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Production of Medium-Chain Length Polyhydroxyalkanoates by *Pseudomonas citronellolis* Grown in Apple Pulp Waste

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Abstract

Background and objective: Apple pulp waste generated by the fruit processing industry is a sugar-rich material with great potential to be used as a feedstock for production of value-added microbial products. The aim of this work was to use this feedstock for the cultivation of *Pseudomonas citronellolis* and production of medium-chain-length polyhydroxyalkanoates, a natural elastomer.

Material and methods: The solid fraction of the apple pulp waste was discarded and the soluble fraction, rich in fructose (17.7 g l^{-1}), glucose (7.5 g l^{-1}) and sucrose (1.2 g l^{-1}), was used for the batch bioreactor cultivation of *Pseudomonas citronellolis* NRRL B-2504.

Results and conclusion: *Pseudomonas citronellolis* reached a polymer content in the biomass of 30% wt and a volumetric productivity of 0.025 g l^{-1} h^{-1}. The polymer was mainly composed of 3-hydroxydecanoate (68% mol) and 3-hydroxyoctanoate (22% mol), with minor contents of 3-hydroxydodecanoate (5% mol), 3-hydroxytetradecanoate (4% mol) and 3-hydroxyhexanoate (1% mol), and had a molecular weight of 3.7×10^5 Da. It presented glass transition and melting temperatures of -12 and 53°C, respectively, and a thermal degradation temperature of 296°C. The polymer's films were dense, ductile and permeable to oxygen and carbon dioxide. These results demonstrated that apple pulp waste is a suitable feedstock for the production of a biopolymer with properties that render it a promising alternative to some synthetic petrochemical-derived polyesters.

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1. Introduction

In the last years, the fruit juice industry has substantially increased its production, becoming one of the main responsible for the generation of large volumes of wastes and low-grade by-products that account for 20-60% (w w^{-1}) of the processed fruit. Apple pomace is a left-over from the extraction of apple juice (represents 8 million tons from the 76 million tons of apple produced in the world), corresponding to approximately 25-35% (w w^{-1}) of the original fruit mass [1,2]. The pomace is mainly composed of peels, core, seeds and solid parts resulting from juice extraction, which is usually made by mechanical pressing. This waste is mostly used as animal feed or crop fertilizer. However, due to its rich content in fermentable sugars (namely glucose, fructose and sucrose) and high moisture content (80% wt), it holds a great potential to be used as a feedstock for production of value-added microbial products, such as polyhydroxyalkanoates (PHAs) [3].

PHAs are biopolyesters that act as intracellular reserve of carbon and energy in bacteria. They have attracted attention towards their excellent material properties, including their biodegradability and ability to be produced from renewable resources, opening the opportunity for thermoplastic, elastomeric and adhesive biomaterials substituting petroleum based polymers in many of their traditional applications [4,5]. Medium-chain-length (*mcl*-) PHAs, com-posed of monomers with 6 to 14 carbon atoms, are of parti-cular interest due to their low tensile strength (4.9-6.5 MPa) and high elongation at break (190-279%)

that render them flexible and ductile properties, and low melting (25-61ºC) and glass transition temperatures (-12 to -47ºC), which are important for processing and final applications [6-8].

Many bacteria of the genus Pseudomonas including *Pseudomonas* (*P.) putida*, *P. resinovorans*, *P. chlororaphis* and *P. citronellolis*, have been described to produce *mcl*-PHA from several food processing wastes and by-products, such as, for example, oil deodorizer distillates, saturated biodiesel fractions and fruit wastes (e.g., grapes, apricots, cherries, etc.) [2,4,6,8,9]. *P. citronellolis* has been reported as a *mcl*-PHA producer, able to use an extensive range of feedstocks, including oil deodorizer distillates, fatty acids by-product and free fatty acids, margarine fat waste, animal-derived wastes and saturated biodiesel fractions [4,8,10-12]. Fruit wastes, namely, those arising from the fruit processing industry, such as apple pulp waste, were never reported as feedstocks for mcl-PHA production by *P. citronellolis*.

In this study, the use of apple pulp waste was evaluated for the first time as a sole carbon source for *mcl*-PHA production by *P. citronellolis* NRRL B-2504. In addition, the polymer was extracted, purified and characterized regarding its composition, molecular mass distribution and thermal properties. Furthermore, stand-alone films were also prepared from *P. citronellolis mcl*-PHA and their morphology, mechanical properties and permeability to gases were determined, in order to infer about potential applications.

2. Materials and methods

2.1. Polymer production

2.1.1. Microorganism and media: The microorganism used in this study was the bacterium *Pseudomonas citronellolis* NRRL B-2504 (Agricultural Research Service Culture Collection, Northern Regional Research Laboratory Database, USA). The culture was preserved in glycerol (20% v v^{-1}) as a cryoprotectant agent, at -80°C. Reactivation from the stock cultures was performed in Luria-Bertani broth (10 g l^{-1} Bacto Tryptone, 5 g l^{-1} yeast extract, 10 g l^{-1} NaCl), which was also used to prepare inocula for bioreactor cultivation. In the bioreactor experiments, *P. citronellolis* was grown on Medium E* [13] modified as described by Freitas et al. [14], using waste apple pulp as the sole substrate. For medium preparation, the waste apple pulp, which had a pH value of 3.74, was diluted with deionized water (1:3 v v^{-1}), for viscosity reduction, and centrifuged (7012 ×g, 30 min). The insoluble solids were discarded and the sugar-rich supernatant was sterilized by autoclaving at 121°C for 30 min. After cooling to room temperature, the autoclaved supernatant was supplemented with Medium E* components and the pH of the solution was set to 7.0 by the addition of NaOH 5 M.

2.1.2. Inocula preparation: The culture was reactivated from the cryopreserved vials by plating on CHROMagar™ Orientation, and incubating during 48 h at 30°C. For preparation of the pre-inocula, a single colony isolated from the CHROMagar™ Orientation plate was inoculated into 20 ml of Luria-Bertani broth, in 100 ml shake flasks, and incubated in an orbital shaker for 24 h (at 30°C, and 150 rpm). Then, 20 ml of the culture were transferred into 200 ml fresh Luria-Bertani broth and further cultivated for 24 h under the same conditions. The inocula for the bioreactor experiments were prepared by transferring 80 ml of the pre-inoculum into 4× 200 ml Luria-Bertani broth (in 500 ml baffled shake flasks) and incubating during 24 h under the same conditions.

