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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 was focused 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 produced. Thus, a bacterial isolation process was carried out from environmental samples of water and mud. Among the isolates, strain S-29 was selected and used in a fed-batch fermentation to generate 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 S-29, genetically identified as a new strain of *Bacillus megaterium*, turned out

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to be interesting not only due to its growth and PHB accumulation kinetics in the investigated cultivation conditions, but also due to the thermal properties of the produced PHB.

Keywords: 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 to develop new biodegradable polymers with plastic-like properties (*1*). Polyhydroxyalkanoates (PHAs) are biodegradable polyesters synthesized by microorganisms as carbon and energy storage materials under 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 re-utilize 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 PHB and its copolymers (*4, 5*). To a lesser extent, *Azahydromonas lata* (formerly known as *Alcaligenes latus*) (*4, 6*), *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

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generate the major part of 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 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 was 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 were further objectives of the study.

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Materials and Methods

Samples and media

The authors collected four water and mud samples, 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 (figure 1). The samples were taken from the surface of the lagoon's shore.

(Please insert figure 1 here)

The medium used for the isolation of the bacteria was HM composed of (g/L): NaCl, 44.5; $MgSO4 \cdot 7H_2O$, 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 the pH value of 7,0 (*13*).

The conventional medium (M) used for PHB production was the minimal mineral medium according to Küng (*14*) which contained (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; trace element solution SL6, 1 mL; glucose, 10 g; adjusted to the pH value of 7,0. SL6 was composed by (mg/L): $ZnSO_4 \cdot 7H_2O$, 100; H_3BO_3, 300 ; $CoCl_2 \cdot 6 H_2O$, 200; $CuSO_4$,6; $NiCl_2 \cdot 6H_2O$, 20; $Na_2MoO_4 \cdot 2H_2O$, 30; $MnCl_2 \cdot 2 H_2O$, 25. The components susceptible to precipitation were sterilized separately (21 min-120 ºC).

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 ºC for 72 hours (*15*). Once 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 colonies, opaque character; motility and shape of cells, sporulation, and other inclusion). For storage, all strains were frozen at -80 ºC.

Sporulation

The observation of endospores produced after 7 days on solid media (HM) was made by means of phase contrast microscopy (Labophot Microscope, Nikon).

Detection of polymer producing bacteria

Nile Blue A (Sigma, 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 ºC for 48 hours. 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 microscopy (Labophot Microscope, Nikon Instruments, New York, USA) (*16*).

Growth in liquid M medium

A scale up to liquid batch cultures was performed by inoculating selected pure colonies from solid medium to 300 mL baffled shaking flasks containing 100 mL of M medium. All strains were incubated at 35 ºC for 48 hours under continuous shaking at 120 rpm.

Polymer production

Fed-batch fermentation in shaking flasks was performed with the selected strain. Seed culture of the same strain was used to prepare the inoculum cultures. Two parallel setups in 300 mL shaking flasks with 100 mL of M medium were inoculated from solid medium and incubated for 24 h at 35 ºC and 120 rpm. 5 mL of selected pre-culture were then used to inoculate 300 mL

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fermentation flasks containing 150 mL of M media for 72 hours at 35 ºC and 120 rpm. Glucose was added as a concentrated solution of 50 % (w/v) 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.

Analytical methods

CDM determination: Samples of 5 mL of culture broth were taken during the fed-batch fermentation and centrifuged at 12000 x g for 20 min (Heraeus Megafuge 1.0 R refrigerated centrifuge, DJB Labcared, Newport Pagnel, United Kingdom). The pellet was frozen, lyophilised and weighed to determine the cell dry mass (CDM).

PHA determination: The PHA in lyophilized biomass samples was transesterificated by acidic methanolysis according to Braunegg's method (*17*). Analyses were carried out with an Agilant Technologies 6850 gas chromatograph (GC) (30-m HP5 column, Hewlett-Packard, USA; Agilant 6850 Series Autosampler, 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 P(3HB-co-19.1-%-3HV) (Biopol, Imperial Chemical Industries London, United Kingdom) was used for 3-hydroxybutyrate (3HB) and 3-hydroxyvalerate (3HV) calibration by external calibration; hexanoic acid was applied as internal standard. The PHA content (% w/w) in cells was defined as the percentage of the ratio between 3HB concentration and CDM. Residual biomass (RB, [g/L]) was calculated as the difference between CDM [g/L] and PHB [g/L] (*5, 8*).

