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Use of Enzymatic Hydrolysate from Agroindustrial *Asparagus* Waste as Substrate for the Production of Polyhydroxyalkanoate by *Bacillus thuringiensis*

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Polyhydroxyalkanoate (PHA) has unique physicochemical and mechanical properties like conventional plastics; however, its high production cost makes it unsuitable for commercial use. Therefore, the purpose of the present study is to use low-cost and bioavailable raw materials such as agro-industrial waste of asparagus husk, as substrate for obtaining PHA by *Bacillus thuringiensis.* The proximal characteristics and structural carbohydrates of the waste were previously determined using HPLC. The pretreatment conditions were optimized using a Plackett-Burman design and response surface of the central compounds, evaluating temperature, %NaOH, time, % solid/liquid and solvent. Likewise, the enzymatic hydrolysates of the optimal conditions of the pretreatment were used, using an enzymatic solution with cellulase activity at 45°C at 100 rpm for 72 h. To produce PHA, a mineral-based medium, supplemented with enzymatic hydrolysate from the optimal pretreatment, was utilized. This study examined the effects of varying initial inoculum concentrations (0.25, 0.5, and 0.75 g/L) and percentages of enzymatic hydrolysate supplement (%, v/v). The process was conducted at 30°C and agitated at 125 rpm for 72 h. Maximum production of PHA was obtained with 0.138 g/L from an initial inoculum of 0.75 g/L of *B. thuringiensis* and a 47% supplement of the enzymatic hydrolysate. The PHA biopolymer was identified by its chemical characteristics by FTIR and

correlated by HPLC with a standard. This study contributes to the use of agro-industrial waste to obtain biologically-based bioplastic through a low-cost process aligned with the circular economy strategy.

Keywords: polyhydroxyalkanoate, *Bacillus*, biotechnology, lignocellulosic, biopolymer, *Asparagus*.

Introduction

Polyhydroxyalkanoate (PHA) is a biopolymer with a polyester and thermoplastic structure with characteristics similar to synthetic plastics (Akinmulewo and Nwinyi, 2019). PHA presents novel properties such as insolubility in water, biodegradability, biocompatibility, higher tensile strength, melting temperature (Tm) up to 166°C and a high crystallinity index (55–70%) (Grigore et al., 2019); this has allowed its use in various fields such as the development of scaffolds in cell regeneration, in the administration of drugs, dressings, in addition to the development of biodegradable plastic films for crop protection, encapsulation of seeds and fertilizers, as well as products of packaging and bags (Sharma et al., 2021). As a result, PHA has become a suitable sustainable replacement alternative for many applications that today meet petroleum-based plastics.

PHA is produced by a wide diversity of microorganisms such as *Cupriavidus necator, Alcaligenes latus, Azotobacter vinelandii, Bacillus megaterium, Pseudomonas oleovorans and Rhodopseudomonas palustris* (Kumar et al., 2020). PHA biogenesis occurs when there is an excess of carbon and a shortage of essential nutrients for cell growth, such as nitrogen, phosphorus and magnesium, allowing the generation of hydroxyacyl-CoA and its subsequent polymerizations into PHA by PHA-synthase (Choi et al., 2020). In this way, there is an intracellular accumulation of the biopolymer in granules as a reserve of carbon and energy (Alves et al., 2022). The PHA is produced commercially; however, it has a higher production cost at the industrial scale level; for this reason, it has been decided to develop new production routes to improve the economy of the process (Chen et al., 2020).

