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Production and characterization of polyhydroxyalkanoates films produced by *Cupriviadus necator* from agro-industrial residues

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ABSTRACT

Polyhydroxyalkanoates (PHA) are a viable alternative to replacing traditional plastics, harmful to nature. The objective of this study was to evaluate the production of PHA by *Cupriviadus necator* using agro-industrial residues, its physicochemical characterization, and of the membranes produced. Cell growth and PHA production were compared in three carbon sources: residual glycerol, whey cheese with and without a mineral solution. After cultivation, the biopolymer was extracted and characterized by FTIR and 1D and 2D NMR experiments. Membranes containing 5.0% of PHA had their physicochemical properties measured for mechanical properties, color, and water vapor permeability. The results demonstrated that the yield of PHA.g^{-1} of biomass was higher for cultivation using residual. Yet the PHA.L^{-1} was higher in cultures with whey cheese. The NMR and FTIR result allowed to characterize the molecular structure of PHA produced. It was calculated 8 carbon atoms in the methylene main chain and a maximum of 13 carbons (Poly-3-hydroxytridecanoato) in the polymer side chain. The membranes showed a strong whiteness tendency as well as a high opacity, expected low water vapor permeability, and rigidity. In conclusion, mechanical properties indicated that the formed PHA membranes could be applicable to plastic packaging in substitution for petroleum derivatives.

Keywords: PHA, residual glycerol, whey cheese, film plastic, characterization

1. INTRODUCTION

Currently, plastic materials have become an integral part of everyday life due to their ease of molding, resistance to biodegradation, and durability; they are also increasingly used in industry [1, 2]. The most common raw material used in plastic manufacturing is non-renewable crude oil fractions. Besides that, plastic durability permits their accumulation in the environment at a high rate of 25 million tons per year [1, 3, 4]. Plastic recycling and incineration are alternatives currently employed but are not efficient and generate toxic compounds with implications regarding environmental perspectives [1].

In response to this emerging problem, there has been a high interest in the development of biodegradable plastics from biosynthesized products [5]. Biopolymers, in particular the group of Polyhydroxyalkanoates (PHA), were referred to as alternative substitutes to petrochemical derivatives in the

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production of plastics [6]. Several organisms from bacteria to plants are producers of polyhydroxyalkanoates, being easily accessible [7].

However, one of the major obstacles to replacing polypropylene with PHAs in the production of plastic materials has been economic, as its production becomes more expensive due to the use of high-cost substrates such as glucose and propionic acid [7]. Therefore, the use of agro-industrial residues such as raw glycerol (biodiesel production residue) and cheese whey (cheese production residue), which are discarded and can generate serious environmental contamination [8, 9], are alternatives to these high costs, making bioplastic more competitive and potentially applied in different branches of industry.

Bacteria *Cupriviadus necator* accumulate in their cytoplasm under unbalanced growth conditions (lack of essential nutrients such as P, N, and S and excess C) PHA granules as a carbon energy source [10]. These can be produced from various substrates, including fats and oils, lignocellulose, industrial and agricultural residues, among others, which has the advantage of tailings recovery [7].

The aim of this study was to evaluate the growth and accumulation of PHA by *C. necator* when cultivated in a different medium that uses residues as alternative sources of carbon, physicochemically characterizes the polymer obtained and synthesize and partially characterize films from PHA.

