High production of poly(3-hydroxybutyrate) from a wild *Bacillus megaterium* Bolivian strain

A. Rodríguez-Contreras[1], M. Koller[2], M. Miranda-de Sousa Dias[2], M. Calafell-Monfort[3], G. Braunegg[2] and M.S. Marqués-Calvo[1]

1 Departament d'Òptica i Optometria, Universitat Politècnica de Catalunya-Barcelona TECH, Terrassa, Barcelona, Spain
2 Institute of Biotechnology and Biochemical Engineering, Graz University of Technology, Graz, Austria
3 Departament d'Enginyeria Química, Universitat Politècnica de Catalunya-Barcelona TECH, Terrassa, Barcelona, Spain

Abstract

Aim: Taking into account that a novel strain of *Bacillus megaterium* was isolated from Uyuni salt lake (Bolivia) in a previous work, the objectives of this new study were to determine the maximal Poly-3-hydroxybutyrate production potential of *B. megaterium* strain uyuni S29 in an industrial conventional media, the possibility that the strain accumulates different types of polyhydroxyalkanoates, the cellular morphology during the biosynthesis process and the characterization of the produced biopolymers.

Methods and Results: The micro-organism was first tested in a 3-L bioreactor obtaining a high specific growth rate of $1.64 \ h^{-1}$. A second fed-batch experiment was carried out in shaking flasks, reaching up to 70% PHB of cell dry mass. The biosynthesized polymers were extracted by two different extraction procedures and characterized. The results showed that all of them were PHB with thermal properties different to the conventional PHB. The micrographs taken by TEM show the different cell morphology during the fermentation process.

Conclusions: In this previous study, the strain not only grew properly in the industrial conditions proposed without spore formation, but also produced and accumulated a large content of PHB, never reached before for its genus. Therefore, if the culture conditions can be optimized, the biopolymer production could be increased.

Significance and Impact of the Study: The impact of the study has related to the area of the biomaterials and their production. The study provides new data related to the high production of PHB from the wild novel strain *B. megaterium* uyuni S29, the highest polymer accumulation for the genus *Bacillus* without spores formation.

Introduction

Nowadays, plastics are one of the most used materials because of their versatility and material properties, especially as packaging materials due to their durability and resistance to degradation. However, these properties have a very high environmental impact. Consequently, biopolymers such as polyhydroxyalkanoates (PHAs) that are produced by several micro-organisms are an attractive alternative to conventional plastics, not just because they are 100% biodegradable, but because they can be produced from renewable resources. This allows their production to be independent of the oil industry. PHAs are also biocompatible with a wide range of applications in medicine, pharmacy, veterinary and food packaging. Poly-3-hydroxybutyrate (PHB) is the simplest and most commonly produced PHA. Because of its competing thermoplastic and mechanical properties, which are similar to plastics or elastomers derived from petroleum, it is gaining interest as a substitute to conventional plastics (Khanna and Srivastava 2005; Koller *et al.* 2010). However, the major commercial drawback of the bacterial

PHB is its high production cost. It makes it substantially more expensive than synthetic plastics. The main factors that increase the polymer production cost are reported by Choi and Lee (1999). Researchers have been focussing on different strategies to overcome this production cost (Koller *et al.* 2010). With the aim of commercializing PHA, a great deal of effort has been devoted to reducing the production cost by the development of better bacterial strains (Koller *et al.* 2010; Quillaguaman *et al.* 2010).

The genus *Bacillus* was identified as one of the first Gram-positive bacteria capable of producing PHB (Lemoigne 1926). This genus has been widely used for a long time in industry and academia, due to the stability of its replication and maintenance of plasmids (Biedendieck *et al.* 2007). Up to now, many species of PHA-producing bacilli have been isolated from various environments (Singh *et al.* 2009). Among them, great polymer producers have been reported (Singh *et al.* 2009; Tian *et al.* 2009), such as *Bacillus cereus,* which can accumulate 48% PHB of Cell dry mass (CDM) in a starch containing medium (Halami 2008), or *Bacillus sp.* IPCB-403, which is able to accumulate PHB in 70% of the CDM in optimum culture conditions (Dave *et al.* 1996). However, there is a drawback to working with *Bacillus* species for large-scale production of biopolymer: sporulation is the reason for low PHB productivity. It is obvious considering the fact that spore formation and PHA accumulation are provoked by similar nutritional stress conditions (Chen 2010). Nevertheless, there are studies on a large-scale PHB production as reported by Valappil *et al.* (2007) with *B. cereus* where the acidic pH used in the medium avoids the spore formation. Hence, it is promising to explore strategies that prevent sporulation by *Bacillus* species to increase the production of PHAs and its efficiency yield from the applied carbon source.

This genus seems to be a potential candidate for the production of PHB due to its better polymer yields and less severe fermentation conditions (Thirumala *et al.* 2010). It is still being widely studied not only because new species with new properties are still appearing (Halami 2008; Rodríguez-Contreras *et al.* 2013), but also because *Bacillus* is used for the production of a variety of different PHAs by utilizing different carbon sources (Thirumala *et al.* 2010). Among the new PHAs-producing *Bacillus*, a wild-type strain of *Bacillus megaterium* was recently isolated from extreme saline environments (Rodríguez-Contreras *et al.* 2013). This novel strain grew well in a conventional medium with low salt content as typically used for industrial production of PHAs. Taking this into account, the main objectives of this new work were to study the maximal PHB production potential of *B. megaterium* strain uyuni S-29 in the conventional media used for industrial biopolymer production and the possibility

that the strain accumulates different types of PHAs. The characterization of the produced biopolymers and the examination of the cellular morphology during the biosynthesis process were further objectives of this study.

Materials and methods

Micro-organism

Bacillus megaterium uyuni S29 was isolated from water and mud samples from the Uyuni hypersaline lake (Bolivia). The new strain was deposited in the Spanish Type Culture Collection (CECT) with the number 7922 (Rodríguez-Contreras *et al.* 2013).

