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Characterization of a polyhydroxyalkanoate obtained from pineapple peel waste using *Ralsthonia eutropha*

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ABSTRACT

Agro-industrial waste can be the production source of biopolymers such as polyhydroxyalkanoates. The aim of this study was to produce and characterize Polyhydroxyalkanoates produced from pineapple peel waste fermentation processes. The methodology includes different pineapple peel waste fermentation conditions. The produced biopolymer was characterized using FTIR, GC–MS and NMR. The best fermentation condition for biopolymer production was obtained using pH 9, Carbon/Nitrogen 11, carbon/phosphorus 6 and fermentation time of 60 h. FTIR analyzes showed PHB group characteristics, such as OH, CH and C=O. In addition, GC–MS showed two monomers with 4 and 8 carbons, referred to PHB and PHBHV. H^1 NMR analysis showed 0.88–0.97 and 5.27 ppm signals, corresponding to CH_3 and CH, respectively. In conclusion, polyhydroxyalkanoate production from pineapple peels waste is an alternative for the treatment of waste generated in Colombia's fruit industry.

1. Introduction

Pineapple (*Ananas comosus*) cultivation in Colombia currently has great importance in the countryís economy, which produces 621,000 tons/year, occupying an area of approximately 14,000 ha (Agronet, 2015). However, pineapple production increase generates agro-industrial wastes from the food industry or from products that were lost in postharvest of around 315,000 tons/year. Then, it becomes important to find applications for these pineapple peel residues.

Pineapple agro-industrial waste has been studied regarding glucose syrup production (Zain and Seker, 2014) and preparation of high surface area activated carbon with pineapple peel (Hameed and Foo, 2012). Regarding food industry applications, Hajar et al. (2012) determined pineapple peel physicochemical properties, and Donkor et al. (2016) and Lambri et al. (2015) used it to develop yogurt and vinegar. Another alternative to the pineapple peel agro-industrial waste is its use in biopolymer production, such as polyhydroxyalkanoates (PHA), by fermentation.

These biopolymers have been produced in laboratory scale through fermentation processes from several waste types, as follows: dairy residues (Bosco and Chiampo, 2010); a co-product stream from soy-based biodiesel production that consists of glycerol, fatty acid soaps and residual fatty acid methyl esters (Ashby et al., 2004); glycerol (Fonseca et al., 2009), and soy cake (Oliveira et al., 2007). Other examples are found in the review on polyhydroxyalkanoate (PHAs) production from waste materials and byproducts by submerged and solid-state fermentation, by Castilho et al. (2009). Other raw materials used for PHAs production that are reported in the literature are fructose (Barbosa et al. 2005) and vegetables oils (Thakor et al., 2005).

PHAs are biopolymers synthesized by many gram-positive and gram-negative bacteria that are stored in intracellular inclusion bodies as an energy reserve, in response to carbon source excess and under nutrient-limited conditions (Mishra et al., 2010). A number of studies reported the production of different PHAs using various strains, such as *Cupriavidus necator* (the same as *Ralstonia eutropha*), *Brevundimonas vesicularis* LMG P-23615b and *Comamonas testosterone* (Thakor et al., 2005).

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For being biodegradable (Lopez-Cuellar et al., 2011), PHAs constitute an important alternative to the environmental concern caused by persistent petroleum-based plastics. PHAs monomeric composition can be related both to the supplied carbon source nature and to the bacteria strain (Simon-Colin et al., 2012). PHAs are R-hydroxyalkanoic acid (HA) thermoplastic or elastomeric polyesters (poly-oxi-esters). Structurally, these polymers are classified based on number of carbons, which ranges from 3 to 14, and on monomeric unit type, producing homopolymers or heteropolymers. PHAs with 3–5 carbon atoms are classified as short chain length PHAs (scl-PHAs), while medium chain length PHAs (mcl-PHAs) contain 6–14 carbon atoms (Tajalli and Roy, 2010).