2. MATERIALS AND METHODS

2.1 Biomass production

Cupriviatus necator DSM 545 was cultured on nutrient agar (3 $g.L^{-1}$ meat extract, 5 $g.L^{-1}$ meat peptone, and 18 g L^{-1} agar) for 24 h at 35 °C. A pre-inoculum was prepared in nutrient broth (3 $g.L^{-1}$ meat extract and 5 $g.L^{-1}$ meatpeptone) for 24h at 35 °C, under constant stirring (250 rpm). The inoculum was prepared at 10% (v/v) in three different culture media: Residual glycerol with mineral salts medium (RG + salts) [(0.3 $g.L^{-1}$ H_3BO_3; 0.2 $g.L^{-1}$ $CoCl_2.6H_20$; 0.1 $g.L^{-1}$ $ZnSO_4.7H_20$; 30 $mg.L^{-1}$ $MnCl_2.4H_20$; 30 $mg.L^{-1}$ $Na_2MoO_4.2H_20$; 10 $mg.L^{-1}$ $CuSO_4.5H_2O$; 105 $\mu L.L^{-1}$ CH_2CO_2H; 60 $mg.L^{-1}$ $(NH_4)_2Fe(SO_4)_2$; 50 $mg.L^{-1}$ $Mg_2SO_4.7H_20$; 10 $mg.L^{-1}$ $CaCl_2.2H_20$; 8.95 $g.L^{-1}$ $Na_2HPO_4.12H_20$; 1.5 $g.L^{-1}$ KH_2PO_4; 5.0 $g.L^{-1}$ $(NH_4)_2SO_4$; 36 $\mu L.L^{-1}$ $C_6H_8O_7$)] containing 20 $g.L^{-1}$ of the crude residual glycerol; mineral salts medium solubilized in clarified cheese whey with 20 $g.L^{-1}$ of lactose (CW + salts); and only clarified cheese whey (CW). After inoculation, the flasks were incubated at 35 °C for 54 h. Collections were carried out over the cultivation time to verify the biomass production. HPLC measured consumption of carbon sources at the end of the cultivation process.

2.2 Biopolymer extraction

Bacterial suspension was centrifuged (4000 x g, 10°C, 20 min), the supernatant was discarded and 0.2 M NaOH was added to the precipitate. Then, the material was stirred under 200 rpm, at 30ºC for 2 h, centrifuged again, the supernatant discarded, and the supernatant washed in ethanol and then in acetone. Then, the precipitate was resuspended in chloroform (20:1) and stirred at 200 rpm for 24 h. It was added an equal volume of distilled water and stirred under 200 rpm for 24 h at room temperature. Then, the mixture was transferred to a separator funnel, and the organic phase was dripped in an equal volume of ethanol at 4 °C (kept in an ice bath). Finally, the precipitate was placed in Petri dishes, for the complete evaporation of the solvents, thus obtaining the PHA [7]. After extraction, the obtained PHA was weighed and the yield was calculated by dividing this final mass of PHA by the initial biomass and express as w/w of biomass.

2.3 Polyhydroxyalkanoates quantification

Fifty milligrams of lyophilized cells were solubilized in 1 mL of chloroform. One milliliter of an acidic methanol solution (97 mL of methanol, 3 mL of H_2SO_4) containing an internal standard (0.3 g of octanoic acid) was added and vortexed for 1 min. For cell lysis, the preparation was incubated for 2 h on ultrasound, polymer chain hydrolysis, and monomer methylation. Then, was added for sample 1 mL of 60 $g.L^{-1}$ Na_2CO_3 solution for neutralization [3]. Then, the two-phase system was subjected to vortex for 2 min to promote the extraction of the methyl esters to the organic phase. After centrifugation at 4500 rpm during 5 min, 400 µL of the organic phase was collected and analyzed in a gas chromatograph (GC) (Agilent Technologies GC7890A) equipped with a flame ionization detector (FID) detector and a capillary column (HP-5 from Agilent J&W Scientific, 30 m in length and 0.32 mm of internal diameter). The oven, injector and detector temperatures were kept constant at 60 °C, 120 °C and 150 °C, respectively. Data acquisition and analysis were performed with the GC Chemstation. Peak identification was achieved using a standard of 3-methyl hydroxybutyrate (Sigma) [3].

2.4 Fourier Transform Infrared (FTIR)

The characterization by Fourier Transform Infrared spectroscopy (FTIR) was performed with 200 mg of PHA, in a VERREX 70 spectrometer (Bruker Optics, USA) under dry air at room temperature (25 °C). The spectra were scanned from 4000 to 500 cm^{-1} with 4 cm^{-1} resolution, from powder samples that were dispersed in KBr pellets.