An alternative to reducing costs is through the use of cheap and highly sustainable carbon sources. The lignocellulosic waste of the agroindustry represents a viable option as a raw material because they are abundant, bioavailable and easily degradable (Pratt et al., 2019). Asparagus (*Asparagus officinalis* L.) is one of the main Peruvian agro-export vegetables in the international market, with an annual turnover of 488.6 and 771.7 million USD in 2020 (Esteve-Llorens et al., 2022). In the production of canned asparagus, peeling provides waste that corresponds to 40–50% of the wet weight and 22% cellulose content (Cruz-Tirado et al., 2019). This residual cellulose could be used by converting it into fermentable sugars such as glucose, with a sustainable form through catalysis or enzymatic hydrolysis (Ojewumi et al., 2022). In the present investigation, agro-industrial waste from asparagus husk was used to obtain fermentable sugars by enzymatic hydrolysis, as the only carbon source to produce polyhydroxyalkanoate. *Bacillus thuringiensis* was used to produce PHA after optimizing residue pretreatment and maximizing enzymatic extraction of fermentable sugars. The PHA was identified through its chemical characteristics by infrared spectrum and by liquid chromatography.

Methods

Conditioning and characterization of agro-industrial waste

The source of asparagus husk waste (AHW) was acquired from the production lines of agroindustrial companies in the region of La Libertad (Peru). The waste was dried at 40°C for 3 h, ground into asparagus waste powder (ARP), and poured into a 0.595 mm diameter sieve. They were stored in hermetic bags at room temperature until use (Pereira et al., 2022).

From ARP, the protein content (total nitrogen) was determined by the Kjeldahl method (Binalshikh-Abubkr et al., 2021), total lignin content (Sluiter et al., 2008), and ash according to the ASTM D-3172-89 method (Stella-Mary et al., 2016). Likewise, the structural carbohydrates of the residue were quantified according to the NREL method (Akter and Zabed, 2020). An HPLC system (Thermo Scientific, Ultimate 3000, USA) equipped with a charged aerosol detector (Thermo Scientific, Corona Veo-RS) and a SUGAR SP0810 column (Shodex) with a SUGAR SH-G 6B safety column (Shodex),

using D-(+)-cellobiose, D-(+)-glucose, D-(+)-xylose, D-(+)-Galactose and D-(-)-Arabinose as analytical standards, was obtained from Sigma Aldrich, USA (99.5% purity).

Conditioned waste pretreatment

To identify the effect of several factors on the residue before the enzymatic digestibility for the extraction of fermentable sugars, 5 independent variables were organized, percentage of sodium hydroxide, temperature, percentage of solid/liquid, time and solvent, with 3 levels at 10 runs according to the Plackett-Burman design (PBD) (Zhang and Wu, 2022). In *Table 1*, the experimental levels of each factor are presented. Then, the pretreated ARP was filtered and washed with distilled water until reaching pH 7. Subsequently, it was dried at 80°C for 24 h and it was hydrolyzed with a cellulase solution under the initial conditions. The yield of reducing sugars was measured as a response to find the optimal conditions of the pretreatment.

Table 1. *Experimental level of the Plackett-Burman design*

Level	NaOH, %	T (°C)	Solid/liquid, %	t (min)	Solvent *
Low	5	80	5	5	–1
Medium	15	100	10	15	0
High	25	120	15	25	1

** Solvents: distilled water (–1), alcohol/distilled water (0) and alcohol (–1).*

According to the PBD statistical analysis, the significant variables of the pretreatment were found from the maximum yield of reducing sugars. Then, a central composite design (CCD) was used to determine the optimal values of the significant variables (temperature and percentage of sodium hydroxide) (*Table 2*). Design Expert software was used to generate the two-factor experimental design with 11 runs, using the reducing sugars and the hydrolysis yield percentage as final response values after enzymatic hydrolysis (Ma et al., 2020). The experiments were performed by duplicate.

The pretreated ARP was added 0.1 g to 9 mL of sodium citrate tampon (50 mM, pH 4.8) in a 50 mL flask and sterilized at 121°C for 15 min. Then, it was allowed to cool to room temperature and 1 mL of sodium azide (0.5 g/L) was previously sterilized by filtration (0.2 µm).