Glucose determination: Carbohydrate (glucose) concentration in sample supernatant was monitored by means of high-performance liquid chromatography (HPLC) equipment consisting of a thermostated Aminex HPX 87H column (thermostated at 75 ºC, Biorad, Hercules, USA), 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*).

Determination of nitrogen source: 2 mL of supernatant were mixed with 50 μL 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

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electrode; the signal was monitored by a voltmeter. The standard curve was calculated measuring ammonium sulphate standard solutions of defined concentrations (*5*).

Polymer extraction:

The cells cultivated in the shaking flask experiments were *in situ* pasteurized for 30 min at 70 ºC. The pasteurized biomass was then centrifuged at 12000 x g for 20 min, frozen and lyophilized for 24 h. The pellet was degreased by overnight stirring with ethanol (12.5 mL ethanol per gram of biomass). Then, PHA was recovered from the dried degreased biomass by overnight Soxleth extraction with chloroform. After removal of the major part of chloroform on a rotary evaporator (Büchi Rotavapor RE111, 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*).

Polymer identification and characterization

The extracted polymer was characterised by means of Fourier transform infrared (FTIR) and nuclear magnetic resonance (NMR). FTIR spectra of biopolymer were collected using a Perkin Elmer Paragon 1000 FT-IR spectrometer (Perkin Elmer, Massachusetts, USA). 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 (Bruker Optik, Ettlingen, Germany). The polymer samples were dissolved in chloroform; a drop of tetra methyl silane (TMS) (internal standard for calibration) was added as reference. 10 mg of sample dissolved in 1 mL of deutered solvent were 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 second. The Gel performance chromatography (GPC) measurements were performed 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, 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 Perkin-Elmer Pyris 1 instrument (Perkin Elmer, Massachusetts, USA) 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

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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 ºC and were ramped at 10 ºC/min to 230 ºC (*3*, *5*).

DNA extraction and bacterial taxonomy 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 of 0.8 % of sodium dodecyl sulphate). The DNA product was amplified by PCR (Bio-Rad iCycler) reaction according to the following procedure: 30 µL reaction mixture contained 6 µL 5xTaq&Go (MP Biomedicals, Carlsbad, USA), 1.5 µL of primer pair mix EubI-forward (5'-GAG TTT GAT CCT GGC TCA G-3') and 907r-reverse (5'- CCG TCA TTT C(AC)T TT(AG) AGT TT-3') both in a concentration of 10 pmol/µL and 20-30 ng template (*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) 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.

Nucleotide sequence accession number

The nucleotide sequence data reported in this paper have been deposited at GenBank (GenBank, NCBI, Bethesda, MD, USA- http://blast.ncbi.nlm.nih.gov/Blast.cgi) with an accession number of **JF508445**.

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 hours of fermentation in shake flasks, samples were fixed with a freshly prepared mixture of 2 % (v/v) glutaraldehyde, 3 % (w/v) of freshly prepared paraformaldehyde, 5 % (w/v) sucrose, and 0.1 M sodium cacodylate buffer, pH value 7.4. Afterwards, bacterial cells were dehydrated using ethanol solutions with

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increasing concentrations, and finally embedded in a low-viscosity embedding resin polymerizing at 60 ºC overnight. Resin-embedded bacteria were sectioned using ultramicrotomy (thickness of 70 nm) (*19*).

Results

Bacterial isolation

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 new solid HM medium, a detailed macro- and microscopic observation was done in order to differentiate the microorganisms. 49 different bacteria were considered for this study, as the sum of clearly distinct microorganisms.

Selection of PHB-accumulating bacteria

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 hours 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.