2.5 NMR characterization

1D and 2D spectra of the PHA sample were recorded using a 600MHz NMR spectrometer (Bruker Biospin, Rheinstetten, Germany) with a triple resonance probe [11]. Approximately 10 mg of the sample was dissolved in 0.5 mL of 99.9% dimethyl sulfoxide-d6 (Cambridge Isotope Laboratory, Cambridge, MA). All spectra were recorded at 323K with HOD (deuterated water exhibiting a peak due to exchange with residual H_2O) suppression by presaturation. For 1D ^1H NMR spectra, 128 scans were recorded, using an inter-scan delay equal to 1s. 2D $^1H/^1H$ TCOSY (total correlation spectroscopy) spectra was recorded with 256 scans and 1024 x 512 points using an increment number setup set to 64 and states-TPPI (time proportion phase incrementation) for quadrature detection in the indirect dimension. Chemical shifts were displayed relative to the DMSO-d6 signal at 2.50 ppm. The maximum size of the methylene side chain was estimated by the ratio between the integral values of the methylene protons found at the side chains identified in PHA and the methylene protons of the α-carbon in a commercial polyhydroxybutyrate (PHB) (Sigma-Aldrich).

2.6 Preparation of plastic film

Plastic film forming solutions were prepared in chloroform with 5% (w/v) pure PHA kept under magnetic stirring (500 rpm) and heating at 80°C for 2h. One milliliter of each plastic film forming solution was spread in a 50 mm diameter glass petri dish. After evaporation of the solvent (5 min), the formed film was stored at room temperature for the mechanical properties' experiments. The film was produced in quintuplicates.

2.7 Colour and opacity

Color and opacity parameters were determined using a digital colorimeter (Konica Minolta, model Chroma Meter CR-400, Osaka, Japan) according to [12]. Calibration was performed on illuminant C with a white pattern. The parameters determined were L * (L * = 0 for black and L * = 100 for white), a * (−a * tendency to green and + a * tendency to red) and b * (−b * tendency to blue and + b * tendency to yellow).

These parameters are those recommended by the International Lighting Commission. The opacity of the film was calculated as the ratio between the opacity of each sample in the black pattern (Yb) and in the white pattern (Yw). Measurements were performed in random quintuplicates and an average of these was used for calculations. The experiment was performed in quadruplicate, and the results were expressed as a percentage and determined by Equation 01:

$$Y(\%) = \left(\frac{Y_b}{Y_w}\right) \times 100 \quad (01)$$

2.8 Thickness

Film thickness was determined by measuring five different random points using a digital micrometer (Digimess, Brazil).

2.9 Water vapor permeability (WVP)

Water vapor permeability (WVP) was determined according to the ASTM E96-92 method [12]. Permeation cells containing distilled water were sealed with the films and placed in a desiccator containing silica. The cells were weighed for 10 h, always at 2 h intervals. The slope of mass loss *versus* time was obtained by linear regression. The WVP was expressed in $g.m^{-2}.s^{-1}.Pa^{-1}$ and calculated using the following equation 02:

$$P = \frac{(WVTR \times L)}{\Delta P} \quad (02)$$

Where, WVTR is the transmission rate of water vapor through the film, L is the average film thickness and ΔP is the partial difference of the water vapor pressure on both sides of the film.

2.10 Water Vapor Transmission Rate

The water vapor transmission rate (WVTR) was determined gravimetrically based on ASTM E96-92 method [13, 14]. Plastic film was sealed on the top of a permeation cell containing distilled water (100% RH; 2337 Pa vapour pressure at 20 °C), placed in a desiccator at 20 °C and 0% RH (0 Pa water vapour pressure) containing silica. The evaluation of the cells was carried out for a period of 10h at regular intervals of 2h. The constant air circulation outside the test cell, maintained through a mini fan inside the desiccator, was assumed to be a steady and uniform water pressure condition [15]. The slope of weight loss versus time was obtained by linear regression. This measurement was made in quadruplicate temple and they were determined using the Equation 03:

$$WVTR = \frac{\Delta w}{\Delta t.A} \tag{03}$$

Where, $\Delta w/\Delta t$ is the moisture gain weight per time ($g.s^{-1}$), A is the exposed surface area of the film (m^2). Results were expressed as mean, standard deviation.

