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Comparison of the performance of an organic acid and an inorganic acid pretreatment by means of enzymatic hydrolysis of coffee husk

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RESEARCH ARTICLE

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ABSTRACT

The study of different lignocellulosic materials for second-generation biofuels is one of the trending topics today because of the high demand for fuels for transportation and electricity generation. Coffee husk is presented as one study option considering that only 10% of the coffee fruit is used for coffee production. The pretreatment of the coffee husk with sulfuric(VI) acid (3 or 6%) and citric acid (6 or 12%) was compared using two methodologies. The first had reaction condition time (50, 70, 90, and 1440 min) and temperature (70 and 90 °C), while the second had autoclave conditions (121 °C, 14.696 psi, 60 min). The comparison was made to find the best methodology for acid pretreatment before enzymatic hydrolysis. The best result of the reduction of sugars (17.017%) and glucose yield (3.882%) was found with 6% $C_6H_8O_7$ in autoclaving (121 °C, 14.696 psi, 60 min) with hydrolysis conditions of 72 h, 150 rpm, 50 °C, and using cellulases from *Trichoderma reesei*.

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1. Introduction

As is well-known, the use of fossil fuels has multiple negative impacts on the environment. One of those impacts can be observed when fossil fuels are burnt to generate combustion inside the vehicle engine. During this process, greenhouse gases, such as nitrogen oxides (NOx), carbon dioxide (CO_2), and atmospheric particulate matter (PM), are released into the environment. These greenhouse gases and their emissions have become alarming, considering the large number of vehicles that circulate hourly around the world. The said emissions have been found to be the main cause of large-scale effects, such as global warming [1].

As an alternative to the use of fossil fuels, biofuels emerge as a possible solution and four types that can be produced on the basis of their raw material. First-generation biofuels, according to reference [2], started to be investigated in Brazil using sugar cane and in the United States using corn in 1997. The large areas used for energetic crops (destined only for the production of first-generation biofuel production) must be previously deforested, generating the emission of large amounts of CO_2. This counteracts the advantages presented by biofuels in relation to the target of reducing greenhouse effects. Another disadvantage is the use of land that can be used to grow food for the population and instead is used to produce biofuels, reducing food availability [3].

In response to the above-mentioned problems, second-generation biofuels emerged as a practical solution. These biofuels use as raw materials the lignocellulosic organic waste of crops and are presented as a better solution due to the large amount of organic waste available [4]. Crops such as coffee, sugar cane and rice generate a large amount of waste with a high cellulose content that can be implemented as a source of energy [5].

In Colombia, coffee production is one of the most important industries, due to coffee crops, which take up approximately 3.3 million hectares and represent between 7 and 10% of export earnings [6]. As coffee demand is high, there is also an increase in coffee residue (coffee husk), however, there is not enough research on how this waste can be exploited to give added value.

The amount of coffee husk generated corresponds to 90% of the coffee plant [7] and is composed of between 85 and 91% water and between 6.2 and 7.4% sugars, consisting of 63% reducing sugars [8]. This sugar content represents a high rate of fermentation which can be used to generate second-generation biofuels by means of hydrolysis.

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In previous research by the author of this article [9], coffee husk was evaluated in subcritical hydrolysis without pretreatment, obtaining the best conditions at 250 °C, 2000 psi, and a retention time of 30 min, giving 9.15% reduction sugars. However, a higher percentage was expected as coffee husk contains 43% cellulose, which is the main element transformed into reducing sugars. During this research, the pretreatment of the coffee husk with organic and inorganic acids was evaluated under different conditions of temperature, time, acid type, and concentration, followed by enzymatic hydrolysis to analyze which pretreatment conditions allowed a better extraction of sugars during hydrolysis.

2. Experimental

pH measurements were performed with a Crison Basic 20 pH metre and a Crison 52 09 pH electrode at room temperature. The phenol content was found according to the Folin-Ciocalteau procedure [10]. The concentration of glucose and total reducing sugar was determined using the enzyme test kit (Biomaxima) and the DNS procedure [11].

The methodology involved four phases: (I) preparation of the raw material, (II) acid pretreatment with citric and sulfuric acid at different concentrations, (III) computational analysis of the hydrolysis results in terms of glucose, reducing sugars, and phenols, and finally (IV) selection of suitable conditions for enzymatic hydrolysis. Since glucose is the predominant component of cellulose, it is assumed that all glucose originates from cellulose, and the other reducing sugars are from hemicellulose.

2.1. Sample collection and mechanical pretreatment

The coffee husk was collected at the coffee plantation, La Arabia, located in Viota, Colombia (June 2021) and vacuum-packed to be transported to Wroclaw, Poland. Once the coffee husk arrived, it was separated into bags of 200 g and frozen to avoid any type of degradation or fermentation. To decrease the particle size of the coffee husk and create a larger surface area in which the reaction can occur, the coffee husk was ground and, later, the material was filtered and dried for 24 hours at 60 °C.