Table 2. *Factors and levels for the design of the central compound*

Factor	Name	Unit	Level low (–1)	Level medium (0)	Level high (1)
A	Temperature	°C	71	85	99
B	Sodium hydroxide	%	1	4	7

Initial conditions of enzymatic hydrolysis

Subsequently, enzymatic hydrolysis was applied using a Celluclast (Novozyme, Denmark) enzyme solution with cellulase activity from *Trichoderma reesei* of 20 fpu/gds (filter paper activity unit per gram of dry substrate) of the enzyme (Agrawal et al., 2018). Hydrolysis was carried out at 45°C at 100 rpm for 72 h, in a shaking incubator. The wastes were separated through centrifugations at 10 000 rpm in an ultracentrifuge for 10 min for the determination of reducing sugars. The reducing sugars of the hydrolysate were determined by the 3,5-dinitrosalicylic acid (DNS) method, and previously a calibration curve with anhydrous glucose (Merck, USA) was performed (Mercado-Pacheco et al., 2020).

Inoculum preparation and fermentative production of PHA

In this study, *Bacillus thuringiensis* SP7-1 isolated from agricultural soils was used through serial dilutions and nutrient-glucose agar, and it was characterized and identified by morphological, biochemical and molecular characteristics, determining a similarity of 99.82% from the aligned sequences of the 16S rRNA gene (Cueva-Almendras et al., 2022). The strain was inoculated in a nutrient medium (1.5% peptone, 0.5% meat extract and 0.5% sodium chloride) at 30°C, 125 rpm for 20 h. From the biomass obtained, a calibration curve was made between the cell dry weight and the optical density at 600 nm using a UV-VIS spectrophotometer (EvolutionTM 260 Bio, USA).

The PHA was produced by fermentation, transferring 10% of inoculum (v/v) of optical density (OD600) between 0.6–0.8 in 30 mL of mineral culture medium with residue hydrolysate containing (g/L): $(NH_4)_2SO_4$ (1.25), $NaHCO_3$ (0.5), KH_2PO_4 (2.0), Na_2HPO_4 (1.6), $MgSO_4x7H_2O$ (0.5), $CaCl_2x2H_2O$ (0.01), $FeNH_4xCitrate$ (0.05), 5 mL of trace elements ($ZnSO_4x7H_2O$ 0.08, $MnCl_2x2H_2O$ 0.03, H_3BO_3 0.3, $CoCl_2x6H_2O$ 0.2, $CuCl_2x2H_2O$ 0.01, $NiCl_2x6H_2O$ 0.02 and $Na_2MoO_4x2H_2O$ 0.03) (Razzaq et al., 2022),

supplemented with the enzymatic hydrolysate of the optimal conditions of the pre-treated ARP. The effect of the initial inoculum concentration (0.25, 0.5 and 0.75 g/L) and the enzymatic hydrolysate supplement (%, v/v) on the production of PHA was evaluated through a complete multilevel factorial, under conditions of incubation at 30°C, 125 rpm and 72 h. The yields were determined ending in each treatment of % PHA accumulation and volumetric production (Qp: PHA/time).

Extraction and quantification of PHA

The intracellular PHA was extracted from the biomass using 4.5% sodium hypochlorite (Merck) at a ratio of 1 g of dry cells per 30 mL of NaClO, at room temperature conditions for 8 h (Montiel-Jarillo et al., 2022). Then, the pellet was separated by centrifugation at 5000 rpm for 10 min and rinsed with a solution of ethyl alcohol (96%) and distilled water. Subsequently, drying was carried out at 50°C and stored for measurement.

PHA content was determined by oxidation to crotonic acid by high-performance liquid chromatography (HPLC) employing UltiMate™ 3000 Rapid Separation (Thermo Scientific, USA) with a SUGAR SH1011 column (Shodex) at sulfuric acid (0.01 N) elution at 1 mL/min 50°C (Pungsungvorn and Wisetsing, 2021).

Characterization of extracted PHA

From 5 mg of extracted PHA, functional groups were studied by Fourier-transform infrared spectroscopy (FT-IR) by attenuated total reflectance (ATR) employing Nicolet IS50-FTIR spectrophotometer (Thermo Fisher, USA) at 4000–550 cm^{-1} with a resolution of 4 cm^{-1} (Muneer et al., 2022).

