



Study of the Pectinase Production from Soursop and Cherimoya Pulp for Agro-Industrial Waste Reduction in Colombia

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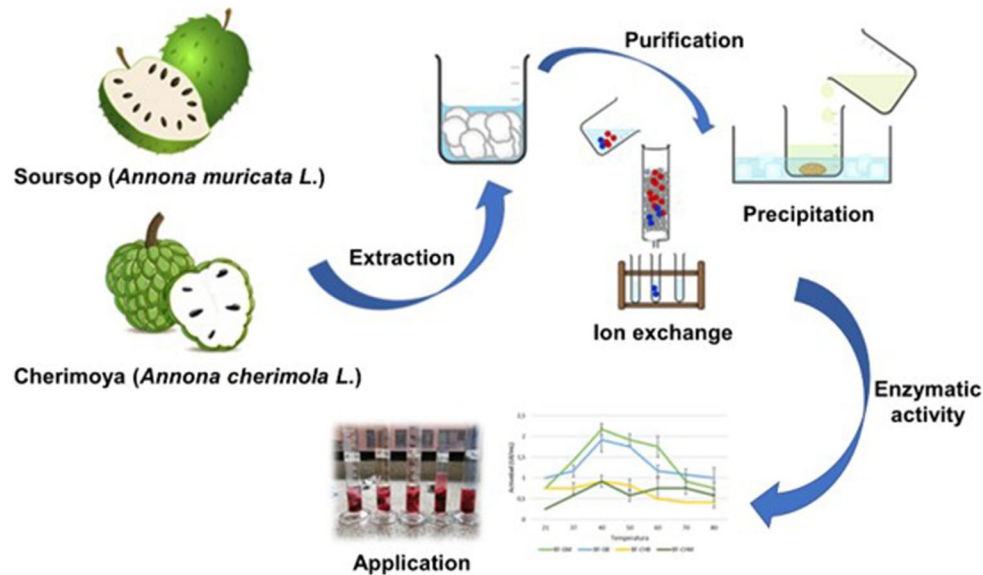
Abstract

Pectic enzymes are an eco-friendly alternative to improve extraction processes for the food industry. The objective of the research was to obtain pectinase from the pulp of soursop (*Annona muricata* L.) and cherimoya (*Annona cherimola* L.), as an alternative to reduce agro-industrial waste in Colombia; involving the study of the factors influencing the protein content of the enzymes extracted from different stages and types of fruits, the conditions for enzymatic activity, and the potential application in the food industry, particularly in extraction processes and juice clarification. A factorial design 2³, spectrometric and potentiometric techniques evidenced that the protein content depends on factors such as fruit stage (suitable “s” and unsuitable “u” fruits for consumption) and fruit type (soursop and cherimoya). The higher protein content was obtained by the precipitation method in comparison to ion exchange chromatography. Cherimoya fruit presented a higher protein content of 326 ± 26 mg by precipitation, however, the soursop samples evidenced higher enzymatic activity, 2.2 ± 0.1 U/mL. The pectinase obtained was an acidic enzyme of type pectin methylesterase with an enzymatic activity of 27.0 ± 0.9 U/mL, under the optimal conditions of pH 4 and temperature of 40 °C. The application in blackberry juice evidenced a decrease in the absorbance and pH changes. From the current research, the pulp of soursop and cherimoya was proven as a cheap source to obtain pectinase with enzymatic activity for juice clarification, and this process is an alternative to reduce the negative impact on the environment and an economical source for the local producers.

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Graphical Abstract



Keywords Annona · Biotechnology · Enzyme · Extraction · Pectinase · Waste

Introduction

The *Annonaceae* family is composed of different species of fruit, soursop (*Annona muricata* L.), anon (*Annona squamosa*) and cherimoya (*Annona cherimola* L.) are some of them. In Colombia, these tropical fruits are cropped at altitudes between 1800 and 2200 m above sea level (masl) [1, 2]. The consumption and their interest as a product of exportation have increased due to their attractive properties such as sweet taste, nice smell, smooth texture, nutritional composition (sugars, polysaccharides, vitamins C and E, and minerals), and bioactive constituents, which have shown some health benefits [3, 4]. However, due to the texture and juice content, the fruit undergoes rapid post-harvest deterioration during transport, handling, storage and distribution, which makes it necessary to convert into value-added products such as jellies, juices, pulp, and others. So, the consumption of fresh pulp from ripe fruit is limited and this should be processed into intermediate products [5, 6]. The fruit juice is another commercial product obtained from the different fruits through extraction, however, this conventional method requires an excessive amount of energy, and part of the juice is not extracted efficiently. So, the enzymatic treatment used before the juice extraction must improve the processing conditions [7].