Growth in liquid M medium

A scale up to liquid fed-batch cultures of the 12 selected bacterial strains using M medium was carried out. The strain from Salar de Uyuni, labelled S-29, grew significantly faster under these conditions compared to the other selected strains. Its optical density (OD_{420}) increased from 0.2 to 10.1 after 48 hours of incubation (growth rate 0.21 h^{-1}). Consequently, strain S-29 was chosen for studies of polymer production.

Polymer production

As a preliminary study of the polymer production capacity of the selected strain S-29, fed-batch fermentation in shaking flasks was carried out in M medium. The purpose was the identification

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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 an M medium nitrogen source at the beginning of the cultivation, and the repeated addition of glucose (carbon source) during the fermentation. Spore-formation was always controlled by bacterial observation under microscope. Figure 2a shows the fermentation pattern by indicating the time curves of CDM, RB, and PHB concentration. The concentration of CDM increased to 4.59±0.12 g/L after 72 hours of fermentation. The maximal concentration of PHA (1.30±0.04 g/L PHB) was reached after 48 hours with a polymer content of 31±0.20 % in CDM. After this time, the polymer concentration decreased again. The time courses for substrates (glucose and inorganic nitrogen) are shown in figure 2b. The nitrogen source (ammonium sulphate) was almost depleted after approximately 48 hours. 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 hours 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 figure 2b). Table 1 summarizes the most significant results of the cultivation of the bacterial strain S-29.

(Please insert figure 2 and table 1 here)

Polymer identification and characterization

FTIR and NMR results showed typical bands and signals of PHB. FTIR transmission spectrum of the PHA extracted from the fermentation with S-29 showed the main bands at 1726, 2960-2850, 1390-1370, and at 1230-1050 cm^{-1} corresponding to the carbonyl group, methyl and methylene groups, the methyl group, and the ester group respectively. ^{1}H 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, GPC and DSC 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

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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 S-29 indicate an X_c and T_g of 36.7±1.2 % and -17.10±0.81 ºC, respectively, and three different T_m peaks at 96.50±3.50, 118.50±3.50 and 134.34±2.51 ºC. These results are shown in the DSC curve of figure 3.

(Please insert figure 3 and table 2 here)

Bacterial identification

Morphological identification: Macroscopic identification of S-29 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 figure 4. Here, it is possible to observe the cells as round-ended rods (1.0x2.0 µm) occurring in pairs and in short chains (7.0 µm). Some polymer inclusions are already visible as clear grey granules after 4 hours of fermentation.

(Please insert figure 4 here)

Taxonomic identification: The strain S-29 has been identified as a new strain of *Bacillus megaterium.* The strain was deposited in the Spanish Type Culture Collection (CECT) with the number 7922 and given the name of *Bacillus megaterium* uyuni S29.

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Discussion

The importance of this work was the discovery of a strain from environmental samples accessible to 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 (*20, 21*) reporting the isolation of important PHA producers from regions with similar extreme conditions such as *Halomonas boliviensis* (*11*), or *Haloferax mediterranei* (*12*). The use of the industrial conditions for polymer production, especially moderate amounts of salt in medium, leads to advantages from two different points of view. Firstly, if the novel strains do not require any additional change of the culture conditions, no additional cost for its adaptation to the production process occurs. 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 the fermentation economically and ecologically improves the production process.

HM medium was used for the isolation of microorganisms in similar studies (*11*, *13*). 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 the biopolymer production. As this constitutes a non-desired 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 liquid M medium. M medium was selected for polymer production because it is a conventional, industrially used medium for the controlled accumulation of PHAs. Its composition, especially the nitrogen content, is optimized in order to induce the polymer accumulation already present after a few hours of fermentation (*5, 8*, *14*). Strain S-29 was selected among the PHA-positive bacteria, because it showed fastest growth in liquid M medium. Taking into account its origin and its promising growth kinetics in M medium at low salt content, strain S-29 can be classified as a halotolerant bacterium, characterized by a tolerance to high NaCl concentrations) (*15*).