Cultures medium

The strain was maintained at 4°C on solid minimal mineral medium (M medium), according to Küng (1982), containing (per litre) $Na_2HPO_4.2H_2O$, 4·5 g; KH_2PO_4, 1·5 g; $MgSO_4.7H_2O$, 0·2 g; NaCl, 0·9; $(NH_4)_2SO_4$, 2 g; $CaCl_2.2H_2O$, 0·02 g; $NH_4Fe(III)$ Citrate, 0·05 g; agar, 15; trace element solution SL6[1], 1 ml; glucose, 10 g. M medium was used to grow the strain in liquid medium for the precultures of the experiments. A modified M medium was used in the fermentation in the 3-L bioreactor containing: KH_2PO_4, 5 g; $MgSO_4.7H_2O$, 0·40 g; NaCl, 0·9; $(NH_4)_2SO_4$, 2 g; $CaCl_2.2H_2O$, 0·02 g; $NH_4Fe(III)$ Citrate, 0·05 g; trace element solution SL6[1], 2 ml; and glucose, 10 g (Rodríguez-Contreras *et al.* 2013). The same M medium was used for fermentation in the shaking flasks experiment, but citric acid 1 mol l^{-1} was added. Sugars and mineral salt solutions were autoclaved separately at 121°C for 20 min (Atlić *et al.* 2011).

Fermentation strategy for bioreactor

Precultures of the strain were first inoculated from solid M medium grown for 24 h. They were incubated overnight in 300-ml shaking flasks containing 100 ml of M medium at 35°C and a pH value of 7·0 in a rotary shaker at 120 rpm. The strain was cultivated until the cell density reached an OD_{420} of 14. The inoculum was prepared with 10 ml of these precultures in 1-l erlenmeyer flasks containing 250 ml of modified M medium. 0·5 l of these cultures with OD_{420} 10·7 and 11·5 was used to inoculate 1 l of modified M medium in the bioreactor. The fermentation was carried out in a stirred tank reactor of Labfors3 (Infors AG. Headoffice, Bottmingen, Switzerland). It had a total volume of 3 l, with a working volume of 1·5 l (0·5 l of inoculum). All relevant fermentation parameters (pH value, flow rate, dissolved oxygen concentration, stirrer speed, consumption of pH

correction solutions, and activity of antifoam probe) were monitored and recorded using IRIS software program. The temperature of the system was maintained by water flow at 35°C. The pH value (Hamilton sensor) was maintained at 7·0 using 10% solution of H_2SO_4/NaOH-NH_4OH. NH_4OH was used in the growing phase, and when the ammonium source was limited, NaOH was used instead. The air inflow rate and agitation speed were used to monitor the cell activity, and they were initially adjusted to 5 l min^{-1} and 500 rpm, respectively. The oxygen partial pressure (pO_2) (Ingold sensor) was kept at about 40% of the saturation concentration of oxygen in water, and oxygen was supplied at 150 l h^{-1} through an absolute filter (Midisart 2000, Sartorius, Goettingen, Germany). Glucose was added from a concentrated solution of 50% (w/v) during fermentation to avoid the carbon source limitation.

Shaking flasks experiment

Precultures were prepared from solid medium and incubated for 24 h at 35°C and 120 rpm. When the precultures reached an OD_{420} of 13·2, pH value 6·7, 10 ml was used to inoculate two parallel set-ups with 250 ml of modified M medium in 1-litre flask. Glucose was added as a concentrated solution (50% w/v) during fermentation to avoid the carbon source limitation. Growth was monitored *via* optical density at λ of 420 nm.

Analytical procedure

Determination of the CDM, residual biomass and PHB content

Samples of 10 ml of culture broth were taken along the fermentations and centrifuged at 2·600 ***g*** for 15 min (Megafuge 1.0R Heraeus Sepatech). The pellet was frozen, lyophilized and weighed to determine the CDM by weight difference. The PHA in lyophilized biomass samples was transesterificated by acidic methanolysis following Braunegg's method (Braunegg *et al.* 1978). Analyses were carried out with Agilent Technologies 6850 gas chromatograph (GC) (30-m HP5 column, Hewlett-Packard, Palo Alto, CA, USA; Agilent 6850 Series Autosampler, Derwood, MD, USA). The methyl esters of PHA constituents were detected by a flame ionization detector with helium as carrier gas (split- ratio of 1 : 10). P(3HB-co-19·1%-3HV) (Biopol; Imperial Chemical Industries, Billingham, UK) was used for 3HB and 3HV calibration and hexanoic acid was used as internal standard. The PHB content (wt%) was defined as the percentage of the ratio of 3HB concentration to CDM. The residual biomass (RB) was then calculated as the difference between cell concentrations (CDM) and the PHB content.

Determination of glucose

Carbohydrate concentration from supernatant was monitored by means of HPLC equipment composed of a thermostated Aminex HPX 87H column (thermostated at 75°C, Bio-Rad, Hercules, CA, USA), a LC-20AD pump, a SIC-20 AC autosampler, a RID-10A refractive index detector and a CTO-20 AC column oven. Also, the LC solution software for registration and evaluation of the data obtained was used. 1·5 ml of liquid media was sterile-filtrated and transferred into vials. Water was used as eluent at a flow rate of 0·6 ml min^{-1}. The standards were prepared with different concentrations of glucose.

Determination of nitrogen source

Two millilitres of supernatant was mixed with 50 μl of alkaline ISAB solution containing 5 mol l^{-1} sodium hydroxide, 10% methanol, 0·05 mol l^{-1} Na_2-EDTA and a colour indicator. The mixture was immediately analysed with an Orion ion selective electrode; the signal was monitored by a voltmeter. The standard curve was calculated measuring different ammonium sulphate standards solutions of defined concentrations.