Enzymes are proteins with specific functions that allow chemical reactions to improve the industrial process by reducing water use and synthetic chemical substances, evidencing an eco-friendly alternative [5]. In the food industry,

these have gained importance due to the benefits of improving some processes such as extraction, clarification, cleaning, fermentation, and others. Industrially the enzymes for the extraction and clarification of juice and wine, are obtained from microorganisms such as bacteria and filamentous fungi [7–9]. Just 25% of the pectinase enzyme used in the market comes from microorganisms, hence the importance of studying alternatives to obtain these enzymes which are widely used in the world market [10]. Studies about the use of agro-industrial residues and plants for the extraction of pectinase have increased. The pectinase involves a set of enzymes that can eliminate insoluble solids and degrade pectin by hydrolysis. The pectic enzymes include pectin lyase (PL), polygalacturonase (PG) and pectin methylesterase (PME) [8, 9, 11], depending on the type of action; but the pectic enzymes also are subdivided according to the optimal pH for their enzymatic activity, as acidic and alkaline pectinase [12].

In this study, the pulp of soursop (*Annona muricata* L.) and cherimoya (*Annona cherimola* L.) in a state of consumption and not suitable for consumption, were a source to extract pectic enzymes, an alternative to reduce agro-industrial waste in Colombia to obtain enzymes with commercial applications. The pH, type and stage of the fruit were evaluated in the extraction process, the protein content was quantified, the optimal conditions of the enzyme were established, and their application was evaluated in commercial pectin as substrate.

Table 1 Protein content of the crude extract of the samples

Sample	Protein content $\pm$ SD (mg/mL) ^a	%Protein $\pm$ SD (%w/w) ^b
Pb-S _s	153 $\pm$ 22	7.8 $\pm$ 1.8
Pb-S _u	186 $\pm$ 43*	10.2 $\pm$ 4.0
Pb-C _s	105 $\pm$ 33*	5.9 $\pm$ 3.1
Pb-C _u	284 $\pm$ 77*	17.9 $\pm$ 7.3
Ab-S _s	143 $\pm$ 11	5.7 $\pm$ 0.3
Ab-S _u	183 $\pm$ 10*	10.3 $\pm$ 1.1
Ab-C _s	122 $\pm$ 32	7.0 $\pm$ 0.7
Ab-C _u	277 $\pm$ 40*	19.5 $\pm$ 4.3

^aTotal volume of crude extract. ^bProtein percentage in 50 g of pulp. Data represents the mean $\pm$ SD ($n=3$). Statistically significant: * $p=0.0002$

Materials and Methods

Materials

The fruits cherimoya (*Annona cherimola* L.) and soursop (*Annona muricata* L.) were selected in two maturation stages, suitable and unsuitable fruits for consumption, according to the Colombian norms NTC 756 - Sampling and NTC 5208 - Fresh fruits. The fruits were acquired in a local fruit market in Bogotá, Colombia. Sodium phosphate dibasic heptahydrate, sodium phosphate monobasic monohydrate, sodium acetate and ethanol were supplied by Pan-Reac (Darmstadt – Germany). Acetone, sodium hydroxide (NaOH), sodium chloride (NaCl), acetic acid, ovalbumin (OVA), and Biuret reagent were obtained by Merck (Darmstadt – Germany). Commercial pectin and pectinase were used. Qualitative filter paper (Whatman of 150 mm) was used. Sephadex G-25 was supplied by Sigma (USA, Saint Louis).