PHA products were identified by high-performance liquid chromatography (HPLC) described above. The PHA produced was identified as crotonic acid dissolved in concentrated H_2SO_4, using crotonic acid and pure PHA digested using H_2SO_4 (cc) as standards (Chekroud et al., 2022).

Statistical analysis

The experimental data were taken in triplicate and analyzed with one-way ANOVA using the statistical software Minitab 18 (USA), with 95% statistical significance. The central composite design (CCD) was obtained using the Design Expert software v13 (Minneapolis, USA) and was analyzed with statistical ANOVA (Vu et al., 2022).

Results and Discussion

Lignocellulosic agroindustrial waste

A proximal analysis of the AHW was determined showing a protein content of 13%, ash at 7.4% and lignin at 11.3%. The protein content and ash (minerals) content are usable for fermentation; however, the lignin content of the ER is a disadvantage due to the inhibitory products that can be formed (Vanmarcke et al., 2021). In turn, the structural sugars of ER, measured by HPLC from total acid hydrolysis, revealed amounts of glucose 21.7, cellobiose 10.9, xylose 5.0, galactose 4.7 and arabinose 2.6%, showing glucose as a higher fermentable sugar due to the content of hydrolyzed structural cellulose from the residue (Tulashie et al., 2021).

Pretreatment of agro-industrial waste

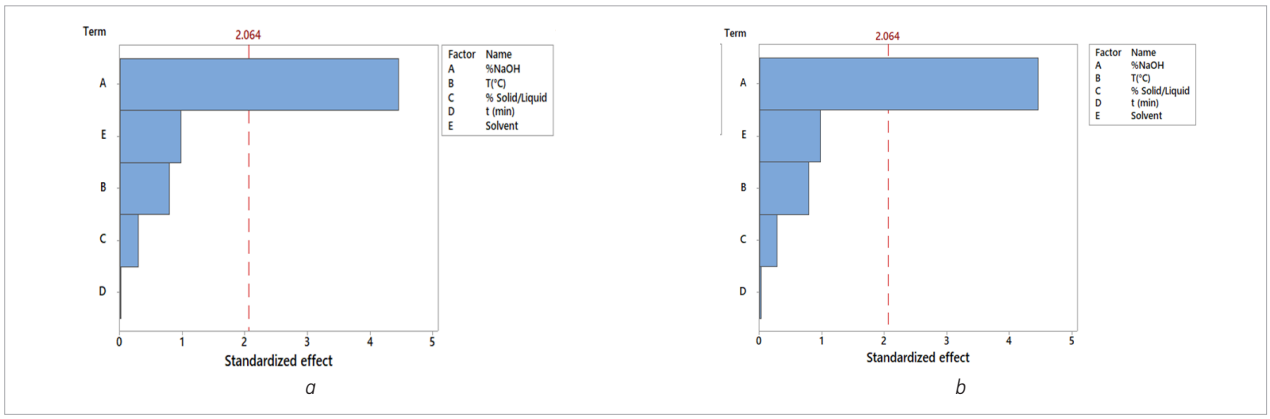
In the Plackett-Burman statistical design of the ARP pretreatment, experimental tests were applied to compare the predicted optimal levels of independent variables (*Table 3*). It was found that the highest yield of enzymatic hydrolysis and reducing sugars was on average 70.76% and 7.11 g/L, respectively, after pretreatment with 15% NaOH, 100°C, 10% solid/liquid ratio, 15 min and solvent with water/alcohol.