2.2. Acid pretreatment

After the drying process, 2 g of coffee husk were poured into a 100 mL capacity reactor with 40 mL of acid. Two types of acids were evaluated: (i) sulfuric(VI) acid with a concentration of 3 or 6 % and (ii) citric acid with a concentration of 6 or 12%. The reaction was analyzed at 50, 70, 90, or 1440 minutes, 150 rpm, and at temperatures of 70 or 90 °C. Reaction times 50, 70 and 90 min were selected to perform a comparison between the proposed pretreatment and autoclave in which the same acid concentrations were analyzed but the parameters were 60 min, 121 °C and 15 psi. This comparison is made, since the use of an autoclave is the most common pretreatment method found in the literature [12], allowing for determination of the effectiveness in biomass decomposition. The parameters with the highest concentration of phenol, glucose and reducing sugars other than glucose, obtained for each acid (either by the proposed methodology or the autoclave procedure) will be used before the enzymatic hydrolysis stage.

At the end of the run, all samples were neutralized using 0.4 M sodium hydroxide until a pH between 6 and 7. After neutralization, the color of the samples turned dark brown, generating interference in the measurements; thus, after neutralization, the coffee husk was filtered four times using a filter paper.

The solid phase (coffee husk pre-treated) and the liquid phase were separated by centrifugation (6000 rpm, 5 min, Hettich Universal 320R Centrifuge). The solid phase was dried at 60 °C for 48 hours to be prepared for enzymatic hydrolysis and the liquid phase was used to measure phenols, glucose, and reducing sugars using the phenol methodology [10], Biomaxima pink reagent, and 3,5-dinitrosalicylic acid (DNS) procedure, respectively [11].

2.3. Computational analysis

For statistical analysis, each parameter (glucose, phenol, and reducing sugars other than glucose-DNS) was analyzed based on the acid used during the experiment, to finally compare the efficiency between the best results of the methodology proposed and the autoclave results. To analyze the proposed methodology, three main tools were used: (i) Pareto chart, where the "α" level indicates statistical signify-cance and determines if the condition (temperature (°C), time (min) and concentration (%)) and its combination with other conditions have a significant effect during the process. This significance can be determined when a parameter crosses the reference line, which is 2.31. These conditions are statistically significant at the 0.05 level [14]. (ii) The main effects plot is based on the differences between the means for one or more conditions and the response mean for each parameter connected by a line. The main effect of a parameter is determined when the line formed by the minimum and maximum values is not horizontal. This graph does not show the interaction between conditions or predictable values [14]. (iii) Contour graphs are presented to evaluate the relationship between conditions by means of contour bands that represent the ranges of the response values. However, if the effect of the combination of the conditions in the Pareto diagram is not significant, the relationship is not being evaluated. Finally, a table with the conditions found to be optimal by the statistical analysis is presented for each parameter, showing the best combination of temperature (°C), time (min), and concentration (%) for the highest parameter concentration.

2.4. Enzymatic hydrolysis

To determine whether the pretreatment with citric acid was able to achieve the same hydrolysis rate as sulfuric acid, the coffee husk that remained from the best pretreatment for each acid was dried at 60 °C for 24 h and hydrolysed using *Trichoderma reesei* cellulase. 1 mL of cellulase was mixed with 40 mL of 0.1 M citrate buffer with pH = 5 and 2 g of dry coffee husk (5% w/v). The reaction was carried out for 72 h at 50 °C and 150 rpm, taking samples at 1, 24, 48, and 72 h to determine glucose and sugar conversion over time and find the highest concentration achieved. Biomaxima pink reagent and DNS procedures [11] were applied to determine total glucose and reduce sugar conversion, respectively.

3. Results and discussion

Tables 1 and 2 present the results for sulfuric acid and citric acid, respectively, having as conditions acid concentration (%), temperature (°C), and time (min). To explore the effects of the conditions chosen and significance level, the results were studied in MINITAB software [13] choosing the factorial design to find the optimal parameters and compare them with the autoclave results.

3.1. Statistical analysis for sulfuric acid

3.1.1. Glucose concentration

The Pareto chart (Figure 1) shows that all conditions (acid concentration, g/L (A); temperature, °C (B); and time, min (C)) and their combinations (AB, BC, AC, ABC) are significant, being the most important condition the time (C) with a standardized effect of 27.

Table 1. Results for the pretreatment of coffee husk with sulfuric acid under the methodology proposed (Concentration: 3 and 6%, temperature: 70 and 90 °C, reaction time: 50, 70, 90 and 1440 min).