PHAs have different applications, such as in biomedicine, food, packaging, textiles and household compliances (Tajalli and Roy, 2010). In biomedicine, PHAs are used in cardiovascular products, tissue regeneration scaffolds, vascular grafts, stents, heart valves, dental and maxillofacial treatments, orthopedics, urology and wound management materials (sutures and dressings) (Zinn et al., 2001).

In order to find a better use to the waste generated in Colombia's fruit industry, especially from pineapple agro-industry, the aim of this study was to research polyhydroxyalkanoate production by fermentation using pineapple agro-industrial waste as substrate, particularly focusing on the fermentation condition effect on biopolymer structural properties.

2. Materials and methods

2.1. Materials

Pineapple peels from the Uraba region, Antioquia, were purchased in Medellin's local market. The pineapple variety used in this study is called "Oro Miel". Peels were characterized to determine moisture, total Ash, fat, total nitrogen and total protein with a coefficient of 6.25 (AOAC, 2005). Total Carbohydrates were calculated by difference, and Mg, K and Zn were determined by atomic absorption.

Before fermentation, pineapple peel samples were washed to remove dirt and grinded in an Oster blender. Afterwards, they were frozen and stored at $-20 \pm 0.5\,^{\circ}C$ until further processing.

Pineapple peel hydrolysis was carried out using sulfuric acid (97% HONEYWELL®). For fermentation, ammonium sulphate (MERK®) and dipotassium phosphate (PANREAC®) were used as nitrogen and phosphorus sources, respectively.

Raslthonia eutropha ATCC 17697 was used as bacterial strain. OXID® was used for bacteria culture broth inoculation. The bacterial strain used in this work was *Ralstonia eutropha* ATCC 17697. This bacterial strain was selected because it is one of those cited in the literature for production of polyhydroxyalkanoates. Biopolymer extraction was conducted by using chloroform (MERK®) and ethanol (PANREAC®).

2.2. Fermentation

Previously to fermentation process, pineapple peels were subjected to hydrolysis using sulfuric acid at 2%, 121 °C and 15 Psi. Subsequently, fermentation was conducted at Lab scale using 250 ml Erlenmeyers, in a shaker Heidolph Unimax 1010. In order to do so, pH was adjusted and Carbon/Nitrogen (C/N) and carbon/phosphorus (C/P) ratios were controlled (Table 1). Then, the resulting suspension was sterilized. Finally, the culture was inoculated to allow for fermentation in Laboratory scale. Three repetitions were used for each condition. Then, the fermentation process is briefly described: initially fermentation volume 250 ml was defined; Inoculum volume was 10% of the final volume (25 ml) and the pre-inoculum volume was 10% of the volume of inoculum (2.5 ml). For the preparation of pre-inoculum, nutritious broth and distilled water were used, taking into account the following relationship: 13 g of nutrient broth diluted in 1000 ml distilled water (this ratio was recommended by the commercial broth OXID®). The broth was sterilized at 121 °C and 15 psi for 15 min. After that, it was stored until further use.

The inoculation of pre-inoculum was carried-out at room temperature. The flask was incubated at 30 °C, under agitation at 200 rpm, for 24 h. Subsequently, the inoculum was also prepared, similarly to pre-inoculum preparation (Barbosa et al., 2005). Finally, the inoculum was sown in hydrolysates waste pineapple peels, previously sterilized and adjusted to the conditions of fermentation shown in Table 1.

2.3. Biopolymer extraction

Once fermentation was completed, the material was collected in 50 ml falcon tubes and centrifuged for 30 min at 4000 rpm. The supernatant was removed and the decanted fraction was freeze-dried. The biopolymer was extracted from the dried biomass using chloroform in a ¼ biomass/chloroform ratio, under vigorous agitation, for 12 h at 30 °C. Subsequently, dispersion was filtered on no. 1 Whatman paper. After filtration, the material was added to a Chloroform/Ethanol (1/4) solution and maintained under stirring for 5 min. Then, it was stored at 4 °C for 3 h, in order to precipitate the biopolymer. Finally, the dispersion was filtered under vacuum using a 25 μm paper, while the solid material, constituted by the biopolymer, was dried in an oven at 65 °C until reaching constant weight. Once the polymer was dry, this was stored in glass containers at room temperature in a dark place, until further use.

