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Production of Polyhydroxyalkanoate by *Bacillus thuringiensis* Isolated from Agricultural Soils of Cascas-Peru

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HIGHLIGHTS

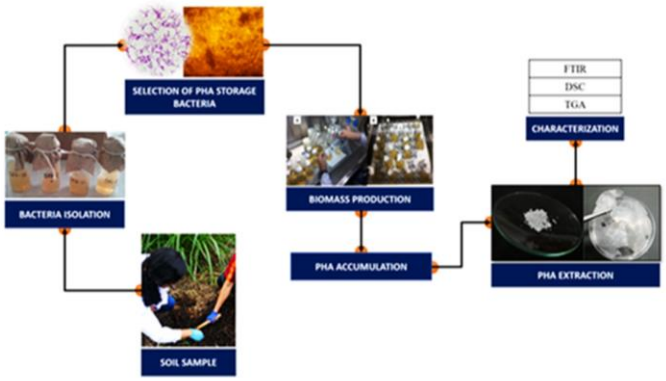
- *Bacillus thuringiensis* SP7-1 from agricultural soils producing polyhydroxyalkanoate.
- The strains SP7-1 with a PHA accumulation of 0.54 g/L and 19 % in dry biomass.
- PHA was characterized with FTIR, DSC and TGA with remarkable chemical properties.
- A thermic degradation in a range of 270-303°C and a Melting Temperature of 166.88°C.

Abstract: Polyhydroxyalkanoates (PHA) are biodegradable biopolymers of microbial origin that can be alternative materials to decrease the extensive use of plastics of petrochemical or synthetic origin. Thus, the selection of microorganisms with potential for PHA production from unexplored natural sources is a strategy to find bacterial species of high value. In the current study, 55 microbial strains related to bacteria were isolated from agricultural soils from Cascas, Peru. Initially, 4 strains were selected by Sudan Black B staining and Nile blue A fluorescence methods, subsequently, they were screened to examine its production capacity of PHA. *Bacillus thuringiensis* SP7-1 strain was selected based on its high production of PHA at 0.54 ± 0.16 g/L with an accumulation of 19 % by weight of cell biomass, during 72 h at 30 ºC. The isolate was characterized by its morphology, biochemical and molecular tests through the 16S rRNA gene. The extracted

PHA was characterized quantitatively by HPLC and qualitatively by FT-IR. Its thermal properties were determined by TGA and DSC, revealing a thermal degradation temperature of up to 270-303 ºC and a melting temperature of 166.88 °C. Therefore, *Bacillus thuringiensis* SP7-1 can be used as a model system for the production of PHA with efficient thermal stability, and optimize its performance in future research, as well as being applied in obtaining of molded bioplastics.

Keywords: Biopolymer; Biotechnology; Bioplastic; Polyhydroxyalkanoate.

GRAPHICAL ABSTRACT



INTRODUCTION

Petrochemical plastic pollution has become a global environmental challenge; because plastic waste generates a negative impact on aquatic life, soil, air and human life, caused by the emission of toxic gases and harmful acids, suffocation due to abundant accumulation, ingestion of microplastics, plastic leaching, among others [1]. Likewise, during the chemical synthesis of plastics, some chemical substances are usually added, such as bisphenol A, phthalates and fire retardants that give it some special properties; however, these substances are toxic and can cause risks to human health, causing breast cancer, prostate cancer, metabolic disorders, sterility, obesity, among others [2].Therefore, it is essential to find eco-friendly substitutes for plastics that have similar and even better physical-chemical characteristics and that can be generated with production costs equal to or lower than traditional plastics [3].

Polyhydroxyalkanoates (PHA) are intracellular polyester biopolymers that can be extracted from various microorganisms as reserve material when they grow under certain stress conditions [4]. Its main characteristics of the PHA are biodegradable, non-toxic, high flexibility, biocompatible, thermostable and crystalline that are competitive with plastics of petrochemical origin [5]. PHA-based bioplastics confer unique and sustainable properties by being highly deformable, resistant to heat and hydrolysis, showing broad similarity to linear low-density polyethylene [6]. This has made it possible to adapt to different types of applications, in electronics, cosmetics, the biomedical sector, agriculture, packaging industries and transparent biofilms [7].

