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Novel Poly[(*R*)-3-Hydroxybutyrate]-Producing Bacterium Isolated from a Bolivian Hypersaline Lake

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Summary

Poly[(*R*)-3-hydroxybutyrate] (PHB) constitutes a biopolymer synthesized from renewable resources by various microorganisms. This work focuses on finding a new PHB-producing bacterium capable of growing in conventional media used for industrial biopolymer production, its taxonomical identification, and characterization of its biopolymer. Thus, a bacterial isolation process was carried out from environmental samples of water and mud. Among the isolates, strain S29 was selected and used in a fed-batch fermentation to generate a biopolymer. This biopolymer was recovered and identified as PHB homopolymer. Surprisingly, it featured several fractions of different molecular masses, and thermal properties unusual for PHB. Hence, the microorganism S29, genetically identified as a new strain of *Bacillus megaterium*, proved to be interesting not only due to its growth and PHB accumulation kinetics under the investigated cultivation conditions, but also due to the thermal properties of the produced PHB.

Key words: poly[(*R*)-3-hydroxybutyrate], biopolymer, bioproduction, new strain, hypersaline environment, thermal properties

Introduction

The rapid increase in human population during the 20th century has raised the global consumption of goods, thus increasing the volume of non-biodegradable residues, especially plastics. These growing piles of resistant waste constitute a severe environmental problem of soaring impact. Therefore, there is a need to study and develop new biodegradable polymers with plastic-like properties (*1*). Polyhydroxyalkanoates (PHAs) are biodegradable polyesters synthesized by microorganisms as carbon and energy storage materials under the conditions of limiting nutrients such as nitrogen, phosphate or oxygen together with an excess of carbon source (*2*). Under these conditions, several microorganisms are able to divert the usual carbon flux (conversion of acetyl-CoA in the tricarboxylic acid cycle for creation of energy and metabolites for biomass formation) towards synthesis of PHA. Such microorganisms can also reutilize the produced PHA as internal carbon substrate when the supply of the growth-limiting nutrient is provided again (*1,3*). The most extensively studied strain for PHA production on an industrial scale is *Cupriavidus necator*; this is due to its versatility in the accumulation of poly[(*R*)-3-hydroxybutyrate] (PHB) and its copolymers (*4,5*). To a lesser extent, *Azahydromonas lata* (formerly known as *Alcaligenes latus*) (*4,6*),

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Azotobacter sp. and recombinant *Escherichia coli* are also used for polymer production (*4*,*6*,*7*). PHAs are biobased, biodegradable and biocompatible biopolyesters which possess thermal properties similar to some petroleum-based polymers such as polypropylene (*4*). PHB is the most frequently occurring PHA and constitutes a linear, unbranched homopolymer consisting of (*R*)-3-hydroxybutyric acid (3HB) units. It is considered very promising as a biodegradable plastic mainly for packaging industries to solve environmental pollution problems (*7*). In addition, it exhibits potential applications in medicine, veterinary practice and agriculture due to its biocompatibility (*7*).

So far, PHAs are not competitive with petroleum-based polymers in economic terms due to their high production costs (*6*). Therefore, efforts are focused on improving the production steps that generate the major part of the costs. Recent studies attempt to solve the most costly factors (feedstock, polymer extraction and microorganism efficiency) by investigating the use of cheaper carbon sources (*8*), novel polymer isolation methods, different fermentation strategies (*9*), and by discovering new microorganisms (*10*). The importance of investigating novel strains lies in the possibility of replacing well-known industrial production strains with new ones, aspiring to a more productive and efficient polymer production process. Current studies report the isolation of new PHA-producing species from extreme environments, and some of them might replace well-established, industrially implemented microorganisms in the near future (*11*,*12*). Taking this into account, the main goal of this work is to isolate a novel potential PHB-producing bacterium from Bolivian hypersaline lake water and mud samples, capable of growing in one of the conventional media for industrial PHB production without excessive salt concentrations. Taxonomical identification of the selected microorganism and the characterization of the produced biopolymer are further objectives of the study.

Materials and Methods

Samples and media

Four water and mud samples were collected, three from three different lagoons and one from a salt lake in Bolivia (Potosí Department): Laguna Colorada, Laguna Hedionda, Laguna de Chiguana and Salar de Uyuni (Fig. 1, *13*). The samples were taken from the surface of the lagoon shore.

