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ORIGINAL PAPER

Characterization and Production of a Polyhydroxyalkanoate from Cassava Peel Waste: Manufacture of Biopolymer Microfibers by Electrospinning

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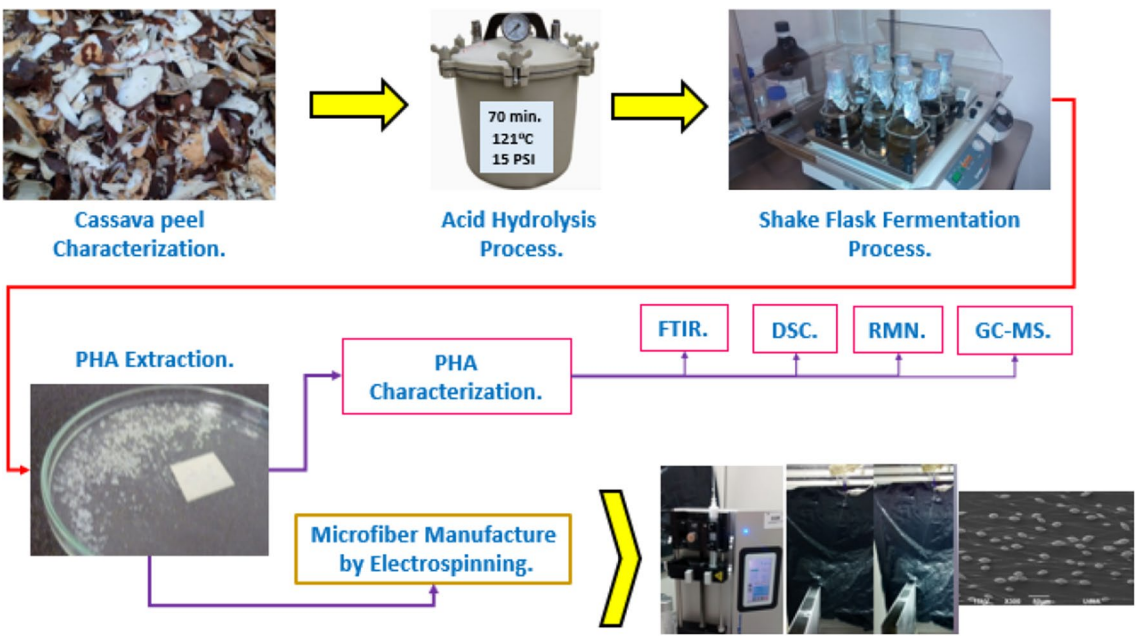
Abstract

Hydrolysate cassava peel obtained from agro-industrial residues was used as a carbon source in the production of polyhydroxyalkanoates (PHAs) by *Cupriavidus necator*. The optimum culture conditions were pH 9, a C/N ratio of 11, and a C/P ratio of 7. Characterization of the PHA polymers by FTIR, DSC and GC–MS revealed copolymers consisting of 3HB and 3HV monomers with melting temperatures (*Tm*) between 100 and 150 °C. Random and aligned microfibers with average diameters of 1.56 ± 0.41 µm and 1.72 ± 0.52 µm, respectively, were produced by electrospinning. In general, it was possible to obtain PHAs from cassava peel, and PHAs could be applied to produce fibers by electrospinning. The above strategy is an alternative use of agro-industrial cassava waste and opens up potential applications in the manufacture of biopolymers for their use in industry and biomedicine.

Graphic Abstract



Keywords Cassava peel · Polyhydroxyalkanoate · Electrospinning · Biopolymers · NMR

Extended author information available on the last page of the article

Introduction

Cassava cultivation (*Manihot esculenta*) is of great importance for Colombia's economy. In fact, 2.1 million tons of cassava are produced annually on a crop area of approximately 230,000 ha [1]. Consequently, cassava production generates approximately 630,000 tons/year of agro-industrial waste [1]. Therefore, developing applications for cassava waste products is of major importance. Some possible applications for cassava waste have already been proposed, including the production of cassava pullulan [2], biofertilizers [3], absorbent Bio-Bio [4] and biogas [5], which is important since only a small amount of agricultural waste is currently used to produce animal feeds, manure, and other valuable products. The production of biopolymers is another valuable alternative for using agricultural waste [6]. In addition, bioplastics are superior to conventional plastics derived from fossil fuels in terms of energy efficiency, carbon emission, and the consumption of petroleum [7]. This work assesses the potential use of cassava waste for the production of polyhydroxyalkanoates (PHAs).

PHAs are biodegradable polyesters synthesized by different bacterial strains that store these polymers in intracellular pockets as energy reserves in response to excess carbon and under nutrient-limited conditions [8]. PHAs degrade completely into nontoxic products [9], constituting an important solution to the environmental problem caused by the use of persistent petroleum-based synthetic plastics. The monomeric composition of PHAs is related to both the nature of the supplied carbon source and to the bacterial strain [10]. PHAs are thermoplastic or elastomeric polyesters (poly-oxoesters) of *R*-hydroxyalkanoic acid (HA) monomers. Structurally, these polymers are classified based on the number of carbon atoms of their monomeric units, which can range from 3 to 14 carbon atoms. PHAs with monomers of 3-5 carbon atoms are short-chain length PHAs (scl-PHAs), and those with monomers of 6–14 carbon atoms are medium-chain length PHAs (mcl-PHAs) [11]. Homo- or heteropolymers can be produced if the polymer consists of a repeating monomer unit or different types of monomers, respectively. The most common PHA is the short-chain homopolymer poly-3-hydroxybutyrate (PHB). PHB has shown enormous potential for applications in bone plates, nails, screws and the treatment of osteomyelitis [12].

Several studies have reported the production of different PHAs by various strains, namely, *Cupriavidus necator (*previously known as *Ralstonia eutropha* [13]*, Brevundimonas vesicularis* LMG P-23615, *Sphingopyxis macrogoltabida* LMG 17324 [14] *or* Comamonas *testosterone* [15]. Carbon sources for PHA production include refined substrates such as fructose [16], or industry and agro-food residues such as milk whey [17], glycerol waste [18], soy cake [13], sawdust [14] or vegetable oil [15]. A review on the production of polyhydroxyalkanoates (PHAs) from waste materials and byproducts by submerged and solid-state fermentation has been published previously [19]. Some authors have reported PHA production from cassava starch using different strains, such as *Cupriavidus sp.* KKU38 [20], *Bacillus tequilensis* MSU 112 [21] and different *Bacillus* species [22]. Likewise, PHAs have been produced from cassava powder using *Bacillus sphaericus* [23], *Bacillus megaterium* MSBN04 using tapioca industrial waste and solid-state cultivation [24]. Finally, the authors were able to produce PHB using a tapioca hydrolysate with an *Alcaligenes eutrophus* culture [25].

PHAs are applied in different fields, including biomedicine, food, packaging, textiles and household compliances [11]. Biomedical applications of PHAs include cardiovascular products, vascular grafts, stents and heart valves, dental and maxillofacial treatments, orthopedics, urology and materials for wound management (sutures and dressings) [26]. Another application of PHAs is the fabrication of scaffolds for tissue regeneration using PHA electrospun fibers [27].