2.11 Mechanical properties: Young's modulus, tensile strength, and elongation-at-break

Young's modulus (YM), tensile strength (TS) and elongation at break (EB) were measured using a TA·HD plus texture analyzer (Serial RS232, Stable Micro Systems, Surrey, United Kingdom), following the guidelines of ASTM D 882-00 (2010). Film strips with a length of 120 mm and a width of 12 mm were used, following the ASTM standard. The claw had an initial separation of 120 mm and the crosshead speed was 5 $mm.min^{-1}$. The YM was determined according to the slope of the elastic region on a stress x strain graph. The TS was calculated by dividing the maximum load (N) by the initial cross-sectional area (m^2) of the sample and was expressed in MPa. EB was calculated as the ratio between the final length at the sample's breaking point and the initial length of a sample (120 mm) and expressed as a percentage. At least five repetitions were performed for each sample. The results were expressed as mean standard deviation.

3. RESULTS AND DISCUSSION

The growth of the bacterial cells in each of the media tested is shown in Figure 1. When cultivating using the cheese whey added of a solution of mineral salts as the carbon source, the growth is up to 14 times higher than when *C. necator* was cultured in crude residual glycerol. It was detected that in the cultures, the whey cheese as a carbon source was totally consumed, however in the culture with glycerol were observed that even after the 54h of culture, it still had about 54.02% glycerol in the cultivation medium.

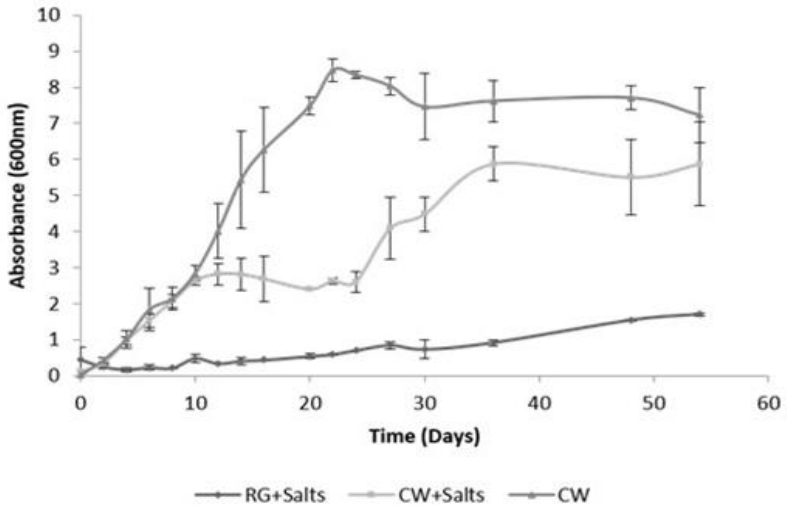

Figure 1: Cell growth of *Cupriviadus necator* in different culture medium compositions.

It was observed that the production of PHA in the cultivation per gram of biomass was higher in the culture with glycerol (0.3 $g.g^{-1}$ of biomass) when compared to the culture with only the cheese whey (0.03 $g.g^{-1}$ of biomass) and the culture with cheese whey and mineral salts (0.08 $g.g^{-1}$ of biomass). Although biomass production is 3.4 and 4.2 x higher in CW and CW+Salts, respectively, which could compensate for the low yield in PHA produced, considering large-scale crops, equivalent amounts would be needed of the solvents used in the extraction that would make the cost high, in this case we suggest the use of residual glycerol for large-scale cultivation.

Microbial PHA is produced by a diversity of species, among which *C. necator* is the most used due its high PHA production yield [1]. One of the main factors in the production of PHA is the limitation of the availability of elements involved in protein synthesis and DNA replication, such as nitrogen, phosphorus and sulfur [7]. This limitation causes a change in cell metabolism, directing the excess of carbon to PHA synthesis [13]. However, we observed that the amount of PHA accumulated when using cheese whey as alternative source of carbon was low, probably indicating a targeted use of carbon present in this residue for the cell multiplication phase.