For the isolation of the bacteria, HM medium was used, composed of (in g/L): NaCl 44.5, $MgSO_4 \cdot 7H_2O$ 0.25, $CaCl_2 \cdot 2H_2O$ 0.09, KCl 0.5, NaBr 0.06, peptone 5, yeast extract 10, glucose 1 and granulated agar 20, adjusted to pH=7.0 (*14*).

The conventional medium used for PHB production was the minimal mineral medium according to Küng (*15*), which contained (in g/L): $Na_2HPO_4 \cdot 2H_2O$ 4.5, KH_2PO_4 1.5, $MgSO_4 \cdot 7H_2O$ 0.2, NaCl 1, $(NH_4)_2SO_4$ 2, $CaCl_2 \cdot 2H_2O$ 0.02, NH_4Fe(III) citrate 0.05, agar 15, glucose 10, trace element solution SL6 1 mL, and adjusted to pH=7.0. SL6 was composed of (in mg/L): $ZnSO_4 \cdot 7H_2O$ 100, H_3BO_3 300, $CoCl_2 \cdot 6H_2O$ 200, $CuSO_4$ 6, $NiCl_2 \cdot 6H_2O$ 20, $Na_2MoO_4 \cdot 2H_2O$ 30 and $MnCl_2 \cdot 2H_2O$ 25. The components susceptible to precipitation were sterilized separately (for 21 min at 120 °C).

Fig. 1. Bolivian map with the location where the samples were taken from. Inside the black circle: Laguna Colorada (1), Laguna Hedionda (2), Laguna Chiguana (3) and Salar de Uyuni (4). The environmental conditions and physicochemical characteristics of these lakes are described by Hurlbert and Chang (*13*)

Bacterial isolation

A procedure for viable counting using serial dilutions of the samples was carried out to obtain the appropriate colony number. The liquid used for the dilutions was sterile saline solution with a dilution factor of 10^5. Aliquots of 0.1 mL of each diluted sample were spread over the surface of agar plates containing HM medium using a sterile Drigalski glass spreader. The cultures were incubated at 35 °C for 72 h (*16*). Once the pure cultures were obtained by propagating single colonies on new HM agar plates, the strains were differentiated by their macro- and microscopic characteristics (shape and colour of the colonies, opaque character, motility and shape of the cells, sporulation, and inclusions). For storage, all strains were frozen at –80 °C. The observation of endospores produced after 7 days on solid HM medium was made by means of phase contrast microscopy (Labophot Microscope, Nikon Instruments, New York, NY, USA).

Bacterial selection

For the detection of polymer-producing bacteria, Nile Blue A (Sigma-Aldrich, St. Louis, MO, USA) solution in dimethylsulfoxide (DMSO; 0.25 mg/mL) was added to the sterile HM medium. Each isolated strain was incubated at 35 °C for 48 h. The agar plates were exposed to UV light (312 nm) after cultivation to detect PHB accumulation in the grown colonies. The cells were observed under fluorescent microscope (Labophot Microscope, Nikon Instruments) (*17*). Afterwards, the detected polymer-producing bacteria were grown in conventional liquid

medium. A scale-up of liquid batch cultures was performed by inoculating selected pure colonies from solid medium to 300-mL baffled shake flasks containing 100 mL of conventional medium. All strains were incubated at 35 °C for 48 h under continuous shaking at 120 rpm. The bacterial strain that grew significantly faster under these conditions was selected for further study.

Bacterial identification

Bacterial genomic DNA was extracted by the lysozyme-proteinase K-sodium dodecyl sulphate method (modified by increasing the reagent concentration to 2.4 mg/mL of lysozyme, 0.5 mg/mL of proteinase K and 0.8 % of sodium dodecyl sulphate). The DNA product was amplified by PCR (Bio-Rad iCycler, Madrid, Spain) reaction according to the following procedure: 30 μL of the reaction mixture containing 6 μL of 5× Taq-&GO™ (MP Biomedicals, Carlsbad, CA, USA), 1.5 μL of primer pair mix EubI-forward (5'-GAGTTTGATCCTGGCTCAG-3') and 907r-reverse (5'- CCGTCATTTC(AC)TTT(AG)AGTTT-3'), both at a concentration of 10 pmol/μL and 20–30 ng of template were used (*18*). The PCR products were purified with GeneClean Turbo Kit (MP Biomedicals) as recommended by the manufacturer. The fragments were sequenced in Molecular Biology-ZMF (Centre for Medical Research in Graz, Austria) using the reverse primer 907r. The fragments were taxonomically identified according to partial 16S rDNA and compared to sequences available in the GenBank databases using BLAST. The nucleotide sequence data reported in this paper have been deposited at GenBank (NCBI, Bethesda, MD, USA; *http://blast.ncbi.nlm.nih. gov/Blast.cgi*), with an accession number of JF508445.