PHAs they present many advantages, however, the production cost is elevated compared to polypropylene, associated in the bioprocessing with the sterilization, low conversion of carbon substrates, low growth of microorganisms and low production yield [8]. For these reasons, strategies have emerged to improve PHA production with the least possible investment, and one of these is the selection of PHA-producing wild microorganisms, due to the fact that it has been reported that the microbiome of environmental samples and the stress generated by the environment, it can increase to 40 times the yield of PHA [9].

La Libertad (Peru) is characterized by its extensive agro-industrial trade in the production of vegetables, fruits, among others, of national importance [10]; it has a diversity of agricultural soils that are rich in cultivable microorganisms, especially of the *Bacillus* genus that are residents of the rhizosphere of many crops [11]. However, an indiscriminate use of inorganic fertilizers and pesticides in agricultural practices has been reported, as well as irrigation water contaminated with heavy metals in La Libertad, Peru [12]; therefore, these abiotic factors affect the physiological, biochemical and molecular parameters of the plant and in turn the stress of the soil microbiome [13]. These limitations constitute potential areas for the search for native microorganisms that produce polyhydroxyalkanoates for their potential application in the development of new materials and scale-up in future research. In the present investigation, microorganisms with potential for PHA

production were explored and selected from agricultural soils of Cascas of the region La Libertad (Peru), and the PHA was characterized by means of high-performance liquid chromatography (HPLC), infrared spectroscopy (IR), Differential Scanning Calorimetry (DSC) and Thermogravimetric analysis (TGA).

MATERIAL AND METHODS

Site description and sample collection

Soil samples were collected to a depth of 15 cm with a sterile inoculating loop/spoon, from three localities of agricultural soils of grapevine cultivation in the last harvest cycle, of the district of Cascas, La Libertad, Peru (Figure 1). They were sifted through a 2-mm mesh to remove the roots and plant's debris. Samples were transported in sterile bags and stored at 4 °C. The color was described according to the Munsell system, the texture was determined using the USDA system. The pH of the soil was also determined [14].

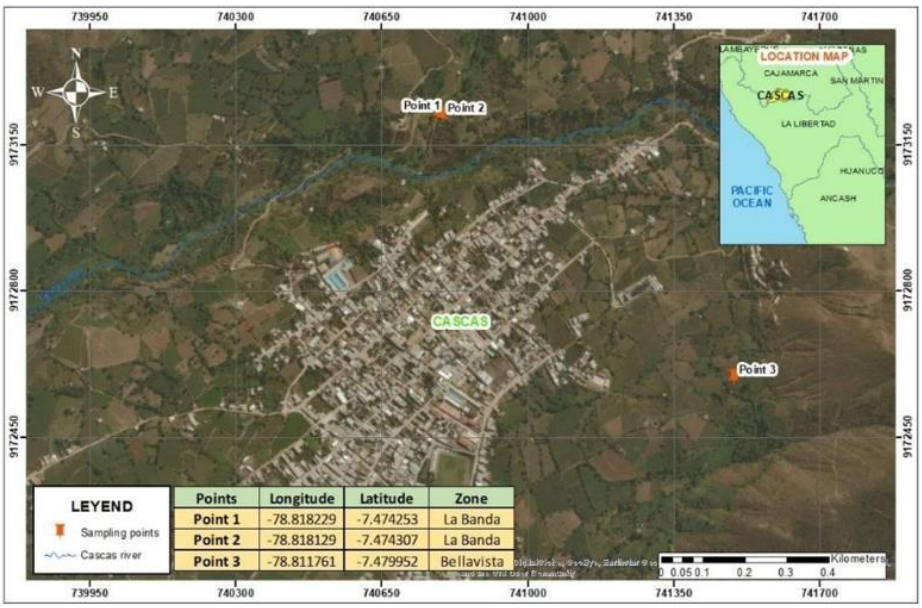


Figure 1. Locations of sampling points of agricultural soils in Cascas - La Libertad - Peru.