2.1.3. Bioreactor cultivation: Biopolymer production was carried out by cultivation of *P. citronellolis* NRRL B-2504 in a 10 l bioreactor (BioStat B-Plus, Sartorius, Germany), with a starting volume of 8 l. A 10% (v v^{-1}) inoculum (800 ml) was used. The bioreactor was operated under a batch mode during 48 h. The temperature and the pH were controlled at 30 ± 0.1°C and 7.0 ± 0.1, respectively. The pH was controlled by the automatic addition of 5 M NaOH or 2 M HCl. A constant air flow rate (4 SLPM, standard liters per minute) was kept during the cultivation run and the dissolved oxygen concentration (DO) was controlled at 30% of the air saturation by automatically adjusting the stirrer speed between 300 and 800 rpm. Foam formation was suppressed by the automatic addition of Antifoam A (Sigma-Aldrich). Samples (24 ml) were collected from the bioreactor. The optical density of the broth was measured at 600 nm. The remaining broth was centrifuged (10956 ×g, 15 min, 4ºC) in polypropylene tubes. The cell-free supernatant was stored at -20ºC for quantification of ammonium and sugars, while the cell pellets were used for OD measure-ments, cell dry mass (CDM) and PHA quantification.

2.1.4. Analytical techniques

For determination of the CDM, the cell pellet obtained as described above was washed with deionized water and lyophilized. The CDM was determined gravimetrically by weighing the lyophilized cell pellets. PHA content in the biomass and its composition were determined by gas chromatography (GC) following the methanolysis method described by Cruz et al. [6], with slight modifications. Briefly, the dried cell samples (5 to 10 mg) were hydrolysed with 2 ml 20% (v v^{-1}) sulphuric acid (Sigma-Aldrich, HPLC grade) in methanol (Fisher Chemical, HPLC grade) and 2 ml of benzoic acid in chloroform (1 g l^{-1}) (Sigma-Aldrich, HPLC grade), at 100°C, for 4 h. Benzoic acid acted as internal standard. The calibration curve was made using home-made *mcl*-PHA with the

following composition, validated by GC-MS (unpublished data): 3% mol 3-hydroxyhexanoate (3HHx), 17% mol 3-hydroxyoctanoate (3HO), 57% mol 3-hydroxydecanoate (3HD), 11% mol 3-hydroxydodecanoate (3HDd) and 12% mol 3-hydroxy-tetradecanoate (3HTd), in concentrations ranging from 0.1 to 2.0 g l^{-1}. The obtained methyl esters were analysed in a Restek column (Crossbond, Stabilwax) at constant pressure (96 kPa) using helium as carrier gas. Splitless injection was used. The oven temperature program was the following: 20°C min^{-1} until 100°C; 3°C min^{-1} until 155°C and, finally, 20°C min^{-1} until 220°C.

Determination of the sugars concentrations was perform-ed by high performance liquid chromatography using a VARIAN Metacarb 87H column, coupled to a refracto-meter. The analysis was performed at 50°C, with sulphuric acid (H_2SO_4, 0.01 N), at a flow rate of 0.6 ml min^{-1}. Glucose (Fluka, 99%), fructose (Scharlau, 99%) and sucrose (Fluka, 99%) were used as standards, at concentrations ranging from 0.0625 to 1.0 g l^{-1}. Ammo-nium concentration was determined by colorimetry using a flow segmented analyzer (Skalar 5100, Skalar Analytical, Netherlands). All measurements were done in replicate analyses. For the determination of total nitrogen was used a kit (LCK 388, LATON®) with a detection range of 20-100 mg l^{-1}. The sample (0.2 ml) was placed into a glass flask, and added with the reagents, as is described in the kit. Afterwards, they were put in a HT 200S (HACH® - LANGE) digester for 15 min at 100ºC. After cooling to room temperature, 0.5 ml of the digested solution was transferred to a new vial and after 15 min the absorbance was read in a DR2800 tm spectrophotometer (HACH®).

2.1.5. Calculations

The maximum specific growth rate ($\mu_{max,}$ h^{-1}) was determined from the linear regression slope of the exponential phase of Ln X_t versus time, where X_t (g l^{-1}) is the rest biomass (i.e., cells without PHA) at time *t* (h). The rest biomass was determined by Eq. 1:

$$X_t = CDM_t - PHA_t \quad \text{(Eq. 1)}$$

where CDM_t (g l^{-1}) and PHA_t (g l^{-1}) are the cell dry weight and the concentration of polymer at time *t* (h). This concentration is given by the polymer accumulated in the cells (calculated on a dry basis, wt%). The polymer yield on a substrate basis ($Y_{p/s}$, g_p g_s^{-1}) was calculated by Eq. 2:

$$Y_{p/s} = \frac{\Delta P}{\Delta S} \quad \text{(Eq. 2)}$$

where ΔP (g l^{-1}) is the PHA produced and ΔS (g l^{-1}) is the total sugars consumed during the cultivation. The volumetric productivity (r_p, g l^{-1} h^{-1}) was calculated by dividing the final PHA concentration (P, g l^{-1}) for the total time of fermentation (Δ*t*, h).

2.2. Polymer extraction and purification

The cultivation broth recovered from the bioreactor at the end of the assay was centrifuged (13131×g, for 20 min) and the biomass pellet was lyophilized. The polymer was extracted from the dried biomass by Soxhlet extraction with chloroform (~10 g biomass for 250 ml chloroform), at 80°C, for 48 h. Cell debris were removed by filtration with syringe filters with a pore size of 0.45 μm (GxF, GHPmembrane, PALL) and the polymer was precipitated in ice-cold ethanol (1:10 v·v^{-1}) under vigorous stirring. The polymer was recov-ered and dried at room temperature.

2.3. Polymer characterization

2.3.1. Composition

The composition of the extracted polymer was determined by GC, as described above, except for the fact that a lower amount of sample was used (~3 mg).

2.3.2. Fourier Transform Infrared Spectroscopy

Fourier transform infrared spectroscopy (FTIR) analysis was conducted with a Perkin-Elmer Spectrum two spectro-meter. The polymer was directly analysed on the FTIR cells. The spectrum were recorded between 400 and 4000 cm^{-1} resolution with 10 scans, at room temperature.