Acid%	Temperature (°C)	Time (min)	Glucose (g/L)	Phenol (g/L)	Reducing sugars (g/L)
3	70	50	0.2079	0.2822	2.8662
3	70	70	0.2198	0.2822	3.4772
3	70	90	0.2237	0.3122	3.7298
3	70	1440	0.3821	0.2802	6.4279
3	90	50	0.2356	0.1650	4.2930
3	90	70	0.2699	0.2025	5.3120
3	90	90	0.2897	0.2127	5.5251
3	90	1440	0.7303	0.4832	9.6412
6	70	50	0.0836	0.1776	2.6871
6	70	70	0.1006	0.1831	3.0487
6	70	90	0.1034	0.1878	3.3376
6	70	1440	0.2851	0.3567	6.7597
6	90	50	0.1844	0.2313	4.7869
6	90	70	0.2126	0.2669	4.9806
6	90	90	0.2315	0.2705	5.3921
6	90	1440	0.3859	0.4336	7.8777

Table 2. Results for the pretreatment of coffee husk with citric acid under the methodology proposed (Concentration: 6 and 12%, temperature: 70 and 90 °C, reaction time: 50, 70, 90 and 1440 min).

Acid%	Temperature (°C)	Time (min)	Glucose (g/L)	Phenol (g/L)	Reducing sugars (g/L)
6	90	50	0.0134	0.2583	0.6028
6	90	70	0.0223	0.2630	0.6701
6	90	90	0.0445	0.2691	0.8458
6	90	1440	0.1439	0.3779	1.9746
6	70	50	0.0074	0.2793	0.3685
6	70	70	0.0178	0.3060	0.3919
6	70	90	0.0267	0.3270	0.4446
6	70	1440	0.0460	0.5868	4.9864
12	70	50	0.1012	0.2546	0.2304
12	70	70	0.0867	0.3017	0.3065
12	70	90	0.0607	0.3313	0.3791
12	70	1440	0.3064	0.3328	0.5121
12	90	50	0.0723	0.4366	0.1344
12	90	70	0.1156	0.4060	0.0996
12	90	90	0.1185	0.3804	0.0861
12	90	1440	0.1185	0.5038	1.0324

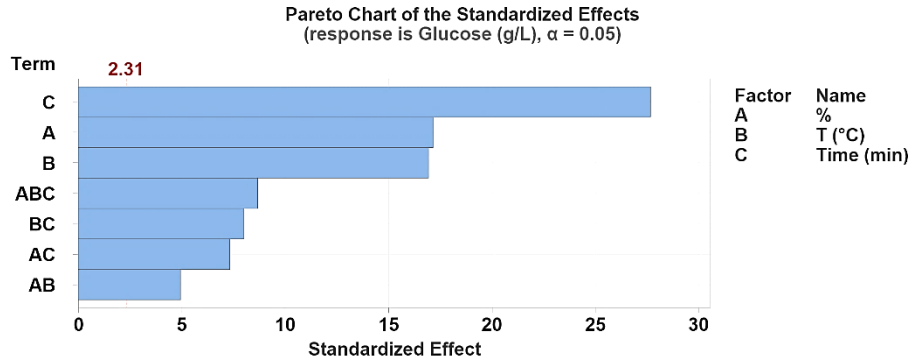

Figure 1. Pareto chart for glucose in sulfuric acid pretreatment. This figure illustrates the statistical significance of the acid concentration (%) A, temperature (°C) B and time (min) C in glucose production for the pretreatment of coffee husk with sulfuric acid.

This is confirmed in the main effects plot (Figure 2), where the highest mean glucose concentration (0.45 g/L) is obtained by time (C), followed by the temperature (B) and the concentration (A) that has the same impact (0.40 g/L) during the process. In the main effects graph (Figure 2), it can also be seen that an increase in acid concentration and a decrease in temperature and reaction time have a negative impact on glucose concentration. Following the above results, the relationships between the contours are plotted and it can be seen that the optimal conditions are found in the relationship between high temperature (85-90 °C) with low acid concentration (3.0-3.7%) (Figure 3a), high temperature (85-90 °C) with long reaction time (1200-1440 min) (Figure 3b), and low acid concentration (3.0-3.7%) with long reaction time (1200-1440 min) (Figure 3c). The statistical analysis for glucose in sulfuric acid pretreatment leads to the conclusion that optimal glucose production requires high temperature, low acid concentration, and long reaction time.

3.1.2. Phenolic compound concentration

The Pareto chart (Figure 4) shows that only the conditions of time, min (C) and temperature, °C (B) are significant during the run, being the most important time (C) with a standardized effect of 16.2. This is confirmed in the main effects plot (Figure 5), where there is a low difference in phenol production influenced by acid concentration (A), making this condition not significant. In the main effects plot (Figure 5), it is observed that time is the condition with the highest mean phenol concentration (0.39 g/L) followed by the temperature (0.34 g/L).

