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Optimization of the enzymatic clarification process of glucose syrups derived from agro-industrial residues

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Abstract
In this work, the application of pectinase enzymes to clarify glucose syrups produced from corn fiber was studied. The enzymatic activity and kinetic parameters of the pectinase enzyme used were quantified. A specific activity of 5,528 U/mg protein, a maximum rate of 19151 U/mL, and a Michaelis-Menten constant of 1,656 mg/mL were obtained. The syrup was prepared by hydrolyzing corn fiber at 50°C and 180 rpm, and a central composite design was performed for the clarification step to find the optimal conditions of enzyme-substrate ratio and agitation. The syrup was heated to a temperature of 50°C, and an enzyme substrate ratio between 2.5 to 4.5 U/mL of syrup and agitation, varying between 215 to 285 rpm, was applied. The conditions to maximize the clarification of corn fiber syrup were determined to be an enzyme substrate ratio of 3.716 U/mL syrup and agitation of 267 rpm. These conditions were validated by taking the syrup from clarity of 65.8% to 88.1%, demonstrating that the methodology used presents advantages in the syrup clarification process. A physicochemical characterization of the corn fiber used to prepare the syrups was carried out, which presented a content of 12.90%, 23.33%, 13.4%, and 0.36% of cellulose, hemicellulose, lignin and pectin, respectively.

Keywords: enzymatic hydrolysis; glucose syrup; pectin; polygalacturonase; enzymatic clarification.

Optimización del proceso de clarificación enzimática de jarabes glucosados a partir de residuos agroindustriales

Resumen
En este trabajo se estudió la aplicación de enzimas pectinasas para clarificar jarabes de glucosa producidos a partir de fibra de maíz. Se cuantificó la actividad enzimática y los parámetros cinéticos de la enzima utilizada. Se obtuvo una actividad específica de 5.528 U/mg de proteína, una velocidad máxima de 19151 U/mL y una constante de Michaelis-Menten de 1.656 mg/mL. El jarabe se preparó hidrolizando fibra de maíz a 50°C y 180 rpm, y se realizó un diseño compuesto central para la etapa de clarificación con el fin de encontrar las condiciones óptimas de relación enzima-sustrato y agitación. El jarabe se calentó a una temperatura de 50°C, y se aplicó una proporción enzima-sustrato entre 2,5 y 4,5 U/mL de jarabe y una agitación que varió entre 215 y 285 rpm. Se determinó que las condiciones para maximizar la clarificación del jarabe de fibra de maíz eran una proporción de sustrato enzimático de 3,716 U/mL de jarabe y una agitación de 267 rpm. Estas condiciones se validaron llevando el jarabe de una claridad del 65,8% al 88,1%, demostrando que la metodología empleada presenta ventajas en el proceso de clarificación del jarabe. Se realizó una caracterización fisicoquímica de la fibra de maíz utilizada para preparar los jarabes, que presentó un contenido de 12,90%, 23,33%, 13,4% y 0,36% de celulosa, hemicelulosa, lignina y pectina, respectivamente.

Palabras clave: hidrólisis enzimática; jarabe glucosado; pectina; poligalacturonasa; clarificación enzimática.

1 Introduction

Currently, industries such as agriculture, sugar refineries, fruit juice, and coffee production present challenges in the disposal of their residues, such as peels, seeds, leaves, and stems. In Colombia, approximately 72×10^6 tons of these wastes are produced annually, taken to landfills, or incinerated [1].

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Researchers have studied strategies to transform these wastes into products of industrial interest, such as fertilizers, food additives, chemical compounds, and furfural used to manufacture plastics, solvents, oil, or the production of liquid and gaseous fuels [2-4].

One of those strategies is the transformation of amylaceous and lignocellulosic residues by enzymatic hydrolysis to produce glucose syrups as a value-added product. These syrups are glucose-rich solutions with the capacity to sweeten and produce energy and with the ability to bring brightness to the manufacture of transparent beverages. They are ideal for animal nutrition or as ingredients for the preparation of medicines [5-8].

The first stage in the production of glucose syrups is the pretreatment. In this stage, compounds such as lignin and hemicellulose are degraded, leading to more access to cellulose or starch by the enzymes used in the subsequent stages. This pretreatment can occur by milling, acids, or high temperature and pressure in the presence of water. The next stage is the application of amylase or cellulase, enzymes that can hydrolyze the bonds of starch and cellulose, obtaining an opaque solution rich in glucose. This solution is then subjected to clarification and discoloration processes to reduce turbidity and color and a concentration process to eliminate part of the water that composes it, obtaining more concentrated solutions with a higher level of sweetness [5-8].