Isolation and screening of PHA production

10 g of soil was suspended in 90 ml of sterile peptone water (g/L): peptone 1 and NaCl 5; it was agitated during 15 min at 150 rpm and let rest during 5 min. From the supernatant, samples were serially diluted and the last three dilutions they were planted by surface in plates with glucose nutrient agar (GNA) (g/L): anhydrous glucose 20; yeast extract 2, beef extract 1, peptone 5, NaCl 5, agar 15; it was adjusted to pH 7 and incubated at 30 °C for 24 hours. The selected colonies were kept inclined in GNA and 15% glycerol for additional study. Isolates were analyzed for determine the PHA production in GNA by staining with Sudan Black B and Nile Blue A for observe intracellular PHA granules [15].

Bacterial isolates characterization

The selected colonies were identified with the biochemical tests shown in Table 1, as described in Bergey's Manual of Systematics of Archaea and Bacteria [16] and by ABIS (Advanced Bacterial Identification Software) [17]. Genomic DNA extraction and purification was performed from a pure 2-day old culture by DNA extraction kit (InnuPREP DNA kit, AnalytikJena, Germany) and the 16S rDNA gene was amplified by polymerase chain reaction (PCR) according to the methodology described by Cruz et al. [18], using the universal oligonucleotides of 27F (5'-AGAGTTTGATCMTGGCTC-3') and 1942R (5'-TACGGYTACCTTGTTACGACTT-3') and the amplified rDNA was characterized on a 1.5% agarose gel. The obtained amplicons were sent to sequence to Macrogen (Seoul, South Korea) using next generation sequencing (Ion Torrent, MR DNA). The partial sequence of the 16S rDNA gene was analyzed with the Mega X software for alignment and phylogenetic trees were constructed; it was compared with the available nucleotide sequences in the EzBioCloud database (https://www.ezbiocloud.net) [19].

PHA scale production

The fermentation to Erlenmeyer scale was done in a culture medium (g/L): glucose 20, peptone 5 and meat extract 3. Fermentation was done in 125 ml flasks with 27 ml of culture medium and 3 ml of bacterial inoculum with optical density (OD) of 0.5 in orbital agitation (Shaking Incubator LBSI-100A) at 30 °C and 250 rpm for 72 hours. It was executed in triplicate to compare the PHA production parameters, evaluating performance every 24 hours from Y: X/S (Biomass dry weight (g)/Reducing sugars (g/L)), Y: P/X (Concentration of PHA produced ((g/L)/Biomass dry weight (g)) and Y: P/S (Concentration of PHA produced (g/L)/ Reducing sugars (g/L)) [20].

The PHA was extracted by digestion of the dry biomass using sodium hypochlorite (NaClO, Merck) (4.5%), in a proportion of 30 ml of NaClO for each 1 g of dry biomass, at room temperature for 8 hours. Subsequently, the precipitate was centrifuged at 5000 rpm for 10 min and rinsed with distilled water and ethanol (96 %). Then, it was dried at 50 °C and stored for later analysis [21].

Biomass production was evaluated by dry weight. Residual glucose consumption was determined by 3, 5-dinitrosalicylic acid method, using a UV/VIS Spectrophotometer (EvolutionTM 260 Bio) [22]. The accumulation of poly-3-hydroxybutyrate was determined by HPLC-UV using a Thermo Scientific Ultimate 3000 Rapid Separation UHPLC and on a Shodex SUGAR SH1011 column, elution was performed with 0.01 N H_2SO_4 at 1 mL/min and 50 °C [23].

Characterization of extracted biopolymer

The main functional groups of the PHA were analyzed by Fourier transform infrared spectroscopy with attenuated total reflectance (ATR) accessory using a Nicolet IS50 FTIR spectrometer (Thermo Fisher) with a spectral 4000 and 550 cm^{-1} range with a 4 cm^{-1} resolution [24].