Polymer extraction

The cells cultivated were *in situ* pasteurized and the culture broth was then centrifuged at 14·500 ***g*** for 20 min (Inula Sorvall RC-5B Refrigerated Superspeed centrifuge), frozen and lyophilized for 24 h in all cases. After degreasing the biomass by shaking overnight with ethanol, the polymer was extracted from the cells *via* chloroform, by stirring 1 g of freeze-dried cell in 100 ml of chloroform for 48 h and purified by re-precipitation with 10 volumes of ice-cold methanol (Hahn *et al.* 1995). The alternative polymer extraction method was performed *via* Soxhlet with acetone under reflux at 56°C for 5 h. Polymer precipitation was carried out by cooling the solution (Jiang *et al.* 2006). The purity of the extracted material was determined by GC.

Polymer characterization

The chemical structure was characterized with a Perkin Elmer Fourier Transform Infrared microscopy using optical Perkin Elmer software. The line-scan spectra were based on 32 scans and a resolution of 4 cm^{-1}. ^{1}H NMR spectra were recorded at 25°C on a Bruker AM300 spectrometer. The polymer samples were dissolved in chloroform and a drop of TMS (tetra methyl silane used as internal standard for calibrating chemical shift for ^{1}H) was added as reference. 10 mg of the sample dissolved in 1 ml of deutered solvent was used. Proton spectra were

recorded at 300·1 MHz with a spectrum of 32 K data points. A total of 64 scans were utilized with a relaxation delay of 1 s. The Gel Performance Chromatography (GPC) measurements were taken immediately after polymer extraction to ensure no further degradation with possible residual organic solvent. Chloroform was utilized as an eluent at a flow rate of 0·80 ml min^{-1} with a stabilization pressure of 35 bars and a sample concentration of 1·5 mg ml^{-1}. A Waters Styragel HT column for mid-range molecular-mass distributions was used, and samples of polystyrene with different molecular masses were used as standard. Differential Scanning Calorimetry (DSC) experiments were performed on a Perkin-Elmer Pyris 1 instrument with a dry nitrogen gas flow of 50 ml min^{-1}. The apparatus was calibrated using indium of high purity. Approximately, 5 mg of the sample was sealed in an aluminium plate and analysed. The melting temperature (T_m), melting enthalpy (ΔH_m) and the glass transition temperature (T_g) were determined by the second heating run of DSC endothermic peaks. The crystallinity degree (X_c) of PHB was calculated assuming that the ΔH_m value of 100% crystalline PHB is 146 J g^{-1} (Barham *et al.* 1984). Scans started at $-30°C$ and were ramped at 10°C per minute to 230°C.

Electron microscopy

Transmission electron microscopy (TEM) observations were achieved with a JEOL 1200 EX-II electron microscope operating at 90 kV, and the PHB granules contained were observed as thin sections prepared following the method in Tian *et al.* (2009). Samples from the fermentation in shaking flasks were taken at 3 different times (4, 12 and 21 h) and were fixed with a freshly prepared mixture of 2% (v/v) glutaraldehyde, 3% (w/v) paraformaldehyde made fresh, 5% (w/v) sucrose and 0·1 mol l^{-1} sodium cacodylate buffer, pH 7·4. Afterwards, bacterial cells were dehydrated using ethanol solutions and then embedded in low-viscosity embedding resin, which polymerized at 60°C overnight. Resin-embedded bacteria were sectioned using ultramicrotome with a thickness of around 70 nm.

Results

Polymer production

Two different fed-batch fermentations were carried out to study the possibilities of the strain *B. megaterium* uyuni S29 of producing and accumulating PHB. Table 1 summarizes the main results obtained from both fed-batch fermentations. Comparing the results of both assays, higher specific growth rate (μ_{max}) and final CDM were

Table 1 Values of the main parameters of the fermentations: bioreactor and shaking flasks

Parameter	Bioreactor	Shaking flasks
Final cell dry mass (g l^{-1})	28·59 ± 0·09	7·14 ± 0·08
Final Poly-3-hydroxybutyrate (g l^{-1})	8·50 ± 0·65	2·35 ± 0·05
Maximal content of PHA in biomass (%)	29·70 ± 0·28	69·20 ± 4·12
Volumetric productivity PHA (g l^{-1} h)	0·25 ± 0·03	0·45 ± 0·01*
μ_{max} (1/h)	1·64 ± 0·01	0·58 ± 0·01
Total consumption of sugars (g l^{-1} h)	1·81	0·51
Yield $_{(PHA/Sugars)}$	0·14 ± 0·02	0·18 ± 0·01
Yield $_{(CDM/Sugars)}$	0·47 ± 0·00	0·56 ± 0·01

*At 12 h of fermentation.

obtained in the growth curve of the bioreactor experiment, while the sugar conversion was higher for the shaking flasks assay as a result of the higher polymer accumulation.

Fermentation in the bioreactor

Fed-batch fermentation in a 3-L bioreactor was first carried out, showing the data a μ_{max} of 1·64 ± 0·01 with a volumetric productivity of 0·25 ± 0·03 g l^{-1} h PHB (Table 1). The strain started to accumulate biopolymer after 15 h of fermentation, and it stayed constant until it was ended. The maximal polymer content reached 29·70 ± 0·28% (w/w) of CDM from glucose as a carbon source. This first fermentation lasted 18 h and the CDM reached a final value of 28·59 ± 0·09 g l^{-1} with 8·50 ± 0·65 g l^{-1} of biopolymer. Figure 1a shows the results of the bioreactor fermentation in terms of CDM, PHB content and RB concentrations.

The ammonium source was added during the growth phase as ammonium hydroxide (25% w/w) together with the adjustment of the pH value. After 15 h of fermentation, this addition was terminated to induce polymer accumulation as indicated in Fig. 1b by the grey arrow. The consumption of ammonium started; however, it was never limited during the entire fermentation. Regarding the carbon source evolution, the consumption and addition of glucose is also shown in Fig. 1b as a result of HPLC analysis. The initial glucose concentration was 10 g l^{-1}, it was totally consumed by the cells after 8·3 h, and then it was added from a concentrated glucose solution (50% w/v) constantly maintained in excess.