Polymer production

Fed-batch fermentation in shake flasks was performed with the selected strain. Seed culture of the same strain was used to prepare the inoculum cultures. Two parallel set-ups in 300-mL shake flasks with 100 mL of conventional medium were inoculated from solid medium and incubated for 24 h at 35 °C and 120 rpm. A volume of 5 mL of selected precultures was then used for inoculation in 300-mL fermentation flasks containing 150 mL of conventional medium for 72 h at 35 °C and 120 rpm. Glucose was added as a solution of 50 % (by mass per volume) during the fed-batch fermentation to avoid the carbon source limitation. Samples of 5 mL were taken at regular time intervals to trace the polymer production.

In order to determine cell dry mass (CDM), samples of 5 mL of culture broth were taken during the fed-batch fermentation and centrifuged at 12 000×*g* for 20 min (Heraeus Megafuge 1.0 R refrigerated centrifuge, DJB Labcared, Newport Pagnell, UK). The pellet was frozen, lyophilised and weighed to determine the CDM.

For PHA determination, the PHA in lyophilized biomass samples was transesterified by acidic methanolysis according to Braunegg's method (*19*). Analyses were carried out with an Agilent Technologies 6850 gas chromatograph (GC) equipped with 30-m HP5 column (Hewlett-Packard, USA) and a 6850 Series Autosampler (Agilent Technologies, Santa Clara, USA). The methyl esters of the PHA constituents were detected by a flame ionization detector with helium as carrier gas (split ratio of 1:10). Pure poly[3-hydroxybutyrate-co-3-hydroxyvalerate] (P(3HB-co-19.1-%-3HV), Biopol; Imperial Chemical Industries, London, UK) was used for 3-hydroxybutyrate (3HB) and 3-hydroxyvalerate (3HV) external calibration; hexanoic acid was applied as internal standard. The PHA content (in % by mass) in cells was defined as the percentage of the ratio of 3HB and CDM concentrations. Residual biomass (RB, in g/L) was calculated as the difference between CDM (g/L) and PHB (g/L) (*5*,*8*).

For glucose determination, carbohydrate (glucose) concentration in the sample supernatant was monitored by means of high-performance liquid chromatography (HPLC) equipment consisting of an Aminex HPX 87H column (BioRad, Hercules, CA, USA) thermostated at 75 °C, a LC-20AD pump, a SIC-20 AC auto-sampler, a RID--10A refractive index detector and a CTO-20 AC column oven. Likewise, the LC solution software for registration and evaluation of the data was used. Water was used as eluent at a flow rate of 0.6 mL/min. The standards were prepared using different glucose concentrations (*5*).

Nitrogen source was determined as follows: 2 mL of supernatant were mixed with 50 μL of alkaline ISAB (ionic strength adjustment buffer) solution containing 5 M sodium hydroxide, 10 % methanol, 0.05 M Na_2-EDTA and colour indicator. The mixture was analysed with an Orion ion selective electrode; the signal was monitored by a voltmeter. The standard curve was calculated measuring ammonium sulphate standard solutions of defined concentrations (*5*).

Polymer extraction, identification and characterization

The cells cultivated in the shake flask experiments were pasteurized *in situ* for 30 min at 70 °C. The pasteurized biomass was then centrifuged at 12 000×*g* for 20 min, frozen and lyophilized for 24 h. The pellet was degreased by overnight stirring with ethanol (12.5 mL of ethanol per g of biomass). Then, PHA was recovered from the dried degreased biomass by overnight Soxhlet extraction with chloroform. After the removal of the major part of chloroform on a rotary evaporator (Büchi Rotavapor RE111, Flawil, Switzerland), the polymer was precipitated by adding cold ethanol and separated from the liquid by filtration. The purity of the extracted material and the completeness of extraction were determined by GC (*5*,*8*).