A thermal analysis was performed to determine the melting temperature (Tm) and the melting enthalpy (ΔHm) in the sample of the obtained biopolymer and the commercial PHA, using the differential scanning calorimeter DSC 250 (TA Instruments). Approximately 5 mg of sample was loaded into a hermetically sealed aluminum pan and then heated from 0-200 °C, at a heating and cooling rate of 20 °C/min in a nitrogen environment with a gas flow of 20 mL/min [25].

The thermal stability of the biopolymer was determined by thermogravimetric analysis through the difference in mass loss between a sample charge in an aluminum crucible and an empty one as a reference for temperature increase. TGA analysis was performed using the TGA550-TA, with a scanning temperature of 25 to 400 °C at a heating rate of 20 °C/min and 200 mL/min of nitrogen flow [26].

Statistic analysis

All experimental essays were performed in triplicate and the values were recorded as mean ± standard deviation using Microsoft Excel software.

RESULTS AND DISCUSSION

Isolation and microbial characterization from agricultural soil

The soils of Cascas, La Libertad (Peru), are considered grape producers and the high demand for cultivation has caused the excessive use of chemical fertilizers and soil erosion [27]. The agricultural soil samples presented a pH of 6.6-7 with a loamy and clayey type texture of yellow brown color, the latter shows a soil with a low profile in organic matter indicating a limitation of microbial nutrient [28]; however, these analyzes do not manifest a complete analysis of contamination.

55 pure microbial cultures were obtained from the three agricultural soil samples (SP1, SP2 and SP7) and using Sudan Black B staining 4 positive isolates were found that showed circular granules of dark colored inside the bacterial cells (Figure 1). This was a preliminary test for the identification of bacterial colonies that accumulate PHA. Subsequently, of the 4 positive isolates, they were stained with the Nile blue A to confirm the identification of PHA production, through fluorescence microscopy was revealed intracellular bright orange fluorescence under ultraviolet (UV) light (Figure 1). The 4 confirmed isolates were coded as SP1-6, SP2-12, SP7-1 and SP7-18; these colonies were subcultured and temporarily preserved at -20 ºC for future use.

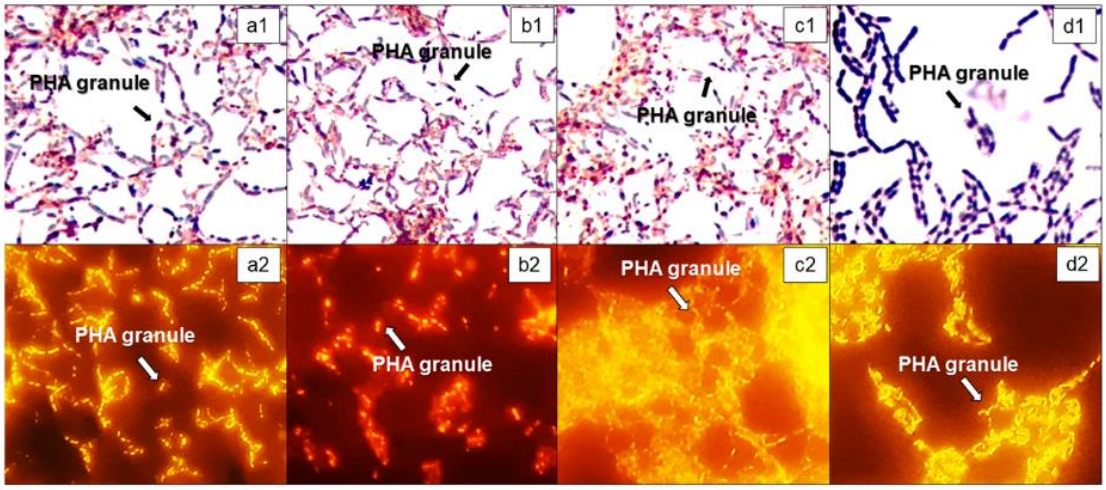


Figure 2. Micrographs using fluorescence microscopy of Sudan Black B staining (a1: SP1-6 strain; b1: SP2-12 strain; c1: SP7-1 strain; d.1: SP7-18 strain) and with Nile blue A staining (a2: SP1-6 strain; b2: SP2-12 strain; c2: SP7-1 strain; d2: SP7-18 strain).