Fermentation in shaking flasks

A total limitation of the nitrogen source was achieved after 12 h of fermentation. Exactly at this moment, the maximal PHB content was reached with a 70% (69·20 ± 4·10% of
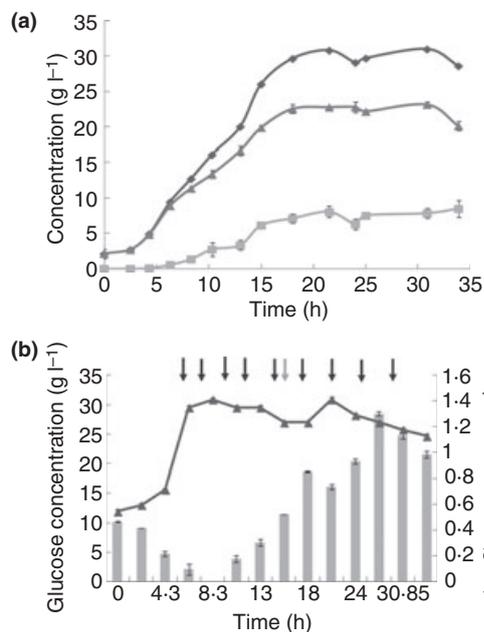
Figure 1 Growth curves of *B. megaterium* strain uyuni S29 under controlled conditions in the bioreactor fermentation: cell dry mass (CDM), polymer content (PHB) and residual biomass (RB) (a). Substrates evolution during fermentation: glucose and ammonium concentration. The grey arrow shows the cut in the addition of nitrogen source (ammonium hydroxide) and the black arrows indicate the addition of glucose (b).The error bars refer to deviations between three samples taken. (a) (–◆–) CDM; (–▲–) RB and (–■–) PHB; (b) (▭) Glucose and (–▲–) Ammonia.

3HB from the gas chromatography after transesterification) of CDM (Fig. 2) obtaining a volumetric productivity of 0.45 ± 0.01 g l^{-1} h PHB. In this fermentation, the RB decreased between 8 and 12 h of fermentation coinciding with a decrease in the nitrogen source. After 12 h of fermentation, the RB increased without any addition of nitrogen. After the maximal polymer concentration was reached, the RB increased and the polymer content decreased. Hence, the polymer and the cell concentrations coincide in the growth curve after 16 h of fermentation. The decrease in PHB concentration in the medium implied the re-utilization of biopolymer by the cells.

Polymer characterization

After polymer extraction with chloroform for both experiments and also with acetone for the bioreactor experiment, three polymers were obtained and characterized by means of Fourier transform Infrared spectroscopy (FTIR), ^{1}H NMR, GPC and DSC.

Fourier transform Infrared spectroscopy results show no differences between the spectra of the three extracted polymers. Carbonyl and ester groups at 1726 and 1330–
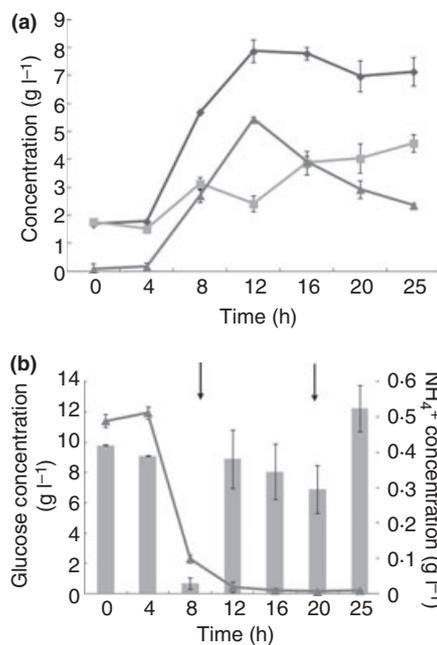

Figure 2 Growth curves of *B. megaterium* strain uyuni S29 in the shaking flasks experiment: cell dry mass (CDM), polymer content (PHB) and residual biomass (RB) (a). Substrates evolution during fermentation: glucose and ammonium concentration. The black arrows indicate the glucose addition (b). The error bars refer to deviations between two parallel experimental set ups. (a) (–◆– CDM; (–■–) RB; (–▲–) PHB; (b) (▭) Glucose and (–▲–) Ammonia.

1050 cm^{-1}, respectively, and methyl group at 1390 cm^{-1} are shown in the three spectra corresponding to the main bands of PHB. ^{1}H NMR spectra show the presence of three groups of signals, characteristics of the homopolymer PHB (Fig. 3): a doublet at 1·29 ppm which is attributed to the methyl group coupled with one proton (signal 3, Fig. 3), a doublet of quadruplet at 2·57 ppm which is attributed to a methylene group adjacent to an asymmetric carbon atom bearing a single proton (signal 2, Fig. 3) and a multiplet at 5·27 ppm characteristic of the methylene group (signals 1, Fig. 3). Two other signals are also observed: one at 3·70 ppm which is due to the methylene group at the end of the chain (signals 4, Fig. 3) and another one at 7·25 ppm which is attributed to chloroform (signals CHCl$_3$, Fig. 3) (Jan *et al.* 1996).