The extracted polymer was characterised by means of Fourier transform infrared (FTIR) spectroscopy and nuclear magnetic resonance (NMR). FTIR spectra of the biopolymer were collected using a PerkinElmer Paragon 1000 FT-IR spectrometer (PerkinElmer, Waltham, MA, USA). The line-scan spectra were based on 32 scans and a resolution of 4 cm^{-1}. The ^1H NMR spectra were recorded at 25 °C on a Bruker AM300 spectrometer (Bruker Optik, Ettlingen, Germany). The polymer samples were dissolved in chloroform; a drop of tetramethylsilane (TMS), internal standard for calibration, was added as reference. A mass of 10 mg of sample dissolved in 1 mL of deutered solvent was used. Proton spectra were recorded at 300.1 MHz with a spectrum of 32 kB data points. A total of 64 scans were utilized with a relaxation delay of 1 s. The gel performance chromatography (GPC)

measurements were done using chloroform as an eluent at a flow rate of 0.80 mL/min with a stabilisation pressure of 35 bars and a sample concentration of 1.5 mg/mL. A Waters Styragel HT column (Waters Corporation, Milford, PA, USA) for mid-range molecular-mass distributions was used; samples of monodisperse polystyrene with different molar masses were applied as standard. Differential scanning calorimetry (DSC) analyses were performed on a PerkinElmer Pyris 1 instrument with dry nitrogen gas flow of 50 mL/min. The apparatus was calibrated using indium of high purity. Approximately 5 mg of sample were sealed in an aluminium pan and analyzed. The melting temperature (t_m), melting enthalpy (ΔH_m) and the glass transition temperature (t_g) were determined from DSC endothermic peaks of the second heating scan. The degree of crystallinity (X_c) of PHB was calculated assuming a ΔH_m value of 100 % crystalline PHB of 146 J/g. Scans started at –30 °C and were ramped at 10 °C/min to 230 °C (*3*,*5*).

Electron microscopy

Transmission electron microscopy (TEM) observations were achieved with a JEOL 1200 EX-II electron microscope (Jeol Ltd., Tokyo, Japan) operating at 90 kV. The cells and their intracellular PHB granules were observed as thin sections prepared as follows: after 4 h of fermentation in shake flasks, samples were fixed with a freshly prepared mixture of 2 % (by volume) glutaraldehyde, 3 % (by mass per volume) of freshly prepared paraformaldehyde, 5 % (by mass per volume) sucrose, and 0.1 M sodium cacodylate buffer, pH=7.4. Afterwards, bacterial cells were dehydrated using ethanol solutions with increasing concentrations, and finally embedded in a low-viscosity embedding resin polymerizing at 60 °C overnight. Resin-embedded bacteria were sectioned using ultramicrotomy (thickness of 70 nm) (*20*).

Results

Bacterial isolation and selection

HM medium was used to isolate the bacteria from the water and mud samples taken from Laguna Colorada, Laguna Hedionda, Laguna de Chiguana and Salar de Uyuni. Several distinguishable colonies were obtained after spreading the four diluted samples on solid HM medium. When the pure cultures were separated by transferring each colony to a new solid HM medium, a detailed macro- and microscopic observation was done in order to differentiate the microorganisms. Forty-nine different bacteria were considered for this study, as clearly distinct microorganisms.

Twenty of these 49 bacteria produced spores within 7 days in HM solid medium. These species were not used for further studies of polymer production. The remaining bacteria were observed by fluorescence microscopy after being stained with Nile Blue A to distinguish the PHB-producers. After 48 h of incubation, the viable colonies that showed bright orange colour under UV light were selected for further studies of polymer production. Altogether 12 bacteria were found that showed polymer inclusions.

A scale-up to liquid fed-batch cultures of the 12 selected bacterial strains using conventional medium was carried out. The strain from Salar de Uyuni, labelled S29 (and later identified as *Bacillus megaterium*), grew significantly faster under these conditions compared to the other selected strains. Its absorbance ($A_{420\ nm}$) increased fifty times from 0.2 after 48 h of incubation (growth rate 0.21 h^{-1}). Consequently, strain S29 was chosen for studies of polymer production and, therefore, the morphological and taxonomical identification of the strain was carried out.

Bacterial identification

Macroscopic identification of the selected new strain showed white, viscous colonies with regular edges and flat elevation. Microscopic observation of the cells showed rod-shaped Gram-positive bacteria. The cells are often found in chains where the walls are joined or slightly separated. TEM micrographs are shown in Fig. 2. Here, it is possible to observe the cells as round-ended rods (1×2 mm) occurring in pairs and in short chains (7 mm). Some polymer inclusions are already visible as clear grey granules after 4 h of fermentation.