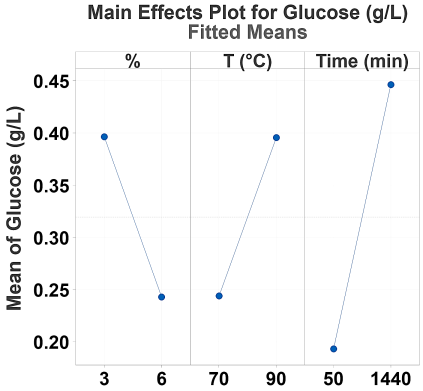

Figure 2. Main effect for glucose in sulfuric acid pretreatment. This figure illustrates how the effect of acid concentration (%), temperature (°C) and time (min) affects the mean glucose concentration for each condition value for the pretreatment of coffee husk with sulfuric acid.

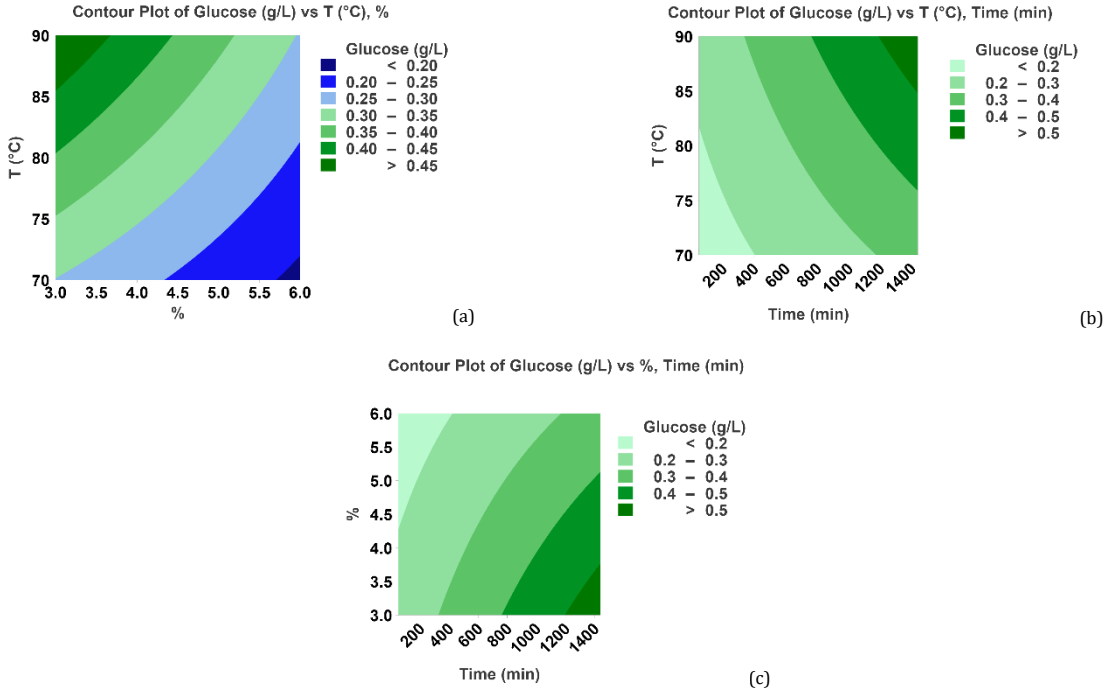

Figure 3. Contour plots for glucose reaction conditions (Temperature, concentration, and time) under sulfuric acid pretreatment. (a) Contour plots temperature *versus* concentration of glucose in sulfuric acid. This figure illustrates the ranges of glucose concentration 0.20-0.45 g/L obtained and classified by colors. Dark green represents the higher glucose concentration in the relationship of temperature (°C) and acid concentration (%). (b) Contour plots the temperature *versus* time for glucose in sulfuric acid. This figure illustrates the glucose concentration ranges obtained 0.2-0.5 g/L and classified by colors. Dark green represents the higher glucose concentration in the temperature (°C) and time (min) relation. (c) Contour plots the concentration *versus* time of glucose in sulfuric acid. This figure illustrates the ranges of glucose concentration of 0.2-0.5 g/L obtained and classified by colors. Dark green represents the higher glucose concentration in the acid concentration (%) and time (min) relation.

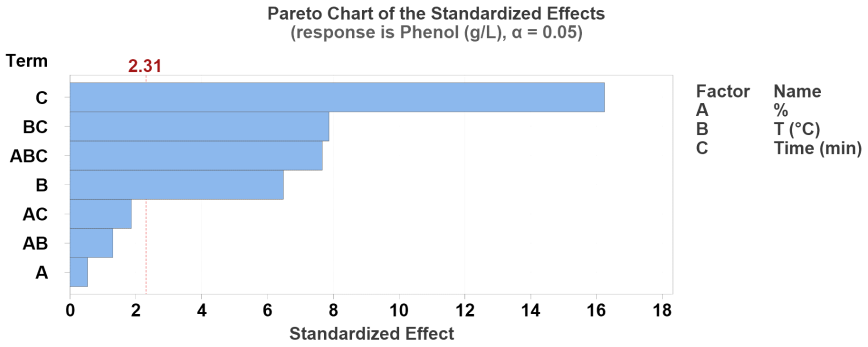

Figure 4. Pareto chart for phenol in sulfuric acid pretreatment. This figure illustrates the statistical significance of the acid concentration (%) A, temperature (°C) B and time (min) C in the production of phenol for the pretreatment of coffee husk with sulfuric acid.