2.4. Biopolymer characterizations

2.4.1. Fourier transform infrared (FTIR)

FTIR spectra were obtained through a Perkin Elmer equipment, Spectrum One model, using a DTGS detector with 16 scans, resolution of 4 cm^{-1} and wave range of (ν) 4000–450 cm^{-1} at 22 °C.

Table 1
Fermentation conditions and biopolymers production from pineapple peels.

Assay	pH	C/N*	C/P*	Fermentation Time (h)	Production PHA (mg/250 ml)	Production PHA (mg/100 g peel)
1	6	3	6	12	7 ± 1 a,b**	14 ± 10
2	8	9	2	12	9 ± 1 b	18 ± 1
3	7	1	4	30	9 ± 2 b	18 ± 1
4	8	9	6	12	7 ± 2 a,b	14 ± 1
5	7	6	1	30	7 ± 1 a,b	14 ± 1
6	9	6	4	30	6 ± 1 a,b	12 ± 1
7	6	9	6	48	5 ± 4 a,b	10 ± 4
8	6	3	2	12	5 ± 1 a	10 ± 1
9	9	11	6	60	22 ± 4 c	44 ± 4

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

Samples were prepared as tablets using KBr with a mass ratio of 100:1 (KBR:sample) and pressure of 5 tons.

2.4.2. Gas chromatography–mass spectrometry (GC–MS)

Gas chromatography analysis was carried out using an GC QP2010 MS Shimadzu equipment with an SH-Rxi-5Sil Ms ultra-Column (30 m, 0.25 mm ID, 0.25 um df), using the following conditions: oven temperature of 60 °C, injector temperature of 120 °C, and detector temperature of 150 °C. Samples were prepared by methanolysis, according to Fonseca et al. (2009).

2.4.3. Nuclear magnetic resonance (NMR)

Nuclear magnetic resonance analysis was carried out using a Bruker Fourier spectrometer at 300 MHz for ^{1}H analysis and at 125 MHz for ^{13}C analysis. Deuterated chloroform was used as a solvent. In order to analyze the spectra, chemical shift reference of 7.26 ppm was used. ^{1}H and ^{13}C spectra were analyzed using NMR Mestre-C version 4.8.6.0 program. 3-hydroxybutyrate (HB) and 3-hydroxyvalerate (HV) relative mole fraction in the copolymers was determined from spectrum ^{1}H NMR using Eq. (1)

$$X = \frac{\gamma_1}{\gamma_1 + \gamma_2} \quad (1)$$

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 in relation to methyne groups resonance in HV and HB, respectively.

2.5. Data analysis

Data was statistically analyzed using an ANOVA test with 95% significance level. In order to do so, Centurion STATGRAPHICS Ver. XVI.I software was used. LSD tests with 95% significance level were carried out to determine significant differences between different fermentation conditions for biopolymer production, considering random experiments and taking fermentation with 9 levels as the only factor. The response variable was the biopolymer amount produced.

3. Results and discussion

3.1. Pineapple peel physicochemical properties

Physicochemical properties of pineapple peel samples used in this study are given in Table 2. It can be observed that moisture, protein and ash had values of 70.97 ± 0.84%, 0.63 ± 0.02%, 1.10 ± 0.08%, respectively; with respect to Mg, K and Zn content, values obtained for pineapple peel residues were of 1.9 ± 0.9 mg Mg/kg, 26.1 ± 0.4 mg K/kg and 298 ± 9 mg Zn/kg, respectively. Protein and moisture content reported in this study are comparable with the corresponding values early reported by Dhanasekaran et al. (2011). Another study that reports determination of physicochemical properties of different fruit peels was made by Macagnan et al. (2015), who evaluated apple, orange and passion fruit peel different physicochemical properties, and reported moisture values from 5.64 ± 0.15 to 8.45 ± 0.83%, ash values from 1.26 ± 0.12 to 6.04 ± 0.19%, protein values from 4.89 ± 0.06 to 6.98 ± 0.32% and lipid values from 1.26 ± 0.02 to 8.19 ± 0.05% for different peel types. Difference in results is due to physicochemical properties variation according to fruit type and place of origin.