This clarification and discoloration stage is commonly performed by decantation, centrifugation, filtration, or adsorption with activated carbon or ion exchange membranes [9-11]. In the decantation process, the mixture that needs to be separated is left undisturbed until the solids dispersed into the liquid settle at the bottom of the recipient thanks to the action of gravity and their difference in density, which then can be separated by pouring the clarified liquid gently. The centrifugation process is quite similar to the decantation process, but instead of gravity, centrifugal force is used to force the solids into the bottom of the recipient. In filtration, the liquid is forced to pass through a porous filter, trapping the solids bigger than the pores on one side while the clarified liquid passes through the filter. Activated carbon and ion exchange membranes are used to remove non-charged and charged particles, respectively, that may impair unwanted colors, flavors, and smells to the clarified product, thanks to the action of intermolecular (van der Waals forces for the Activated carbon) and intramolecular forces (Ionic bonds for the ion exchange membranes) that bond those molecules to the solid media.

These stages' objective is to remove dissolved substances such as cell wall debris, proteins, and fats not degraded during hydrolysis, which scatter the light that enters these products, contributing color and turbidity to the syrups [12-17]. Nevertheless, most of the clarification methods have drawbacks: more viscous fluids need longer clarification times and faster speeds or more pressure to have an efficient process, decantation, and centrifugation, despite being simple, present material losses of around 50%; filtration, despite the good results, requires the use of a stepwise process with expensive equipment; and adsorption, even with a high capacity to remove colored compounds, also absorbs around 14.4% of the total reducing sugars in the syrups and the syrup may end with a darker coloration if the carbon cannot be removed entirely [15-19].

Among the residues that affect turbidity is pectin, a complex polysaccharide presents in the cell wall of higher plants, composed of long linear chains of polygalacturonic acid linked by α-bonds (1-4) and branched regions of various sugars linked by up to 20 different types of bonds [20-23].

The removal or degradation of pectin is a process of industrial interest due to the ability of pectin to form gels. The linear regions of pectin interact, forming three-dimensional networks where other solid particles are trapped, increasing the turbidity and viscosity of the solutions, giving a less desirable appearance for the consumers of the final product, and hindering processes dependent on fluid viscosity [24-27].

A clarification method studied in the fruit juice and wine industry is the use of pectolytic enzymes for clarification because these enzymes could modify and degrade pectin, obtaining a more significant amount of a more precise product during the extraction processes, in addition to facilitating the transport and processing of these products by reducing their viscosity [28-36]. This work proposed a strategy for clarifying glucose syrups using pectic enzymes. An experimental design utilizing response surface methodology was developed to determine the optimal agitation and enzyme-substrate (E/S) ratio for the clarification stage. Before this, the raw material was characterized, and the enzymatic activity was measured along with the estimation of kinetic parameters of the pectic enzyme.

2 Materials and methods

2.1 Materials

Corn fiber was used as a substrate to produce glucose syrups, consisting of the remains of the endosperm of corn kernels resulting from corn maceration and starch extraction [32-36]. The enzyme pectinase/polygalacturonase (EC 3.2.1.15) produced from *Aspergillus niger* was used in the enzymatic hydrolysis. The enzyme preparation consists of a brown, clear solution with glycerol as a base, stored at temperatures between 2 and $8°C$ for preservation. According to the supplier, the enzyme solution reported an enzymatic activity $> 5\ U/mg$ of enzyme units per milligram of protein. One enzyme unit corresponds to the amount of enzyme that produces one µmol of galacturonic acid per minute at a pH of 4.0 and a temperature of $45°C$.

2.2 Characterization of the raw material

To determine the chemical composition of the corn fiber, it was subjected to a characterization following the protocols proposed by the National Renewable Energy Laboratory (NREL) [37-41] modified by the laboratory of processes and reactive flows of the Universidad Nacional de Colombia-Medellín (BIOFRUN). Moisture content and total solids were determined by drying the samples in a Binder convection oven at $105°C$ (NREL TP-510-42621), ash content by calcining the samples in a muffle with a temperature ramp up to $575°C$ (NREL TP-510-42622), and extractives in water, ethanol, and hexane by heating the solvent to boiling point, (NREL TP-510-42619). Then, their

structural carbohydrate, cellulose, hemicellulose, and lignin content was determined by acid hydrolysis (NREL TP-510-42618 and TP-510-42617) and their starch content using amylase enzymes to degrade this compound (NREL TP-510-42624), measuring the amount of sugar produced to calculate the proportion of these polysaccharides in the sample.