Table 3. *Experimental model of Plackett-Burman design on reducing sugars and enzymatic hydrolysis yield of asparagus waste*

Run	NaOH %	T (°C)	Solid/ liquid, %	T (min)	Solvent	Reducing sugars (g/L)	Enzymatic hydrolysis yield, %
1	5	80	5	25	1	4.51	44.97
2	25	80	5	5	–1	5.52	54.98
3	5	120	5	5	1	4.64	46.25
4	25	120	5	25	–1	6.15	61.27
5	5	80	15	25	–1	4.10	40.78
6	25	80	15	5	1	6.33	63.07
7	5	120	15	5	–1	4.56	45.39
8	25	120	15	25	1	6.26	62.31
9 (C)	15	100	10	15	0	7.07	70.35
10 (C)	15	100	10	15	0	7.15	71.18

The graphic demonstration of the statistical design is given in the Pareto diagram (*Fig. 1*), which details the importance of the main effect to find to what extent these factors are different from zero; it was observed that NaOH was positively affecting the yield of obtaining reducing sugars and the yield of enzymatic hydrolysis, with a $P < 0.05$ value of 0.0165 of significance. These results agree with Tsegaye and coauthors (2019) who demonstrate the positive effect of NaOH as a pretreatment to release the maximum cellulose and reduce the lignin content in lignocellulosic residues. Likewise, it is observed that the temperature did not present a significant difference; however, *Table 1* shows an increase in hydrolysis up to 100°C and then begins to decrease, which may be because the structure of the reducing sugar could deteriorate at one point (Chriswardana et al., 2021).

Fig. 1. *Pareto diagram of standardized effects on (A) Reducing sugars (g/L) and (B) % Yield of enzymatic hydrolysis of asparagus waste*



For the optimal composition of the residue pretreatment and to improve the enzymatic hydrolysate, the DCCR was used as a statistical analysis tool. The carried-out experiments were calculated based on the percentage of NaOH and temperature. The regression equation of the highest result of reducing sugar production and hydrolysis yield of the pretreatment was derived from the statistical analysis shown in *equation (1, 2)*:

$$\text{Reducing sugars} = -14.21 + 0.33X + 2.34Y - 0.02X^*Y - 0.001X^2 - 0.09Y^2 \quad (1)$$

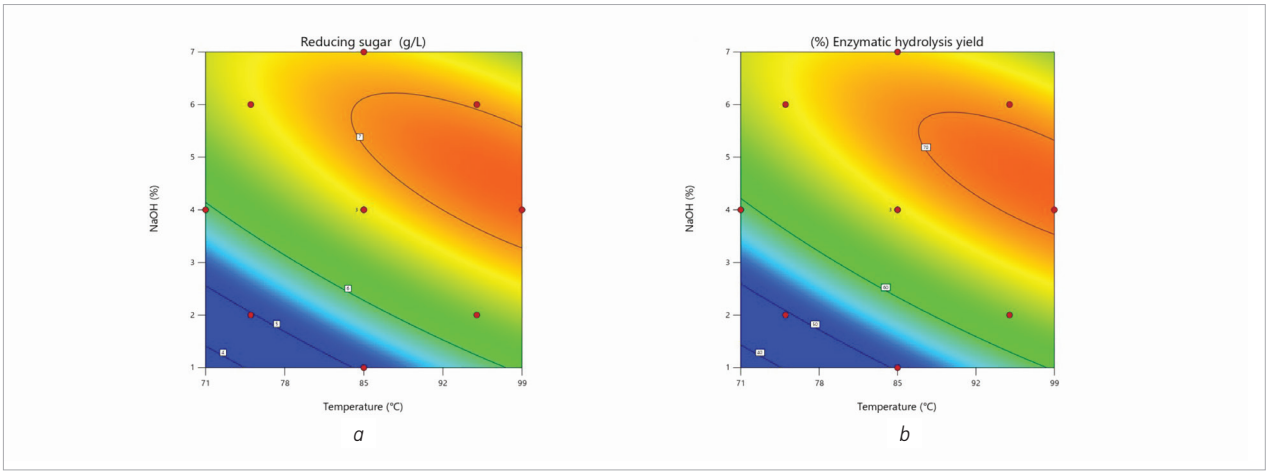
$$\text{Hydrolysis yield} = -140.95 + 3.25X + 23.23Y - 0.16X^*Y - 0.01X^2 - 0.88Y \quad (2)$$