Table 2
Results of physicochemical analysis of pineapple peel.

Parameter	Value*
Moisture (g/100 g)	70.97 ± 0.84
Total Ash (g/100 g)	1.10 ± 0.075
Fat (g/100 g)	0.21 ± 0.085
Total Protein (N × 6.25; g/100 g)	0.63 ± 0.02
Total Carbohydrate (g/100 g)	27.08
Mg (mg Mg/kg)	1.894 ± 9.756
K (mg K/kg)	26.096 ± 0.446
Zn (mg Zn/kg)	298.184 ± 9.756

* All values are in wet basis.

3.2. Biopolymer production

Different PHB production values are reported in the literature, varying according to substrate, origin and type of strain and fermentation conditions. In that sense, Yezza et al. (2007), achieved PHB production values between 2.15–3.26 g/L. Koller et al. (2005) studied surplus materials and reported different PHA, 3-PHB and 3-PHV production amounts achieving values of 16, 1.6 and 2 g/L, respectively, in a total time of 168 h. Some of these values corroborate what was found in this paper.

3.3. Biopolymer characteristics

3.3.1. Infrared spectroscopy analysis

Spectra determined by FTIR analysis are shown in Fig. 1. All spectra showed typical functional group band characteristics of a PHA, such as OH (3200–3500 cm^{-1}), COC (1185–1228 cm^{-1}) (Wang et al., 2011), CH (2922–2929 cm^{-1}) CO (1000–1300 cm^{-1}) (Silverstein et al., 2005) and C=O (1600–1850 cm^{-1}) (Hong et al., 1999), with similar values to commercial PHBs. Oliveira et al. (2007) and Lopez-Cortes et al. (2010) also reported OH extension for PHB and PHAs obtained from soy cake and canola oil, reporting vibration between 3444 cm^{-1} and 3449 cm^{-1}, respectively. Likewise, Barbosa et al. (2005) and Hong et al. (1999) reported that 2928 cm^{-1} bands corresponded to C—H group vibrations or extensions for PHB and PHAs obtained from fructose and different cell types. Lopez-Cortes et al. (2010), Lopez-Cuellar et al., 2011; and Misra et al. (2000) reported that the 1724 cm^{-1} band was the C=O extension, which

Table 3
Chemical shift for NMR H^{1} spectra of PHB-PHV obtained for pineapple peel and simulation signals.

Chemical shift (ppm)	H^{+} and possible PHA	Number Carbon	SignalIntensity[a]	Chem Draw[b]	ACD/Labs[b]	Marvin Sketch[b]
0.88–0.97	CH_3 (HV)	18	M	0.89	0.94	0.94
1.26–1.28	CH_3 (HB)	11,13,24	M	1.16; 1.32	1.25;1.31	1.28;1.29
1.61–1.68	CH_2 (HV)	17	M	1.55	1.73;1.79	1.46;1.68
2.02	CH_3	26	M	2.10	2.16	2.18
2.31–2.37	CH_2 coupled to CH	4,8,16	DD	2.59;2.70	2.30;2.66	2.59;2.39
2.57–2.65	CH_2 coupled to CH	23	DD	2.54;2.79	2.85;2.88	2.69;2.71
3.65	CH_3—COR	1	M	3.41	3.28	3.39
4.17–4.27	O—CH_2-R	3	M	4.84	3.90	4.03
5.27	CH (15) HV	15	M	5.20	5.11;5.14	5.12
5.34	CH (7, 22) HB	7,22	M	5.30;4.89	5.24	5.28

[a] M: multiplet, DD: double doublet.
[b] Standard deviation: ±0.01 ppm.