2.3.3. Molecular mass distribution

The polymer (15 mg) was dissolved in 3 ml of chloroform, for 18 h at room temperature. Then, the solution was filtered with a glass fibber filter 47 mm (PALL) and analysed by a Size Exclusion Chromatography (SEC) System (Waters Millenium) with support SEC: PLgel 5 μm Guard; Polymer Laboratories; 50×7.5 mm, PLgel 5 μm 10^4 Å; Polymer Lab-oratories; 300×7.5 mm, PLgel 5 μm 500 Å; Polymer Labor-atories; 300×7.5 mm, using a temperature of equilibration of 30°C, with a flow rate of 1 ml min^{-1}, degassing and chloroform as the mobile phase. Samples were stored at 4°C before injecting 100 μl in the SEC circuit. A RI detector (Waters 2410) was adopted for polymer detection using the sensitivity 512 and a collect duration of 25 min. The relative molecular weight (MW) of the polymers were determined adopting monodisperse polystyrene standards with MW ranging between 800 Da to 504 kDa. SEC Water software relying upon the universal calibration method has been adopted to calculate the relative MW of *mcl*-PHA.

2.3.4. Thermal analysis

Differential Scanning Calorimetry (DSC) analysis was performed using a differential scanning calorimeter DSC 131 (Setaram, France). The samples were placed in aluminium crucibles and analysed in the temperature range between -90 and 120°C, with heating and cooling speeds of 10°C min^{-1}. Two heating cycles were performed and data was collected from the second heating cycle. Thermogravimetric Analysis (TGA) was performed using a thermogravimetric equipment Labsys EVO (Setaram, France). Samples were placed in aluminium crucibles and analysed in the temperature range between 25 and 500°C, at 10°C min^{-1}.

The glass transition temperature (T_g, ºC) was taken as the midpoint of the heat flux step and the melting temperature (T_m, ºC) was determined at the minimum of the endothermic peak.

2.4. mcl-PHA films

2.4.1. Films preparation: The polymer (1.0 g) was dissolved in chloroform (20ml) (BIOCHEM Chemopharma, HPLC grade), under stirring, at room temperature, until complete dissolution. The solutions were transferred into glass petri dishes (with a diameter of 9.5 cm), which were placed in a desiccator and kept at room temperature until complete and slow solvent evaporation.

2.4.2. Morphological characterization: The morphology of the obtained films was assessed by Scanning Electron Microscopy (SEM) using an Energy Dispersive Spectroscopy (SEM-EDS). The *mcl*-PHA films were placed in a desiccator until completely dry, frozen in liquid nitrogen and fractured in small pieces followed by coating with a thin layer of Au/Pd. The films were analysed using an analytical JEOL 7001F scanning electron microscope (FEG-SEM, JEOL, USA Inc.) equipped with a field emission gun operated with an acceleration voltage of 15 kV. All samples were visualized on their surface and cross-section.

2.4.3. Water contact angles: The contact angle was measured by the sessile drop method, where a drop of distilled water was manually deposited on the film's surface with a small syringe. The software acquired ten images per sample and the tangent was determined by fitting the drop shape to a known mathematical function. Multiple replicates were performed, and the mean angle was determined. All images were acquired by CAM2008 (KSV Instruments Ltd, Finland).

2.4.4. Swelling in water: *mcl*-PHA film samples with a size of 1.0×1.0 cm^2 were weighed and their thickness was measured with a micrometer (Elcometer, England). The samples were immersed in 15 ml deionized water, in a closed vial, and kept at 30ºC during 24 h. The swelling degree in terms of mass of the samples was calculated with the Eq. 3:

$$\text{Swelling Degree} = \frac{X2-X1}{X1} \times 100\% \qquad \text{(Eq. 3)}$$

where X_1 and X_2 are, respectively, initial and final mass (g), respectively, of the samples measured at a different time period. After the immersion period (24 h), the films were cleaned with paper tissue and their thickness was measured with a micrometer (Elcometer, England).

2.4.5. Gas permeability: Gas permeation tests were performed with pure CO_2 and O_2 using a gas permeation setup composed of a stainless steel cell with feed and permeate compartments that were separated by the *mcl*-PHA film. The experimental apparatus was placed in a thermostatic bath (Julabo GmBH ED, Germany, ± 0.1 K), at a constant temperature of 30ºC. The experiment was initiated with both compartments pressurized with the pure gas (CO_2 or O_2), and establishing a pressure of 0.7 bar before opening the permeate valve and instantly closing it. The pressure variation in the compartments of the cell was measured by two pressure transducers (Druck PCDR 910, models 99166 and 991675, UK, ± 0.008 bar). The films' permeability (P, $m^2\ s^{-1}$) for pure CO_2 and O_2 gases was calculated according the following equation:

$$\frac{1}{\beta} \ln \frac{\Delta_{po}}{\Delta_p} = P\ \frac{t}{l} \qquad \text{(Eq. 4)}$$

Where Δ_p (bar) corresponds to the difference of the pressures in the feed and permeate compartments, t (s) is the time and l (m) is the film's thickness. Δ is a geometric parameter characteristic of the cell (m^{-1}), and was obtained using Eq. 5:

$$\beta = A \left(\frac{1}{V_{feed}} + \frac{1}{V_{perm}}\right) \qquad \text{(Eq. 5)}$$

Where A is the film's area (cm^2) and V_{feed} and V_{perm} are the volumes (bar) of the feed and permeate compartments, respectively. The gas permeability P was obtained from the slope when representing $\frac{1}{\beta} ln \frac{\Delta_{po}}{\Delta_p}$ as a function of $\frac{t}{l}$. In order to compare the results with those available in the literature a conversion was made (1 Barrer = 1×10^{-10} cm^3 (STP) cm cm^{-2} $cmHg^{-1}$ s^{-1} = 8.3×10^{-13} $m^2{\cdot}s^{-1}$) [15].