For the determination of pectin content, the procedure described by the Mexican Official Standard F-347-S-1980 [42] was used: boiling $50g$ of the material in $400mL$ of distilled water, precipitating the pectin with $1N$ sodium hydroxide, $1N$ calcium chloride, and $1N$ acetic acid, and filtering the sample through a Whatman No. 41 filter paper, to measure the change in weight that this filter undergoes after this process.

2.3 Production of syrup from corn fiber

The methodology proposed in the BIOFRUN laboratory patents produced syrups [7,8]. It starts with a thermal pretreatment of the raw material, adding distilled water and corn fiber until a percentage of solids of 15% is obtained. The solution then was autoclaved, subjecting it to high pressure (15-20 PSI) and temperature ($121°C$) conditions for 40 minutes. The solids obtained were washed with distilled water and dried following the NREL TP-510-42620 protocol [43].

Once the drying process was completed, enzymatic hydrolysis was performed by adding $28g$ of the material and $375g$ of 0.1 M acetate buffer with a pH of 5.0. Subsequently, $3.6\% v/v$ cellulase was added. The resulting solution was incubated at a temperature of $50°C$ and $180rpm$ agitation for 48. Finally, the glucose-rich solution was subjected to a boiling water bath to denature the enzyme.

To verify the degradation of the corn fiber, the dissolved solids ($°Brix$) present in the solution were monitored by taking $1mL$ of the solution and depositing it in a PAL-BX/RI pocket refractometer for $48h$. The final pectin content of the syrup was also measured using the procedure mentioned in section 2.2 above.

2.4 Determination of the enzymatic activity of pectinases.

For the determination of the enzymatic activity of the pectinase, the methodology proposed by Li et al. [44] was followed. Initially, a calibration curve was constructed with 1/6000, 1/9000 and 1/12000 dilutions of the enzyme, which were incubated at $50°C$ for $30min$, in a $5g/L$ solution of apple pectin, allowing to determine the amount of galacturonic acid released using the 3,5-dinitrosalicylic acid (DNS) method. The curve generated by these points was used to determine the enzyme concentration that releases 0.4 g/L of galacturonic acid, which can be used in Eq (1) to find the activity of the original enzyme solution.

$$Activity_{Enzime} = Dilution\ Factor \frac{0.687\ U}{mL} \quad (1)$$

Where 0.687 U/mL corresponds to the theoretical amount of enzyme that releases 0.4 g/L of galacturonic acid. According to the assays of Li et al. [53], the product release curve presents a linear region between 0.2 and 0.6. This central point was selected.

To determine the amount of protein in the enzymatic solution, the method proposed by Bradford [45] was used, measuring the absorbance at 595 nm by mixing the solution with a dye prepared from Coomassie Brilliant Blue G-250 and comparing the absorbance of the samples against a calibration curve prepared from bovine serum albumin in an interval of 0.2 to 2.0 g/L.

2.5 Modified DNS method

A modified version of the DNS method proposed by Li et al. [44] was used. The DNS reagent was prepared by diluting $1g$ of 3,5-dinitrosalicylic acid and $1.6g$ sodium hydroxide in $100mL$ distilled water. $3mL$ of these reagents were added to 1 ml of the studied solutions in $25mL$ test tubes, which were heated in a boiling water bath for $5min$ and cooled in an ice bath for $10min$ to develop the color. Finally, 16 mL distilled water was added to make an initial dilution.

To obtain a standard against which the amount of sugar in the above samples could be compared, a calibration curve was prepared using galacturonic acid solutions from 0.125 to $20.0g/L$. Finally, the absorbance of the samples was measured at 540 nm in a Thermo Scientific™ Evolution 60S UV-visible spectrophotometer, performing a second dilution of the samples if the measured absorbance exceeded a value of 0.8.