GPC and DSC analyses were carried out to determine the molar mass distribution and the thermal properties of the biopolymer, respectively. The molar masses from GPC analyses did not vary considerably in the polymers from both experiments and with both extraction methods. The results presented two main peaks in all cases that correspond to two different molar masses: one peak represents a molecular mass of 600 kDa, while the other represents a molecular mass of 125 kDa with a polydispersity index of 1·2 and 1·5, respectively. From the DSC thermal analyses,
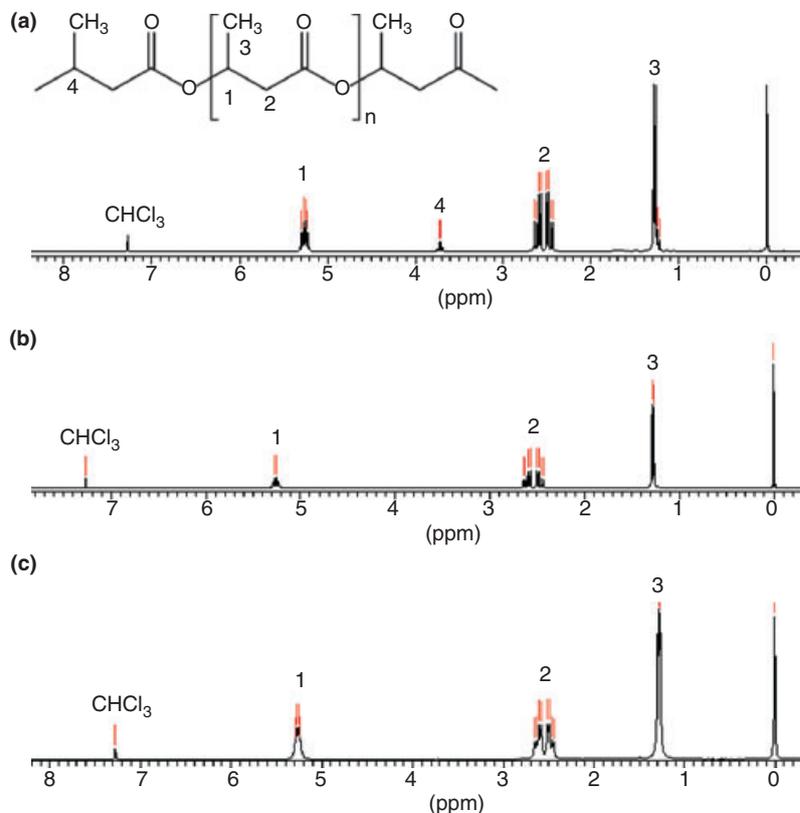
Figure 3 ^{1}H NMR spectra of polymer extracted from fermentation in the bioreactor with acetone (a), with chloroform (b) and from shaking flasks experiments (c). The chemical shift is expressed in parts per million (ppm). Signal 1 is characteristic of the methylene group, signal 2 corresponds to the methylene group adjacent to an asymmetric carbon atom bearing a single proton, signal 3 corresponds to the methyl group coupled to one proton, and signal 4 corresponds to the methylene group at the end of the chain.

Table 2 Main thermal parameters of DSC analyses for the bioreactor and shaking flasks experiments

		Bioreactor		Shaking Flasks
	2nd heating	Acetone	Chloroform	Chloroform
T_m (°C)	1st peak	93	100	–
	2nd peak	115	122	151
	3rd peak	131	136·8	161
T_g (°C)		−17	−16	−11
X_c (%)		37	35	65·95

differences in the thermal properties of the extracted PHBs were observed. Table 2 shows different T_m, T_g and X_c for the three PHB samples characterized.

Cellular morphology

Figure 4 shows TEM micrographs taken at different fermentation times. The evolution of the cells and their polymer inclusions can be observed. Micrographs of samples after 4 h of fermentation show long, growing cells, while 12-h grown cells are already synthesizing polymer. Micrographs taken from samples of 21 h of fermentation show shorter and wider cells deformed due to the pressure of the big internal inclusions. In this last case, several difficulties were encountered in cutting off the samples with the microtome due to the high amount, and hence, resistance of the intracellular inclusions. Consequently, the cellular walls of several cells were fractured because of the jump of some internal inclusions. This can be observed in the micrographs of the samples at 12 and 21 h of fermentation (black arrow in Fig. 4).

Discussion

PHB production

In a previous study (Rodríguez-Contreras *et al.* 2013), *B. megaterium* uyuni S29 showed the formation of intracellular PHA granules. The granules were processed for analysis and the proton displacements and chemical shifts observed in the ^{1}H NMR spectrum confirmed the chemical structure of PHB homopolymer. The polymer
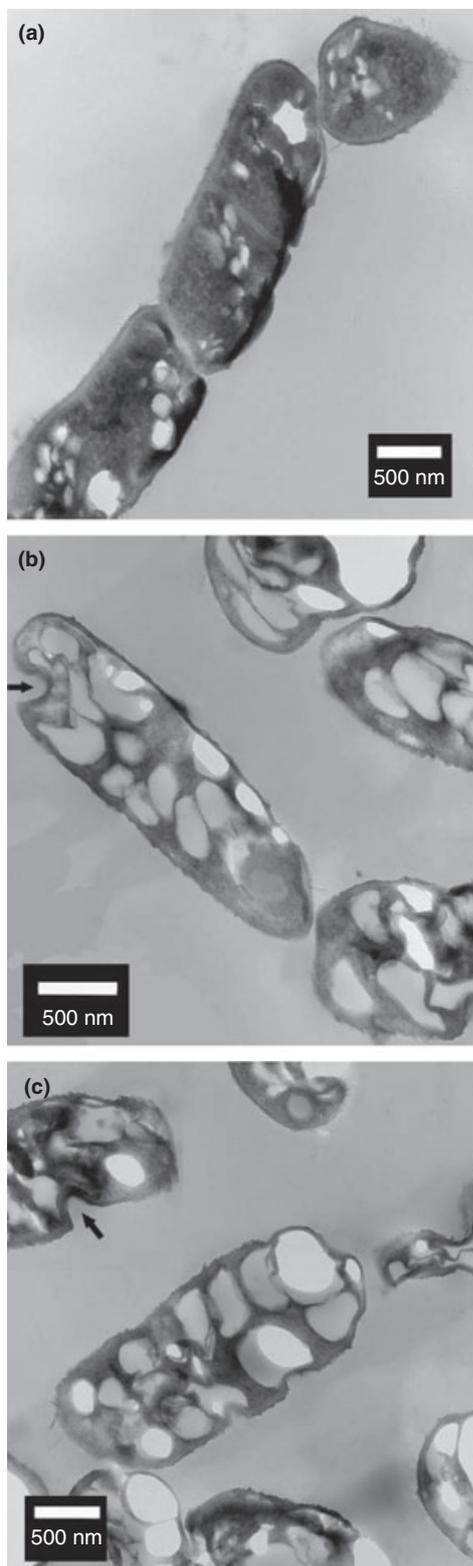
Figure 4 Micrographs of *B. megaterium* strain uyuni S29 after 4 hours (a), 12 hours (b) and 21 hours (c) of fermentation. Black arrows indicate the jump of a polymer inclusion.

biosynthesis was carry out utilizing a conventional medium with low salt content as typically used for industrial production of PHAs (Atlić *et al.* 2011; Küng 1982). Considering the involved advantages of using conditions used industrially (Rodríguez-Contreras *et al.* 2013), the same medium was used in both fed-batch experiments.