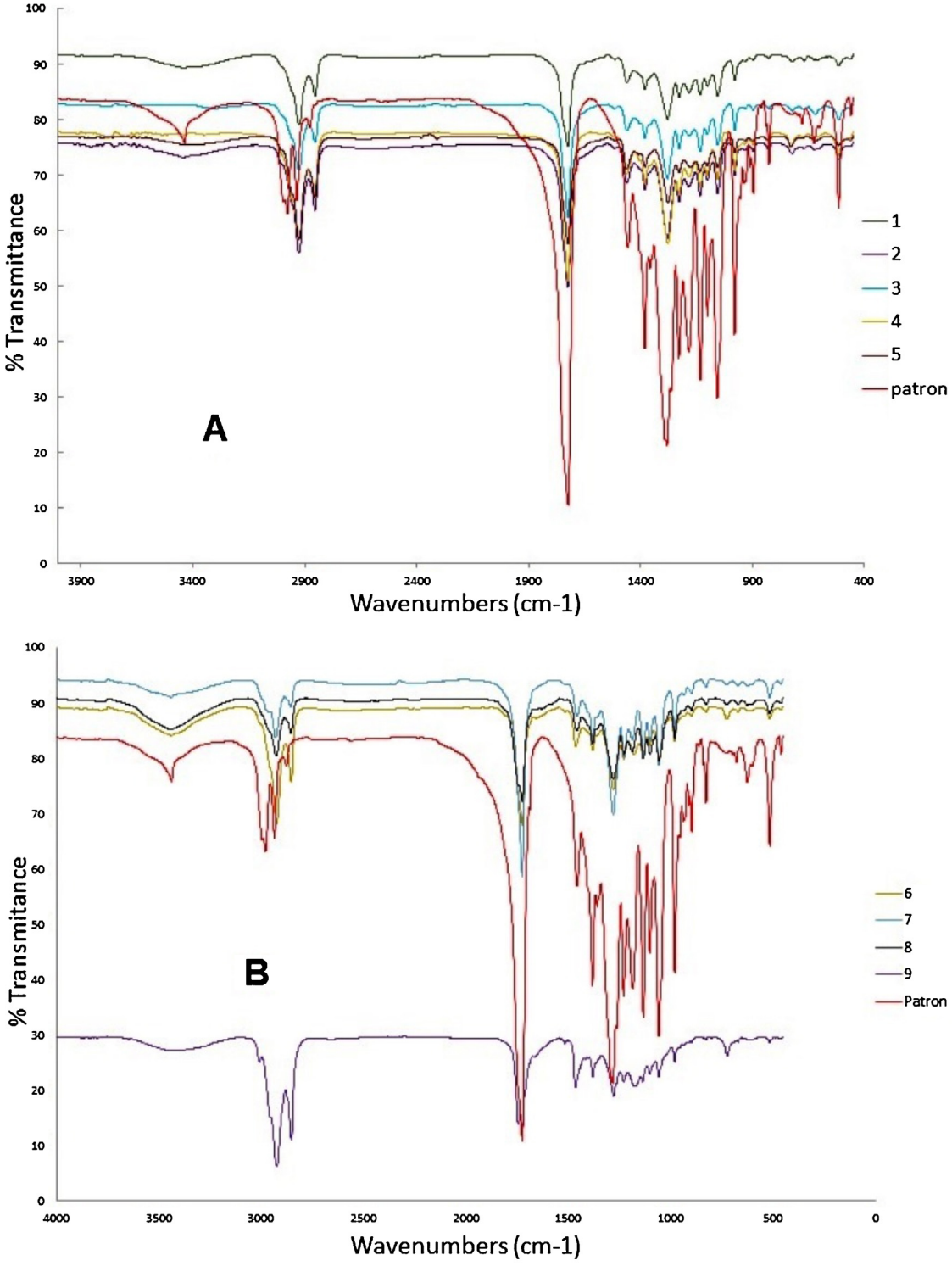

Fig. 1. FTIR spectra of PHA samples. (A) Samples 1–5 and patron (B) samples 6–9 and patron.

was to what was found in this investigation. Moreover, asymmetric CH bending, found in this study at 1452–1457 cm^{-1} and 1379–1384 cm^{-1}, corresponded to CH_2 and CH_3 groups, respectively, and was also reported by Oliveira et al. (2007) and Gáscue et al. (2000). Finally, groups associated with spectra vibrations found for each polyhydroxyalkanoate extracted from each fermentation condition corresponded to the values given by (Silverstein et al., 2005).