2.6 Optimization of the clarification process by statistical analysis

To find the optimal conditions of agitation and E/S ratio, a composite central design generated with the Minitab® 2015 program was performed, the intervals and axial points ($\boldsymbol{\alpha}$) for E/S ratio and agitation used in this central design are reported in Table 1.

While the temperature is an important factor in enzymatic reactions, this parameter was discarded as an option because the temperature interval recommended by the enzyme manufacturer and used by the different researchers that studied this kind of enzyme was only from 40 to 50 °C. This short work interval may not show any appreciable improvement in the clarification, so the other two not studied were chosen to reduce the number of experiments needed for this study.

The design obtained corresponds to 4 experiments using the axial points, 4 experiments combining the ends of the intervals, and 6 central points, for a total of 14 experiments.

During these trials, $100mL$ syrup centrifuged at 4500 rpm was used, and clarification was performed at $50°C$ for two hours using the studied parameters. Once this period was over, syrup clarity was measured, expressed as the percentage transmittance

Table 1.
Central composite design.

Variable	Value				
	α-	**-1**	**0**	**+1**	**α+**
E/S ratio (U/mL)	2,08	2,50	3.50	4,50	4,91
Agitation (rpm)	200,5	215,0	250,0	285,0	299,5

Source: The authors.

at $600nm$ (%T 600) using a Thermo Scientific™ Evolution 60S UV-Visible spectrophotometer, pH with a pH-meter Titulador 848 Titrino plus, total solids ($°Brix$) with a PAL-BX/RI pocket refractometer and reducing sugars using the DNS method. These data were analyzed by analysis of variance (ANOVA) using the R program (version 4.2.0).

There are other methodologies to evaluate the efficiency of clarification like: measuring the change in the turbidity of the syrup [13], comparing the absorbance at certain wavelengths [14] or checking how much solids were removed from the syrup [15]. %T 600 was chosen as the measuring method since it was the most common method to measure the clarification of fruit juices using pectinases.

2.7 Determination of the kinetic parameters of the enzyme

To determine the kinetic parameters of the pectinase, maximum velocity (V_{max}), and the Michaelis-Menten constant (K_m), a Lineweaver-Burk diagram was used, preparing $300mL$ apple pectin solutions with concentration between 0.01-0.15$\%w/w$, which were treated with the optimum E/S ratio and stirring determined in the design of experiments. The kinetics of the process were determined by taking a $5mL$ sample every $5min$, and inactivating the enzyme in a boiling water bath. The amount of galacturonic acid released was then measured using the DNS method.

Subsequently, the amount of sugar released in moles was plotted against time, and the slope generated by the first 5 experimental data was taken to determine the initial velocity (V) of the process. These velocity values are used to create a Lineweaver-Burk plot, plotting the inverse of the velocity ($1/V$) against the inverse of the substrate concentration ($1/S$), which allows the determination of V_{max} and K_m.

2.8 Kinetics of the clarification process

The change in clarity and concentration of reducing sugars over time of enzymatic hydrolysis treated syrups were monitored by subjecting $300mL$ of syrup to the clarification process, using the optimum enzyme concentration and agitation at $50°C$. Every $20min$ $10mL$ of solution were removed, inactivating the enzyme in a boiling water bath at $95°C$ for $5min$ and cooling in an ice bath, then centrifuged at $4500rpm$ for $5min$. Employing the DNS method, the amount of galacturonic acid released and the transmittance at $600nm$ was measured to determine the clarity in the syrups produced.

3 Results and Discussion

3.1 Characterization of the raw material

Due to the complexity of the lignocellulosic materials, the NREL characterization methodologies have a total composition range from 90 to 110%. Table 2 presents the results of the characterization of the corn fiber; with these tests, it was determined that 12.90% of the material was cellulose, which cellulases can hydrolyze, and 0.36% was pectin, which ends up in the syrups by the processes of heating and hydrolysis of cellulose.

Comparing the results obtained with those presented by Gáspár et al., Kálmán et al., Kaur et al., and Zhang et al. [32-36], similar values for cellulose and ash can be seen in the work of Kálmán et al [34]. which reports a value of 15% for cellulose and 1% for ash. The value of hemicellulose was lower compared to this work, where they report a value of 37.1%, closer to the value reported by Kaur et al. [35] where a value of 22.4% is presented. The article by Gáspár et al. [33] is the only one that reports a lignin value, obtaining a value of 12.2%, quite similar to that obtained in this study. A possible source of variation is the presence of starch reported by these authors; the residue used in this test has undergone several processes to remove the starch and other valuable compounds, while the literature reports corn fiber in nature from which the starch has not yet been removed or has only undergone the starch removal process.