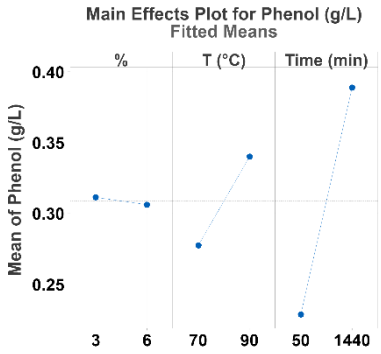

Figure 5. Main effect for phenol in sulfuric acid pretreatment. This figure illustrates how the effect of acid concentration (%), temperature (°C) and time (min) affects the mean phenol concentration for each condition value for the pretreatment of coffee husk with sulfuric acid.

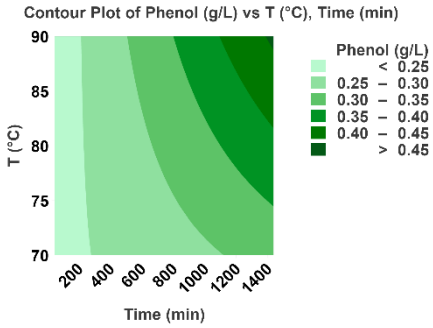

Figure 6. Contour plots temperature *versus* time for phenol in sulfuric acid pretreatment. This figure illustrates the 0.25-0.45 g/L phenol concentration ranges obtained and classified by colors. Dark green represents the higher concentration of phenol in the temperature (°C) and time (min) relation.

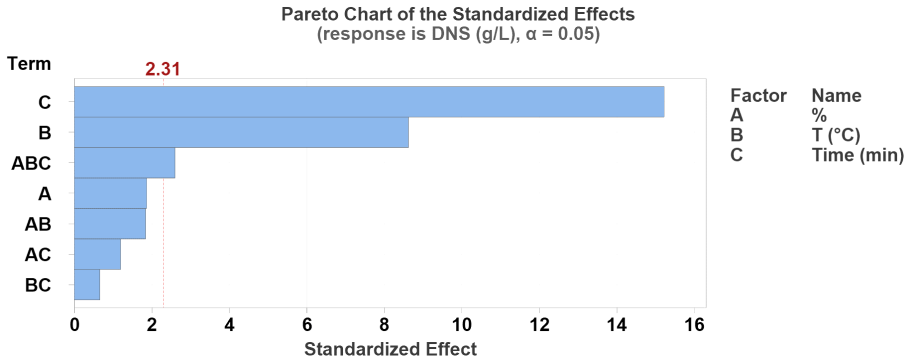

Figure 7. Pareto diagram for DNS (reducing sugars) in sulfuric acid pretreatment. This figure illustrates the statistical significance of the acid concentration (%) A, temperature (°C) B and time (min) C in the production of reducing sugars for the pretreatment of coffee husk with sulfuric acid.

For phenolic compounds obtained from sulfuric acid pretreatment, increasing the acid concentration and decreasing the reaction time and temperature negatively affect the final phenol concentration. The only significant relationship found in the Pareto diagram (Figure 4) is BC, which under optimal conditions has the relationship between high temperature (82-90 °C) and long reaction time (1200-1440 min) (Figure 6).

3.1.3. DNS (Reducing sugars)

The Pareto chart (Figure 7) shows that only the conditions of time, min (C) and temperature, °C (B) are significant during the run, being the most important time (C) with a standardized effect of 15.7. This is confirmed in the main effects graph (Figure 8), where there is a low difference in the production of reducing sugars based on acid concentration, which makes this condition not significant. In the main effects plot (Figure 8), it is observed that time is the condition with the highest mean reducing sugar concentration (7.8 g/L) followed by temperature (6.9 g/L). For the reducing sugars obtained from a sulfuric acid pretreatment, increasing the acid concentration and decreasing reaction time and temperature negatively affect the concentration of the reducing sugars. Following the results found in the main effects plot (Figure 8), it is expected to have the highest reducing sugar production at a long reaction time, low acid concentration, and high temperature, simultaneously.

Based on the previous individual analysis of coffee husk pretreated with sulfuric acid, it can be concluded that the combination of the three conditions (time, acid concentration, and temperature) has a significant impact on obtaining optimal values of glucose, reducing sugars, and phenols. For optimal conditions, increasing acid concentration and decreasing reaction time and temperature negatively affect the final concentration of glucose, reducing sugars, and phenol. For the three parameters evaluated, the optimal conditions are found at 3% H_2SO_4, 90 °C, and 1440 min, these values will be further compared with the results of the autoclave conditions.