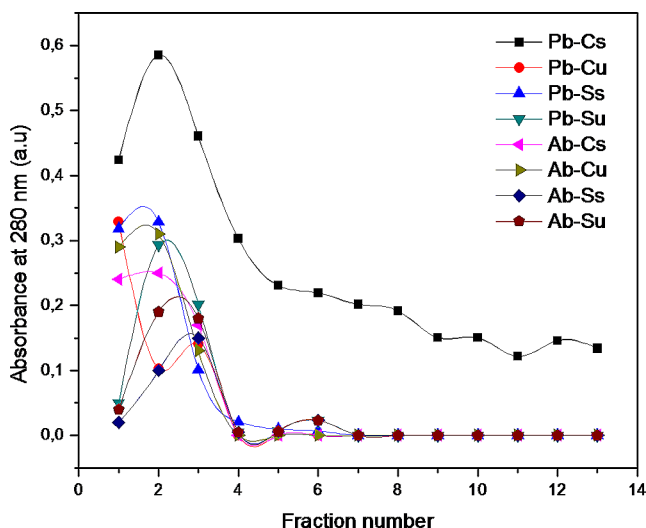


Fig. 1 Elution profile of protein on Sephadex G25. Ab: acetate buffer. Pb: phosphate buffer. C_u and S_u: cherimoya and soursop pulp unsuitable for consumption. C_s and S_s: cherimoya and soursop pulp suitable for consumption

Extraction and Purification of the Pectic Enzymes

A factorial design 2³ was used, the pH (5.3 and 7.1), type of fruit (soursop “S” and cherimoya “C”) and the stage of the fruit (suitable “s” and unsuitable “u” fruits for consumption) were the factors analyzed. The pulp was obtained from the fruit, and the feeds were withdrawals. 50 g of each sample was mixed with 30 mL of the respective buffer, phosphate buffer “Pb” pH 7.1 and acetate buffer “Ab” pH 5.3 at the concentration of 0.1 M. The mixture was homogenized at 4 °C in an ice bath for 1 h, with the help of a stirring rod. After that, the samples were centrifugated for 10 min at 5000 rpm in a Thermo Scientific CL10 Centrifuge. The supernatant was filtered [13–16]. The samples were stored at 4 °C in an amber bottle.

To identify the samples, the following nomenclature was established: Pb-S_s, Pb-S_u, Ab-S_s, Ab-S_u; Pb-C_s, Pb-C_u, Ab-C_s, Ab-C_u.

The purification was made by precipitation with acetone (1:2, crude extract: solvent) at 4 °C. The precipitate was dried at room temperature in a desiccator until constant weight. Also, ion exchange chromatography (IEC) was used as a purification method. Sephadex G-25 was equilibrated with 0.02 M Pb pH 7.5 / azide 0.02%. The Sephadex was added in a syringe with 4 cm of height and 16 mm of width, and this was equilibrated for one day with the buffer. The samples were applied at a concentration of 0.025 mg/mL and two fractions of 4 mL were eluted. After that, six fractions of 4 mL were eluted with 0.02 M Pb pH 7.5/NaCl 0.05 M. Three fractions were obtained with 0.02 M Pb pH 7.5/NaCl 0.1 M. Finally, four fractions were recollected with a solution of 0.02 M of Pb pH 7.5/NaCl 0.3 M [13, 15, 17, 18].

Determination of the Protein Content and the Enzymatic Activity of the Pectic Enzymes Purified

To determine the protein content of the samples purified by precipitation, an OVA calibration curve and the Biuret reagent were used. The calibration curves were prepared in water, Pb pH 7.1 and Ab pH 5.3 at the concentration of 0.1 M. Each sample of OVA and an aliquot of 1 mL of the sample purified 0.02% (w/v) were treated with 0.5 mL of Biuret reagent for 5 min at room temperature, and the measurements were made at 540 nm on a RIGOL Ultra-3660 spectrophotometer [9, 10, 17, 19]. Each sample was measured by triplicate. However, the protein content of the samples purified by IEC was done by UV-Vis at 280 nm [20, 21].

The enzymatic activity was analyzed at different pH (4–8) and temperatures (20–80 °C).