The electrospinning process uses an electrical field as driving force to direct a polymer solution or melted polymer from a syringe tip towards a metal collector, producing thin fibers with micro- to nanoscale diameters. These fibers are usually organized in a nonwoven mesh, forming a porous structure [28]. Different types of micro- and nanofiber meshes have been fabricated with different polymers by electrospinning and assessed for different applications [29, 30].

This research aimed to study polyhydroxyalkanoate production by fermentation using cassava peel agro-industrial waste as a substrate to develop an application for cassava waste use in Colombia. The emphasis of this study was an evaluation of the fermentation conditions and their effect on the biopolymer structural properties. Finally, the fermentation conditions that allowed the highest biopolymer productivity were used to produce random and aligned microfiber meshes by electrospinning.

Materials and Methods

Microorganism

Cupriavidus necator DSM 531 *(Ralstonia eutropha* ATCC 17697) was selected because it demonstrated to be a versatile strain able to grow in different carbon sources and an excellent PHB producer [31].

Chemicals

All chemicals were of analytical grade and commercially available from the major suppliers. Ammonium sulfate ($(NH_4)_2SO_4$, MERCK®) and dipotassium phosphate (K_2HPO_4, PANREAC®) were used as nitrogen and phosphorus sources, respectively. The complex medium OXOID® (Thermo Scientific) was used as the growth medium for the inoculum. Biopolymer extraction was conducted by using chloroform (MERCK®) and ethanol (PANREAC®), while dichloromethane (Fluka Analytical®) was used to dissolve the polymer for electrospinning. The commercial PHB sample was supplied by BIOMERCK®.

Cassava (Manihot esculenta) Peels

Cassava peels from the Uraba region, Antioquia-Colombia, were purchased from Medellin's local market. The cassava variety used in this study was ICAP13. Cassava peel samples were washed to remove dirt and ground in an Oster blender (Oster, 600 W, model BRLY07, Mexico). Afterwards, they were frozen and stored at − 20.0 ± 0.5 °C until further processing. Peels were characterized by measuring moisture content, ash, fat, total nitrogen and total protein content (using the 6.25 coefficient) [32]. Additionally, the Mg, K and Zn contents were determined by atomic absorption. The total carbohydrate content $\%CH$ was calculated according to Eq. 1:

$$\%CH = 100\% - \sum (\%Moisture + \%Ash + \%Fat + \%Protein) \quad (1)$$

Pretreatment of Cassava Peels

Cassava peel hydrolysis (121 °C and 15 Psi for 70 min) was carried out using a 2% (v/v) sulfuric acid solution (97n % HONEYWELL®) and a solid load of 20% (w/v) (100 gr cassava peel/500 ml of 2% H_2SO_4 solution). The hydrolysate was stored at 4 ± 1 °C until further use. The liquid and solid fractions were not separated after the treatment.

Strain Storage and Inoculum Preparation

The lyophilized strain was activated according to the protocol established by ATCC, and then conserved in cryovials with glycerol at − 20 °C. The inoculum volume was 10% of the final volume (50 ml), and the preinoculum volume was 10% of the inoculum volume (5 ml). For preinoculum preparation, nutritious broth and distilled water were used with 13 g of nutrient broth diluted in 1000 ml distilled water as recommended by the commercial broth OXOID®. The broth was sterilized at 121 °C and 15 psi for 15 min. Then, the broth was stored until further use. The inoculation of the preinoculum was carried out at room temperature. The flask was incubated at 30 °C under agitation at 200 rpm for 24 h [14, 31].

Culture Conditions

Shake Flask Assays

Shake flask assays were conducted in 500 ml Erlenmeyer flasks to test the use of the pretreated cassava peels as substrates for cell growth and polymer production, with a working volume of 250 ml. The hydrolysate pH was adjusted with 1 N NaOH. The carbon/nitrogen (C/N) and carbon/phosphorus (C/P) mass ratios were tuned to achieve the desired values (Table 2). A 10% (v/v) inoculum was used [33]. The flasks were placed in a reciprocal shaker (Heidolph Unimax 1010) at 30 °C and 200 rpm. Different cultivation times were tested. Each condition was tested in triplicate. During the fermentation process, the pH was not controlled. Tests using similar conditions of C/N and pH have been previously reported [33–35]. The fermentation time was between 12 and 60 h.

Sugar Quantification in the Hydrolysate

Glucose and fructose were quantified using an HPLC Agilent 1200 Series equipped with a refractive index detector. A Supelcogel C610H chromatographic column (300 mm × 7.8 mm) was used. The mobile phase was 0.008 N sulfuric acid with a flow of 0.6 ml/min. The oven temperature was 35 °C, and the injection volume was 20 µl. The retention times were 9.5 and 10.3 min for glucose and fructose, respectively.

Biopolymer Extraction and Characterization

Biopolymer Extraction

The culture broth was collected in 50 ml Falcon tubes and centrifuged for 30 min at 4000 rpm in a centrifuge (C-28 BOECO®, Germany). The supernatant was removed, and the pellet was freeze-dried. The biopolymer was extracted from the dried pellet using chloroform in a 1:4 biomass/chloroform ratio under vigorous agitation for 12 h at 30 °C. Subsequently, the dispersion was filtered on Whatman filter paper No. 1 to remove the solid fraction. After filtration, the dissolved biopolymer was slowly poured into 4 flasks containing cooled (4 °C) 96% ethanol, and the solution was gently stirred for 5 min. Afterwards, the polymer was allowed to precipitate for 3 h at 4 °C. Finally, the precipitated polymer suspension was filtered under vacuum using 25 µm filter

paper ADVANTEC® for polymer separation. Subsequently, to remove solvent residues, the polymer was dried at 65 °C until reaching a constant weight and then stored in glass containers at room temperature in the dark until further use [16, 36].

Fourier Transform Infrared (FTIR) Spectroscopy

FTIR spectra were obtained from a total of 16 scans at a resolution of 4 cm^{-1} within a wavenumber range of (ν) 4000–450 cm^{-1} at 22 °C using a Perkin Elmer Spectrum One model equipped with a DTGS detector. The samples were prepared as KBr pellets using a 100:1 KBr:sample mass ratio [37].

Differential Scanning Calorimeter (DSC)

A differential scanning calorimeter (DSC) (TA Instruments, Model Q100, New Castle-United States) was used to analyze the thermal properties of the polymer. Biopolymer samples of 3.4 mg were weighed in standard aluminum capsules. DSC measurements of the samples were performed at a rate of 20 °C/min in the range from 25 to 80 °C, followed by a heating cycle at the same rate to 150 °C. Subsequently, the samples were cooled to − 80 °C at a cooling rate of 20 °C/min. Finally, a second heating cycle was performed in the range from − 80 to 200 °C at a heating rate of 10 °C/min [38]. Glass transition temperatures (Tg) and Tm were taken from the second heating cycle. Tg was recognized as the midpoint of the changes in the baseline, and the melting temperature (Tm) was taken at the maximum peak. The Tg was determined with TA Instrument Software to DSC Equipment.