The fed-batch fermentation study was carried out with S-29 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 initiator for 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 figure 2 imply that the polymer is already accumulated during the exponential phase of bacterial growth. This can also be concluded from figure 4 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 strain S-29 in shaking flasks, inducing oxygen limitation. At the end of the fermentation, the RB increased after 48 hours without any addition of nitrogen source. This indicates that the cell autolysis could be occurring (*23*). Also during this time, the PHB

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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 the bacterial strain S-29 (table 1) are not far from other published results (*8*, *12*). For instance, Koller et al. (*8*) used the same medium M 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 of 0.03 to 0.05 and 0.08 to 0.29, respectively. S-29 showed a higher growth rate growing in M medium (0.1±0.14 1/h). Quillamanan et al. (*11*) reached a high PHB content and volumetric productivity, 88% of CDM and 1.1 g/Lh 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 strain S-29 through the control and fine-tuning of the fermentation conditions (pH value, dissolved oxygen, temperature, providing sub-optimal 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 bacterium S-29 enabled the identification and the 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 strain S-29 exclusively accumulates PHB homopolymer using M medium with glucose as a substrate. Wild type bacteria typically produce PHB molar masses between 3,000-10 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 bacterium S-29 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 ºC and T_m about 160-180 ºC (*25*). In contrast, PHB extracted from bacterium S-29 shows significantly lower values for these thermal parameters (table 3). 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

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can be explained by the fact that the thermal behavior 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 molar masses (different degrees of polymerization).

(Please insert table 3 here)

The phylogenic 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 (*26*): 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 are given by Retting (*27*). A previous classification of *B. megaterium* indicates that it is halotolerant (*28*), thus coinciding with the initial conclusion of S-29. Although *B. megaterium* uyuni S29 is a member of the Bacillaceae family which are characterised by their endospore formation in unfavourable environments (*29*), no endospores were detected throughout the study. The culture conditions used probably induced the production of the biopolymer but were not favourable for the spores formation. Recently, some studies have shown many strains from this family that can be great polymer producers with high industrial potential (*30*) such as *B. cereus* which can accumulate 48 % PHB in CDM with starch as a carbon source (*31*) or *Bacillus* sp. IPCB-403 which can accumulate PHB in 70 % of the CDM at optimum culture conditions (*32*). Thus, it is possible to avoid sporulation during polymer synthesis if the required conditions are found.

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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 S-29 indicates 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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References

1. G. Braunegg, R. Bona., F. Schellauf, E. Wallner, Solid waste management and plastic recycling in Austria and Europe, *Polym.-Plast. Technol.43* (2004a) 1755-1767.

2. Y. C. Xiong, Y. C. Yao, X. Y. Zhan, G. Q. Chen, Application of Polyhydroxyalkanoates Nanoparticles as intracellular sustained drug-release vectors, *J. Biomaterials Sci. 21* (2010) 127-140.

3. M. Koller, R. Bona, C. Hermann, P. Horvat, J. Martinz, J. Neto, L. Pereira, P. Varila, G. Braunegg, Biotechnological production of poly(3-hydroxybutyrate) with *Wautersia eutropha* by application of green grass juice and silage juice as additional complex substrates, *Biocat. Biotransform. 23* (2005) 329-337.

4. T. Volova, Polyhydroxyalkanoates, plastic materials of the 21st century, Maya Columbus, New York, USA (2004).

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

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

7 C.S.K. Reddy, R. Ghai, V.C. Rashmi, Kalia, Polyhydroxyalkanoates: an overview, *Bioresource Technol. 87* (2003) 137-146.

8. M. Koller, P. Hesse, R. Bona, C. Kutschera, A. Atlić, G. Braunegg, Potential of various archaea- and eubacterial strains as industrial polyhydroxyalkanoate producers from whey, *Macromol. Biosci. 7* (2007) 218-26.

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

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

11. J. Quillaguaman, H. Guzman, D. Van-Thuoc, R. Hatti-Kaul, Synthesis and production of polyhydroxyalkanoates by halophiles: current potential and future prospects, *Appl. Microbiol. Biotechnol. 85* (2010) 1687-96.

12. J.G. Lillo, F. Rodriguez-Valera, Effects of culture conditions on Poly(3-hydroxybutyric acid) production by *Haloferax mediterranei*, *Appl. Environ. Microb*. (1990) 2517-2521.