Effect of the Temperature on Enzymatic Activity

To evaluate the enzymatic activity of the pectic enzymes, 50 mg of the commercial pectin was dissolved in 10 mL of NaCl 0.2 M, two drops of phenolphthalein were added, and the pH was adjusted to 7.0 with NaOH 0.05 M. The enzyme purified by precipitation 0.3% (w/v) was added to the pectin solution, at the respective temperature in the range of 20–80 °C. The enzyme was inactivated in an ice bath for 10 min. After that, the solution was titled with NaOH 0.05 M. To determine the activity in each temperature, Eq. 1 was used [16, 18, 22, 23]. A pectin solution without enzyme was used like a blank. All samples were analyzed in triplicate.

$$\frac{\text{Activity unit}}{\text{mL}} = \frac{\text{mLNaOH}_{\text{sample}} * \text{NaOH concentration} * 1000}{\text{Tiempo (min)} * \text{mL sample}} \quad (1)$$

Effect of the pH on Enzymatic Activity

To study the enzymatic activity at different pH (4–8) [18], different buffers were used, Pb 0.02 M and Pb 0.02 M/Citric acid 0.02 M [24]. 50 mg of the commercial pectin was dissolved in 10 mL of the respective buffer with pH 4, 5, 6, 7 and 8 at 40 °C; and two drops of phenolphthalein were added. The enzyme purified by precipitation 0.3% (w/v) was added to the respective pectin solution and this was stirred for 1 min. Then, this was inactivated in an ice bath for 10 min. After that, each solution was titled with NaOH 0.05 M [25]. To determine the activity at the different pH, Eq. 1 was used [26]. A pectin solution without enzyme was used like a blank. All samples were analyzed in triplicate.

Application of the Pectic Enzymes

Pectic enzymes have some specific functions, according to the enzyme type, these mainly include pectin lyase (PL), polygalacturonase (PG) and pectin methylesterase (PME). To establish the behavior of the enzyme obtained in this study, the following procedures were developed.

Hydrolysis of a Pectin Solution

50 mg of the commercial pectin was dissolved in 10 mL of NaCl 0.2 M and the pH was adjusted to 7.0. The enzyme purified by precipitation 0.3% (w/v) was added to the pectin solution and the mixture was stirred at 37 °C. The pH was measured at different times. The temperature of 40 °C was not used because the speed of the reaction was faster, and precise data could not be taken with the pH meter.

Table 2 Protein content of the purified samples

Sample	Protein content $\pm$ SD (mg) ^a	%Protein content $\pm$ SD (%w/w) ^b	Activity $\pm$ SD (U/mL) ^c	Protein content IEC (mg) ^d
Pb-S _s	95 $\pm$ 12*	19.0 $\pm$ 2.4	1.9 $\pm$ 0.3**	4.1
Pb-S _u	92 $\pm$ 17	18.3 $\pm$ 3.3	2.2 $\pm$ 0.1	1.6
Pb-C _s	274 $\pm$ 35*	54.9 $\pm$ 7.0	0.9 $\pm$ 0.1**	12.5
Pb-C _u	326 $\pm$ 26*	65.2 $\pm$ 5.1	0.9 $\pm$ 0.1	6.8
Ab-S _s	121 $\pm$ 8	24.2 $\pm$ 1.7	1.5 $\pm$ 0.3**	1.0
Ab-S _u	90 $\pm$ 8	17.9 $\pm$ 1.7	1.8 $\pm$ 0.1	1.2
Ab-C _s	333 $\pm$ 13*	66.6 $\pm$ 2.7	1.6 $\pm$ 0.1	6.9
Ab-C _u	315 $\pm$ 9*	63.0 $\pm$ 1.8	0.7 $\pm$ 0.1**	6.2

^aAfter purification by precipitation. ^bRespect to 50 g of pulp to the samples purified by precipitation. ^cMeasurement at 40 °C and pH 7. ^dIEC: ion exchange chromatography. Data represents the mean $\pm$ SD ($n=3$). Statistically significant: * $p=0.000$, ** $p=0.000$

Juice Clarification

The action of the enzyme in the clarification of blackberry juice was analyzed by reading the absorbance at 630 nm on a RIGOL Ultra-3660 spectrophotometer. 10 g of blackberry pieces were added in graduated cylinders of 100 mL. Then, 17 mL of deionized water and 3 mL of purified enzyme (0.3% (w/v) in NaCl 0.2 M) were added. The samples were incubated for 30 min at 40 °C in a water bath. The pH and absorbance were measured in triplicate [22, 25].

Statistical Analysis

All data are presented as mean $\pm$ standard deviation ($\pm$ SD) from at least three measurements. Means were compared between groups by one-way analysis of variance (ANOVA) and two groups by Student's t-test. A $p < 0.05$ value was considered statistically significant. Calculations were done using the software Statgraphics Centurion XVI.I.