Nuclear Magnetic Resonance (NMR)

Nuclear magnetic resonance analyses were carried out using a Bruker Fourier spectrometer at 300 MHz for ^1H analysis. Deuterated chloroform was used as a solvent. The chemical shift at 7.26 ppm was used as reference to analyze the spectra. ^1H spectra were analyzed using the program NMR Mestre-C version 4.8.6.0. The 3-hydroxybutyrate (HB) and 3-hydroxyvalerate (HV) relative mole fractions in the copolymers were determined from the ^1H NMR spectrum using Eq. (2):

$$X = \frac{\gamma_1}{\gamma_1 + \gamma_2} \tag{2}$$

where X is the HV mole fraction and γ_1 and γ_2 correspond to the signal areas at 5.24 and 5.36 ppm, respectively, which further correspond to the methyl group resonance in HV and HB, respectively [39].

Gas Chromatography-Mass Spectrometry (GC–MS)

The biopolymer was subjected to hydrolysis and methylation to identify the monomers according to the protocol described by Rosengart et al. [40]. A 4% (w/v) polymer solution in chloroform was mixed with 1.0 ml of an acidic methanol solution containing hexanoic acid as the internal standard (97 ml of methanol, 3 ml of H_2SO_4 (95–97%) and 0.3 g of hexanoic acid). After vortexing for 1 min, the mixture was incubated for 5 h at 100 °C in a Memmert GmbH oven (Model 200). After cooling, the sample was neutralized by adding 1.0 ml of a 60 g/l solution of Na_2CO_3 and then vortexed for 1 min. The mixture was subsequently centrifuged at 2800 g for 5 min. The organic phase containing the methyl esters was analyzed with a Shimadzu QP2010 GC–MS equipment. The compounds were separated by a fused-silica capillary column Rxi-5Sil MS column (30 m × 0.25 mm ID). The oven and the injection temperatures were 60 °C and 120 °C, respectively. Nitrogen was used as carrier gas. Mass spectrometry was performed in electron-impact ionization mode; the electron energy was 70 eV. Peak assignment was archived by the MS library.

PHA Electrospinning and Fiber Processing

PHA in dichloromethane solutions 4% (w/v) were electrospun in an apparatus described elsewhere [27], which employed a spinneret with 0.51 mm inner diameter that was connected by a Teflon tube to a syringe pump (KDSLegato210) set to 1–2 ml/h under an applied voltage of 10–15 kV (Glassman, l ES/EL40P01), and the fibers were collected either on a flat stainless copper plate or a parallel stainless steel plate, both placed 15–20 cm below the spinneret to obtain randomly oriented or aligned PHA fibers.

Scanning Electronic Microscopy (SEM)

The microfibers produced by electrospinning were imaged to determine the dimensions of the fibers; a JEOL JSM-6490LV (Japan) SEM was used. The samples were fixed on graphite tape, underwent a thin coating of gold (Au) (equipment DENTON VACUUM Desk IV) and analyzed under high vacuum to obtain high-resolution SEM images. The secondary electron detector EDX was used to evaluate the morphology and the topography of the samples.

Experimental Design

A completely randomized design was employed to analyze the data. The different fermentation conditions for biopolymer production corresponded to the independent variable studied at nine levels (Table 1), and the biopolymer amount produced corresponded to the dependent variable.

Table 1 Physicochemical characterization of cassava peel

Parameter	Value on a wet basis	Value on a dry basis
Moisture (g/100 g)	78.05 ± 0.1	–
Total Ash (g/100 g)	1.24 ± 0.07	5.7
Fat (g/100 g)	0.08 ± 0.02	0.4
Total protein (N × 6.25; g/100 g)	0.88 ± 0.04	4.0
Total carbohydrate (g/100 g)	19.75	90.0
Mg (mg Mg/kg)	1.9 ± 0.9	8.7
K (mg K/kg)	32.3 ± 0.4	147.4
Zn (mg Zn/kg)	509 ± 0.01	2320.2

ANOVA was performed with a level of significance of 95% to determine the statistical significance of the differences. A multiple range test was performed by a Fisher LSD test to determine the difference between the treatments.

For the analysis of the images of the fibers obtained by electrospinning, 50 random measurements of the diameter of the fibers were taken, determining the average diameter of the fibers as well as the average length and diameter of the beads.

All statistical analyses were performed using STAT GRAPHICS Centurion XVI software (Statistical Graphics Corporation, Version 16.0.07, Rockville, USA).

Results and Discussion

Cassava Peel Physicochemical Properties

The physicochemical properties of the cassava peel samples used in this study are presented in Table 1. These values are slightly different than the values for cassava peel composition previously reported by Ki et al. [41], Moshi et al. [42] and Sudaryanto et al. [43]. In general, the cassava peels used in this work exhibit lower protein and ash contents than the values reported by other authors. These differences could be attributed to variations in the type of soil, cassava variety, crop growing climatic conditions and harvesting methods. It is worth noting that the carbohydrate content (calculated by difference) of these agro-industrial residues is very high (ca. 90% dry basis) and is thus a sugar source in biological products. The differences in the proximal composition of the different varieties of cassava could affect the production of PHA, depending on the content of available carbohydrates, which are susceptible to transformation into available sugars, a fundamental raw material for bacterial fermentation processes.

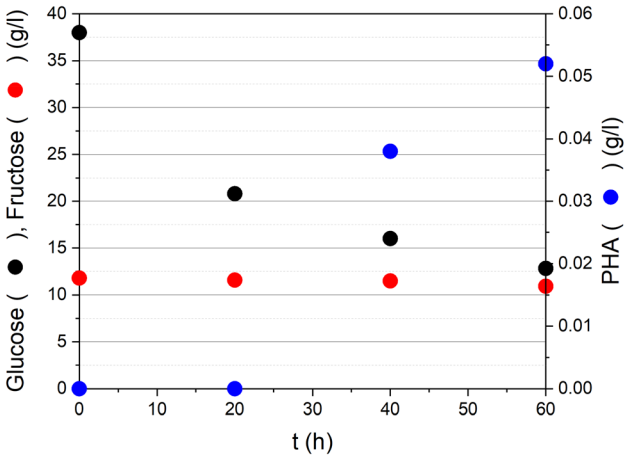


Fig. 1 Consumption Glucose, Fructose and PHA production from cassava peel residues for experiment 8 (Table 1) at pH 9, ratio C/N 11 and ratio C/P 7

Sugar Composition of the Cassava Peel Hydrolysate

The sugar composition of the cassava peel hydrolysate was determined by HPLC and had glucose and fructose concentrations of 38.90 ± 0.38 g/l and 11.30 ± 0.05 g/l, respectively. These values correspond to 17% and 5% of the total carbohydrates in cassava peel. It has been reported that hydrolysis of cassava peels or pulps may yield glucose, galactose, rhamnose, mannose, arabinose and xylose as reducing sugars [44]. Glucose values between 23 and 27 g/l for a cassava pulp hydrolysate were estimated after testing different levels of microwave irradiation treatment. Later, the same authors reported values of approximately 19–52 g/l glucose in microwave-assisted hydrolysis of cassava pulp [45]. Additionally, cassava peel hydrolysis with sulfuric acid resulted in a total amount of reducing sugars between 40 and 60 g/l [46].