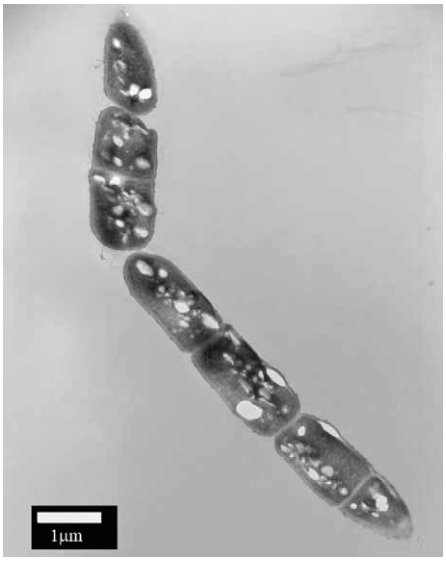

Fig. 2. TEM micrograph of *Bacillus megaterium* uyuni S29 after 4 h of fermentation

The strain labelled S29 has been identified as a new strain of *Bacillus megaterium.* It was deposited in the Spanish Type Culture Collection (CECT) with number 7922 and given the name of *Bacillus megaterium* uyuni S29.

Polymer production, identification and characterization

As a preliminary study of the polymer production capacity of *Bacillus megaterium* uyuni S29, fed-batch fermentation in shake flasks was carried out in conventional medium. The purpose was the identification and characterization of the accumulated biopolymer. Limitation of nitrogen source (ammonium sulphate) in the presence of a surplus of carbon source was used to provoke the strain to accumulate biopolymer. These conditions were

realized by providing a conventional medium nitrogen source at the beginning of cultivation, and the repeated addition of glucose (carbon source) during the fermentation. Spore formation was always controlled by bacterial observation under microscope. Fig. 3a shows the fermentation pattern by indicating the time curves of CDM, RB, and PHB concentrations. The concentration of CDM increased to (4.59±0.12) g/L after 72 h of fermentation. The maximal concentration of PHB of (1.30±0.04) g/L was reached after 48 h with a polymer content of (31±0.20) % in CDM. After this time, the polymer concentration decreased again. The time courses for substrate consumption (glucose and inorganic nitrogen) are shown in Fig. 3b. The nitrogen source (ammonium sulphate) was almost depleted after approx. 48 h. This coincides with the time when the maximal polymer concentration was obtained. Regarding the sugar consumption, the bacteria were consuming it throughout the fermentation to grow and to synthesise biopolymer. The total consumption of sugar after 48 h was (7.55±0.36) g/L, thus the polymer and CDM yields at this point were (17±5) and (55±3) %, respectively. The glucose concentration at the end of the fermentation was higher than 10 g/L (initial concentration) because 12 g/L of glucose were added after 48 h (black arrow in Fig. 3b). Table 1 summarizes the most significant results of the cultivation of *Bacillus megaterium* uyuni S29.

FTIR and NMR results showed typical bands and signals of PHB. FTIR transmission spectrum of the PHA

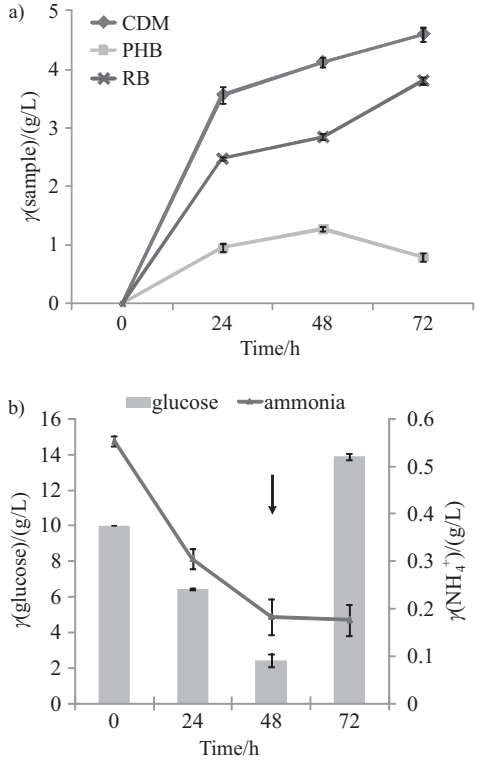

Fig. 3. Time curves of fermentation with *Bacillus megaterium* uyuni S29: a) cell dry mass (CDM), polymer (PHB) and residual biomass (RB) concentrations, b) substrate (carbon and nitrogen source) concentrations. The arrow indicates the time of refeeding with glucose (50 % by mass). The error bars indicate the deviation between two parallel set-ups

Table 1. Average and standard deviation (S.D.) values of the main parameters resulting from the fermentation with *Bacillus megaterium* uyuni S29