Results and Discussions

Extraction and Purification of the Pectic Enzymes

Most of the studies have been focused on obtaining pectic enzymes from bacteria, fungi, and yeast; species such as *Aspergillus* and *Bacillus* are the most explored [20, 21, 27, 28]. We have studied the pulp of *Annonaceae* family fruits as an alternative to reuse agro-industrial wastes, reduce environmental impact and contribute to sustainable production of these fruits.

After extraction, the crude extract was analyzed. Table 1 shows the protein content obtained for each sample. The statistical analysis evidenced differences between the means. The samples Pb-C_s, Ab-C_s, Ab-S_s, and Pb-S_s showed a

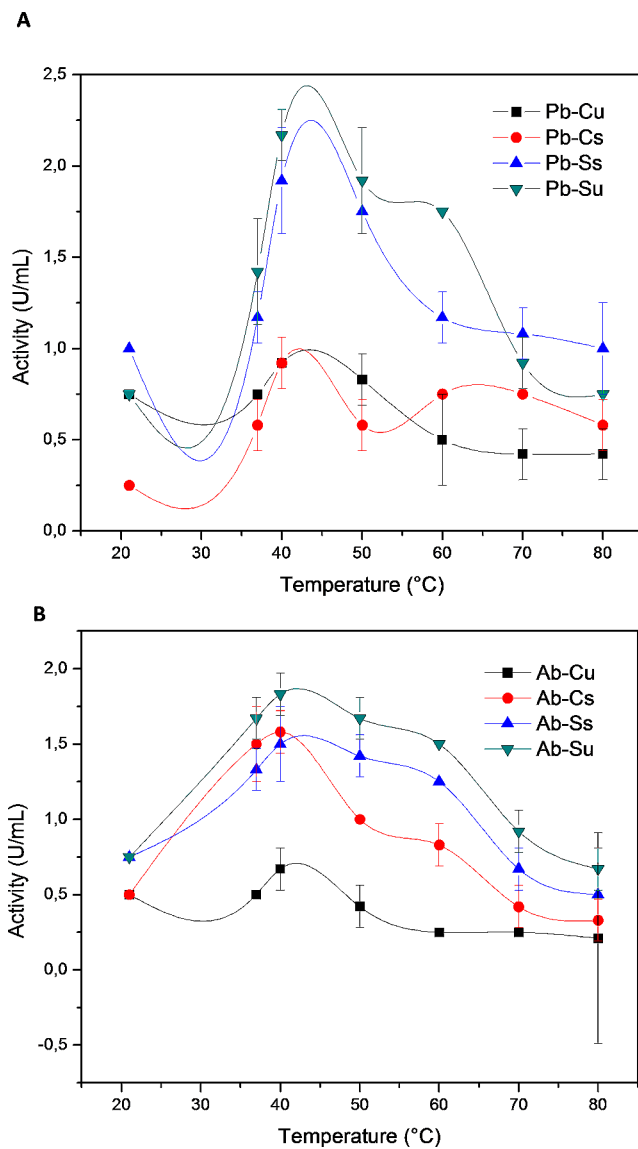


Fig. 2 Effect of the temperature on enzymatic activity. Ab: acetate buffer. C_u and S_u : cherimoya and soursop pulp unsuitable for consumption. C_s and S_s : cherimoya and soursop pulp suitable for consumption

homogeneous behavior; similarly, the group of the samples: Ab- C_s , Ab- S_s , Pb- S_s , Ab- S_u , Pb- S_u ; meaning that the pH did not affect the protein content, however, the stage of the fruit (suitable “s” and unsuitable “u” fruits for consumption) and the fruit type were important factors in the extraction process due to the protein content was higher in the “ C_u ” samples, 284 ± 77 mg/mL. In comparison with other studies, the results have evidenced differences, Joshi, et al., (2011) in their study obtained a protein content of 62 mg/mL of pectic enzyme obtained by fermentation of apple pomace using *Aspergillus niger*. Prajapati et al., (2021) extracted pectinase from *Bacillus subtilis* obtaining 122.72 mg of total protein. A previous study where pectinesterase extracted from soursop (*Anona muricata*) pulp using ammonium sulfate