2.4.6. Mechanical properties: The *mcl*-PHA films were cut into 2.5×1.5 cm strips, which had an average thickness of 100 μm, measured using a digital micrometer (Mitutoyo, Japan). Tensile tests were performed at ambient temperature (22°C) using a TA-XT plus texture analyser (Stable Micro Systems, Surrey, England) equipped with a 5 kg load cell. The films' strips were attached on tensile grips A/TG and stretched at 0.5 mm s^{-1} in tension mode until break. The stiffness of the membranes was determined by measuring the Young modulus (MPa), determined as the slope of the linear initial section of the stress-strain curve. The tensile stress at break (MPa) was calculated as the ratio of the maximum force to the films initial cross-sectional area. The elongation (strain) at break (-) was determined as the ratio of the extension of the sample upon rupture by the initial gage length. Three film replicas were analyzed.

3. Results and discussion

3.1. Polymer production

P. citronellolis was able to use the apple pulp waste for cell growth and polymer synthesis under the batch cultivation conditions used in this study (Figure 1, Table 1). The culture grew at a specific cell growth rate of 0.24 h^{-1} (Table 1). This value is considerably higher than the ones

reported by Muhr et al. [8] for cultivation of *P. citronellolis* in a fed-batch bioreactor run using tallow-based biodiesel (0.08-0.10 h^{-1}), thus demonstrating apple pulp waste provided a more suitable feedstock for the culture's cell growth. A rest biomass concentration of 1.9 g l^{-1} was reached within 8 h of cultivation, when the available ammonia had been depleted (Figure 1a). However, there was still nitrogen present in the medium (62 mg l^{-1}) (Figure 1a), which supported cell growth, although at a lower rate (0.05 h^{-1}). This nitrogen was present in the apple pulp waste and allowed the culture to attain a maximum of rest biomass concentration of 2.8 g l^{-1} at 31 h of cultivation (Figure 1a). No significant cell growth was noticed thereafter.

Polymer production was initiated by the time ammonia was depleted and the cell growth rate was reduced (Figure 1a). A polymer concentration of 1.2 g l^{-1} (Table 1) was attained at the end of the experiment. This corresponds to an overall volumetric productivity of 0.025 g l^{-1} h^{-1}. This value is lower than that reported by Muhr et al. [8] (0.067-0.1 g l^{-1} h^{-1}), which was obtained in a fed-batch bioreactor cultivation wherein a higher CDM was reached. The polymer content in the biomass was 30% wt, which is higher than the values reported for *P. citronellolis* grown on different feedstocks, 3-27% wt, and within those reported for other *mcl*-PHA producers (15-36% wt), such as *P. resinovorans* [6,10] and *P. chlororaphis* [4].

The apple pulp waste had a total sugars content of 31.0 g l^{-1}, out of which 25.2 were accounted as the monosaccharides fructose (17.7 g l^{-1}) and glucose (7.5 g l^{-1}). Sucrose was also detected at low concentrations (below 2.0 g l^{-1}). Glucose was depleted within around 20 h of cultivation (Figure 1b). Fructose, on the other hand, was not completely consumed and only 2.5 g l^{-1} of fructose was consumed until the end of the run. Sucrose was not consumed (Figure 1b). Considering the simple sugars available in the apple pulp based medium, glucose and fructose, there was an overall consumption of 10.0 g l^{-1}. The corresponding polymer yield on a sugar basis was 0.12 g_p g_s^{-1} (Table 1).

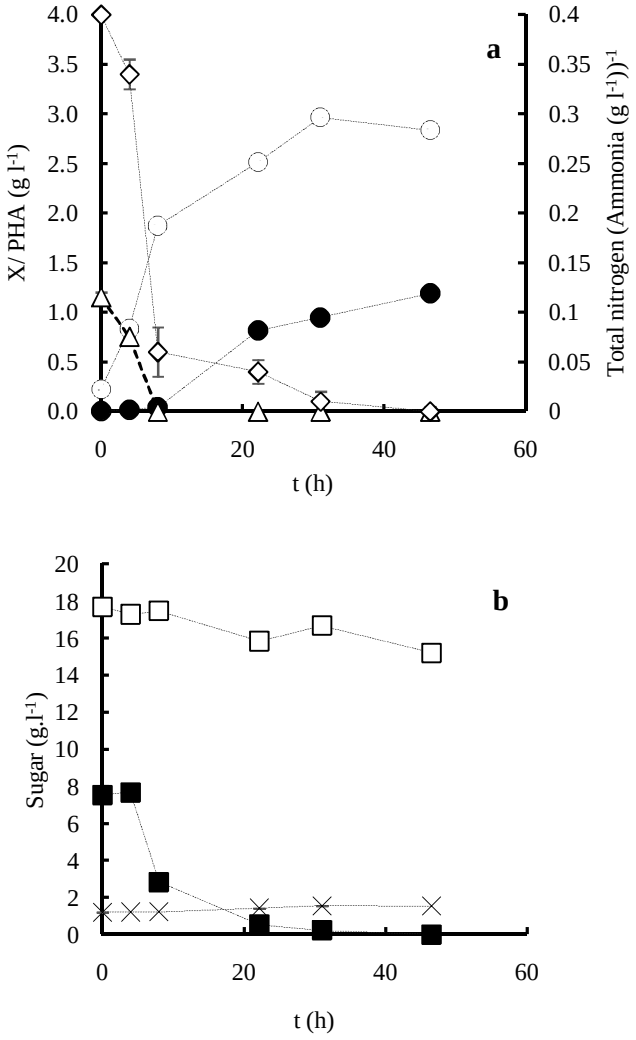

Figure 1. Cultivation profile of *P. citronellolis* NRRL B-2504 using apple pulp waste as the sole carbon source, a) concentration of active biomass (○), PHA (●), total nitrogen (◊), ammonia (Δ), b) glucose (■), fructose (□) and sucrose (x).