3.3.2. Gas chromatography–mass spectrometry (GC–MS)

GC–MS analyzes showed that PHB characteristic peaks appearance times were between 6, 7, 9 and 12 min, which may be indicative of PHA monomer chains with 4, 8 and 10 carbons, capable of forming PHB, PHBHV or mcl-PHA (Wang et al., 2014). With respect to mass spectra peaks, it was observed that mass/charge (m/z) ratios, 117 and 131, which correspond to PHB and PHV, respectively, agreed with reports by Choi and Lee (1997). The m/z

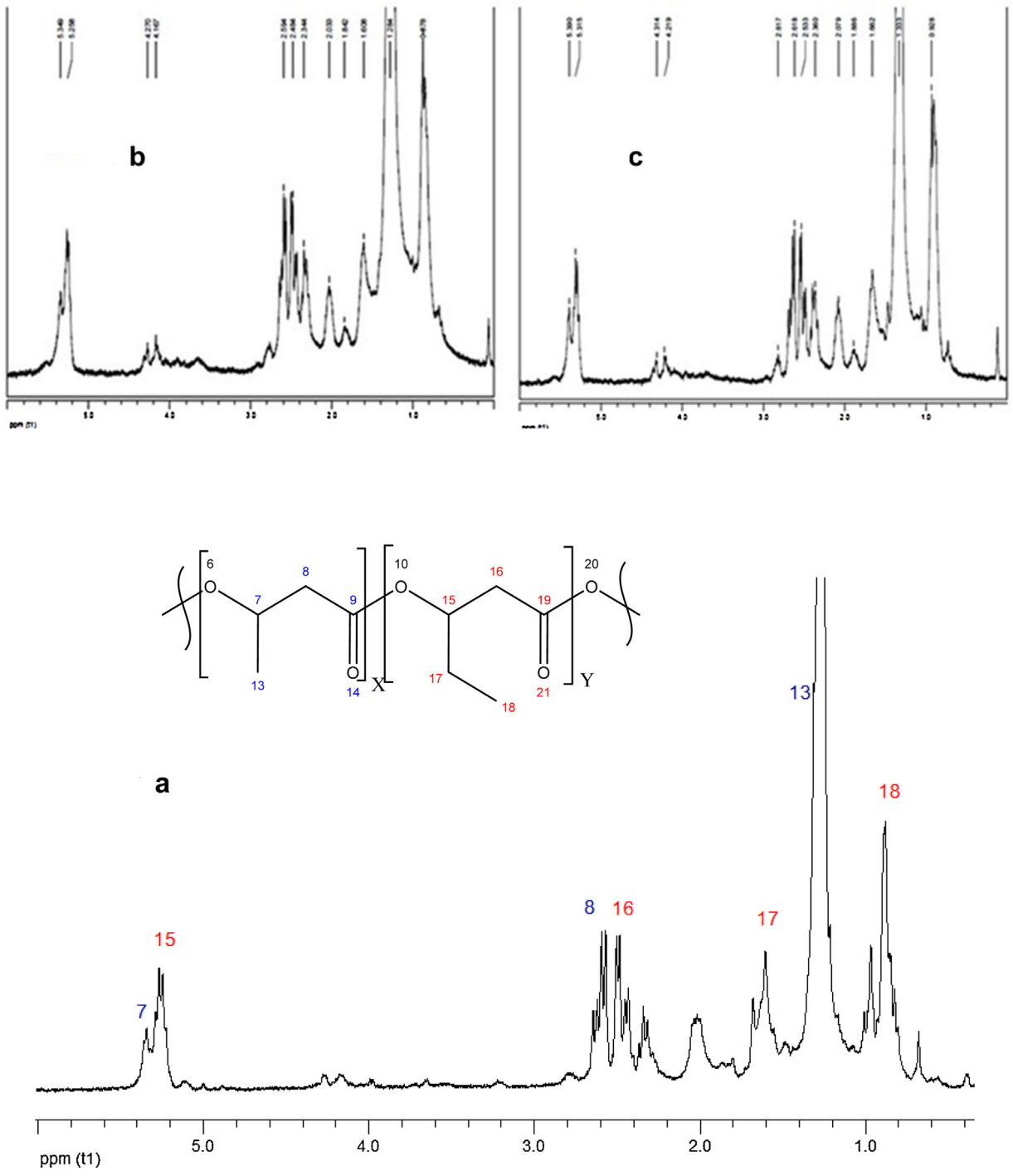

Fig. 2. 300-MHz ^{1}H NMR spectra of PHB-PHV units, from pineapple peel residues for fermentation conditions 9 (a), 1 (b) and 3 (c).

ratio with the highest relative abundance were of 59, 75 and 117 for PHB. For PHV, highest relative abundance was of 59 and 131. Ashby et al. (2005) reported similar retention times and *m*/*z* ratios to those found in this study for a PHA obtained with glucose.

3.3.3. Nuclear magnetic resonance (NMR)

^{1}H NMR analysis results are shown in Table 3. Some NMR spectra examples can be seen in Fig. 2, where resonance signals associated to PHB were of 1.26–1.28 ppm and 5.34 ppm, corresponding to CH_3 and CH groups, respectively. In the PHV case, resonance signals associated with CH3 and CH groups were given at 0.88–0.97 ppm and 5.27, respectively. Other important signals were observed between 2.31–2.37pmm and 2.57–2.65 ppm, which corresponded to CH_2 group coupled to CH.

In general, signals were consistent with results reported by Doi et al. (1986) for a PHBV copolymer. Other studies reported similar resonance signals to those obtained in this study. PHAsMCL NMR H^1 spectrum obtained from *P. guezennei* biovar. *tikehau* on coprah oil, reported by Simon-Colin et al. (2008), showed methane proton (CH) signals at 5.2 ppm. Likewise, those authors found that resonance at 0.89 ppm was associated to the terminal methyl group (CH3). These authors assigned multiplet resonance at 2.47–2.6 ppm to methylene protons (CH2).