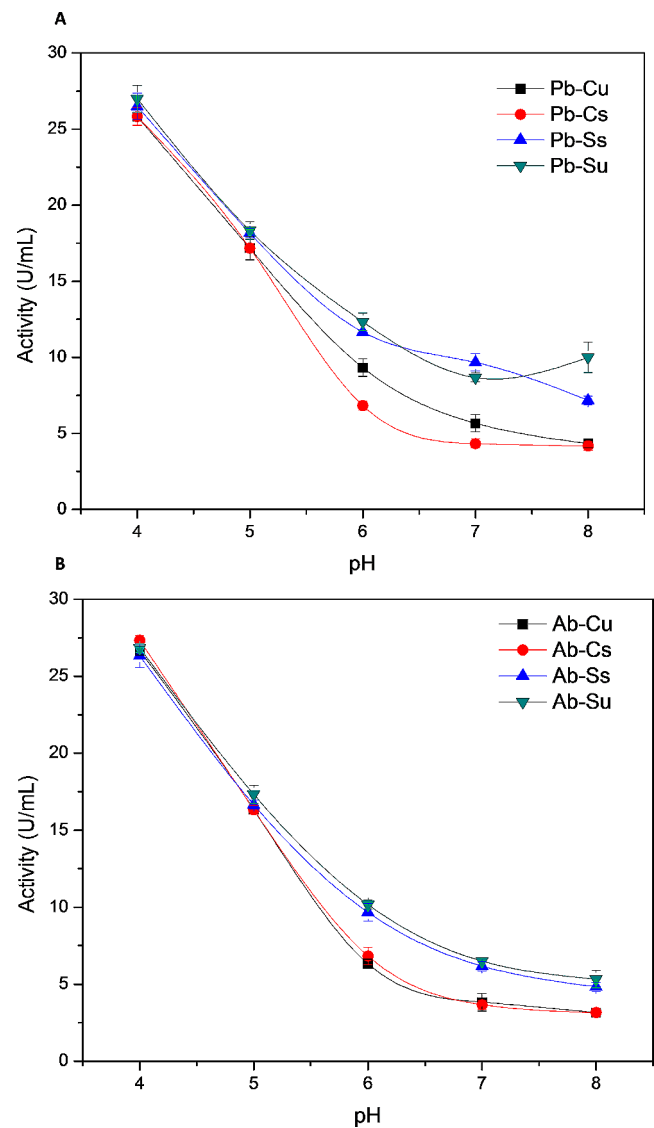


Fig. 3 Effect of the pH on enzymatic activity. Pb: phosphate buffer. C_u and S_u : cherimoya and soursop pulp unsuitable for consumption. C_s and S_s : cherimoya and soursop pulp suitable for consumption

got a protein content of 4522 mg [29]. This means that the extraction source and conditions are determining factors in the yield extraction [27].

Determination of the Protein Content and the Enzymatic Activity of the Pectic Enzymes Purified

The calibration curves of OVA presented a linear regression in the three media: Water: $Y = 0.2864X + 0.0766$, $R^2 = 0.9989$. Phosphate buffer “Pb” pH 7.1: $Y = 0.3343X + 0.0689$, $R^2 = 0.954$. Acetate buffer “Ab” pH 5.3: $Y = 0.2659X + 0.1116$, $R^2 = 0.9828$.

Table 2 shows the content protein of the samples purified by precipitation and IEC. The statistical analysis evidenced

differences between the means in the samples purified by precipitation. The samples Ab-S_u, Pb-S_u, Pb-S_s, and Ab-S_s showed a homogeneous behavior; similarly, the group: Ab-C_u, Pb-C_u, and Ab-C_s. However, Pb-C_s presented a significant difference concerning the other samples; meaning that the pH did not affect the protein content obtained, but the fruit type was an important factor in the protein content. The cherimoya fruit evidenced a higher protein content, Ab-C_u, Ab-C_s, Pb-C_u, and Pb-C_s. However, the enzymatic activity was higher in the samples extracted from soursop “S” with Pb at pH 7.1, 2.2 ± 0.1 U/mL. This could be explained by the presence of interfering compounds and other enzymes that increase the protein content in the quantification process [27]. Some studies have evidenced how the total protein decreased when several purification steps were carried out, thus obtaining a higher specific activity. Pectinase obtained from *Bacillus subtilis* was purified by precipitation with ammonium sulfate and Sephadex G-10, decreasing the protein total from 122.72 mg to 8.72 mg, and the specific activity increased from 12.89 to 128.66 U/mg [28]. Similar results were observed in the pectinesterase extracted from soursop pulp and purified by precipitation with ammonium sulfate and CM-Sephadex; this presented a total starting protein of 4522 mg and after the purification of 38.5 mg; and the specific activity increased from 1.92 until 129.6 U/mg [29]. Possibly, the soursop pulp contains fewer interfering compounds allowing it to highlight the properties of the pectinase.