Parameter	Average±S.D.
γ(final cell dry mass)/(g/L)	4.59±0.12
$\gamma(PHA_{max})$/(g/L)	1.30±0.04
$w(PHA_{max}(biomass))$/% at 48 h	31.00±0.20
μ_{max}/h^{-1}	0.10±0.04
r_P(PHA)/(g/(L·h))	0.03±0.03
γ(glucose)/(g/L) from 0 to 48 h	7.55±0.36
Y(PHA/sugars) from 0 to 48 h	0.17±0.05
Y(CDM/sugars) from 0 to 48 h	0.55±0.03

$\gamma(PHA_{max})$=maximum PHA content, $w(PHA_{max}(biomass))$=maximum PHA content in biomass, r_P(PHA)=volumetric productivity of PHA, μ_{max}=maximum specific growth rate, Y=yield

extracted from the fermentation with *B. megaterium* uyuni S29 showed the main bands at 1726, 2960–2850, 1390–1370 and 1230–1050 cm^{-1} corresponding to the carbonyl group, methyl and methylene groups, the methyl group, and the ester group, respectively. The ^1H NMR results showed a spectrum with the presence of three groups of signals characteristic of the PHB homopolymer: at 1.29 ppm attributed to the methyl group, at 2.57 ppm for the ethylene group adjacent to an asymmetric carbon atom, and at 5.27 ppm characteristic of the methylene group. The signal observed at 7.25 ppm corresponds to the residual chloroform. For polymer characterization, DSC (Fig. 4) and GPC analyses were carried out in order to determine the molar mass distribution and the thermal properties of the biopolymer. Table 2 shows the results of the GPC analyses. The measurements of the refraction index show three main peaks, corresponding to different molar masses. Their proportions are also indicated in Table 2. The thermal analyses of the PHB extracted from *B. megaterium* uyuni S29 indicate an X_c and t_g of (36.70±1.20) % and (–17.10±0.81) °C, respectively, and three different t_m peaks at (96.50±3.50), (118.50±3.50) and (134.34±2.51) °C. These results are shown in the DSC curve of Fig. 4.

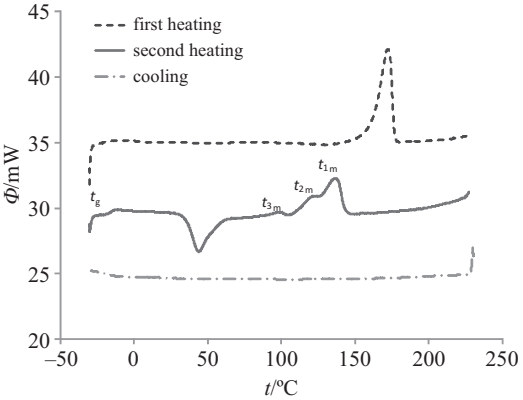

Fig. 4. DSC curve (heat flow *vs.* temperature) of PHB extracted from *Bacillus megaterium* uyuni S29. Melting temperatures: t_{1m}=(134.34±2.51) °C, t_{2m}=(118.50±3.50) °C, and t_{3m}=(96.50±3.50) °C, glass transition temperature t_g=(–17.10±0.81) °C, and the degree of crystallinity X_c=(36.70±1.20) %

Table 2. Molecular mass distribution of polymer extracted from *Bacillus megaterium* uyuni S29 and the proportion of each different molecular mass

Peak	Fraction	M_n	M_w	PDI	M_p
	%	kDa	kDa	(M_w/M_n)	kDa
1st	76	705	795	1.12	740
2nd	13	135	190	1.40	174
3rd	11	27.0	39.6	1.47	31.3

M_n=average number of the molecular mass, M_w=average mass of the molecular mass, PDI=polydispersity index and M_p=maximum of the molecular mass

Discussion

The importance of this work is the discovery of a strain from environmental samples available for cultivation in a conventional medium with low salt content which is already used in the industrial production of PHAs. The samples were taken from Bolivian hypersaline lakes, based on previous studies (*13*,*21*) reporting the isolation of important PHA producers from regions with similar extreme conditions such as *Halomonas boliviensis* (*11*) or *Haloferax mediterranei* (*12*). The advantages of the use of industrial conditions for polymer production, especially moderate amounts of salt in the medium, are twofold. Firstly, if the novel strains do not require any additional change of the culture conditions, there is no additional cost for its adaptation to the production process. Secondly, high salinity levels make the fermentation medium quite corrosive and might incur higher investment cost for the bioreactor equipment used in large scale. Salt in the medium must be concentrated and recycled in order to reduce the overall process cost as well as to minimize ecological drawbacks implicit in the disposal of post-fermentation residues (*8*,*11*). Therefore, avoiding the use of high salt concentrations during fermentation improves the production process economically and ecologically.