Table 1. Kinetic and stoichiometric parameters for *mcl*-PHA production by *Pseudomonas citronellolis* NRRL B-2504 using several wastes and by-products as feedstocks (μ_{max}, maximum specific cell growth rate; CDM, cell dry mass; X, rest biomass; r_p, volumetric productivity; $Y_{p/s}$, polymer yield on a substrate basis; n.a., data not available)

Feedstock	Cultivation mode	μ_{max} (h^{-1})	CDM (g l^{-1})	X (g l^{-1})	PHA (wt %)	PHA (g l^{-1})	r_p (g l^{-1} h^{-1})	$Y_{P/S}$ (g_p g_s^{-1})	References
Apple pulp waste	Batch bioreactor	0.24±0.01	4.0±0.08	2.8±0.06	30±1.7	1.2±0.05	0.025±0.001	0.12±0.007(*)	This study
Saturated biodiesel fraction	Fed-batch bioreactor	0.08-0.10	11.2-14.1	8.4-11.2	20-27	2.8-2.9	0.067-0.1	n.a.	[8]
Olive oil distillate	Shake flask	n.a.	4.8	4.3	10	0.5	0.008	0.08	[10]
Fatty acids by-product	Shake flask	n.a.	3.5	3.4	3	0.1	0.004	0.02	[10]
Tallow free fatty acids	Shake flask	n.a.	1.7	1.6	3	0.05	0.0008-0.0012	n.a.	[12]
Margarine waste	Shake flask	n.a.	6.3	5.8	8	0.5	0.007	n.a.	[11]

(*) Calculated considering the consumption of sugars, glucose and fructose, *mcl*-PHA= medium-chain-length polyhydroxyalkanoates

3.2. Polymer characterization

3.2.1. Composition

The *mcl*-PHA produced by *P. citronellolis* from apple pulp waste was mainly composed of 3-hydroxydecanoate (3HD), 68% mol, and 3-hydroxyoctanoate (3HO), 22% mol (Table 2). It also had minor contents of 3-hydroxydodecanoate (3HDd), 5% mol, and 3-hydroxytetradecanoate (3HTd), 4% mol, while only traces of 3-hydroxyhexanoate (3HHx), 1% mol, were detected. Comparing the composition of the polymer with that of other *mcl*-PHA synthesized by *P. citronellolis* (Table 2), it can be seen that, although the same monomers are present, their relative content is significantly different. Indeed, 3HD and 3HO were also the main comp-onents, but 3HD was the dominant monomer in the *mcl*-PHA produced from apple pulp waste, while both monomers were present in similar amounts or 3HO had a higher content for the biopolymers produced from saturated biodiesel fraction [8], olive oil distillate, fatty acids by-product and tallow fatty acids [10,12]. The observed differ-ences may be related with the different carbon sources used.

When compared to the *mcl*-PHA produced by other *Pseudomonas* sp., it is clear that the composition of the synthesized polymer is highly dependent, not only from the producing strain, but also from the feedstock used (Table 2). For example, *P. mediterranea* CFBP 5447 produced a *mcl*-PHA with a monomer composition similar to that of *P. citronellolis* produced from apple pulp waste using glycerol as carbon source, while the polymer synthesized by *P. resinovorans* from olive oil distillate or the one produced by *P. oleovorans* from octanoic acid had completely distinct composition [10,16,17].

3.2.2. Fourier Transform Infrared Spectroscopy

The FTIR spectrum of the *mcl*-PHA polymer produced by *P. citronellolis* (Figure 2) shows an intense absorption peak at 1727 cm^{-1} corresponding to the stretching band of the ester carbonyl group (C=O). This band is the strongest peak in the spectra, corresponding to a characteristic band of PHA. Near 2961-2854 cm^{-1} three peaks can be identified that can be attributed to the asymmetric methyl group (the peak at 2961 cm^{-1}), to the stretching vibration due to asymmetric CH_2 of the lateral monomeric chains (the peak at 2924 cm^{-1}) and to the symmetrical methyl group (the peak at 2854 cm^{-1}) (Figure 2). Between 1000 and 1500 cm^{-1}, a group of peaks is observed, indicating the presence of several characteristic structures within *mcl*-PHA. For example, the peak at 1260 cm^{-1} corresponds to asymmetric C-O-C stretching vibration. The region between 1010 and 1161 cm^{-1} shows series of absorption bands, which can be assigned to C-O and C-C stretching vibration in the amorphous phase of the *mcl*-PHA. The band nearby 802 cm^{-1} is attributed to the CH_2 bonds in the side-chain of the polymer [18,19].

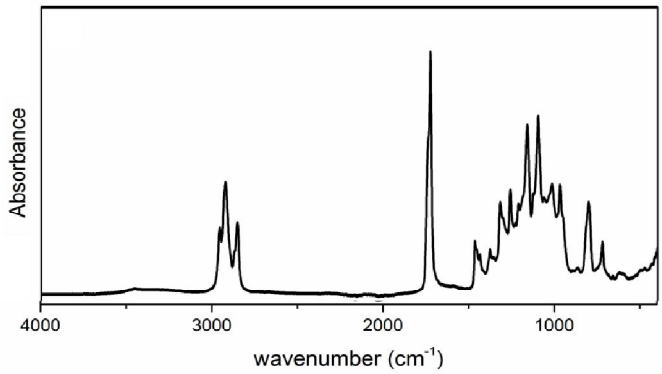

Figure 2. FTIR-ATR spectra of the mcl-PHA polymer produced by *P. citronellolis* from apple pulp waste.

Table 2. Physical-chemical properties of the medium-chain-length polyhydroxyalkanoates, produced by *Pseudomonas citronellolis* NRRL B-2504 and other *Pseudomonas* sp. (3HHx, 3-hydroxyhexanoate; 3HO, 3-hydroxyoctanoate; 3HD, 3-hydroxydecanoate; 3HDd, 3-hydroxydodecanoate; 3HTd, 3-hydroxytetradecanoate; Mn, molecular number; Mw, molecular weight; PDI, polydispersity index; T_g, glass transition temperature; T_m, melting temperature; T_{deg}, degradation temperature; n.a., data not available)

Bacteria	Feedstock	Composition (%mol)					Mw ($\times 10^5$ Da)	Mn ($\times 10^5$ Da)	PDI	T_g (ºC)	T_m (ºC)	T_{deg} (ºC)	References
		3HHx	3HO	3HD	3HDd	3HTd							
P. citronellolis NRRL B-2504	Waste apple pulp	1	22	68	5	4	3.7	1.7	2.1	-12	53	296	This study
	Saturated biodiesel fraction	5-6	40-46	36-40	7-9	n.a.	0.7-2.0	0.4–0.8	1.9–2.5	-47 to -44	48-54	295	[8]
	Olive oil distillate	14	43	32	12	<1	0.3	0.2	1.5	-14	25	n.a.	[10]
	Fatty acids by-product	10	36	40	14	<1	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	[10]
	Tallow fatty acids	10	48	28	10	4	0.9–1.6	0.4–0.7	2.2–2.6	n.a.	n.a.	n.a.	[12]
P. resinovorans NRRL B-2649	Olive oil distillate	19	44	28	9	<1	0.2	0.3	1.5	-16	36	n.a.	[10]
P. mediterranea CFBP 5447	Glycerol	4	17	61	6	n.a.	0.5	n.a.	1.3	-47	41	230	[16]
P. oleovorans ATCC 29347	Octanoic Acid	8	93	n.a.	n.a.	n.a.	3.9	2.1	1.8	-33	61	n.a.	[17]