13. M. Niño de Guzmán, V. Vargas, H. Antezana, M. Svoboda, Lipolytic enzyme production by halophilic/halotolerant microorganisms isolated from Laguna Verde, Bolivia, *Rev. Bol. Quim. 25* (2008) 14-23.

14. W. Küng, Growth and poly[(R)-3-hydroxybutyrate] accumulation by *Alcaligenes latus*, PhD thesis, Graz University of Technology, Austria (1982) (in German).

15. M.T. Madigan, J.M. Martinko, J. Parker, Brock Biology of microorganisms, Ed. Prentice Hall, New Jersey, USA (2000).

16. P. Spiekermann, B.H.A. Rehm, R. Kalscheuer, D. Baumeister, A. Steinbüchel, A sensitive, viable-colony staining method using Nile red for direct screening of bacteria that accumulate polyhydroxyalkanoic acids and other lipid storage compounds, *Arch. Microbiol. 171* (1999) 73-80.

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

18. D. Tagu, C. Moussard, Techniques for Molecular Biology, First edition, Science Publisers. Enfield, New Hampshire, USA (2006).

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19. J. Tian, A.J. Sinskey, J. Stubbe, Kinetic studies of polyhydroxybutyrate granule formation in *Wautersia eutropha* H16 by Transmission Electron Microscopy, *J. Bacteriol. 187* (2005) 3814-3824.

20. S.H. Hurlbert, C.C. Chang, The distribution, structure, and composition of freshwater ice deposits in Bolivian salt lakes, *Hydrobiologia 158* (1988) 271-299.

21. A. Spyros, R. H. Marchessault, Segmental Dynamics in Poly(3-hydroxybutyrate-co-4-hydroxybutyrate)s above the Glass Transition Temperature: ^{13}C Nuclear Magnetic Relaxation in the Amorphous Phase, *Macromolecules*, *29* (1996) 2479-2486.

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

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

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

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

26. D.B. Drucker, D.K. Whittaker, Microstructure of colonies of rod-shaped bacteria, *J. Bacteriol.* (1971) 515-525.

27. S.L. Rettin, B.F. Jones, F. Risacher, Geochemical evolution of brines in the Salar of Uyuni, Bolivia, *Chem. Geol. 30* (1980) 57-59 .

28. S.A. Smith, J.N. Benardini, J.L. Strap, R.L. Crawford, Diversity of aerobic and facultative alkalitolerant and halotolerant endospore formers in soil from the Alvord Basin, Oregon*, System. Appl. Microbiol. 32* (2009) 233-244.

29. D.A.A. Mossel, J.L. Shennan, E.H. Meursing, H. Slot, H.J. Pouw, The enumeration of „all" spore-bearing cells of Bacillaceae, *Antonie van Leeuwenhoek, 39* (1973) 656.

30. M. Singh, S.K.S. Patel, V.C. Kalia, *Bacillus subtilis* as potential producer for polyhydroxyalkanoates, *Microbial Cell Factories, 8* (2009) 38.

31. P.M. Halami, Production of polyhydroxyalkanoate from starch by the native isolate *Bacillus cereus* CFR06, World J. Microb. Biot. *24* (2008) 805-812.

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

33. A. El-Hadi, R. Schnabel, E. Straube, G. Müller, S. Henning, Correlation between degree of crystallinity, morphology, glass temperature, mechanical properties and biodegradation of poly(3-hydroxyalkanoate) PHAs and their blends, *Polym. Test. 21* (2002) 665-674.

34. S.P. Valappil, S.K. Misra, A.R. Boccaccini, T. Keshavarz, C. Bucke, I .Roy, Large-scale production and efficient recovery of P(3HB) with desirable material properties, from the newly characterized *Bacillus cereus* SPV, *J. Biotechnol. 132* (2007) 251-258.

35. B. Hazer, A. Steinbüchel, Increased diversification of polyhydroxyalkanoates by modification reactions for industrial and medical applications, *Appl. Microbiol. Biotechnol.* 74 (2007) 1-12.