Table 2.
Characterization of corn fiber

Compounds	Percentage (%)
Humidity	8.5 ± 0.1
Total Solids	91.5 ± 0.1
Cellulose	16.3 ± 0.1
Hemicellulose	27.5 ± 0.1
Lignin	13.4 ± 0.1
Pectin	0.4 ± 0.3
Starch	0.0 ± 0.1
Protein	18.5 ± 0.1
Ash content	0.6 ± 0.1
Total extractives	32.4 ± 0.1
Total	109.2 ± 0.1

Source: The authors.

3.2 Production of syrup from corn fiber

Table 3 shows the characteristics of the syrups produced for the clarification tests. Observing the development of dissolved solids, this was very similar in all the trials, doubling during the first $24h$ after the enzyme was added but remaining approximately constant until $48h$ were reached. These five solutions were centrifuged at 4500 rpm for 5 min to eliminate the larger solids and combined, obtaining a syrup with 4.8 $°Brix$ and a pectin concentration of 0.13 ± 0.02 %.

Table 3.
Characteristics of the syrups

Vessel	1	2	3	4	5
Solids ($\pm 0.0001\ g$)	28.29	29.20	28.25	28.05	28.02
Buffer ($\pm 5g$)	375	375	375	375	375
%Solids ($\%w/w$)	7.01	7.22	7.01	6.96	6.95
%Enzyme ($\%v/v$)	3.70	3.70	3.70	3.70	3.70
Initial Brix($\pm 0.1°Brix$)	0.8	0.8	0.8	0.8	0.8
Enzyme Brix ($\pm 0.1°Brix$)	2.3	2.4	2.6	2.6	2.6
Brix $24h$ ($\pm 0.1°Brix$)	5,2	5,0	5,0	5,1	4,9
Brix $48h$ ($\pm 0.1°Brix$)	5,4	5,1	5,0	5,0	5,0

Source: The authors.

3.3 Determination of the enzymatic activity of the pectinase.

The amount of galacturonic acid released by the enzymatic solutions with a dilution factor of 6000, 9000, and 12000 was 0.472, 0.385, and 0.314,g/L respectively, plotting these results against their corresponding dilution factor yielded a line with a coefficient of determination R^2 of 0.996 and Equation (2):

$$Dilution\ factor = -37830\ Gal.Acid + 23759 \quad (2)$$

Replacing the value of $0.4g/L$ in this equation shows that the enzyme concentration that releases $0.4g/L$ of galacturonic acid has a dilution factor of 8627; using this data in Equation (1) it was determined that the original enzyme solution has an activity of 5.926 $U/\mu L$.

The albumin calibration curve between 0.2 to 2.0 g/L used for protein determination has a coefficient of determination R^2 of 0.968 and is represented by Equation (3):

$$Concentration\ (g/L) = 40.5826Abs - 0.0439 \quad (3)$$

Where Abs is the absorbance of the sample, and concentration (g/L) is the amount of protein present in the sample. Measuring the absorbance at $595nm$ of $1\mu L$ of enzyme solution dissolved in $0.999mL$ of distilled water, we found an absorbance of 0.027 ± 0.001, which corresponds to a concentration of 1.072 $mg/\mu L$ protein in the enzyme solution, which allows us to find that the specific activity of the enzyme has a value of 5.528 U/mg protein, which agrees with the value reported by the supplier of > 5 U/mg protein.

3.4 Optimization of the clarification process by statistical analysis

The results of clarity, pH, galacturonic acid released and dissolved solids for the 14 trials, a blank without enzymatic treatment, and uncentrifuged syrup are shown in Table 4.

Table 4.
Design of experiments for the clarification step.