The results of both fed-batch fermentations show that better growth conditions were achieved in the bioreactor experiment, while higher polymer content was achieved in fed-batch experiments in shaking flasks (Table 1). This is possible because the bioreactor allows the total control of fermentation parameters, while the shaking flasks experiment indicates the tendency of the behaviour (Büchs 2001).

In the first fed-batch experiment carried out in a 3-L bioreactor, no limitation of nitrogen was achieved in the entire fermentation. Because all other nutrients were under control, the system was likely to be in suboptimal conditions for biopolymer accumulation, and no high amount of polymer content could be attained. Thus, there was a margin for further improvement on the PHB production. Consequently, a second fed-batch experiment was carried out to totally restrict the nitrogen source. This time, nitrogen limitation was achieved after 12 h of fermentation, attaining the maximal polymer content at this point ($69.20 \pm 4.10\%$ PHB). In this fermentation, the RB increased without any addition of nitrogen after 12 h, indicating that cell autolysis could occur (Wang and Yu 2007).

In the literature, studies with *B. megaterium* for PHB production were initially reported to show a maximal polymer content of 40% of CDM in a glucose medium containing acetate (Macrae and Wilkinson 1958). Later, a polymer yield of 48.13% with *B. megaterium* Y6 was reached (Yilmaz *et al.* 2005), and 42% in a fed-batch experiment using sugar cane molasses and urea as cheap carbon sources under nitrogen limiting conditions (Kulpreecha *et al.* 2009). Most recently, it was found that *B. megaterium* strain OU303A yielded a maximum of 62.43% DCM polymer in the medium containing glycerol as a carbon source (Reddy *et al.* 2009). Table 3 shows a summary of the main values related to PHB production by different strains of *B. megaterium* reported in the literature. As mentioned before, sporulation is the cause of low PHB productivity in *Bacillus* genus. *B. megaterium* produces spores under similar conditions to PHA formation, mainly as a consequence of the depletion of essential nutrients, leading to a decrease in the accumulated PHB (Omar *et al.* 2001). This could be the reason why such a low PHB content was reached by this genus before. To our knowledge and compared with the percentages found in the literature, the 70% of PHB ($69.20 \pm 4.10\%$ PHB) reached by *B. megaterium* uyuni S29 is up to now the highest polymer content obtained by a *Bacillus* genus.

Table 3 Comparative values of Poly-3-hydroxybutyrate (PHB) production by different *Bacillus megaterium strains*

PHB producing strains	Carbon substrate	Culture	PHB (%)	Volumetric productivity (g l^{-1} h)	Mw (KDa)	Tm (°C)	Tg (°C)	Reference
B. megaterium uyuni S29	Glucose	Fed-batch	30	0·45	600–125	161	−11	This work
B. megaterium uyuni S29	Glucose	Fed-batch	70	0·25	600–125	136·8	−16	This work
B. megaterium KM	Glucose	Batch	40	–	–	–	–	Macrae and Wilkinson 1958;
B. megaterium P7	Yeast extract peptone	Batch	14·04	–	–	–	–	Yilmaz *et al.* 2005;
B. megaterium BA-019	Molasses	pH-stat fed-batch	42·1	1·27	3900	174	3·9	Kulpreecha *et al.* 2009;
B. megaterium OU303A	Glycerol	Batch	62·43%	–	510	175·23	8·85	Reddy *et al.* 2009;
B. megaterium OU303A	Glucose	Batch	58·63%	–	519	178	8·50	Reddy *et al.* 2009

Moreover, the results from the experiments carried out in this study with *B. megaterium* uyuni S29 indicate that PHB production and accumulation takes place when the nitrogen source is limited, reaching a high polymer concentration with no visible spore formation.

In addition and considering an already industrial scaled strain such as *Azahydromonas lata* (formerly known as *Alcaligenes latus*) which reaches its high PHB content (88% of CDM) with ammonium limitation (Quillaguamán *et al.* 2008), *B. megaterium* uyuni S29 is not far from this result. It has to be considered that *A. lata* has been studied for a long time, and its optimal fermentation conditions are being constantly improved. In comparison, the percentage reached with strain uyuni S29 (70% of CDM) was obtained from a shaking flask experiment with feed-batch fermentation, where the conditions were not completely optimal. Therefore, it is very important to continue the research with bacterial strain *B. megaterium* uyuni S29 to further improve its PHB content.

Furthermore, it is possible to consider *B. megaterium* strain uyuni S29 for polymer production on an industrial scale: first, because the 70% of PHB obtained in this fermentation is higher compared with the 60% of polymer that is necessary for considering a strain suitable to be used in an industrial process (Macrae and Wilkinson 1958; Reddy *et al.* 2003); and second, because industrial fermentation conditions (the used conventional medium and moderate salt content) were already taken into account for the bacterium selection in the previous study (Rodríguez-Contreras *et al.* 2013), those conditions were maintain in this work. Thus, no additional changes in the culture conditions are needed to adapt *B. megaterium* strain uyuni S29 to the required industrial conditions for biopolymer synthesis.