Under the growth conditions proposed in this study, we observed that *C. necator* was not efficient in metabolizing the residual crude glycerol, presenting a low cell growth rate compared to when cheese whey was used as a carbon source. This cheese whey is rich in lactose, lipids and proteins, having all the essential elements for bacterial metabolism [16] and, even without salt supplementation, it promoted rapid cell multiplication, reaching the stationary phase with only 22 h of culture. After this period, it was possible to observe the accumulation of PHA.

PAULA *et al.* [17] and ALBUQUERQUE *et al.*, [7] studying the synthesis of PHA using residual and commercial glycerol, observed a greater production when they used residual glycerol. FIGUEIREDO et al., [18] demonstrated that the production of PHA from residual glycerol reached 22.22 % with 72 h of cultivation. Such results were inferior to those obtained in this work, which reached a yield of 30 % of PHA production in 54 h. During this time, the presence of residual glycerol in the culture was still detected, meaning that the maintenance of bacterial growth for more days would further increase the yield when the entire carbon source has been consumed.

POVOLO *et al*. [16], evaluating the production of PHA in *Sinorhizobium melioti* and *Hidrogenophaga pseudoflava* using clarified cheese whey obtained a yield of 3.5% and 4.4% of accumulated PHA after 96 h of cultivation. Similar results were obtained in our work with *C. necator*, whose cultivation in CW and CW + salts, yielded 3.0 and 8.0% of PHA, respectively. Although preliminary, the results obtained here indicate that the use of cheese whey, a highly contaminating reject, as a substrate for an accelerated PHA production (shorter time and higher yield), seems to be efficient.The biopolymer extracted was characterized by FTIR and ^{1}H NMR to assess their chemical structure. The FTIR technique estimates the chemical interactions of the molecule and its possible modifications, being essential in the study of polymers. The FTIR analysis shown in Figure 2 shows bands and PHA type signals. Observing the spectrum, one can notice the presence of a peak that is characteristically attributed to the carbonyl group stretching vibration (C═O) in esters (around 1715 cm^{-1}), which can also be observed in previous works [1, 18, 19]. In the region between 2800 and 3000 cm^{-1} there are peaks attributed to the C–H stretching vibration and in the regions between 3100 and 3500 cm^{-1} broadband attributed to the O–H stretching vibration was observed.

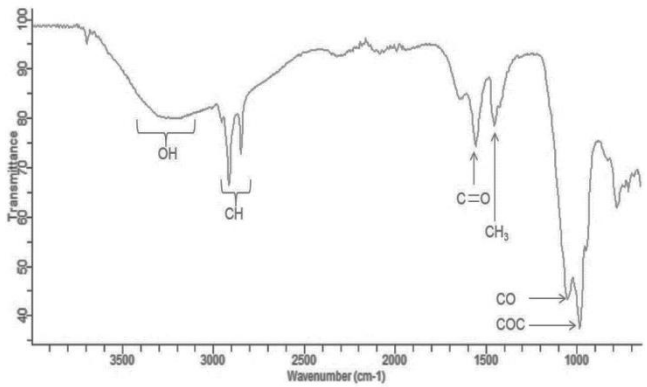

Figure 2: Fourier Transform Infrared (FTIR) spectra of the PHAs obtained by cultivating *Cupriviadus necator* using agro-industrial waste as a carbon source.

The ^{1}H NMR spectrum of the PHA produced by *C. necator* (Figure 3) was characterized in reference to the internal standard dimethyl sulfoxide-d6 (δ 2.5 ppm). The ^{1}H spectrum of a commercial PHB was used as a model to calculate the maximum of methylene that could be found in the side chain of PHA. The observed triplet peaks 1 (δ 5.33 ppm) and multiplet peaks number 2 (δ 2.75-2.17 ppm) are assigned to methane and methylene protons of the β and α-carbon, respectively [20–23]. A weak signal "x" at 5.32 ppm showed the presence of a small percentage of side chains containing the –CH═CH– sequences. At 2.06-2.00 ppm a multiplet signal "y" related to methylenes linked to double bonds was also observed. According to some literature assigning, the observed "x and y" signals 5.32 and 2.06-2.00 ppm, respectively, are related to contaminant hydrophobic lipids such oleate and palmitate found in cellular membrane that scape during the purification process [20, 24]. Peak number 4 (δ 1.51 ppm) is assigned to methylene protons adjacent to the β-carbon in the sidechains [20-23]. Peak number 5 (δ 1.25 ppm) and triplet number 3 (δ 0.87-0.84 ppm) are assigned to the methylene protons and terminal methyl proton of the side-chains, respectively [20-23].