Test	E/S ratio	Agitation	Clarity (%T 600)	pH	Released sugars (g/L)	Solids ($°Brix$)
1	α+	0	74.4	4.85	25.26	4.7
2	0	0	79.7	4.84	24.55	4.6
3	0	0	78.5	4.83	24.51	4.6
4	-1	+1	77.2	4.82	24.70	4.5
5	+1	-1	77.7	4.85	25.31	4.6
6	0	α-	71.4	4.83	23.18	4.6
7	0	0	79.1	4.83	24.65	4.6
8	-1	-1	69.7	4.86	24.22	4.6
9	0	0	80.1	4.83	24.41	4.6
10	0	0	79.3	4.84	24.32	4.6
11	α-	0	67.5	4.87	23.94	4.5
12	+1	+1	77.8	4.85	25.12	4.6
13	0	0	76.9	4.84	24.51	4.6
14	0	α+	77.1	4.75	24.70	4.6
Control	-	-	62.6	4.89	22.66	4.5
Raw syrup	-	-	11.8	4.91	22.37	4.5

Source: The authors.

Table 5.
ANOVA for clarity.

Coefficients	Values	*p* Values
R^2	0.878	0.001707
$R^2_{adjusted}$	0.802	0.001707
Constant term	78,933	5.138e-14
E/S ratio	2.29476	0.006049
Agitation	1.95763	0.013480
(E/S ratio)^2	-3.24167	0.001026
Agitation^2	-1.59167	0.039002
(E/S ratio) (Agitation)	-1.85000	0.068000
Lack of fit	-	0.064043

Source: The authors.

An analysis of the results shows that a higher enzyme ratio releases more galacturonic acid, which leads to a reduction in pH, and also shows a slight increase in dissolved solids as more enzyme is applied, but when studying the interaction between the enzyme ratio and agitation, and the effect that these variables have on clarity, it is difficult to find a clear result, so these data were processed with R (version 4.2.0). Table 5 displays the ANOVA results.

The second-order model generated by the ANOVA analysis shows that the first and second-order coefficients for the enzyme ratio and agitation are significant, with a confidence level higher than 95%, presenting a p-value lower than 0.05. Still, the interaction of these two coefficients shows a *p*-value higher than 0.05, which cannot be assumed to be significant at this confidence level. The following polynomial expression can express this empirical model:

$$\begin{aligned} Clarity\ (\%T600) = 78.933 &+ 2.295\ E/S\ ratio \\ &+ 1.958 Agitation \\ &- 3.242\ (E/S\ ratio)^2 \\ &- 1.59167 (Agitation)^2 \end{aligned} \quad (4)$$

The performance of this model can be verified with the values of the coefficient of determination (R^2), adjusted coefficient of determination ($R^2_{adjusted}$), and the p-value of the lack of fit. The data used to generate the model are adjusted to this, so the value of R2 and adjusted R^2 are close to 1 and present a p-value (0.001) less than 0.05. On the other hand, the lack of fit to the model has a value of 0.06, greater than 0.05, showing that this model is acceptable for representing these points and predicting new values. Plotting these results (Fig. 1), the clarity presents a maximum when applying 3.716 units of enzyme per ml of syrup (0.627 μL of enzyme solution per mL of syrup) and a stirring of 267 rpm. When using a lower amount of enzyme, the pectin residues are not entirely removed, and a lower clarity is obtained. In comparison, if a higher amount of enzyme is used, the presence of these dissolved compounds causes the light transmittance to be lower, resulting in fewer clear syrups.

To validate the results of this model, clarification tests were performed in triplicate at the optimum point (3.7143 U/mL and $267.4991 rpm$), one with high agitation and high enzyme ($4.9142 U/mL$ and $299.4975 rpm$), and one with low agitation and low enzyme (2.0858 U/mL and $200.5025 rpm$), comparing the results obtained with those predicted. Table 6 shows the results.

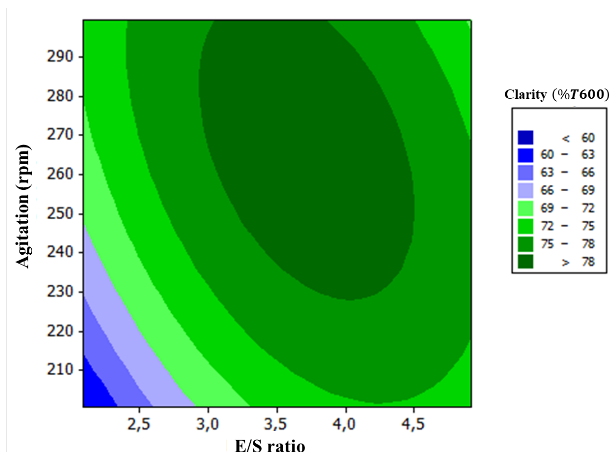

Figure 1. Contour plot, clarity concerning agitation and E/S ratio.
Source: Author made

Table 6.
Validation of the central composite design.