Polymer extraction and characterization

Two extraction methods have been carried out in this work in order to study the possibility of the strain to produce other qualities of PHA besides PHB. Extraction with acetone by Soxhlet was used in previous works to extract medium-chain-length PHA (Jiang *et al.* 2006). These are PHA biopolymers that contain 6 to 14 carbons in their subunits and possess attractive properties such as low melting points, high elasticity and biodegradability (Jiang *et al.* 2006). However, the main bands and peaks of the FTIR and ^{1}H NMR spectra from the polymers extracted using both extraction procedures (*via* chloroform and *via* acetone) correspond to the characteristic ones of PHB homopolymer, a short-chain-length (scl) PHA, according to the literature (Oliveira *et al.* 2007). These results together with the GC analysis indicate that *B. megaterium* uyuni S29 produces and accumulates not other PHA but PHB homopolymer from glucose as sole carbon substrate, matching with the results from the preliminary study of polymer characterization with strain *B. megaterium* strain uyuni S29 (Rodríguez-Contreras *et al.* 2013).

The molecular masses of PHB produced from wildtype bacteria are usually in the range of 10 and 3000 kDa with a polydispersity around 2 (Sudesh *et al.* 2000). The results of the GPC analysis showed that the values for the biopolymer production by *B. megaterium* strain uyuni S29 are within this range. The values of the two molar masses concur with the results obtained in previous works with this bacterium (Rodríguez-Contreras *et al.* 2013).

Usually, scl-PHAs, especially PHB, constitute highly crystalline materials, although they are amorphous within the bacterial cell. The crystallization rapidly occurs after disruption of cells when the polymer is extracted. A common X_c is typically found between 60 and 80%, the T_g about 4°C and the T_m about 160–180°C (Sudesh *et al.* 2000; Khanna and Srivastava 2005; Valappil *et al.* 2007). In this study, the three PHB samples extracted from *B. megaterium* uyuni S29 showed very different thermal properties compared with the usual PHAs. The biopolyesters extracted from both fed-batch experiments (bioreactor

and shaking flasks) showed lower thermal parameters compared with the common ones from PHBs. These results coincide with the thermal properties of the biopolymer extracted in initial studies with this strain (Rodríguez-Contreras *et al.* 2013). Particularly, the extracts from the bioreactor experiment showed even lower X_c and T_g (Table 2). The obtained thermal values confirmed that different fermentation processes and extraction techniques can influence the thermal properties, corresponding to the findings reported by Valappil *et al.* (2007). Low thermal properties shown by the chloroform extraction technique could be explained by the presence of possible impurities. The method may allow lipids, fatty acids and other hydrophobic cellular materials to be extracted along with PHB. However, the fact that the polymer extracted *via* Soxhlet-acetone also showed low thermal properties confirms that this is a property of the biopolymer synthesized by *B. megaterium* uyuni S29.

On the other hand, these uncommon thermal properties could be the result of the blend of the different PHBs fractions, as the two different molar masses resulted from the GPC analyses show. Therefore, the results obtained from DSC and GPC for these extracted PHBs could be connected to each other. The thermal behaviour of some polymers is influenced by the chain length and molecular mass of the polymer used (Rojas de Gáscue *et al.* 2002). As already reported by Lundgren *et al.* (1965), lower molar masses tend to melt at lower temperatures, because the end groups act as impurities. The different melting point values are related to polymer fractions which have undergone different degrees of degradation, yielding polyester with a relatively large fraction of chain ends (low degree of polymerization). The different peaks of the melting points from DSC results could be a consequence of these different chain lengths. Thus, there is evidence that the synthesized PHB features a blend of different PHB fractions with different molar masses (different degrees of polymerization). This matches with the DSC results obtained from previous studies with this strain (Rodríguez-Contreras *et al.* 2013).

The crystallinity of a polymer is known to play a major role in the degradation of a polymer: the amorphous regions in polymers degrade at a much faster rate compared with crystalline regions (Iannace *et al.* 2001). The relatively lower crystallinity of the PHB isolated from *B. megaterium* uyuni S29 can be an advantage, because it will depend on the application needs. Otherwise, the thermal properties of the polymers influence their mechanical properties (Odian 2004).Thus, the relatively low thermal properties of the PHBs isolated from *B. megaterium* uyuni S29 can be reflected on a decrease in the strength and on an increase in the extensibility of the material, enlarging its possible applications.

Conclusions

Strain uyuni S29 is a wild type of *B. megaterium* never tested before for PHB or any other PHA biosynthesis. In this work, the strain not only grew properly in the industrial conditions proposed without spore formation, but it also produced and accumulated a large content of PHB never reached before for its genus (70% w/w of CDM). Consequently, *B. megaterium* uyuni S29 could be considered for polymer bioproduction on an industrial scale. Additionally, the different thermal properties of the PHB produced by the strain could make the use of the material in a wider range of applications possible.

Acknowledgements

References

Atlić, A., Koller, M., Scherzer, D., Kutschera, C., Grillo-Fernandes, E., Horvat, P., Chiellini, E. and Braunegg, G. (2011) Continuous production of poly([R]-3-hydroxybutyrate) by *Cupriavidus necator* in a multistage bioreactor cascade. *Appl Microbiol Biotechnol* **91**, 295–304.

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

Biedendieck, R., Gamer, M., Jaensch, L., Meyer, S., Rohde, M., Deckwer, W.D. and Jahn, D. (2007) A sucrose-inducible promoter system for the intra- and extracellular protein production in *B. megaterium*. *J Biotechnol* **132**, 426–430.

Braunegg, G., Sonnleitner, B. and Lafferty, R.M. (1978) A rapid gas chromatography method for the determination of poly-*β*-hydroxybutyric acid in microbial biomass. *Eur J Appl Microbiol* **6**, 29–37.

Büchs, J. (2001) Introduction to advantages and problems of shaken cultures. *Biochem Eng J* **7**, 91–98.

Chen, G.-Q. (2010) Plastics from bacteria: Natural functions and applications. Microbiology monographs. Alexander Steinbüchel Series editor, Germany.

