyalkanoate by *Bacillus thuringiensis* under non-limiting nitrogen conditions, Int. J. Biol. Macromol. 78 (2015) 9–16.

[26] J.M. Cavalheiro, M.C.M. de Almeida, C. Grandfils, M.M.R. da Fonseca, Poly(3-hydroxybutyrate) production by *Cupriavidus necator* using waste glycerol, Process Biochem. 44 (5) (2009) 509–515.

[27] J.M. Cavalheiro, R.S. Raposo, M.C.M. de Almeida, M.T. Cesário, C. Sevrin, C. Grandfils, M.M.R. da Fonseca, Effect of cultivation parameters on the production of poly (3-hydroxybutyrate-co-4-hydroxybutyrate) and poly (3-hydroxybutyrate-4-hydroxybutyrate-3-hydroxyvalerate) by *Cupriavidus necator* using waste glycerol, Bioresour. Technol. 111 (2012) 391–397.

[28] Y. Kawata, S.I. Aiba, Poly(3-hydroxybutyrate) production by isolated *Halomonas* sp. KM-1 using waste glycerol, Biosci. Biotechnol. Biochem. 74 (1) (2010) 175–177.

[29] R.D. Ashby, D.K. Solaiman, T.A. Foglia, Bacterial poly(hydroxyalkanoate) polymer production from the biodiesel co-product stream, J. Polym. Environ. 12 (3) (2004) 105–112.

[30] R.D. Ashby, D.K. Solaiman, T.A. Foglia, Synthesis of short-/medium-chain-length poly(hydroxyalkanoate) blends by mixed culture fermentation of glycerol, Biomacromolecules 6 (2005) 2106–2112.

[31] R.D. Ashby, D.K. Solaiman, G.D. Strahan, C. Zhu, R.C. Tappel, C.T. Nomura, Glycerine and levulinic acid: renewable co-substrates for the fermentative synthesis of short-chain poly (hydroxyalkanoate) biopolymers, Bioresour. Technol. 118 (2012) 272–280.

[32] T.V.N. Ramachander, D. Rohini, A. Belhekar, S.K. Rawal, Synthesis of PHB by recombinant *E. coli* harboring an approximately 5 kb genomic DNA fragment from *Streptomyces aureofaciens* NRRL 2209, Int. J. Biol. Macromol. 31 (1) (2002) 63–69.

[33] D.T. Johnson, K.A. Taconi, The glycerin glut: options for the value-added conversion of crude glycerol resulting from biodiesel production, Environ. Prog. 26 (4) (2007) 338–348.

[34] J.A. López Jiménez, Polímeros de Interés Industrial, Síntesis y Caracterización de Polihidroxibutirato (PHB), Universidad Nacional del Sur, Bahía Blanca, Buenos Aires, Argentine, 2011 (Tesis Doctoral).

[35] J. Pochon, L. Tardieu, Techniques d'analysisenmicrobiologie du sol, De la Tourelle, Paris, 1962.

[36] K.L. Burdon, J.C. Stokes, C.E. Kimbrough, Studies of the common aerobic spore-forming Bacilli staining for fat with Sudan Black B-stain, J. Bacter. 43 (1942) 717–724.

[37] S.Y. Lee, Bacterial polyhydroxyalkanoates, Biotechnol. Bioeng. 49 (1996) 1–14.

[38] A.B. Schaeffer, M. Fulton, A simplified method of staining endospores, Science 77 (1933) 194.

[39] A. Manna, R. Banarjee, A.K. Paul, Accumulation of poly(β-hydroxybutyric acid) by some soil *Streptomyces*, Curr. Microbiol. 39 (1999) 153–158.

[40] S.P. Valappil, S.K. Misra, A.R. Boccaccini, T. Keshavarz, Large-scale production and efficient recovery of PHB with desirable material properties, from the newly characterized *Bacillus cereus* SPV, J. Biotechnol. 132 (2007) 251–258.

[41] S. Bloembergen, D.A. Holden, G.K. Hamer, T.L. Bluhm, R.H. Marchessault, Studies of composition and crystallinity of bacterial poly(β-hydroxybutyrate-co-(-hydroxyvalerate), Macromolecules 19 (1986) 2865–2871.

[42] R Core Team, R: A Language and Environment for Statistical Computing, R Foundation for Statistical Computing, Vienna, Austria, 2015. http://www.r-project.org/.

[43] S. Kulpreecha, A. Boonruangthavorn, B. Meksiriporn, N. Thongchul, Inexpensive fed-batch cultivation for high poly(3-hydroxybutyrate) production by a new isolate of *Bacillus megaterium*, J. Biosci. Bioeng. 107 (2009) 240–245.

[44] M. Zafar, S. Kumar, S. Kumar, J. Agrawal, A.K. Dhiman, Valorization of glycerol into polyhydroxyalkanoates by sludge isolated *Bacillus* sp. RER002: experimental and modeling studies, Chem. Prod. Process Model. 9 (2014) 117–131.

[45] S. Ståhl, O. Olsson, Temperature range variants of *Bacillus megaterium*, Arch. Microbiol. 113 (1977) 221–223.