Figure 1 shows glucose, fructose and PHA concentrations during the fermentation of cassava peel hydrolysate by *Cupriavidus necátor* at pH 9. The glucose concentration ranged from 38 g/l to 12.8 g/l after 57 h of fermentation, which corresponds to an average glucose consumption of 66.25%. Figure 1 shows that the highest glucose consumption occurred in the first 20 h, probably because the strain was growing. Several authors have reported sugar consumption between 62 and 68% in the production of PHAs using *Ralstonia eutropha* [47, 48].

Biopolymer Production

The results of biopolymer production are shown in Table 2. The results were analyzed by ANOVA using the LSD-Fisher method, with a significance level of 95%. Cultivation assays 1 and 8 (Table 2), which yielded the highest

Table 2 Cultivation conditions and biopolymer production from cassava peel waste at 30 °C

Condition	Assay condition				Replica	Production PHA (g/l)	g PHA/100 g Cassava	PHA average production (g/l)	PHA average production (g)
	pH	C/N*	C/P*	Time (h)					
1	7	11	4	30	1	0.0516	0.0258	0.055 ± 0.002 (c)	0.014 ± 0.002 (c)
					2	0.064	0.032		
					3	0.0484	0.0242		
2	7	1	4	30	1	0.05	0.025	0.036 ± 0.003 (a, b)**	0.0089 ± 0.003 (a, b)
					2	0.0352	0.0176		
					3	0.0224	0.0112		
3	7	6	1	30	1	0.0404	0.0202	0.034 ± 0.001 (a)	0.0085 ± 0.001 (a)
					2	0.0308	0.0154		
					3	0.0312	0.0156		
4	7	6	4	30	1	0.0316	0.0158	0.036 ± 0.001(a, b)	0.0089 ± 0.001 (a, b)
					2	0.0428	0.0214		
					3	0.0332	0.0166		
5	9	6	4	30	1	0.0232	0.0116	0.031 ± 0.002 (a)	0.0078 ± 0.002 (a)
					2	0.0288	0.0144		
					3	0.0416	0.0208		
6	6	3	2	12	1	0.0232	0.0116	0.04 ± 0.003 (a, b, c)	0.0099 ± 0.003 (a, b, c)
					2	0.042	0.021		
					3	0.0536	0.0268		
7	7	6	4	60	1	0.0272	0.0136	0.034 ± 0.002 (a)	0.0084 ± 0.002 (a)
					2	0.0392	0.0196		
					3	0.0344	0.0172		
8	9	11	7	60	1	0.0492	0.0246	0.052 ± 0.001 (b, c)	0.013 ± 0.001 (b, c)
					2	0.0464	0.0232		
					3	yy0.0608	0.0304		

*Ratio carbon/nitrogen (C/N) and carbon/phosphorous (C/P). **a, b, c: different letters mean that there were significant differences $p < 0.05$ between treatments for the average

polymer production (approximate final biopolymer titers of 0.056 and 0.052 g/l, respectively), corresponded to the assays with the highest carbon to nitrogen ratio (C/N = 11). These values are similar to those obtained by Khanna and Srivastava [49], who tested different substrates for polymer production by *Ralstonia eutropha* and reported the production of 0.031 g/l of PHB when using 20 g/l glucose as the substrate in shake flask assays.

Table 2 shows that fermentation condition 1 exhibited significant differences compared with the other conditions ($p < 0.05$), except for condition 8; in fermentation conditions 2, 3, 4, 5, and 6, the average production of extracted PHAs ranged from approximately 0.032 g/l to 0.04 g/l. According to Table 2, alkaline pH influenced the biopolymer production values; the more alkaline the pH was, the better the polymer accumulation by the bacteria, which was similar to a previously reported study [50].

In general, biopolymer production was affected by the fermentation time and the C/N ratio. Longer fermentation time and the higher C/N ratio increased the biopolymer production. At higher C/N ratios, nitrogen is consumed first, which increases the consumption of the available carbon source, promotes cell growth, and produces more PHA. This behavior is evident in fermentation condition 8.

The production values shown in Table 2 are lower than those reported in some previous studies using similar raw materials but use different strains and cultivation conditions. PHA titers were reported as 0.161 g/l [23] when PHA was produced from cassava powders. Higher PHA titers were reported when PHA was produced from cassava starch, and ranged from 0.27 g PHA/l to 2.81 g PHA/l [20] or from 0.10 g PHA/l to 1.07 g PHA/l [22]. Additionally, in batch cultivations, using cassava starch and the strain *Bacillus tequilensis* MSU 112, PHA titers from 0.02 to 3.3 g/l were attained [21]. The values in the present work are above the inferior limit of this range.

Despite the high glucose content of the cassava peel hydrolysate (approximately 40 g/l), the low polymer concentration values attained in this work and the incomplete glucose consumption are likely caused by an unbalanced cultivation medium composition. The medium for *C. necator* growth and production was obtained by supplementing the hydrolysate with a source of N or P and corrected for the pH with NaOH. There is a potential for the pH to decrease during fermentation due to a possible formation of acids and the consumption of ammonium ions by the bacteria. Although the pH was not monitored during cultivation, the reason for low polymer production is likely attributed to the lack of a

pH buffer in the medium [31]. The decrease in pH during the current assays is unfavorable for the development of bacteria and for the PHA production.

Biopolymer Characterization

Infrared Spectroscopy Analysis

Spectra determined by FTIR analysis for assays 1, 2, 3, 4, 5, 6, 7, and 8 and PHB standards (Patron) are shown in Fig. 2a, b. All spectra showed typical functional group band characteristics of PHA, such as OH (3200–3500 cm^{-1}),