From the equation, X represents the temperature levels used and Y represents the NaOH concentration. The statistical model was analyzed using ANOVA. The results of the reducing sugars obtained and the enzymatic hydrolysis yield showed that the model was significantly adjusted at 95% reliability, and the prob > F value of the significant model terms for both models was significant with $R^2 = 0.878$ and 0.877 for reducing sugars and the enzymatic hydrolysis yield, respectively. The contour plot was plotted showing the effect of optimal temperature and NaOH concentration (*Fig. 2*). From the results of the statistical analysis, the optimal values of the temperature and NaOH (%) were 99°C and 4%, respectively, with a value of 7.3 g/L of reducing sugars and 73% of enzymatic hydrolysis yield. The obtaining of reducing sugars and the yield of the enzymatic hydrolysis increased from 30 and 31%, respectively, as the NaOH concentration (1 to 4%) and the temperature (71 to 99°C). From the equation, it was predicted that the optimal pretreatment of asparagus residue could produce reducing sugars and a hydrolysis yield of 6.77 g/L and 67.29%.

PHA production from waste

The effect of the initial inoculum at 0.75 g/L of *B. thuringiensis* and the use of 47% supplementation of the enzymatic hydrolysate of the pretreated residue in the fermentative medium were found optimal for the maximum production of PHA with 0.138 g/L under conditions of 30°C, 125 rpm and 72 h (*Table 4*). The bacteria showed a maximum accumulation of PHA in 5.4% from 2.6 g/L of dry biomass in the optimized culture conditions; the increase in dry cell biomass indicates the use of the enzymatic hydrolysate of the residue as the only carbon source for its growth by 71%.

Fig. 2. *The environment plot of the DCCR statistical design of the interactive effect of the temperature and percentage of NaOH on the pretreatment of asparagus residue for obtaining reducing sugars (A) and enzymatic hydrolysis yield (B)*



Our findings stand out from other reports of PHA production from residual sources, such as corn straw yielding 0.048 g for 192 h (Verdini et al., 2022), residual wheat grains 1.4 mg/L PHA for 96 h by *Bacillus sp.* (Sirohi, 2021) and cheese whey fermented with dilute acetic acid of 77.2 mg/L PHA by *Acetobacter pasteurianus* (Chang et al., 2021). However, *Bacillus thuringiensis* strains have been seen to reach up to 4 g/L from mango peel at 37°C for 48 h (Gowda and Shivakumar, 2014). Therefore, the study could be improved by optimizing the fermentative conditions; however, it is important to reduce the formations of inhibitors during the pretreatment of the waste capable of delaying the synthesis of PHA using a large inoculum, more dilute hydrolysate, or tolerant microbial strains (Al-Battashi et al., 2019).

Table 4. *Effect of the initial inoculum of Bacillus thuringiensis and the enzymatic hydrolyzed supplement of the pretreated wastes of asparagus on the production of PHA, % accumulation and Qp*

Run	Initial bacterial inoculum (g/L)	Enzymatic hydrolysate supplement (%, v/v)	Cell biomass (g/L)	PHA (g/L)	PHA accumulation, %	Qp (mg/h)
1	0.25	30	3.15	0.121	3.84	0.05
2	0.25	40	2.68	0.124	4.65	0.05
3	0.25	47	2.34	0.120	5.11	0.05
4	0.5	30	3.18	0.112	3.53	0.04
5	0.5	40	2.77	0.131	4.74	0.05
6	0.5	47	2.54	0.137	5.40	0.06
7	0.75	30	2.56	0.134	5.28	0.06
8	0.75	40	2.28	0.123	5.41	0.05
9	0.75	47	2.55	0.138	5.40	0.06