The morphological characteristics of the isolates SP1-6, SP2-12, SP7-1 and SP7-18 were observed in pure cultures from agar after incubation at 37 ºC for 24 h. Their morphological characteristics are shown in Table 1, all the strains were proved gram-positive, which brings with it an advantage in the study by not producing endotoxins in the outer membrane of the lipophilic granules like those present in the gram-negative [29]. The SP7-1 and SP2-12 strains showed rod cell shape, while SP1-6 and SP7-18 showed a coccoid morphology. Likewise, the SP7-1 strain presented white colonies unlike SP2-12 which showed to be cream; at the same time SP1-6 presented white colonies and SP7-18 cream; this indicates the possible variability of different species among the isolates obtained. Presumptive identification for SP7-1 and SP2-12 strains was achieved using the free software ABIS, being inconclusive for SP7-18 and SP1-6 strains (Table 1). In a similar work, the same morphological and biochemical study was carried out to identify cultures of the genus *Bacillus* in soils [30]. In the literature, other studies have been reported on the use of *Bacillus sp.* isolated from agricultural soils of sugar cane, to synthesize and extract PHA [31]. Likewise, it has been found that they possess hydrolytic enzymes, such as amylase and protease that can be exploited for the economic production of PHA, being typical of *Bacillus* species [32]. Likewise, *Bacillus* species have been selected as PHA-producing representatives due to their genetic stability, higher growth rate, ability to secrete various hydrolytic enzymes, and the absence of an outer layer of lipopolysaccharides that facilitates their simple extraction [33].

Table 1. Morphological and biochemical characteristics of *B. thuringiensis* strains isolated from agricultural soils in Cascas-La Libertad-Peru.

Characteristic	**SP7-1**	**SP2-12**	**SP7-18**	**SP1-6**
Colony color	White	Cream	White	Cream
Gram stain	+	+	+	+
Cell shape	Cane shaped	Cane shaped	Cocci shaped	Cocci shaped
Spores	+	+	-	+
Catalase	+	+	-	+
TSI	K/A	K/A	NF	K/A
Glucose	+	+	+	+
Saccharose	-	-	-	-
Arabinose	-	+	-	+
Xylose	-	-	+	+
Mannitol	-	-	+	-
Citrate	+	-	+	+

Cont. Table 1

Ornithine	-	+	+	+
Urea	-	+	+	+
7% NaCl growth	+	-	+	-
Amylases	+	+	-	+
Gelatin	+	+	-	-
Proteases	+	+	+	+
Oxidase	-	-	-	-
Indole	-	-	-	-
Motility	+	+	-	+
Methyl red	-	-	-	+
Voges Proskauer	+	+	-	+
Nitrate reduction	+	-	-	+

The SP7-1 strain showed a higher performance of PHA production in this study (Table 2), so it was molecularly identified by sequencing the 16S rRNA gene with a fragment size of 1205 base pairs (bp). The 16S rRNA gene sequences aligned, using the MUSCLE algorithm with default settings, indicated that the SP7-1 strain had a similarity of 99.82% with *Bacillus thuringiensis* ATCC 10792, and based on the phylogenetic tree showed an evolutionary relationship between the SP7-1 bacterial strain with 52 other 16S rRNA sequences closely related belonging to *Bacillus* species retrieved from the EzBioCloud database (Figure 3). Therefore, according to the phylogenetic tree constructed with the similarity of the 16S RNA (%), the strain *Bacillus thuringiensis* SP7-1 was identified as *Bacillus thuringiensis* ATCC 10792.