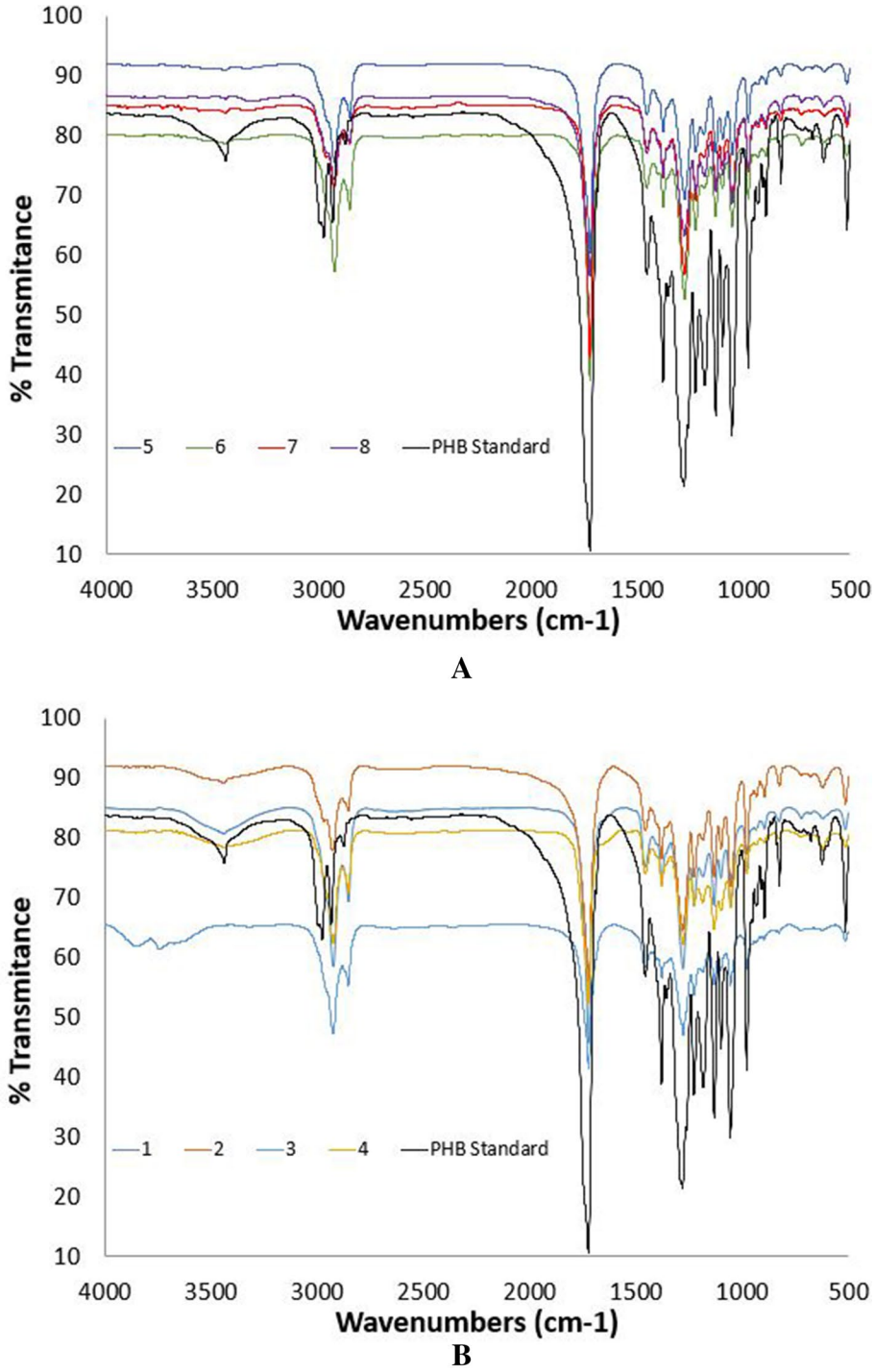


Fig. 2 FTIR spectra of PHA samples. **a** Spectra: 5, 6, 7, 8 and PHB standard. **b** Spectra 1,2,3,4 y and PHB standard

COC (1185–1228 cm^{-1}) [51], CH (2922–2929 cm^{-1}), CO (1000–1300 cm^{-1}) and CO (1600–1850 cm^{-1}) [37], with similar values compared to commercial PHB. Additionally, OH extension was reported between 3444 and 3449 cm^{-1} in commercial PHB (Aldrich Chemical) and PHAs obtained from canola oil, respectively [52, 53]. Likewise, 2928 cm^{-1} bands corresponded to CH group vibrations of PHAs produced from fructose by different strains [16, 54]. The 1724 cm^{-1} band attributed to the C = O extension was reported by Luo et al. [38] and Misra et al. [55], and was also found in the PHA samples produced in the current study. Moreover, asymmetric CH bending at 1452–1457 cm^{-1} and 1379–1384 cm^{-1} was found in this study and corresponded to CH_2 and CH_3 groups, respectively, which was also reported by Oliveira et al. [13] and Kansiz et al. [56]. In general, the different fermentation conditions shown in Table 2 allowed the production of biopolymers containing the characteristic functional groups of commercial PHB. However, the spectra did not change because the same substrate was used.

Differential Scanning Calorimetry (DSC)

Figure 3a shows the DSC thermograms of the PHA samples obtained from fermentation conditions 1, 2, and 4 as well as the thermogram of the commercial PHB sample (standard). Similar thermograms were obtained for the polymers produced from fermentation with conditions 1–8; the samples showed three melting temperatures and one glass transition temperature. The presence of different melting temperatures, as evidenced in the thermograms, is related to the presence of different polymer chains with different sizes and compositions [57].

The melting temperature (T_m), glass transition temperature (T_g), and fusion enthalpy (ΔH) obtained for the polymer produced under each fermentation condition are shown in Table 3. The second melting temperature for the polymers obtained from fermentation conditions 1 to 6 ranged between 100.5 °C and 109.7 °C and were similar to the T_m of the PHA sample obtained in assay 7 (T_m = 111.9 °C). These values of T_m are considerably lower than the T_m of the standard PHB (160 °C), suggesting the production of polymer chains with a very different composition. Enthalpies of fusion were estimated between 2.01 J/g to 44.87 J/g for the polymer from the fermentation of cassava peel hydrolysate, while a value of 71.42 J/g was obtained for the commercial PHB. Higher T_m values of PHBs have been previously reported, which were approximately 172 °C for PHBs obtained from sunflower husk, soybean, sawdust and paddy rice using *Ralstonia*

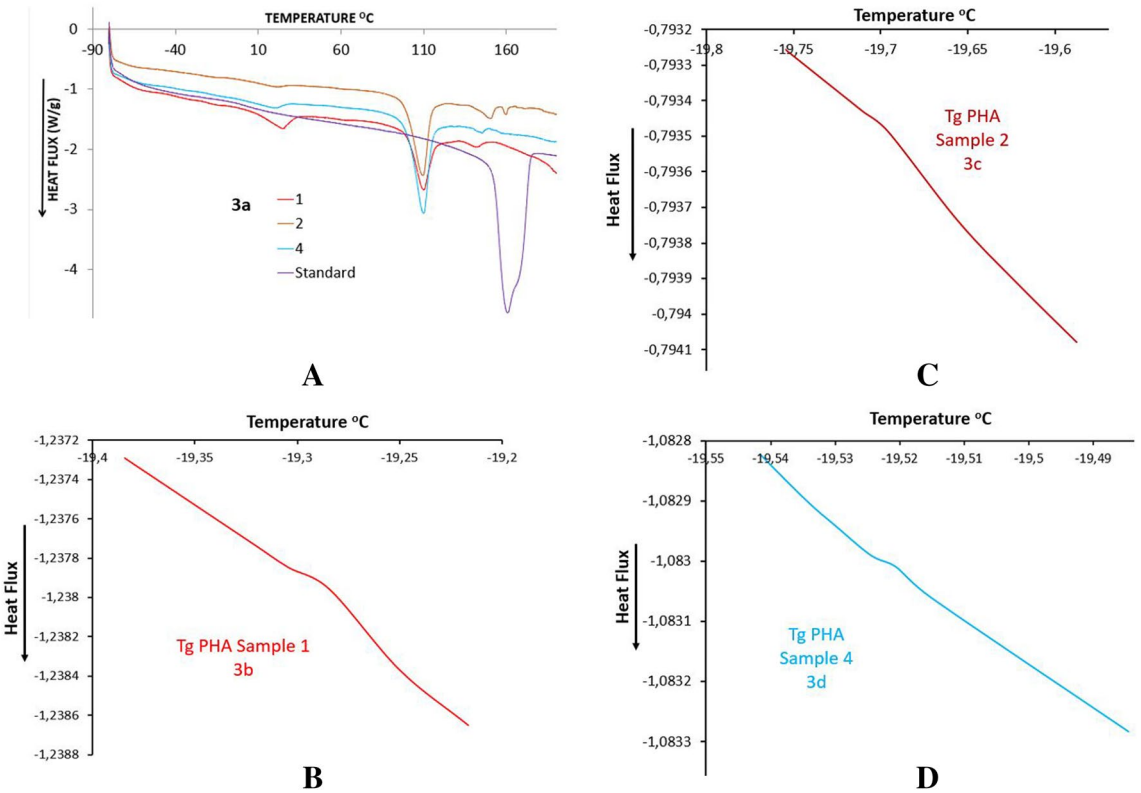


Fig. 3 **a** DSC thermograms of PHA samples (cultivations 1, 2, 4) and of PHB standard. **b**, **c**, and **d** correspond to the thermograms of Tg of PHA samples (cultivations 1, 2, 4), respectively