[46] K. Todar, Todar's Online Textbook of Bacteriology: The Genus *Bacillus*, Department of Bacteriology, University of Wisconsin, Madison, USA, 2005.

[47] K.H. Schleifer, Phylum XIII. Firmicutes Gibbons and Murray 1978, 5 (Firmacutes [sic] Gibbons and Murray 1978, 5). Bergey's Manual of Systematic Bacteriology SE –3, 5 (2009) 19–1317.

[48] M. Thirumala, S.V. Reddy, S.K. Mahmood, Production and characterization of PHB from two novel strains of *Bacillus* spp. isolated from soil and activated sludge, J. Ind. Microbiol. Biot. 37 (2010) 271–278.

[49] N. Prabhu, M. Santimano, S. Mavinkurve, S. Bhosle, S. Garg, Native granule associated short chain length polyhydroxyalkanoate synthase from a marine derived *Bacillus* sp. NQ-11/A2, Anton. Leeuw. Int. J. G. 97 (2010) 41–50.

[50] L.A. Kominek, H.O. Halvorson, Metabolism of poly-hydroxybutyrate and acetoin in *Bacillus cereus*, J. Bacteriol. 90 (1965) 1251–1259.

[51] S.P. Valappil, A.R. Boccaccini, C. Bucke, I. Roy, Polyhydroxyalkanoates in gram-positive bacteria: insights from the genera *Bacillus* and *Streptomyces*, Anton. Leeuw. Int. J. G. 91 (2007) 1–17.

[52] C. Chien, C. Chen, M. Choi, S. Kung, Y. Wei, Production of poly-β-hydroxybutyrate (PHB) by *Vibrio* spp. isolated from marine environment, J. Biotechnol. 132 (2007) 259–263.

[53] A. Shrivastav, S.K. Mishra, B. Shethia, I. Pancha, D. Jain, S. Mishra, Isolation of promising bacterial strains from soil and marine environment for polyhydroxyalkanoates (PHAs) production utilizing Jatropha biodiesel byproduct, Int. J. Biol. Macromol. 47 (2010) 283–287.

[54] M.A. Porras, M.A. Cubitto, M.A. Villar, A new way of quantifying the production of poly(hydroxyalkanoate)s using FTIR, J. Chem. Technol. Biotechnol. 91 (5) (2015) 1240–1249.

[55] H.E. Valentin, D. Dennis, Production of poly(3-hydroxybutyrate-co-4-hydroxybutyrate) in recombinant *Escherichia coli* grown on glucose, J. Biotechnol. 58 (1997) 33–38.

[56] R. Luo, J. Chen, L. Zhang, G. Chen, Polyhydroxyalkanoate copolyesters produced by *Ralstonia eutropha* PHB-4 harboring a low-substrate-specificity PHA synthase PhaC2Ps from *Pseudomonas stutzeri* 1317, Biochem. Eng. J. 32 (2006) 218–225.

[57] K. Tajima, T. Igari, D. Nishimura, M. Nakamura, Isolation and characterization of *Bacillus* sp. INT005 accumulating polyhydroxyalkanoate (PHA) from gas field soil, J. Biosci. Bioeng. 95 (2003) 77–81.

[58] P. Barham, A. Keller, E.L. Otun, P. Holmes, Crystallization and morphology of a bacterial thermoplastic: poly-3-hydroxybutyrate, J. Mater. Sci. 19 (1984) 2781–2794.

[59] S.O. Kulkarni, P.P. Kanekar, J.P. Jog, P. a. Patil, S.S. Nilegaonkar, S.S. Sarnaik, P.R. Kshirsagar, Characterisation of copolymer, poly (hydroxybutyrate-co-hydroxyvalerate) (PHB-co-PHV) produced by *Halomonas campisalis* (MCM B-1027), its biodegradability and potential application, Bioresour. Technol. 102 (2011) 6625–6628.

[60] T.R. Shamala, S.V.N. Vijayendra, G.J. Joshi, Agro-industrial residues and starch for growth and co-production of polyhydroxyalkanoate copolymer and α-amylase by *Bacillus* sp. CFR-67, Braz. J. Microbiol. 43 (2012) 1094–1102.

[61] S.V. Otari, J.S. Ghosh, Production and characterization of the polymer polyhydroxy butyrate-co- polyhydroxy valerate by *Bacillus megaterium* NCIM 2475, Curr. Res. J. Biol. Sci. 1 (2009) 23–26.

[62] S. Shahid, R. Mosrati, J. Ledauphin, C. Amiel, P. Fontaine, J.L. Gaillard, D. Corroler, Impact of carbon source and variable nitrogen conditions on bacterial biosynthesis of polyhydroxyalkanoates: evidence of an atypical metabolism in *Bacillus megaterium* DSM 509, J. Biosci. Bioeng. 116 (3) (2013) 302–308.

[63] S. Gogolewski, M. Jovanovic, S.M. Perren, J.G. Dillon, M.K. Hughes, Tissue response and in vivo degradation of selected polyhydroxyacids: polylactides (PLA), poly (3-hydroxybutyrate) (PHB), and poly (3-hyroxybutyrate-co-3-hydroxyvalerate) (PHB/VA), J. Biomed. Mater. Res. 27 (1993) 1135–1148.