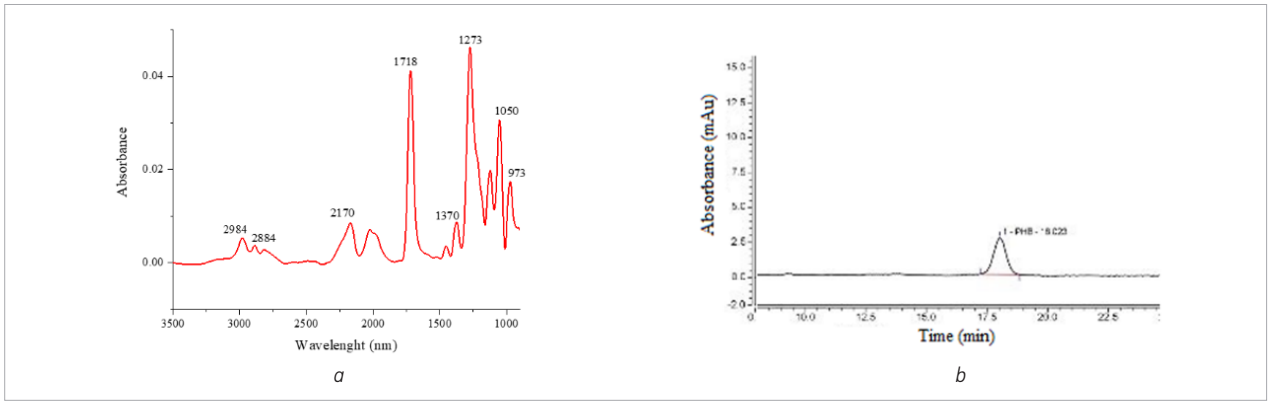
PHA characterization

The biopolymer obtained after extraction was recorded by infrared spectra in the range of 4000–600 cm^{-1} (*Fig. 3A*). Absorption bands were found at 2984 and 2884 cm^{-1} due to CH stretching vibrations and asymmetry of the methyl ($-CH_3$) and methylene ($-CH_2$) groups, respectively (Khamkong et al., 2022). Likewise, a peak was observed around

1718 cm^{-1}, indicating the presence of a carbonyl ester group (C=O) associated with the homopolymer nature of the PHA (Martínez-Herrera et al., 2021). Moreover, several signals observed between 1400–800 cm^{-1} were assigned to vibrational bonds at C-O-C, C-O, CH, and C-C (Kumar et al., 2017). Therefore, it is inferred that from the identified functional groups of the extracted polymer, it is PHA.

HPLC analysis of the extracted biopolymer showed a characteristic peak at a retention time of 18 min (*Fig. 3B*). They were compared with PHA standards when converted to crotonic acid. These peaks indicate the production of PHA from *Bacillus thuringiensis* SP7-1. Comparable results were found in the study by Duvigneau and coauthors (2021), who reported the detection of crotonic acid product of the hydrolysis of PHA copolymers around 18–19 min with an isocratic eluent. Meanwhile, Saeed et al. (2022), detected a PHA retention time of around 13 min at a purity of 98.6%. This variation may be due to the dehydration conversion yields and composition of the extracted polymer (Yu et al., 2005).

Fig. 3. *Chemical characterization of PHA obtained from Bacillus thuringiensis SP7-1 using enzymatic hydrolysates of asparagus waste through A) infrared spectroscopy and B) chromatographic analysis by HPLC*



Conclusions

The agroindustrial waste of asparagus husks showed to be a promising source for the production of PHA biopolymers by *Bacillus thuringiensis* SP7-1, with a maximum value of 0.138 g/L and 5.4% of PHA accumulated after 72 h of incubation at 30°C with 125 rpm. The maximum content of reducing sugars obtained from the enzymatic hydrolysate was from the optimized pretreated residue at 99°C and 4% NaOH. The extracted biopolymer showed chemical characteristics associated with PHA, with bands in the IR spectrum around 1718 cm^{-1} indicating the presence of a carbonyl ester group, in addition to its confirmation by HPLC as crotonic acid. Therefore, an economic process for PHA biosynthesis has been established through the use of enzymatic hydrolysate from the agroindustrial waste of asparagus husk, in the same way, contributing as a start for its low-cost production at industrial levels.

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