Table 3 Glass transition temperatures, melting temperatures and melting enthalpies for the PHA samples obtained from cassava peel hydrolysates

PHAs samples	T_m1 (°C)	T_m2 (°C)	T_m3 (°C)	T_g(°C)	ΔH_1 (J/g)	ΔH_2 (J/g)	ΔH_3 (J/g)
1	24.38	109.34	141.90	− 19.33	9.49	22.65	1.46
2	18.25	109.02	150.36	− 19.70	2.01	29.12	3.30
3	19.09	108.83	149.34	− 19.49	4.33	25.75	6.05
4	19.22	109.67	–	− 19.52	2.43	32.60	–
5	19.06	100.49	147.31	− 21.71	3.41	31.71	5.41
6	25.23	106.15	–	− 20.47	4.97	23.43	–
7	–	111.87	–	− 21.97	–	44.87	–
8	18.32	69.48	–	− 24.19	2.28	3.44	–
		T_m (°C)			T_g (°C)		ΔH (J/g)
Standard PHB		160.83			2.47		71.42

eutropha [47] or 170 °C for PHBs obtained from soybeans and soy molasses as C sources in the solid-state fermentation [13]. T_m values between 133-170 °C are reported by [11]. Values of T_m similar to those found in this work were reported by [58] and ranged from 134 to 151 °C in PHAs comprising 3-hydroxybutyrate, 3-hydroxyoctanoate and 3-hydroxydecanoate obtained from *Ralstonia eutropha* cultures.

Figure 3b–d show the Tg of the PHA samples obtained from fermentation conditions 1, 2, and 4. The T_g of the polymer samples obtained in the current work ranged between − 19.3 °C and − 24.2 °C, with the T_g for the standard estimated at 2.47 °C. A T_g between − 0.3 and − 0.2 °C of PHB produced by *Cupriavidus necator* from molasses and soybean cake was estimated by [13]. Similar values of T_g were found by Chen et al. [58], who reported Tg values of approximately − 24 °C for copolymers of PHAs obtained from the strain *Ralstonia eutropha*. These authors claimed that a low Tg value corresponded to a polymer with amorphous characteristics. The lower and multiple T_m values of the produced PHA samples are also indicative of a blend of polymer chains with a high amorphousness degree [18].

The results indicated different PHA melting temperatures, resulting from the presence of short-chain organic acids, which were likely produced in the hydrolysis process [59]. Given that the organic acids are consumed by the bacteria in the presence of glucose, different polymer chains with different melting temperatures can be generated [60]. The melting temperatures found in this work are lower than those of commercial PHBs, which leads to the assumption that the obtained PHA has both crystalline and amorphous behaviors. The characteristics associated with the presence of PHV were evidenced by the Tg values shifting to the left, which suggests that the PHA obtained from cassava waste has amorphous and elastomeric characteristics.

Nuclear Magnetic Resonance (NMR)

A ^{1}H NMR spectrum of the PHA sample obtained from fermentation condition 8 is shown in Fig. 4. Resonance signals at 1.29–1.31 ppm and 5.37 ppm, corresponding to CH_3 and CH groups, respectively, are associated with the 3HB monomer. Other resonance signals at 0.86–0.91 ppm and 5.28 ppm were also observed, and are associated with the CH_3 and CH groups, respectively, in the 3HV monomer. Other important signals were observed between 2.35–2.37 ppm and 2.53–2.65 ppm, which correspond to the CH_2 group coupled to CH. In general, the signals were consistent with results reported for a PHBV copolymer by [39]. Other studies reported similar resonance signals to those obtained in this study [8, 10, 33]. Likewise, another study found that the resonance signal at 0.89 ppm was associated with the terminal methyl group (CH_3) of the 3HV monomer [10].

The literature reports that the addition of propionate is required to obtain a PHBHV copolymer by *Ralstonia eutropha* [60]. However, in this work, propionate was not added. Some researchers report that the acid hydrolysate of vegetable and fruit waste can contain several fatty acids [59]. Likewise, studies using acid hydrolysis processes applied to food waste report the presence of fatty acids such as propionic, butyric and acetic acids [61]. The hydrolysis of peel residues of potatoes and onions found the presence of volatile fatty acids such as acetic, propionic and butyric acids [62]. Given the above, there is a possibility that the acid hydrolysis applied to cassava shell residues could have generated volatile fatty acids such as propionic acid and, in the presence of glucose *Ralstonia eutropha*, the copolymer PHBHV could have been synthesized.

The molar fractions of the 3HB and 3HV units were quantified from the ^{1}H NMR spectrum using Eq. (1), with two signals for each monomer type, but both with similar proportional intensities. The selected signals correspond to