Figure 1 shows the elution profile of the samples purified by IEC. According to these profiles, the protein was detected in the fractions 1–4. The protein was eluted with a solution of 0.02 M of Pb pH 7.5–NaCl 0.05 M.

The protein content obtained by IEC was lower in comparison to the samples purified by precipitation, for example, to the sample Pb-C_s were 12.5 mg and 274 ± 35 mg, respectively. It is important to highlight what some authors have mentioned about the purification processes. The purification strategy depends on the final application, pure samples are required for diagnostic and therapeutic applications,

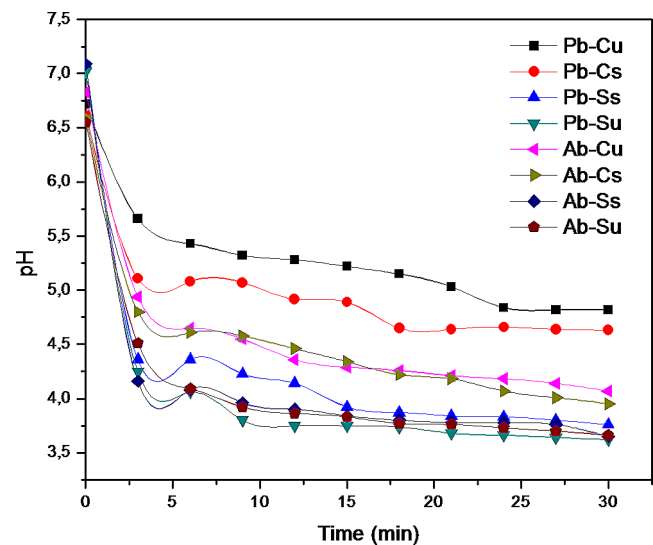


Fig. 4 Effect of the pectic enzymes on pectin solution. Ab: acetate buffer. Pb: phosphate buffer. C_u and S_u: cherimoya and soursop pulp unsuitable for consumption. C_s and S_s: cherimoya and soursop pulp suitable for consumption

generally, these are obtained in low quantity, while industrial applications require high quantity but at low quality [27]. IEC is a technique cleaner and specific than the precipitation, for that reason, other interfering compounds and enzymes can be eliminated and separated getting pure samples at low quantities [12, 27]. Due to the low amount of protein obtained by IEC and given the potential application in juice clarification; the samples obtained by precipitation were used for the other processes.

As was previously mentioned, the main source of extraction of commercial pectinase is using the fungus *Aspergillus niger* with different carbon sources by a fermentation process. Pomelo peels as substrate allowed the obtention of pectinase with a content protein of 2800 mg for the crude extract with a specific activity of 0.81 U/mg. After purification by precipitation was obtained a content protein of 172.5 mg with a specific activity of 11.41 U/mg [30]. On the other hand, banana peels as substrate produced a crude extract with a content protein of 5165 mg and a specific activity of 0.572 U/mg; after purification by precipitation was obtained 853.8 mg of content protein with a specific activity of 0.611 U/mg [31]. In this study, we have obtained pectinase from the species *Annonaceae*, a novel source of agro-industrial waste. The crude extracts showed values between 70 and 360 mg of content protein, Table 1, which means a 2.5–12% respect to the commercial pectinase. The specific activity evidenced values between 0.6 and 2.3 U/mL, meaning that the enzyme obtained by this methodology and source can be used in the food industry, contributing to the biotechnological sector to produce enzymes of type acidic pectinases [32].