Test	Predicted value	Expected value	%Error
High	74.9 ± 2.9	74.9	0.1
Maximum	78.6 ± 0.6	79.7	1.4
Low	70.3 ± 1.7	71.1	1.1

Source: The authors.

Table 7.
Initial velocities and concentration.

Substrate concentration (% w/w)	Initial velocity ($g/L/min$)
0.010	0.012
0.015	0.017
0.030	0.035
0.090	0.112
0.150	0.186

Source: The authors.

Low error values (<2%) indicate that the model obtained can be used and that the optimum is around 3.7143U/mL and 267.4991 rpm.

3.5 Determination of enzyme kinetic parameters.

The apple pectin solutions used for this determination were 0.010, 0.015, 0.030, 0.090, and 0.150% w/w, the initial velocity obtained by plotting the amount of galacturonic acid released against time, taking the slope obtained in the first 25 minutes can be seen in Table 7. Plotting the inverse of these initial velocity values and the inverse of the pectin concentration in moles, the Lineweaver-Burk diagram is obtained, which has a coefficient of determination of 0.998 and is explained by the equation:

$$I/V_i = 6.68E - 0.4/[pectin] + 0.0522 \quad (5)$$

This diagram found that the enzyme is 2439.7 $mg/mL/min$ and an K_m of 1.656 mg/mL, similar to that obtained by Dalagnol et al. [46]. A comparison between these results can be made because, in this study, the activity of pectin enzymes produced by the fungus Aspergillus niger, using apple pectin as substrate, is also sought. The presence of polymorphisms in the enzymes of the pectinase family and the use of other factors at the time of finding the parameters, such as the use of ultrasound or the application of other methods of obtaining and purification, are possible reasons to explain the difference obtained in the work of Dalagnol et al. [46] and the current work.

3.6 Kinetics of the clarification process

A new syrup sample was prepared for these tests following the same steps as in the previous experiments. This solution initially presented a clarity of 65.8 %T 600 nm, 0.051 ± 0.002 % pectin, and a reducing sugars concentration of 17.636 g/L. After two hours of treatment, the increase in clarity reached a value of 88.1 %T 600 nm, following a curved line with a determination coefficient R^2 of 0.993, which is described by the polynomial equation:

$$Clarity\ (\%T\ 600nm) = 66 + 0.15t + 2.66E - 4t2 \quad (6)$$

Reducing sugars reached a value of 20.901g/L, and this property also followed a curved line, which can be described by the following equation, presenting a value R^2 of 0.987.

$$Sugar\ (g/L) = 17.5 + 0.0445t - 1.28E - 4t^2 \quad (7)$$

Comparing these results with those obtained during the previous clarification tests shown in Table 4, a greater clarity of the syrup was obtained. Still, a lower amount of galacturonic acid was released. One possible explanation for these results is the lower initial amount of pectin, which allowed the enzyme to remove most of this compound, resulting in a final syrup with less pectin that is clearer and, as less pectin is degraded, less galacturonic acid is released. As the tridimensional net of galacturonic acid is degraded by the enzyme, the solids trapped by it get released. Since both the pectin and the trapped solids contribute to the haziness of the syrup, the clarification process starts fast and then begins to slow down as pectin becomes scarce.

Figure 2. Untreated and treated syrup.
Source: The authors.

4 Conclusions

In this work, a methodology for clarifying glucose syrups using pectolytic enzymes was proposed. The characterization of the corn fiber used for syrup preparation determined the presence of 16.51% cellulose, 27.83% hemicellulose, 13.4% lignin, 0.36% pectin, 0.6% ash, and 32.45% extractives. An ANOVA analysis determined that the amount of enzyme and agitation that generated the highest response in the clarity of the syrup were around 3.716 U/mL and an agitation of 267 rpm, obtaining a syrup with a notably more transparent appearance and a lighter color (as shown in Fig. 2), which may be more desirable for consumption. Additionally, a characterization of the enzyme from *Aspergillus niger* was carried out, which presents an activity of $5.528U/mg$ and a V_{max} of $19151U/mL$ and K_m of 1,656 mg/mL, when corn fiber is used as a substrate of the process. From the different results, we will seek to study the modeling of the clarification process by simulating the pectin degradation process and see how this affects the clarity of the syrup over time.

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