3.2.3. Molecular mass distribution

The *mcl*-PHA obtained exhibited an average molecular weight (M_w) of 3.7×10^5 Da with a polydispersity index (PDI) of 2.1 (Table 2). The polymer's Mw was higher than the values attained for the *mcl*-PHA produced by the same strain cultivated in different substrates, such as saturated biodiesel fraction (0.7×10^5-2.0×10^5 Da) [8], olive oil distill-ate (0.3×10^5 Da) [10] and tallow fatty acids (0.9×10^5-1.6×10^5 Da) [12], a feature that reflects the polymers composition and properties. The observed differences may be due to the different production conditions, specifically, the composition of the feedstock, cultivation mode and the stage of growth when the cells were harvested [12]. Muhr et al. [8] reported a higher Mw (2.0×10^5 Da) for the polymer produced in a cultivation run that lasted 45 h compared to the Mw (0.7×10^5 Da) obtained for a longer cultivation run (72 h). Those results might indicate that extending the cultivation run might lead to a reduction of the polymer's Mw. In this study, the short cultivation time might explain the observed higher Mw value, besides the fact that a different feedstock was also used. The high Mw of the *mcl*-PHA produced from apple pulp waste, together with its low PDI, renders it interesting for many applications, such as coatings, medical devices, drug delivery, and scaffolds.

3.2.4. Thermal properties

The *mcl*-PHA produced by *P. citronellolis* from apple pulp waste presented glass transition (T_g) and melting (T_m) temperatures of -12°C and 53°C, respectively (Table 2). Regarding the polymer's thermal stability, its decom-position showed a single weight loss of approximately 99%, with a maximum degradation rate at 296°C (Figure 3). These values, namely, the T_m and T_{deg} displayed by the *mcl*-PHA produced from apple pulp waste, were similar to those reported for the polymer produced by the same strain using saturated biodiesel fraction (48-50°C and 296°C, respectively), but the polymer had considerably lower T_g values (-47 to -44°C) [8]. The polymer's T_g was similar to that of the *mcl*-PHA produced by *P. citronellolis* from olive oil distillate (-14°C), but the later had a much lower T_m (25°C) [10]. The *mcl*-PHA produced by *P. mediterranea* CFBP 5447 from glycerol, which had a composition similar to that of the polymer obtained from apple pulp waste, displayed considerably lower T_g (-47ºC) and T_m (41ºC) values [16]. The observed difference may be related with the fact that *P. mediterranea mcl*-PHA had a considerably lower Mw (0.5×10^5-0.6×10^5 Da) than that produced by *P. citronellolis* from apple pulp waste (3.7×10^5 Da).

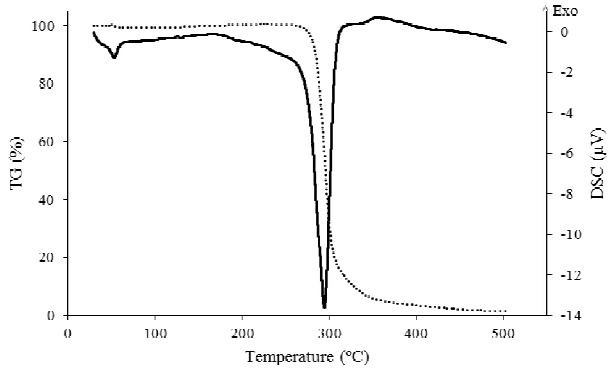

Figure 3. Differential scanning calorimetric (DSC) (straight line) and thermal gravimetric analysis (TGA) (dotted line) curves obtained from *mcl*-PHA produced by *P. citronellolis* NRRL B-2504 from apple pulp waste.

3.3. *mcl*-PHA films

3.3.1. Morphological characterization

The *mcl*-PHA produced by *P. citronellolis* from apple pulp waste was used to prepare films (Figure 4) by solvent casting. Slow solvent evaporation was performed in a saturated chloroform atmosphere to avoid the formation of cracks in the films and to produce homogeneous structures. The films thus obtained were translucid, colourless and flexible, and were used for mechanical tests.

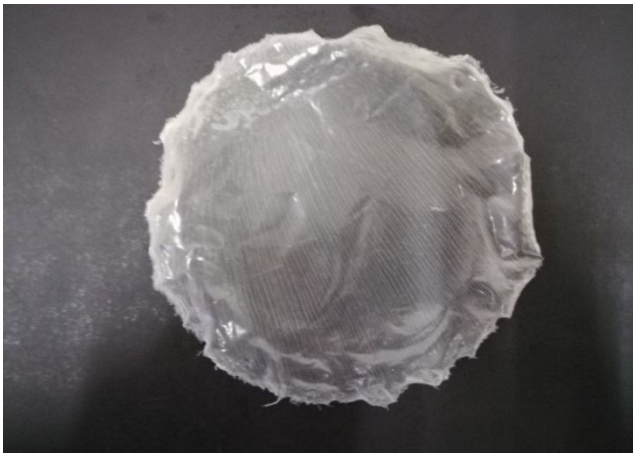

Figure 4. Film obtained with the *mcl*-PHA produced by *P. citronellolis* using apple pulp waste.

The morphology of the *mcl*-PHA membranes was investigated using scanning electron microscopy (SEM). Figures 5 (a) and (b) show the air-oriented surface and the cross-section, respectively, of the *mcl*-PHA films. The films exhibited a homogeneous and rough surface (Figure 5(a)), and were dense with no visible pores or cracks (Figure 5(b)). These findings were confirmed upon magnification (Figure 5 (c) and (d)) of the *mcl*-PHA films' surface and cross section images.