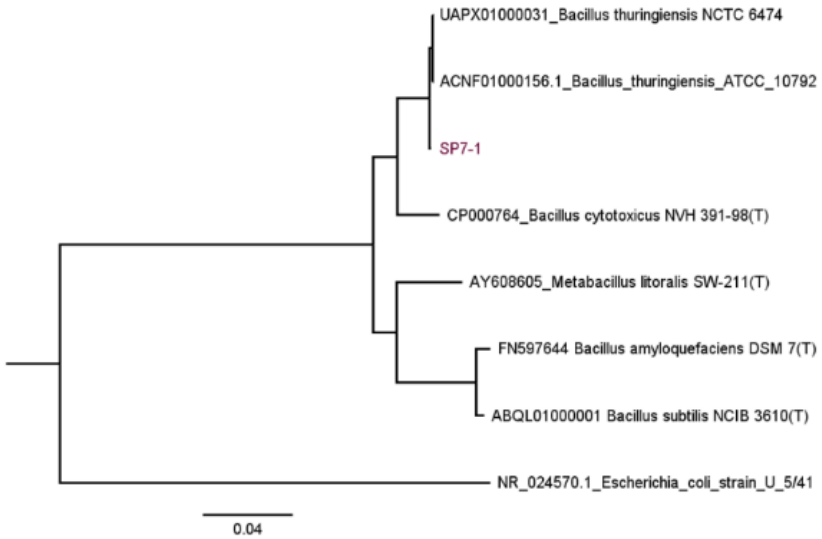


Figure 3. Phylogenetic tree using the Neighbor-Joining method based on 16S rRNA gene sequences showing the position of strain SP7-1. Evolutionary distances were calculated using Kimura's 2 parameter method. *Escherichia coli* was used as an outgroup (NR_024570.1).

Erlenmeyer scale production, PHA performance, and selection of PHA-producing bacteria

The PHA production study revealed a higher performance of the *Bacillus thuringiensis* SP7-1 strain of 0.54 g/L with a cell biomass of 2.81 g/L for 72 hours from 20 g/L of glucose, through the method of sodium hypochlorite extraction (Table 2). This result differs from the other 3 isolated strains SP2-12, SP7-18 and SP1-6 that showed performance of 0.17, 0.20 and 0.14 g/L of PHA respectively, with a maximum difference of 60%. There are several reported that supporting PHA production, such as 0.33 g/L for *B. thuringiensis* SBC4 [34], 3.6 g/L for *Bacillus thuringiensis* E101 [35], and 0.36 g/L for *Bacillus sp*. [36].

On the other hand, it was found that the SP7-1 strain accumulated 19% of PHA in dry weight of the cell biomass. This result last is found in the range reported by Baikar and coauthors [37] between 14-23% of three isolated cultures of from a biogas plant; however, have been found in other studies high values of up

to 79% with other species of *Bacillus cereus* [38]. In addition, there have been reported *Bacillus thuringiensis* strains that can accumulate up to 58.5 % in glucose (20 g/L) due to nitrogen deficiency [39]. Although it is true, specific limiting factors can enhance PHA production, such as nitrogen which improved PHA productivity in *Cupriavidus necator*, or phosphorus in *Pseudomonas putida*, but in other cases without nutritional limitations PHA production was higher in *Azohydromonas lata* [40]. At the present investigation, the preliminary essays were made with a limiting nitrogen factor (peptone) for the production of PHA; nonetheless, it is estimated that with other nitrogen sources, as ammonium sulfate and ammonium Nitrate, the production can increase, as Zakaria [41] demonstrated. For this reason, is important to identify the several conditions of nutrient limitation (potassium, sulfur and phosphate) to optimize PHA production from *Bacillus thuringiensis* SP7-1 for further studies.

Table 2. Kinetic parameters of biomass fermentation and polyhydroxyalkanoate production in Erlenmeyer scale of 24 hours.