Table 3 Effect of the pectic enzymes in the absorbance and pH of a blackberry juice

Sample	pH _i ^a	pH _f ^b	Absorbance 630 nm (a.u.) ± SD
Pb-S _s	4.2	3.5	0.10 ± 0.02*
Pb-S _u	3.8	3.7	0.13 ± 0.02*
Pb-C _s	3.9	3.7	0.14 ± 0.01
Pb-C _u	3.8	3.5	0.15 ± 0.03
Ab-C _s	4.3	3.8	0.11 ± 0.00*
Blank	2.8	2.8	0.15 ± 0.03
Commercial pectinase	4.0	2.7	0.08

^apH_i measurement after enzyme addition. ^bpH_f measurement after absorbance reading. Data represents the mean ± SD ($n=3$). Statistically significant: * $p=0.0028$

Effect of the Temperature and pH on Enzymatic Activity

The pectic enzymes obtained from cherimoya and soursop pulp and purified by precipitation were significantly active in the range of temperature 35–50 °C and pH 4.0–5.5, with optimum temperature and pH at 40 °C and 4.0, respectively; Figs. 2 and 3. These results indicate that the pectic enzymes are acidic and thermostable. These properties have potential as acid pectinase in the clarification and degradation of pectic substances in the processing of juices and wines [28]. Under the optimal conditions, pH 4 and temperature of 40 °C, the enzymatic activity value was higher in the sample Pb-S_u, 27.0 ± 0.9 U/mL, processed data from Fig. 3.

Consistent with what was previously observed, the pectinase samples obtained from soursop pulp showed higher activity compared to the cherimoya samples.

Application of the Pectic Enzymes

Hydrolysis of a Pectin Solution

The mode of action of the pectic enzymes can be evaluated through the reaction with pectin as substrate. Generally, the change in the viscosity of the pectin is a method to evaluate the catalytic action of the pectinase [33–35]. In this case, a simple method allowed to observe the behavior of the hydrolysis of the pectin. The samples were used at the same concentration and conditions. Figure 4 shows that the samples obtained from soursop “S” pulp presented a more marked decrease in pH in less time. It means that the enzyme obtained from this source has higher activity allowing a greater action with the substrate. According to previous studies, the pectinesterase evidences their action through the hydrolysis of the methoxyl bonds of pectin, which can be detected by the decrease of pH due to the formation of carboxyl groups [33, 34]. So, in this study, it was evidenced that the enzymes obtained are acidic pectinesterases.

Juice Clarification

According to the behavior observed in Figs. 2 and 3 for the activity, and the results presented in the hydrolysis of the pectin, Fig. 4. The samples with higher activity (Pb-S_s, Pb-S_u, Pb-C_s, Pb-C_u, Ab-C_s) were used for the clarification of blackberry juice. The statistical analysis evidenced differences between the means. The samples Pb-S_s, Pb-S_u and Ab-C_s showed a significant change in the absorbance with respect to the blank, Table 3; it was observed a decrease in the absorbance of 33.3%, 13.3% and 26.7%, respectively; and the commercial pectinase showed a decrease of 46.7%. These values were close to the study where black mulberry showed a reduction of turbidity of 46.5% [28]. The results

evidenced an enzymatic action, which was also consistent with the decrease in pH; due to the pectinase action, pectin molecules are broken down to form galacturonic acid units, decreasing the pH in the solution [28]. So, the pectinase extracted from soursop pulp suitable for consumption allowed to reduce the turbidity of blackberry juice.

Conclusions

The results of this study evidenced the extraction of pectinase from fruit waste of the species *Annonaceae*, a novel source to obtain an enzyme with great commercial value in the food industry. A simple extraction method to obtain pectinase was used, this being an alternative to processing agro-industrial waste in Colombia, and as a possible economical source. The pectinase obtained was an acidic enzyme with potential application as pectin methylesterase. The optimal temperature and pH of the purified enzyme by precipitation were 40 °C and 4.0, respectively. The comparative study about the extraction conditions such as pH, stage, and type of the fruit, evidenced significantly ($p < 0.05$) that the stage and type of the fruit are factors influencing the extraction process. The pectinase obtained from the soursop pulp in the stage unsuitable for consumption presented a lower protein content (92 ± 17 mg) but a higher enzymatic activity (2.2 ± 0.1 U/mL), showing its potential use in the juice clarification.

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Author Contributions Undergraduate students Nora García and Nelcy Cely developed the extraction, purification, and application; and they interpreted some results. PhD Paula Méndez designed the study, interpreted some results, and drafted the manuscript.

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Declarations

Conflict of interest All authors have approved the manuscript and agreed with the submission. The authors have no conflicts of interest to declare.

Consent to Participate The authors express their consent to cooperate in this article.

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