HM medium was used for the isolation of microorganisms in similar studies (*11*,*14*). Regarding the work at hand, a high diversity of bacterial species was found in the Bolivian samples. However, some of them formed endospores under conditions of nutrient depletion, coinciding with the requirement to induce biopolymer production. As this constitutes an undesired competition for the external carbon source between both metabolic processes (*22*), endospore-producing strains were eliminated from the study.

Bacteria without visible spore formation that showed PHB inclusions after staining with Nile Blue A were grown in conventional liquid medium, which was selected for polymer production because it is industrially used medium for the controlled accumulation of PHAs. Its composition, especially the nitrogen content, was optimized in order to induce the polymer accumulation already present after a few hours of fermentation (*5*,*8*,*15*). *B. megaterium* uyuni S29 was selected among the PHA-positive bacteria, because it showed fastest growth in conventional liquid medium. Taking into account its origin and its promising growth kinetics in conventional medium at low salt content, *B. megaterium* uyuni S29 can be classified as a halotolerant bacterium, characterized by a tolerance of high NaCl concentrations (*16*).

The fed-batch fermentation study was carried out with *B. megaterium* uyuni S29 in order to produce PHA biopolymer for further characterization. The strain was able to accumulate 31 % of PHB in CDM in this first experiment. This value can be considered promising, if compared to other novel bacteria described in recent works (*10*). The maximal polymer production took place when the nitrogen was limited. Nitrogen limitation acts as the initiator of PHA production because the formation of proteins (residual biomass) stops and the flux of carbon is directed to polymer synthesis (*8*). However, the values presented in Fig. 3 imply that the polymer is already accumulated during the exponential phase of bacterial growth. This can also be concluded from Fig. 2, which shows both cell division and polymer granules at the same time. These results are commonly observed when the depletion of other element(s) in the medium, besides nitrogen, restricts cell multiplication. Most likely, the agitation speed (120 rpm) was insufficient for optimum cultivation of *B. megaterium* uyuni S29 in shake flasks, inducing oxygen limitation. At the end of fermentation, the RB increased after 48 h without any addition of nitrogen source. This indicates that the cell autolysis could have occurred (*23*). Also during this time, the PHB concentration decreases, indicating that the strain is degrading and consuming the biopolymer again, as a result of the decrease of the carbon source concentration. Consequently, further studies are required in order to optimise the biopolymer production process and to determine the potential of the strain as PHB-producer. However, the results achieved by this preliminary study of polymer production by *B. megaterium* uyuni S29 (Table 1) are not far from other published results (*8*,*12*). For instance, Koller *et al.* (*8*) used the same conventional medium to compare the potential of three different wild-type bacteria, among them the halophilic archaeon *Haloferax mediterranei*, as industrial scale PHA producers. The maximum specific growth rate and the volumetric productivity were obtained in the range from 0.03 to 0.05 h^{-1} and 0.08 to 0.29 g/(L·h), respectively. *B. megaterium* uyuni S29 showed a higher growth rate growing in conventional medium ((0.1±0.14) h^{-1}). Quillaguamán *et al.* (*11*) reached a high PHB content and volumetric productivity, 88 % of CDM and 1.1 g/(L·h) respectively, using a complex strategy for PHB production by *H. boliviensis*. Therefore, it could be possible to capitalize on the maximal PHB production potential of *B. megaterium* uyuni S29 through the control and fine-tuning of the fermentation conditions (pH value, dissolved oxygen, temperature, providing suboptimal concentrations of nitrogen during PHA accumulation phase) by means of a laboratory bioreactor. In addition, the application of a bioreactor enables the investigation of different strategies for PHB production, such as PHA copolymer production for tailoring the polyester properties or continuous production mode for enhanced productivity.

This first fermentation with *B. megaterium* uyuni S29 enabled the identification and characterization of the produced biopolymer. The main bands and peaks of the FTIR and ^{1}H NMR spectra correspond to PHB, according to those found in the literature (*9*). These results together with the GC analysis show that *B. megaterium* uyuni S29