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Fig. 1. Bolivian map with the location where the samples were taken from. Inside the black circle: L. Colorada (1), L. Hedionda (2), L. Chiguana (3) and Uyuni Salt Lake (4). The environmental conditions and chemical-physical characteristics of these lakes are described by Hurlbert and Chang (21).

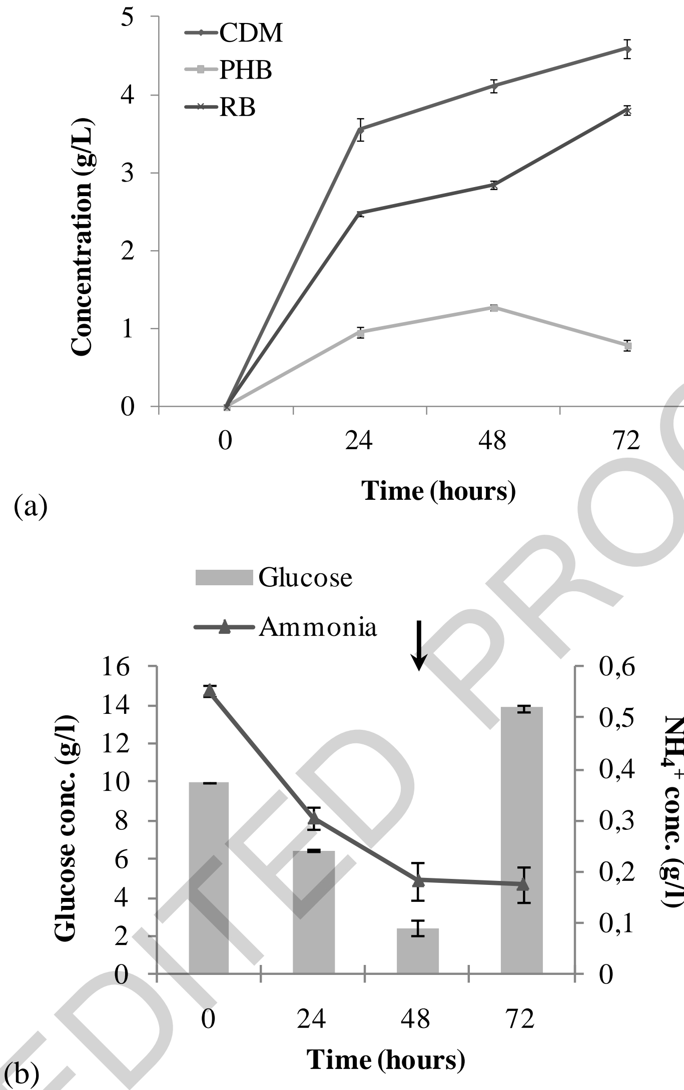

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Fig. 2. Fermentation time curves during 72 hours with *B. megaterium* uyuni S29. Cell dry mass (CDM), polymer (PHB) and residual biomass (RB) concentrations (a). Time curves of substrates (carbon & nitrogen source) of the fermentation with *B. megaterium* uyuni S29. The arrow indicates the time of re-feeding with glucose (50 % w/w) (b). The error bars indicate the deviation between two parallel set-ups