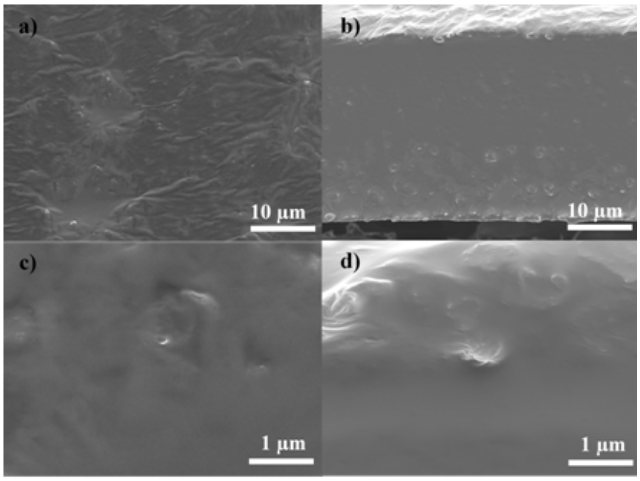

Figure 5. Surface (a, c) and cross-section (b, d) amplified 500× and 5000×, respectively images obtained by Scanning Electron Microscopy (SEM) analysis of the prepared *mcl*-PHA films. *mcl*-PHA= medium-chain-length polyhydroxyalkanoates

3.3.2. Swelling and contact angles of mcl-PHA films

To evaluate the swelling degree of the films prepared with the *mcl*-PHA produced from apple pulp waste, they were immersed in deionized water, at 30ºC for 24 h. There was no significant change in the mass and in the volume of the film samples after immersion. They exhibited a negligible swelling index of around 2%. These results indicate that the *mcl*-PHA films showed no interaction with water, under the conditions of the test.

The air-oriented surface of the *mcl*-PHA films presented a surface contact angle (θ) of 101° (Table 3). The contact angle depends on several factors, namely, the film's roughness, procedure for surface preparation, and surface cleanliness. Given that a surface is considered hydrophobic if the contact angle value of higher than 90° when using water [20], the films obtained with the polymer produced from apple pulp waste are hydrophobic. The surface contact angle obtained for the *mcl*-PHA is similar to that reported for natural rubber (θ = 92°) [21] and silicone rubber (θ = 110°) [21], two known hydrophobic materials.

Table 3. Water contact angles for the *mcl*-PHA films prepared with the polymer produced by *P. citronellolis* NRRL B-2504 from apple pulp waste and comparison with values reported for different materials (P(3HB); Poly (3-hydroxybutyrate); PLA, polylactic acid)

Material	Water contact angle (θ)	Reference
P. citronellolis mcl-PHA	101 ± 0.8	This study
P(3HB)	63-68	[23]
Silicone rubber	110	[21]
Natural rubber	92	[22]
PLA	74	[22]

P. = *Pseudomonas*, *mcl*-PHA= medium-chain-length polyhydroxyalkanoates

The demonstrated *P. citronellolis mcl*-PHA film's hydrophobicity renders the *mcl*-PHA films the ability to compete with other natural polymers in applications such as packaging. Other natural biodegradable polymers, like cellulose and starch, for example, due to their hydrophilic, water wettability or swelling nature, are less technologically suitable for food packaging, where water resistance is crucial.

3.3.3. Gas permeation

The pure gas permeation, CO_2 and O_2, through *P. citronellolis mcl*-PHA films was evaluated and the results obtained are presented in Table 4, together with the permeability values reported for several different synthetic and natural polymers. The permeability of the *mcl*-PHA films was 1.1×10^{-09} $cm^3.cm\ cm^{2}.s.cmHg$ for O_2, and 5.3×10^{-09} $cm^3.cm\ cm^{2}.s.cmHg$, for CO_2. The permeability to O_2 is similar to the value reported for natural rubber (2.4×10^{-09} $cm^3.cm/cm^2.s.cmHg$), which is commonly used as the principal raw material in tyre manufacturing [24]. The value is higher than those described for the biodegradable polyesters P (3HB) (3.0×10^{-12} $cm^3.cm\ cm^{2}.s.cmHg$), P (HBHV) (2.1×10^{-11} $cm^3.cm\ cm^{2}.s.cmHg$) and PLA (3.0×10^{-11} $cm^3.cm\ cm^{2}.s.cmHg$) and also higher than those of the synthetic polyesters PET (5.0×10^{-12} $cm^3.cm\ cm^{2}.s.cmHg$) and polystyrene (1.2×10^{-10} $cm^3.cm/cm^2.s.cmHg$) (Table 4) [25,26]. Moreover, *P. citronellolis mcl*-PHA films had lower permeability to both O_2 and CO_2 than silicone rubber (6.0×10^{-8} and 3.2×10^{-7} $cm^3.cm/cm^2.s.cmHg$, respectively) [26]. These polymers are frequently used in the production of plastic bottles.

Table 4. Oxygen and carbon dioxide permeability values for *P. citronellolis* NRRL B-2504 *mcl*-PHA films and for different natural and synthetic materials for both gases (PET, polyethylene terephthalate; PLA, Polylactic acid; P(3HB), Poly (3-hydroxy-butyrate); P(HBHV), poly (3-hydroxybutyrate-*co*-3-hyd-roxyval-erate); n.a., data not available)

Materials	Permeability ($cm^3.cm/cm^2.s.cmHg$)		References
	O_2	CO_2	
P. citronellolis mcl-PHA	1.1×10^{-09}	5.3×10^{-09}	This study
P(3HB)	3.0×10^{-12}	n.a.	[25]
P(HBHV)	2.1×10^{-11}	n.a.	[25]
PET	5.0×10^{-12}	n.a.	[25]
PLA	3.0×10^{-11}	n.a.	[25]
Silicone rubber	6.0×10^{-8}	3.2×10^{-7}	[26]
Natural rubber	2.4×10^{-9}	n.a.	[26]
Polystyrene	1.2×10^{-10}	n.a.	[26]

P. citronellolis= *Pseudomonas citronellolis*, *mcl*-PHA= medium-chain-length polyhydroxyalkanoates

The gas permeability of polymers is an essential factor regarding their application, for example as barrier materials and membranes for gas separation, in food packaging, as protective coatings and in biomedical materials. The gas separation of polymer membranes depends on the selectivity of a particular gas by the membrane over other gases. Gas transport properties depend on numerous factors related to membrane structure, such as the permeant size and shape, Mw, functional groups, density and polymer structure and crosslinking.