Table 3. Thermal properties of different PHAs

Polymer	Species	M_n/kDa (PDI)	t_m/°C	t_g/°C	X_c/%	Ref.
PHB from glucose	*B. megaterium* uyuni S29	705 (1.12)	134.34	–17.10	36.7	present paper
PHB from glucose	*C. necator* DSM 545	665 (2.6)	178	2.9	68	(*5*)
PHB from molasses	*C. necator*	220	173	–	55	(*26*)
PHB from sugars	*C. necator*	230	150	–	60	(*26*)
PHB from glucose	*B. cereus* SPV	1100 (1.75)	169.71	2.04	57.66	(*27*)
PHB from glucose	*B. cereus* SPV	882 (2.6)	160.83	–2.45	54.42	(*27*)
PHB from glucose	*B. cereus* SPV	885 (3.1)	171.71	2.72	64.08	(*27*)
P3HV	*H. pseudoflava*	–	110–112–118	–15	56	(*28*)
P(3HB-co-6 %3HV) from whey sugar	*H. mediterranei*	1057 (1.5)	150.80–158.90	6	–	(*8*)
P(3HB-co-12 %3HV) from molasses	*C. necator*	245	165	–	45	(*26*)
P(3HB-co-20 %3HV)	–	–	145	–1	–	(*28*)
P4HB	*H. pseudoflava*	–	53	–40	–	(*28*)
P4HB	–	–	56	–48	55	(*21*)
P(3HB-co-18 %4HB)	–	–	165	–4	30	(*21*)
P(3HB-co-69 %4HB)	–	–	50	–36	–	(*21*)

M_n=average number of molecular mass, PDI=polydispersity index, t_m=melting temperature, t_g=glass transition temperature, X_c=degree of crystallinity

exclusively accumulates PHB homopolymer using conventional medium with glucose as a substrate. Wild-type bacteria typically produce PHB of molecular masses between 10–3000 kDa, with a polydispersity index (PDI) of about 2 (*4*,*24*). The results of the GPC analysis show that the values for PHB produced by *B. megaterium* uyuni S29 are within this range. However, three different fractions could be identified, with the highest proportion corresponding to the highest molecular mass. Regarding the thermal analyses, X_c is generally reported in a range between 60–80 %, t_g about 4 °C and t_m about 160–180 °C (*25*). In contrast, PHB extracted from *B. megaterium* uyuni S29 shows significantly lower values of these thermal parameters (Table 3; *5*,*8*,*21*,*26–28*). The thermal properties that are closer to the PHA copolymers than to PHB might be a consequence of a formation of a blend of the three different PHB fractions, as determined by GPC analyses. This can be explained by the fact that the thermal behaviour of some polymers is influenced to a high extent by their chain length (*9*). The polymer fractions with a low degree of polymerization show a relatively large quantity of chain ends; here, the end groups act as 'impurities', lowering the polymer melting points (*25*). Therefore, there is evidence that the synthesised biopolymer features a blend of different PHB fractions with different molecular masses (different degrees of polymerisation).

The phylogenetic analyses of the 16S rDNA from the isolate showed that a new strain, *B. megaterium* uyuni S29, has been found. Thus, its macro- and microscopic characteristics coincide with those found for other strains of *B. megaterium* in literature (*29*): an aerobic, Gram-positive, rod-shaped bacterium with flat and viscous colonies. This is the first time that a wild-type strain of *B. megaterium* has been isolated from an extreme environment with high salinity as is the case with the Salar de Uyuni. The compilation of the chemical analyses of brine samples from Salar de Uyuni is given by Rettig *et al.* (*30*). A previous classification of *B. megaterium* indicates that it is halotolerant (*31*), thus coinciding with the initial conclusion for S29. Although *B. megaterium* uyuni S29 is a member of the Bacillaceae family, which are characterised by their endospore formation in unfavourable environments (*32*), no endospores were detected throughout the study. The used culture conditions probably induced the production of the biopolymer but were not favourable for the spore formation. Recently, some studies have shown many strains from this family that can be great polymer producers with high industrial potential (*33*) such as *B. cereus*, which can accumulate 48 % PHB in CDM with starch as a carbon source (*34*), or *Bacillus* sp. IPCB-403, which can accumulate 70 % PHB in CDM under optimum culture conditions (*35*). Thus, it is possible to avoid sporulation during polymer synthesis if the required conditions are obtained.

Conclusion

This study describes for the first time the isolation of a wild-type strain of *B. megaterium* (uyuni S29) from extreme saline environments. The novel strain grew well in a conventional medium with low salt content as typically used for industrial production of PHAs. The data presented in this preliminary study about PHB production by strain S29 indicate a high potential of this bacterial isolate as PHB-producer. The biosynthesised PHB features thermal properties differing from conventional PHB (lower crystallinity, glass transition temperature and melting point) probably due to polymer fractions of different molecular masses. Therefore, further studies are needed in order to optimise the biopolymer production process and to assess the potential of the material for different applications.

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