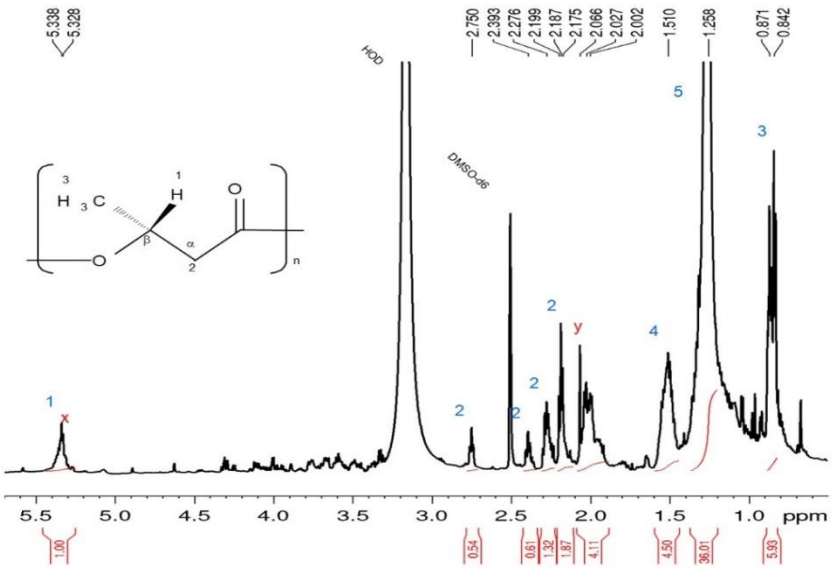

Figure 3: ^{1}H NMR spectra of PHA obtained by *Cupriavidus necator*.

The maximum size of the methylene side chain $(–CH_2–)_n$ calculated was n ≈ 8. Counting the carbonyl, the α and β-carbon at the main chain, the methylene adjacent to the β-carbon and the methyl groups at the side chains, estimated a PHA with a maximum of 13 carbons (Poly-3-hydroxytridecanoato) per polymer chain. Based on the inter-proton correlations observed in the ^{1}H/^{1}H TOCSY spectrum (Figure 4A) the proposed structure for this PHA was shown in Figure 4B. This structure is close to a PHA produced by *Pseudomonas putida*, which grown with glycerol as carbon source [20, 24].

The colour parameters and opacity of the plastic film showed that the studied film was bright with a strong whiteness tendency as presented by L*coordinate values (94.28 ± 0.20), and a blueness appearance represented by b*coordinate (-5.00 ± 0.36) and redness for a* values (1.56 ± 0.14). Plastic film showed a high opacity (Y (%) = 57.02 ± 9.02).