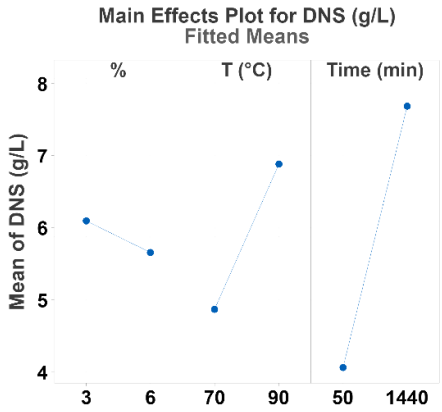

Figure 8. Main effect for DNS (reducing sugars) in sulfuric acid pretreatment. This figure illustrates how the effect of acid concentration (%), temperature (°C) and time (min) affects the mean DNS (reducing sugars) concentration for each condition value for coffee husk pretreatment with sulfuric acid.

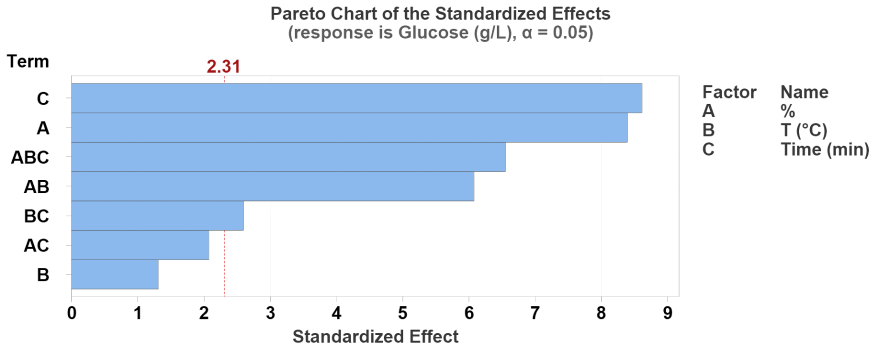

Figure 9. Pareto chart for glucose in citric acid pretreatment. This figure illustrates the statistical significance of the acid concentration (%) A, temperature (°C) B and time (min) C in glucose production with the pretreatment of coffee husk with citric acid.

3.2. Statistical analysis for citric acid

3.2.1. Glucose concentration

The Pareto chart (Figure 9) shows that only the conditions of time, min (C) and acid concentration, % (A) are significant during the run, being the most important time (C) with a standardized effect of 8.7. This is confirmed in the main effects plot (Figure 10), where there is a low difference in glucose production based on temperature, making this condition not significant. In the main effects plot (Figure 10), it is observed that the highest mean glucose concentration is obtained by time (0.152 g/L) followed by concentration (0.150 g/L). Both conditions have almost the same effect on glucose production, making time more influential. For glucose obtained from a citric acid pretreatment, increasing the temperature and decreasing the acid concentration and reaction time have a negative effect on the final glucose concentration. Significant relationships found in the Pareto chart (Figure 9) are concentration-temperature-AB (Figure 11a) and temperature-time-BC (Figure 11b). The highest concentration of glucose was obtained at high concentrations (11.5-12.0 %) at low temperatures (70-74 °C) and low temperature (70-80 °C) with long reaction times (1200-1440 min). The highest concentration of glucose for pretreatment with citric acid requires simultaneously low temperatures, high acid concentration, and a long reaction time.

3.2.2. Phenolic compound concentration

The Pareto chart (Figure 12) shows that only the time min (C) and the temperature, °C (B) conditions are significant during the run, having the same standardized effect of 8.9. This is confirmed in the main effects graph (Figure 13), where there is a low difference in mean phenol production based on acid concentration, making this parameter not significant. In the main effects plot (Figure 13), it is observed that time and temperature have the same effect on the final phenol concentration with a mean phenol concentration of (0.45 g/L). For the phenolic compounds obtained from a citric acid pretreatment, the decrease in the acid concentration, temperature, and reaction time has a negative effect on the final concentration. The significant relationships found in the Pareto graph (Figure 12) are the concentration time AC (Figure 14a), and the temperature time BC (Figure 14b). The relationships of the contours were plotted, finding that the highest concentration of phenol could be obtained at low acid concentration (6-9 %) with long reaction times (1200-1440 min), and high temperature (85-90 °C) with long reaction times (1200-1440 min). The highest concentration of phenolic compounds for pretreatment with citric acid is obtained at high temperatures and long reaction times simultaneously.

3.2.3. DNS (Reducing sugars)

The Pareto chart (Figure 15) shows that all conditions: acid concentration, g/L (A), temperature, °C (B) and time min (C) and their combinations (AB, BC, AC, ABC) are significant as they all have a value greater than 2.31. The most important condition for this parameter is time (C) with a standardized effect of 42. This is confirmed in the main effects plot (Figure 16), where the highest mean reducing sugar concentration (2.2 g/L) is obtained by time (C) followed by acid concentration (A) (2.0 g/L) and temperature (B) (1.6 g/L).