Choi, J. and Lee, S.Y. (1999) Factors affecting the economics of polyhydroxyalkanoate production by bacterial fermentation. *Appl Microbiol Biotechnol* **51**, 13–21.

Dave, H., Ramakrishna, C. and Desai, J.D. (1996) Production of PHB by petrochemical activated sludge and *Bacillus sp*. IPCB-403. *Indian J Exp Biol* **34**, 216–219.

Hahn, S.K., Chang, Y.K. and Lee, S.Y. (1995) Recovery and characterization of poly(3-hydroxybutyric acid) synthesized in *Alcaligens eutrophus* and recombinant *Escherichia coli*. *Appl Environ Microbiol* **61**, 34–39.

Halami, P.M. (2008) Production of polyhydroxyalkanoate from starch by the native isolate *Bacillus cereus* CFR06. *World J Microbiol Biotechnol* **24**, 805–812.

Iannace, S., Maffezzoli, A., Leo, G. and Nicolais, L. (2001) Influence of crystal and amorphous phase morphology on hydrolytic degradation of PLLA subjected to different processing conditions. *Polymeros* **42**, 3799–3807.

Jan, S., Roblot, C., Courtois, J., Courtois, B., Barbotin, J.N. and Shguin, J.P. (1996) ^{1}H NMR spectroscopic determination of poly 3-hydroxybutyrate extracted from microbial biomass. *Enz Microbiol Technol* **18**, 195–201.

Jiang, X., Ramsay, J.A. and Ramsay, B.A. (2006) Acetone extraction of mcl-PHA from *Pseudomonas putida* KT2440. *J Microbiol Methods* **67**, 212–219.

Khanna, S. and Srivastava, A.K. (2005) Recent advances in microbial Polyhydroxyalkanoates. *Process Biochem* **40**, 607–619.

Koller, M., Salerno, A., Dias, M., Reiterer, A. and Braunegg, G. (2010) Modern biotechnological polymer syntesis: a review. *Food Technol Biotechnol* **48**, 255–269.

Küng, W. (1982) Wachstum und Poly-D-(-)-hydroxybuttersäure Akkumulation bei *Alcaligenes latus*. Diploma Thesis, Graz University of Technology, Austria.

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

Lemoigne, M. (1926) Produits de deshydration et the polymerization de l'acide *b*-oxybutirique. *Bull Soc Chim Biol* **8**, 770–782.

Lundgren, D.G., Alper, R., Schnaitman, C. and Marchessault, R.H. (1965) Characterization of Poly-3-Hydroxybutyrate extracted from different bacteria. *J Bacteriol* **89**, 245–251.

Macrae, R.M. and Wilkinson, J.F. (1958) Poly-*β*-hyroxybutyrate metabolism in washed suspensions of *Bacillus cereus* and *Bacillus megaterium*. *J Gen Microbiol* **19**, 210–222.

Odian, G. (2004) *Principles of Polymerization*. Hoboken, NJ, USA: Wiley-interscience.

Oliveira, C.O., Dias, M.L., Castilho, L.R. and Freire, D.M.G. (2007) Characterization of poly(3-hydroxybutyrate) produced by *Cupriavidus necator* in solid-state fermentation. *Bioresour Technol* **98**, 633–638.

Omar, S., Rayes, A., Eqaab, A., Voß, I. and Steinbüchel, A. (2001) Optimization of cell growth and poly (3-hydroxybutyrate) accumulation on date syrup by a *Bacillus megaterium* strain. *Biotechnol Lett* **23**, 1119–1123.

Quillaguaman, J., Guzman, H., Van-Thuoc, D. and Hatti-Kaul, R. (2010) Synthesis and production of polyhydroxyalkanoates by halophiles: current potential and future prospects. *Appl Microbiol Biotechnol* **85**, 1687–1696.

Quillaguamán, J., Doan-Van, T., Héctor, G., Guzmán, D., Martín, J., Everest, A. and Hatti-Kaul, R. (2008) Poly (3-hydroxybutyrate) production by *Halomonas boliviensis* in fed-batch culture. *Appl Microbiol Biotechnol* **78**, 227–232.

Reddy, C.S.K., Ghai, R. and Rashmi Kalia, V.C. (2003) Polyhydroxyalkanoates: an overview. *Bioresour Technol* **87**, 137–146.

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

Rodríguez-Contreras, A., Koller, M., Miranda-de Sousa Dias, M., Calafell, M., Braunegg, G. and Marqués-Calvo, M.S. (2013) Novel Poly[(R)-3-hydroxybutyrate]-producing bacterium isolated from a Bolivian hypersaline lake. *Food Technol Biotechnol* **51** (in press).

Rojas de Gáscue, B., Prin, J.L., Guerra, D. and Urbina de Navarro, C. (2002) Relationship between the thermal properties and the morphology in high density polyethylenes. *J Therm Anal Calorim* **67**, 343–347.

Singh, M., Patel, S.-K.S. and Kalia, V.-C. (2009) *Bacillus subtilis* as potential producer for Polyhydroxyalkanoates. *Microb Cell Fact* **8**, 38.

Sudesh, K., Abe, H. and Doi, Y. (2000) Synthesis, structure and properties of Polyhydroxyalkanoates: biological polyesters. *Prog Polym Sci* **25**, 1503–1555.

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

Tian, P.Y., Shang, L., Ren, H., Mi, Y., Fan, D.D. and Jiang, M. (2009) Biosynthesis of polyhydroxyalkanoates: current research and development. *Afr J Biotechnol* **8**, 709–714.

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

Wang, J. and Yu, H.-Q. (2007) Biosynthesis of polyhydroxybutyrate (PHB) and extracellular polymeric substances (EPS) by *Ralstonia eutropha* ATCC 17699 in batch cultures. *Appl Microbiol Biotechnol* **75**, 871–878.

Yilmaz, H., Soran, H. and Beyatli, Y. (2005) Determination of poly-*b*-hydroxybutyrate (PHB) production by some *Bacillus spp*. *World J Microbiol Biotechnol* **21**, 565–566.