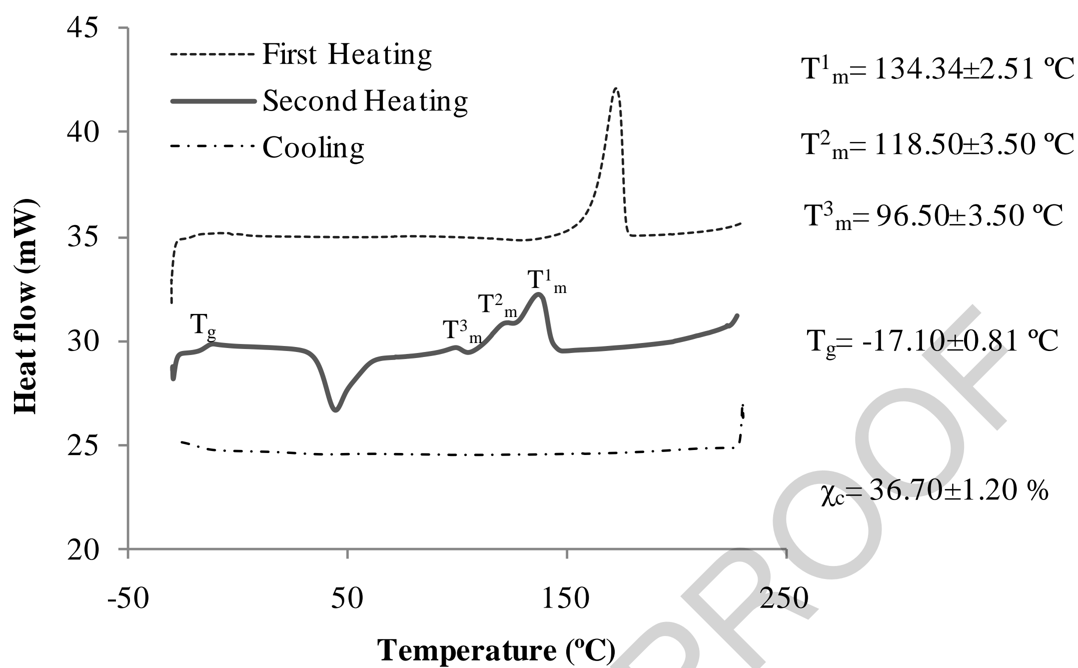

Fig. 3. DSC curve of PHB extracted from *B. megaterium* uyuni S29

Fig. 4. TEM micrograph of *B. megaterium* uyuni S29 after 4 hours of fermentation

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Table 1. Average and standard deviation values of the main parameters resulting from the fermentation process with *B. megaterium* uyuni S29

Parameter	Average
Final cell dry mass/ (g/L)	4.59±0.12
Maximum PHA/ (g/L)	1.30±0.04
Maximum Content of PHA in biomass [%] at t= 48 h	31±0.20
Maximum specific growth rate $\mu_{max\ (1/h)}$	0.10±0.04
Volumetric Productivity PHA/ (g/L·h)	0.03±0.03
Total consumption of glucose from t = 0 to 48 hours/ (g/L)	7.55±0.36
Yield $_{(PHA/Sugars)}$ from t = 0 to 48 hours	0.17±0.05
Yield $_{(CDM/Sugars)}$ from t = 0 to 48 hours	0.55±0.03

Table 2. Molar mass distribution of polymer extracted from *B. megaterium* uyuni S29 and the proportion of each different molar mass. Mn is the average number of the molar mass, Mw is the average mass of the molar mass, PDI is the polydispersity index and Mp is the peak-maximum of the molar mass.

	Percentage fraction	Mn (KDa)	Mw (KDa)	PDI Mw/Mn	Mp (KDa)
1st peak	76	705	795	1.12	740
2nd peak	13	135	190	1.40	174
3rd peak	11	27.0	39.6	1.47	31.3

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Table 3. Thermal properties from different PHAs

Polymer	Species	Mw-PDI (KDa)	T_m (ºC)	T_g (ºC)	X_c (%)	Reference
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	(*33*)
PHB from sugars	*C. necator*	230	150	-	60	(*33*)
PHB from glucose	*B. cereus* SPV	1100-1.75	169.71	2.04	57.66	(*34*)
PHB from glucose	*B. cereus* SPV	882-2.6	160.83	-2.45	54.42	(*34*)
PHB from glucose	*B. cereus* SPV	885-3.1	171.71	2.72	64.08	(*34*)
P3HV	*H. pseudoflava*	-	110-112-118	-15	56	(*35*)
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	(*33*)
P(3HB-co-20 %3HV)	-	-	145	-1	-	(*35*)
P4HB	*H. pseudoflava*	-	53	-40	-	(*35*)
P4HB	-	-	56	-48	55	(*21*)
P(3HB-co-18 %4HB)	-	-	165	-4	30	(*21*)
P(3HB-co-69 %4HB)	-	-	50	-36	-	(*21*)

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