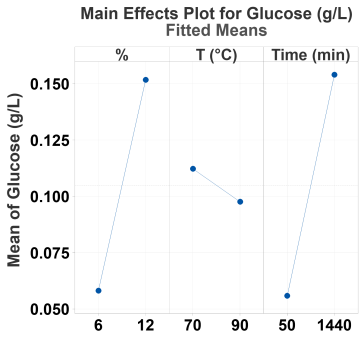

Figure 10. Main effect for glucose in citric acid pretreatment. This figure illustrates how the effect of the acid concentration (%), temperature (°C), and time (min) affects the mean glucose concentration for each condition value with coffee husk pretreatment with citric acid.

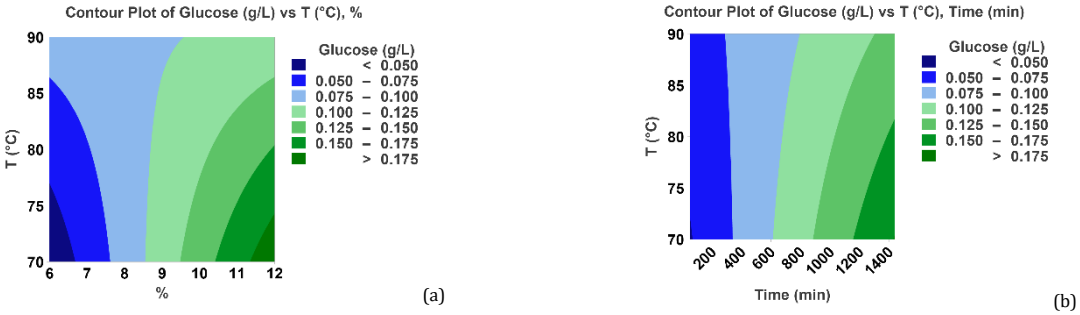

Figure 11. Contour plots for glucose reaction conditions (Temperature, concentration, and time) under citric acid pretreatment. (a) Contour plots temperature *versus* concentration for glucose in citric acid. This figure illustrates the ranges of glucose concentration obtained (0.050-0.175 g/L) and classified by colors. Dark green represents the higher glucose concentration in the temperature (°C) and acid concentration (%) relation. (b) Contour plots temperature *versus* time for glucose in citric acid. This figure illustrates the ranges of glucose concentration obtained (0.050-0.175 g/L) and classified by colors. Dark green represents the higher glucose concentration in the temperature (°C) and time (min) relation.

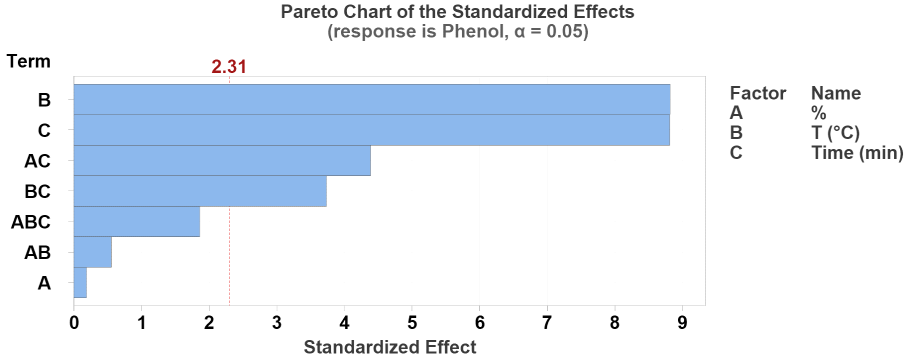

Figure 12. Pareto chart for phenol in citric acid pretreatment. This figure illustrates the statistical significance of the acid concentration (%) A, temperature (°C) B, and time (min) C in the phenol production for coffee husk pretreatment with citric acid.

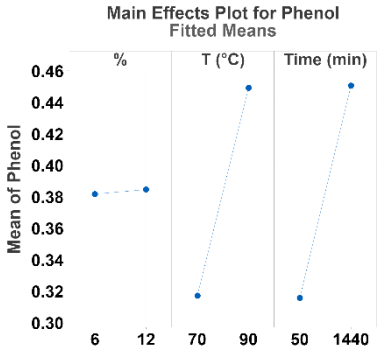

Figure 13. Main effect of phenol in citric acid pretreatment. This figure illustrates how the effect of the acid concentration (%), temperature (°C), and time (min) affects the mean phenol concentration for each condition value for coffee husk pretreatment with citric acid.