Kinetic parameters	SP7-1	SP2-12	SP7-18	SP1- 6
Cellular biomass (g/L)	2.81±0.19	1.29±0.30	1.57±0.11	0.99±0.02
Consumed glucose (g/L)	10.33±1.15	4.34±1.34	2.52±1.15	3.50±0.97
PHA accumulation (g/L)	0.54±0.16	0.17±0.08	0.20±0.04	0.14±0.04
PHA accumulation (%)	19.22±5.84	13.18±6.92	12.74±2.70	14.14±4.05
$Y_{X/S}$ (g/g)	0.293±0.05	0.083±0.03	0.090±0.01	0.060±0.002
$Y_{P/S}$ (g/g)	0.055±0.01	0.011±0.006	0.012±0.003	0.008±0.002
$Y_{P/X}$ (g/g)	0.195±0.07	0.127±0.03	0.127±0.014	0.140±0.03

Characterization of extracted biopolymer

PHA extracted from *Bacillus thuringiensis* SP7-1 was characterized by FTIR spectroscopy. The analysis of the functional groups was carried out in the range of values of the wavenumber of 4000-550 cm^{-1} (Figure 4). A stretching of the band in 1723 cm^{-1} was found belonging to the carbonyl bond (C=O) caused by the PHA [42]. In turn, other common bands were identified between 1100 -1300 cm^{-1} showing a stretching of the C-O bond of the ester group [43]. The spectrum obtained was compared with the commercial PHA in the Figure 4.

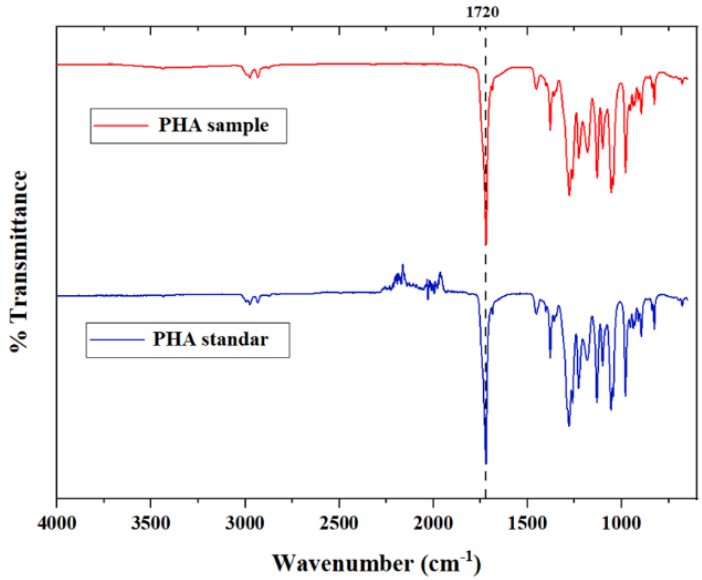


Figure 4. Infrared spectrum of the PHA extracted from the SP7-1 strain within the transmittance range of 4000-550 cm^{-1}. Characteristic peaks of the functional groups are shown and compared to the peaks of the standard polymer.

The thermal properties of the PHA extracted of the SP7-1 strain were performed by DSC and TGA analysis (Figure 5 and 6). The differential scanning calorimetry of the extracted PHA, revealed a Melting Temperature (Tm) peak of 166.92 °C with a Fusion Enthalpy of 84.57 J/g. Likewise, in the standard PHA a melting temperature of 174.63 °C was observed. The Tm of the PHA of SP7-1 is lower than standard PHA; however, values of Tm around 168 ºC produced by *Bacillus megaterium* [44] and 138.24 ºC by *Bacillus thuringiensis* [45] have been reported. This variation of Tm depends on the proportion of 3-hydroxyvalerate (3HV) units, which shape the biopolymer of PHA, in the same way; it affects the resistance and flexibility properties of the polymer [46].