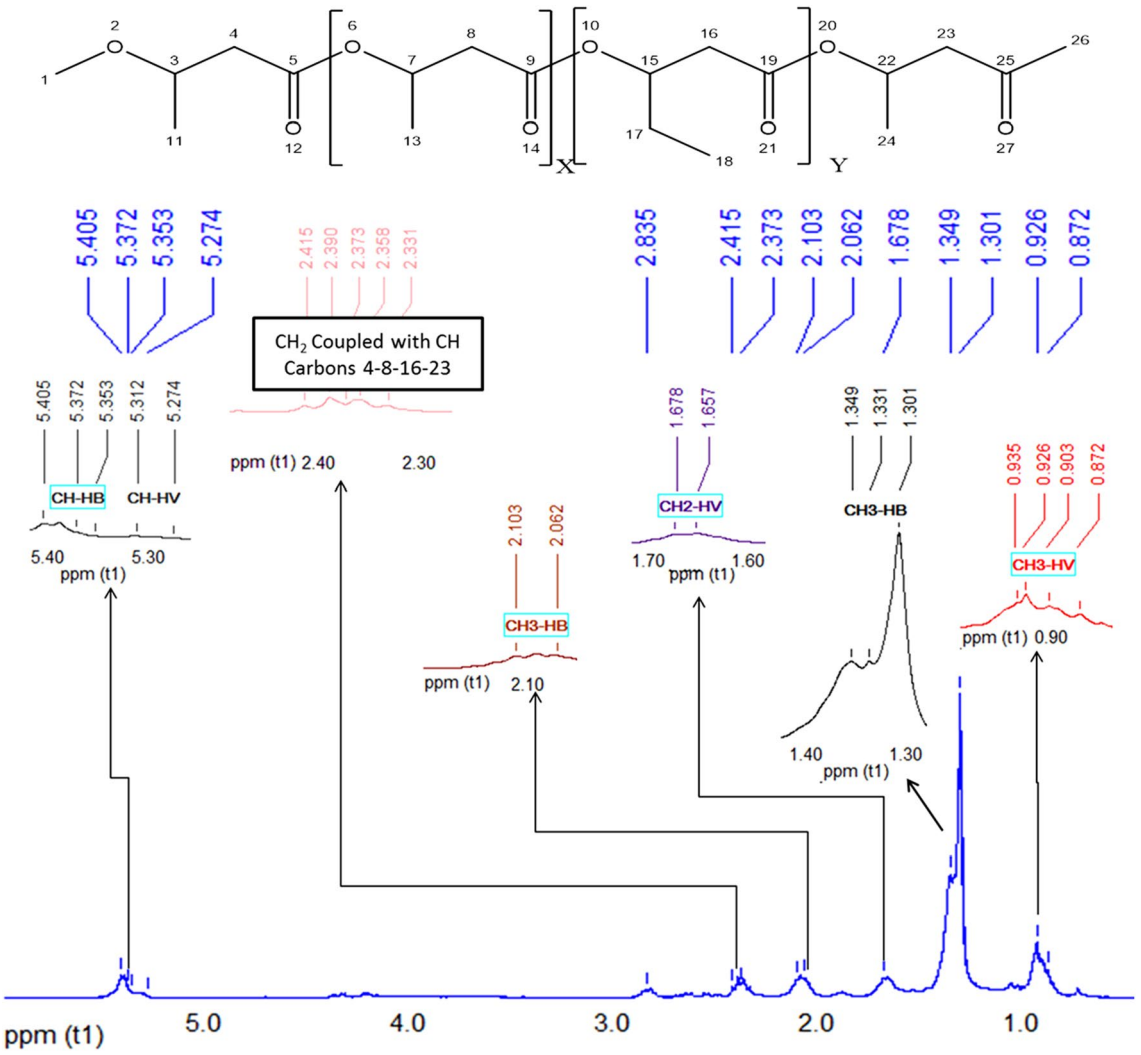


Fig. 4 300-MHz ^{1}H NMR spectra of PHB-PHV units, from cassava peel residues for fermentation conditions 8

CH groups with chemical shifts of 5.36 ppm and 5.24 ppm for 3HB and 3HV, respectively [39]. The molar fractions of 3HV in the produced polymer samples were calculated according to Eq. (1) and are given in Table 4. The results show that the 3HV molar fraction was higher than 60% (mol/mol) in most of the analyzed samples.

Table 4 3HV molar fraction in the different PHAs samples obtained from the produced cassava peels hydrolysate

Assay	% HV
1	72.6
2	16.7
3	82.0
4	76.1
5	73.1
6	67.4
7	78.3
8	73.1

The higher HV content in the produced copolymers is consistent with the DSC results, where much lower T_g values were observed (− 24.2 to − 19.3 °C) compared to the PHB standard (T_g = 2.5 °C), indicating that the polymers had a higher degree of amorphous phase.

Gas Chromatography-Mass Spectrometry (GC–MS)

Mass spectra of some of the PHA samples produced with cassava residue hydrolysates and of the PHB standard are shown in Fig. 5. Figure 5c shows the mass/charge ratios (m/z) obtained for the PHB standard after hydrolysis and methylation. A spectrum with m/z ratios of 59, 75 and 117, similar to the m/z ratios reported for PHB, was obtained by [63]. The same m/z ratios are shown in Fig. 5a, b, corresponding to the 3HB monomer of the PHA samples obtained in assays 1 and 7, respectively. Figure 5d shows the MS spectra for another molecular species obtained after methylation

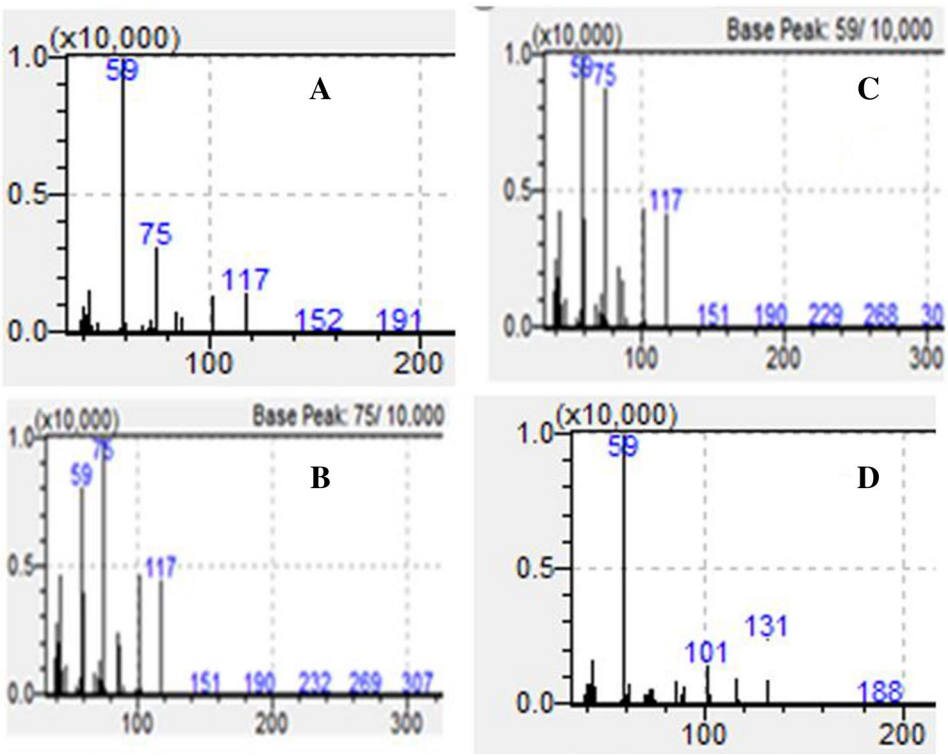


Fig. 5 Mass spectra for PHAs obtained from cassava residues. **a**, **b**, and **d** correspond to the mass spectra of the methylated monomers of the produced PHAs samples; **c** mass spectrum of the 3HB methylated monomer obtained from the PHB standard

of the polymer produced in assay 7. The m/z value with the highest relative abundance was 59, and a small intensity m/z ratio appeared at 131, which corresponds to the typical spectra of the 3HV methylated monomer [63]. Thus, the PHA polymers obtained in this work produced typical MS spectra of the 3HB monomer (m/z ratio with the highest relative abundance of 59, 75 and 117) and the 3HV monomer (relative abundance for m/z ratios 59 and 131). Similar results of the m/z ratio in the characterization of short-/medium-chain-length poly(hydroxyalkanoate) blends have been reported [64]. We concluded that the PHAs produced by *C. necator* from cassava residue hydrolysates contain two types of monomer units, 3HB and 3HV, reinforcing the hypothesis originating from DSC and ^{1}H and ^{13}C NMR analyses.