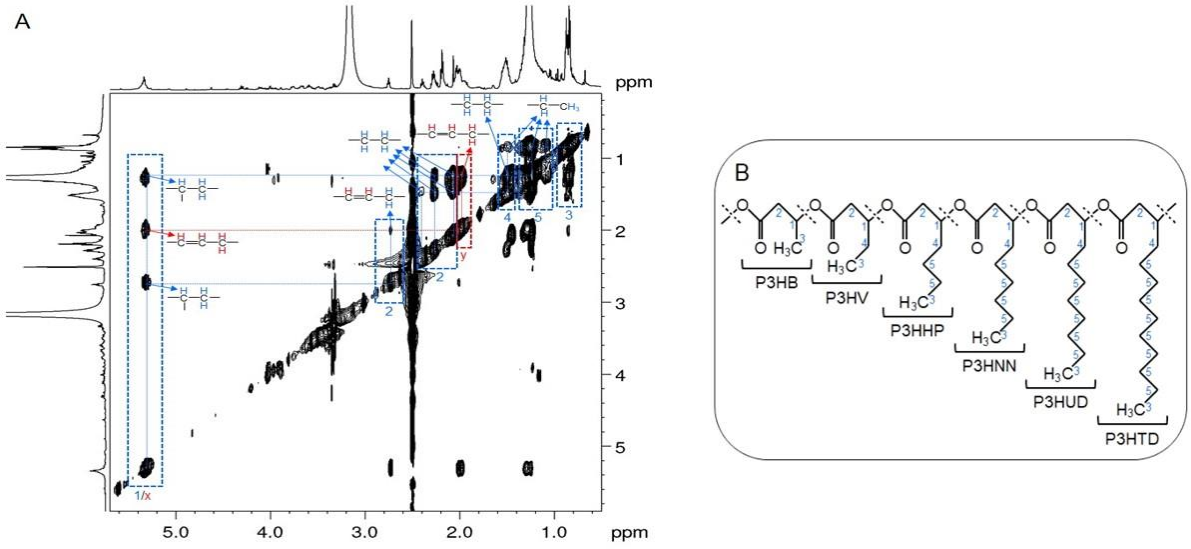

Figure 4: $^{1}H/^{1}H$ 2D TCOSY spectra (A) of the PHA obtained by cultivating *Cupriavidus necator* using agro-industrial waste as a carbon source; (B) is shown the proposed structure for this PHA.

In materials developed for packaging, the water vapor transfer rate is an essential property and indicates the barrier properties of the polymer and thus the possible applications that type of material may be applied [25]. WVTR value was 11.09 ± 2.46 x 10^{-7} ($g^{-1}.m^{-2}.day^{-1}$), with Thickness = 1.46 ± 0.27 x (10^{-5} m) and WVP value of 1.97 ±0.50 ($10^{-11}g.m^{-2}.s.Pa^{-1}$) for a plastic film with 5% pure PHA, and this rate indicates lower material permeability. This result is lower than the values reported by BUGNICOURT *et al.* [26] for PHA films (5 – 19 x 10^{-3} g^{-1} m^{2} day-^{1}). The Small water vapor transmission rate confirms nature very hydrophobic the polymer.

As for the mechanical properties were analyzed Young´s modulus (YM) was analyzed, which measures the intrinsic rigidity of the film, being more rigid or malleable (that deforms), the higher the values obtained for the Young's modulus, the more rigid the material. The analyzed plastic film had a YM of 897.21 ± 57.92 Mpa, indicating that this is a high rigidity film. Another evaluated parameter was the Tensile Strenghr (TS), which indicates the maximum tensile that the film is capable of withstanding, and this parameter is directly related to the chemical composition of the film [27]. The TS of the film was 4.98 ± 0.54 Mpa. This data indicates that the film withstands little before deforming, indicating that as soon as the material begins to deform, it quickly breaks. The elongation at break (EB), measures the flexibility of the film, which was 0.61 ± 0.05%, confirming the previous data that the plastic film produced has littleelastic. However, some works reported mechanical properties at the same magnitude order for the PHA [25, 28].

The films obtained here using the bench casting technique can be easily produced on a large scale using the technique called continuous casting, without losing their intrinsic characteristics. The technique can allow the formation of the film from the same filmogenic solution used in bench casting, in a faster production process with strict thickness control. Continuous casting is already a widespread technique in the conventional plastics, paper and ceramics industry.

4. CONCLUSIONS

Cupriavidus necator grows and is able to accumulate poly-3-hydroxytridecanoate from the carbon sources tested. The use of residual glycerol from the biodiesel industry is a promising carbon source for *C. necator* in produce PHA, that represents the conversion of potentially polluting waste into high added-value products. The films of PHA produced by *C. necator* presents mechanical and barrier with potential for use in the development of biodegradable packaging for the replacement of petrochemical derivative plastics.

5. ACKNOWLEDGMENTS

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