3.3.4. Mechanical Properties

As shown in Table 5, *P. citronellolis mcl*-PHA films had a tensile strength at break of 4.9±0.68 MPa, deforming almost three times its original length until breaking (279±12%), associated with a Young Modulus of 7.8±1.58 MPa. Values of the same order of magnitude have been reported for other *mcl*-PHA, namely the ones synthesized by *P. mediterranea and P. oleovorans* from glycerol and octanoic acid, respectively [16,17] (Table 5). As expected, the *mcl*-PHA films were more ductile and less resistant to deformations than other natural polyesters, such as P (3HB) [27], P(HBHV), P(HBHHx) and PLA. These materials typically have higher tensile strength at break (29.0 -61.0 MPa) and Young Modulus (38-2500 MPa) values, as well as lower elongation at break (1.3-67.3 %), meaning they are more rigid with a higher resistance to deformation.

4. Conclusion

Apple pulp waste from the fruit processing industry demonstrated to be a suitable and prospective feedstock for the production of *mcl*-PHA by *P. citronellolis.* Although the production process needs to be optimised to improve the polymer's volumetric productivity and yield, the results obtained in this study evidenced its potential. Strategies for optimising polymer production will include, for example, increasing the biomass concentration by controlling the pH with ammonium or operating the bioreactor under a fed-batch mode. The resulting polymer was also shown to be a suitable material for the preparation of films with interesting properties, comparable to those of other synthetic polymers, with the considerable advantage of being biodegradable and biocompatible. Therefore, it can be anticipated that *P. citronellolis mcl*-PHA might developed into a material useful for applications ranging from commodity packaging products to high-value biomedical biomaterials.

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6. Conflict of interest

The authors declare no conflict of interest.

Table 5. Mechanical properties of films prepared by solvent casting using the *mcl*-PHA produced by *P. citronellolis* NRRL B-2504 from apple pulp waste and comparison with other natural and synthetic polymers (P(3HB), poly (hydroxybutyrate); P(HBHV), poly (3-hydroxybutyrate-*co*-3-hydroxyvalerate); P(HBHHx), poly (3-hydroxybutyrate-*co*-3-hydroxyhexanoate); PLA, Polylactic acid; n.a., data not available)

Material	Tensile stress at break (MPa)	Strain at break (%)	Young Modulus (MPa)	Reference
P. citronellolis mcl-PHA	4.9 ± 0.68	279 ± 12	7.8 ± 1.58	This Study
P. mediterranea mcl-PHA	6.5 ± 0.35	195 ± 46	5.3 ± 1.14	[16]
P. oleovorans mcl-PHA	n.a.	n.a.	100 ± 4.00	[17]
P(3HB)	16.0-16.4	1.3-4.0	1000-1317	[27,28]
P(HBHV)	28.0-29.0	3-14	2500 ± 0.20	[29,30]
P(HBHHx)	11.3	67	38	[28]
PLA	61.0	5	1904	[31]

P= *Pseudomonas*, *mcl*-PHA= medium-chain-length polyhydroxyalkanoates

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تولید پلی‌هیدروکسی‌آلکانوآت‌های متوسط زنجیره توسط رشد *سودوموناس سیترونلولیس* رشد داده شده روی ضایعات پالپ سیب

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چکیده

سابقه و هدف: ضایعات پالپ سیب تولید شده در صنعت فرآوری میوه ماده‌ای غنی از قند است که توانایی زیادی برای استفاده به عنوان ماده اولیه در تولید فرآورده‌های میکروبی با ارزش افزوده دارد. هدف این کار استفاده از این ماده اولیه برای کشت *سودوموناس سیترونلولیس* و تولید الاستومر طبیعی پلی‌هیدروکسی‌آلکانوآت‌های با زنجیره متوسط بود.

مواد و روش‌ها: بخش جامد پالپ سیب دور ریخته شد و بخش محلول، که غنی از فروکتوز (17/7 $g{\cdot}l^{-1}$)، گلوکز (7/5 $g{\cdot}l^{-1}$) و سوکروز (1/2 $g{\cdot}l^{-1}$) می‌باشد، برای کشت ناپیوسته *سودوموناس سیترونلولیس* NNRL B-2504 مورد استفاده قرار گرفت.

یافته‌ها و نتیجه‌گیری: *سودوموناس سیترونلولیس* میزان بسپار[1] در توده زیستی را به 30 درصد وزن تر و بهره‌وری حجمی را به $g{\cdot}l^{-1}{\cdot}h^{-1}$ 0/025 رساند. بسپار به طور عمده از 3- هیدروکسی دکانوآت (68 %mol) و 3- هیدروکسی اکتانوآت (22 %mol)، با میزان کمی 3- هیدروکسی دودکانوآت (5 %mol)، 3- هیدروکسی تترادکانوآت (4 %mol) و 3- هیدورکسی هگزانوآت (1 %mol) تشکیل شده بود و وزن حجمی Da $3/7{\times}10^5$ داشت. انتقال شیشه‌ای و درجه حرارت ذوب آن به ترتیب 12- و 53°C، و درجه حرارت تجزیه حرارتی 296°C بود. فیلم‌های بسپار چگال، انعطاف‌پذیر و نفوذ پذیر نسبت به اکسیژن و دی اکسید کربن بودند. نتایج نشان داد ضایعات پالپ سیب ماده اولیه‌ای مناسب برای تولید زیست بسپاری است به لحاظ خواصی که دارد می‌تواند جایگزین امیدوانه کننده‌ای برای بسپارهای مصنوعی برپایه پتروشیمی باشد.

تعارض منافع: نویسندگان اعلام می‌کنند که هیچ نوع تعارض منافعی مرتبط با انتشار این مقاله ندارند.

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واژگان کلیدی

- ضایعات پالپ سیب
- پلی‌هیدروکسی‌آلکانوآت‌های متوسط زنجیره (*mcl*-PHA)
- *سودوموناس سیترونلولیس*
- با ارزش کردن ضایعات

***نویسنده مسئول**

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[1] polymer