Fiber Electrospinning

Figure 6 shows the SEM images of the fiber meshes that were obtained using the PHA produced in this work (assay 8). Random organized fibers, presented in Fig. 6a, b, were obtained using the flat cupper collector, and aligned fibers, presented in Fig. 6c, d, were obtained using two parallel vertical stain steel plates. The meshes contained two types of structures, fibers and beads; the fibers are continuous and structure the mesh, and the beads are elliptical structures that are formed within the fibers, most likely due to instability and incomplete stretching of the whip-shaped polymer jets formed during the electrospinning process. To obtain mesh-free beads, additional optimization of the electrospinning process is required: attaining a better balance between the applied electrical field and surface tension at the syringe tip (e.g., through optimization of solution viscosity); attaining a better control of the solvent evaporation during fiber formation through controlling the environmental conditions (e.g., humidity and temperature); or selecting a different solvent composition [65]. A similar average size for the random and aligned fibers was estimated at values of 1.7 and 1.6 µm, respectively (Fig. 6e, f). These types of dimensions are interesting concerning the use of the meshes as scaffolds for cell culture and tissue engineering. Considering mammalian cell sizes, topologies at the dimensions obtained in this work have the potential to be recognized by cells, and therefore, the scaffold could have an impact on cell morphology and organization. The average diameter and length of the beads were 6.2 and 14–15 µm, respectively, and were similar for both meshes, and similar values were reported in the literature [27–29]. A higher percentage of beads is found for meshes of fibers of random orientation. The electrospun fiber size and mesh organization are affected by several parameters, including polymer solution concentration and viscosity, solvent system, applied voltage, needle size, needle-to-collector gap, collector type, humidity, and temperature.

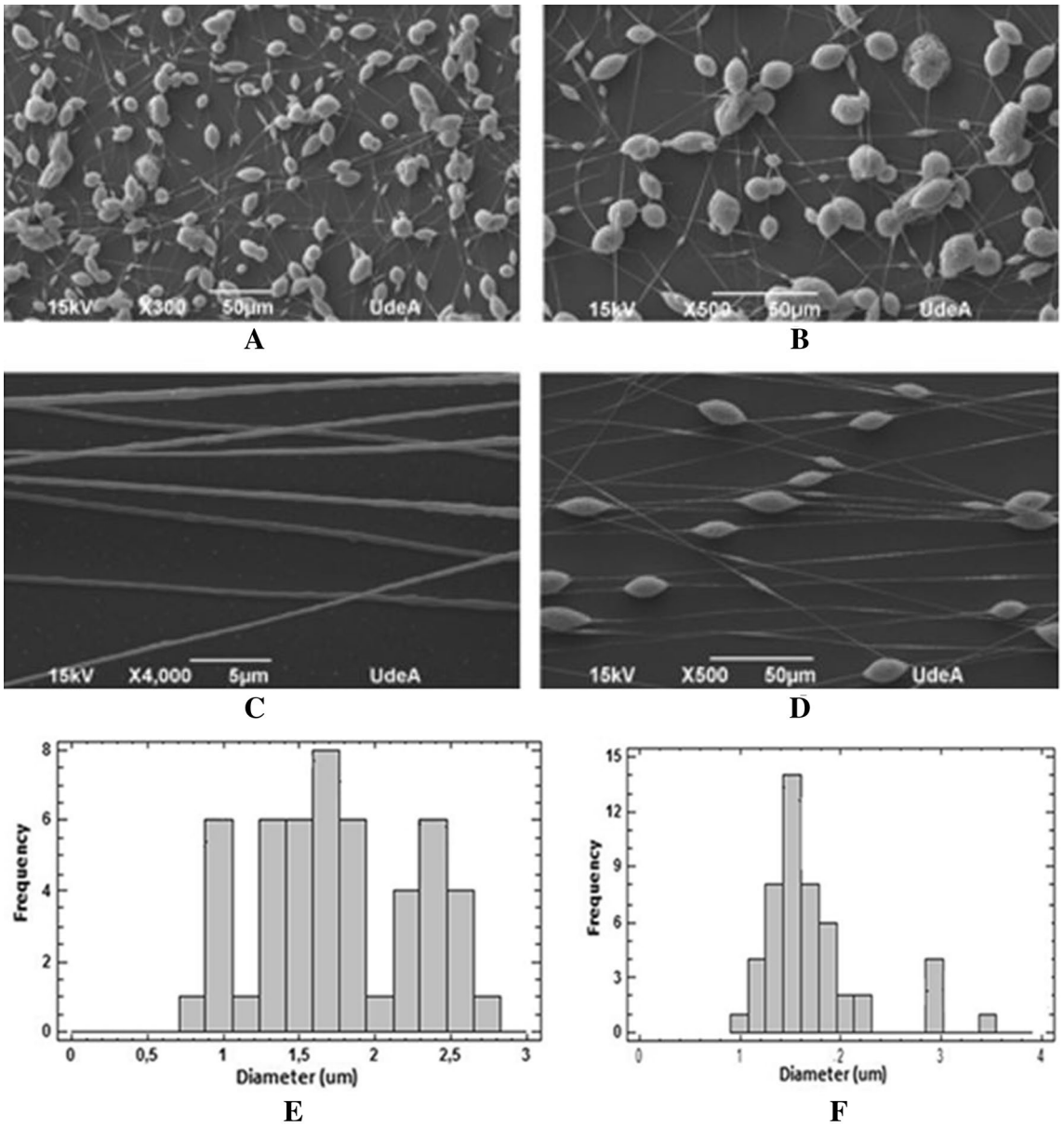


Fig. 6 SEM images of the random (**a**, **b**) and aligned (**c**, **d**) fibers obtained from PHAs cassava residues peels. **e** Distribution of the random fiber diameter; **f** Distribution of aligned fiber diameter

Conclusions

The current study shows that polyhydroxyalkanoates can be produced from cassava peel residues through fermentation processes using the *Cupriavidus necator DSM 531* strain. The PHAs obtained from waste cassava hydrolysates exhibit typical FTIR spectra with the following characteristic functional groups: OH, C–H, C=O, COC and CO at 3200, 2920, 1724, 1200 and 1100 cm^{-1}, respectively. The obtained PHAs presented two to three melting temperatures, as detected by DSC, was is indicative of a blend of polymer chains with different monomer compositions; these values ranged from 106 to 110 °C, 141 to 148 °C and 160 °C. The GC–MS analysis showed different m/z ratios of 59,75, 117 and 131. The FTIR, DSC, 1H NMR, 13C NMR and GC–MS analyses suggest that the obtained biopolymer was a copolymer of P3HB-3HV. Finally, the PHA samples were processed into electrospun meshes of random and aligned microfibers, allowing the development of different porous nonwoven structures. The average size of the random and aligned fibers was 1.7 and 1.6 µm, respectively. Electrospinning is an interesting technique when envisioning the preparation of scaffolds for use in the context of tissue engineering. Therefore, this study notes an alternative route to the management

of agro-industrial waste generated by the agribusiness of cassava, opening potential applications for such waste in the manufacture of biopolymers to be used in industry and biomedicine.

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