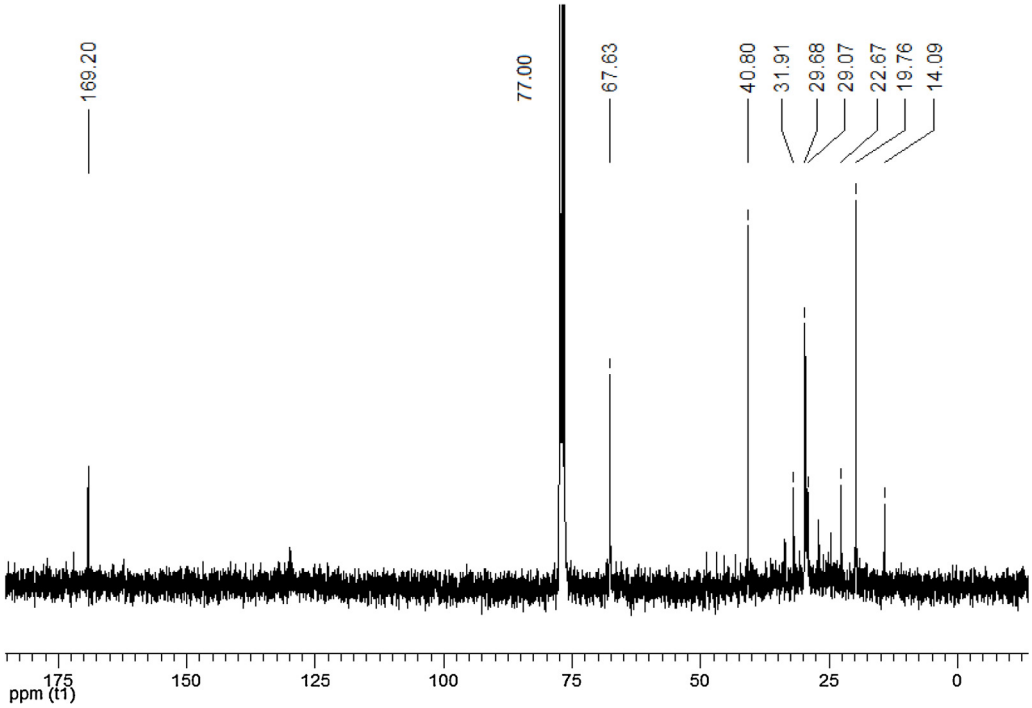

Fig. 3. 125-MHz ^{13}C NMR spectrum of PHB-PHV units, from pineapple peel residues, for fermentation condition 9.

Table 4
Chemical shift for NMR C^{13} spectra of PHB-PHV obtained for pineapple peel.

Chemical shift (ppm)	Carbon Number	Chemical shift (ppm)	Carbon Number
14.11	18	40.80	4
19.80	11	40.88	23
22.70	13	67.63	1
27.22	24	77.20	22
29.07	17	169.1	7
29.34	26	169.2	15
29.68	16	169.14	3
31.91	8	–	

Mishra et al. (2010) reported PHA 1H NMR spectra isolated from SM-P-3M and SM-P-1S compared with the standard PHB (Sigma) and found resonance signals of —HC CH at 5.30 ppm and —CH2—COOH at 2.50 ppm, with methylene groups ranging from 1.25 to 1.57 ppm and a terminal-CH3 at 0.9 ppm. Those authors concluded that signals were similar to the standard PHB.

Finally, in Table 3, a different signal obtained from simulation can be observed, which is in agreement with signal resonance spectra and proposed structure results, as can be seen in Fig. 2a.

C^{13} NMR analysis results are shown in Table 4, and spectra are shown in Fig. 3. These spectra show PHB polymer characteristic signs and other signs that show sample possible contamination. The results agreed with those reported by Doi et al. (1986) and Abd-El-haleem et al. (2007). Terminal unit signals (C 1, 3,4,5, 23, 26) were not observed, possibly due to their low molar ratio in the copolymer. Thus, these results allowed for suggesting a structure model for polyhydroxyalkanoates in this study, as shown in Fig. 2a.

In order to quantify the molar ratio of PHB and PHV units, two exclusive monomer signals were selected, keeping proportional intensities; these signals correspond to the CH group, corresponding to chemical shifts of 5.36 ppm and 5.24 ppm for PHB and PHV, respectively (Doi et al., 1986). Molecular quantification results show that most of the analyzed samples contained approximately 60% more PHV composition; the lowest PHV composition percentage was observed in the 5th fermentation condition, with 55.2%. In general, PHV composition to 1, 2, 3, 4, 5, 6, 8 fermentation conditions was of 64%, 68% 64%, 70.2% 55,2% 68,2% and 74, 2%, respectively. Meanwhile, PHB content for the same fermentation conditions was of 36%, 32%, 36%, 29,8%, 44,8%, 31,8% and 25,5%; these values were calculated according to Eq. (1).

4. Conclusions

In general, it can be concluded that it is possible to obtain Polyhydroxyalkanoates from pineapple peel residues through fermentation using *Ralsthonia eutropha* ATCC 17697 strain. Polyhydroxyalkanoates produced from pineapple peel waste have typical functional groups, such as OH, CH, CH_2, CH_3, C=O and COC, and typical structures of PHB and PHV. Molecular quantification results showed that most of the analyzed samples contain approximately 60% more PHV composition. It also can be concluded that different fermentations have a significant effect on the biopolymer amount produced, but without biopolymers structure effect.

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