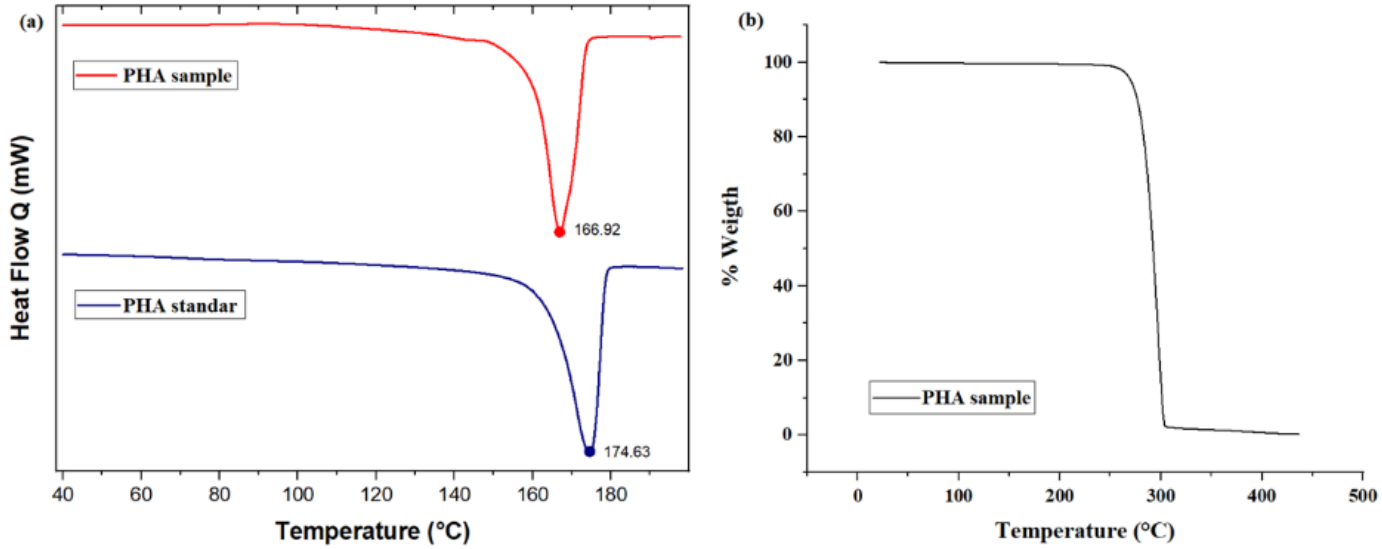


Figure 5. (a) DSC thermogram of PHA extracted from SP7-1 and standard PHB. (b) Thermogravimetry of a sample of PHA obtained from the SP7-1 strain.

The thermal degradation temperature (Td) for the extracted PHA produced by the SP7-1 strain was in the range of 270 - 303 ºC, which was slightly higher than the thermal degradation temperature of the standard PHA (234 - 302 ºC), in addition, it was higher than what was reported by Lorini and coauthors [26] who found temperatures in the range of 248 – 258 ºC for a PHA extracted from *Pseudomonas putida* LS46123 and from Suguna and coauthors [45] that reached temperatures up to 269.36 ºC for a PHA from *Bacillus thuringiensis*. Its thermal stability of the extracted polymer is also related to a more crystalline morphology in its chemical structure [47]. We infer that the PHA obtained in this study has better thermal stability and this can allow its application in obtaining molded products like sheets, films, scaffolds, among others.

CONCLUSION

In the present study, different bacterial isolates were obtained from Cascas, Peru and analyzed for PHA production using glucose as carbon source. *Bacillus thuringiensis* SP7-1 producer of polyhydroxyalkanoate was obtained for the first time from agricultural soils in Cascas La Libertad (Peru). The PHA yield was greater than 0.54 g/L and a PHA accumulation of 19% of the dry weight. The strain was characterized and identified by morphological, biochemical and molecular characteristics, determining a similarity of 99.82% from the aligned sequences of the 16S rRNA gene. The PHA produced showed remarkable chemical and thermal properties, characterized by FTIR, DSC and TGA, reporting the characteristic band at 1723 cm^{-1} due to the presence of the carbonyl bond and the band between 1100-1300 cm^{-1} due to the stretching of the C-O bond of the ester group. As well as a thermal degradation in the range of 270 °C to 303 °C and a melting temperature of 166.92 °C. All this places this strain of *Bacillus thuringiensis* in an ideal position for its possible application as a source of bioplastic.

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Conflicts of Interest: The authors declare no conflict of interest.

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