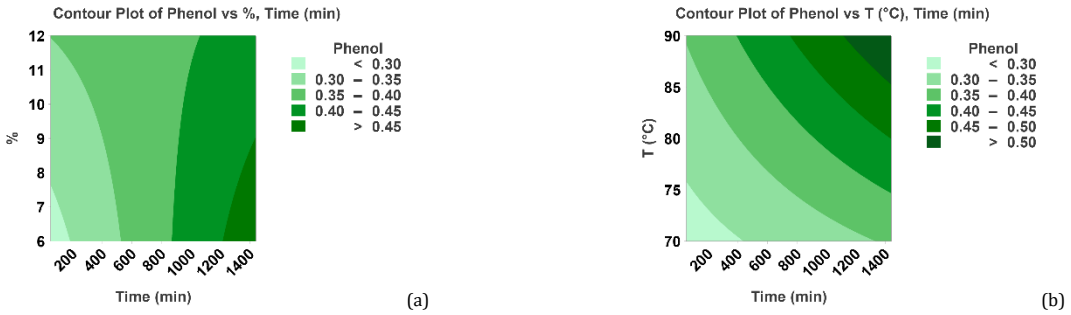

Figure 14. Contour plots for glucose reaction conditions (Temperature, concentration, and time) under citric acid pretreatment. (a) Contour plots concentration *versus* time for phenol in citric acid. This figure illustrates the ranges of phenol concentration (0.30-0.45 g/L) obtained and classified by colors. Dark green represents the higher phenol concentration in the acid concentration (%) and time (min) relation. (b) Contour plots temperature *versus* time for phenol in citric acid. This figure illustrates the ranges of phenol concentration (0.30-0.50 g/L) obtained and classified by colors. Dark green represents the higher phenol concentration in the temperature (°C) and time (min) relation.

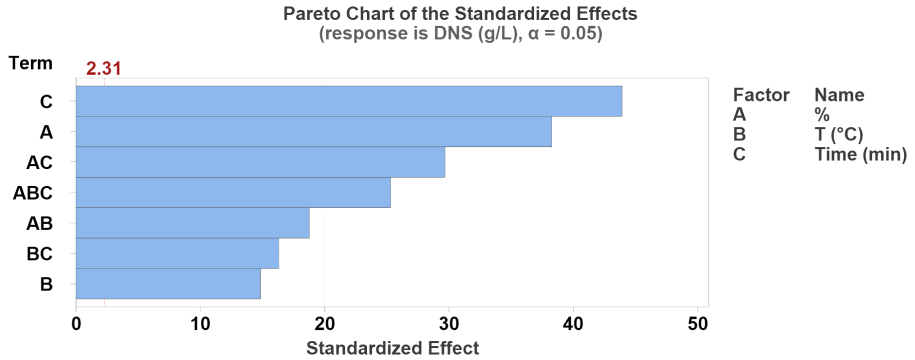

Figure 15. Pareto chart for DNS (reducing sugars) in citric acid pretreatment. This figure illustrates the statistical significance of the acid concentration (%) A, temperature (°C) B, and time (min) C in the reducing sugars production for coffee husk pretreatment with citric acid.

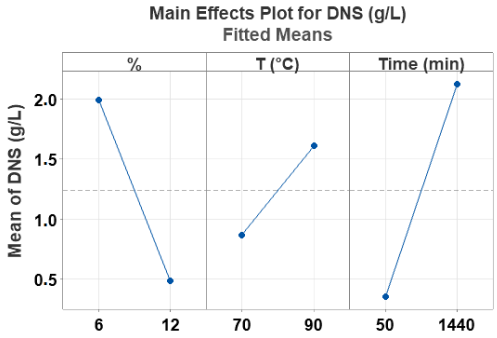

Figure 16. Main effect for DNS (reducing sugars) in citric acid pretreatment. This figure illustrates how the effect of the acid concentration (%), temperature (°C), and time (min) affects the mean DNS (reducing sugars) concentration for each condition value for coffee husk pretreatment with citric acid.

In the main effects graph (Figure 16), it can also be seen that an increase in acid concentration and a decrease in temperature and reaction time have a negative impact on the reduced sugar concentration. The significant relationships found in the Pareto diagram (Figure 15) are the concentration time AC (Figure 17a), the temperature time BC (Figure 17b), and the concentration temperature AB (Figure 17c). The relationships of the contours were plotted, finding the highest concentration of reducing sugars obtained at low acid concentrations (6.0-7.5%) with high temperatures (80-90 °C), high temperatures (85-90 °C) with long reaction time (1200-1440 min), and low acid concentrations (6.0-7.0%) with long reaction time (1200-1440 min). The highest concentration of reducing sugars for pretreatment with citric acid is obtained at high temperatures, low acid concentrations, and long reaction times, simultaneously.

In conclusion for citric acid pretreatment, it can be observed that the conditions can change significantly for the investigated parameters. For example, the highest concentration of glucose (0.306 g/L) requires a low temperature (70 °C), but the highest concentration of reducing sugars (4.986 g/L) requires a high temperature (90 °C). However, among the three parameters analysed, time is the most significant parameter. Longer reaction times when citric acid is used in the pretreatment of coffee husk can have a better effect on the evaluated results.

For sulfuric acid, the highest concentration of glucose (0.831 g/L) and reducing sugars (10.988 g/L) were found in the autoclave (Table 3) and for phenol (0.483 g/L) was found in the proposed methodology (Table 1, 90 °C and 1440 min). However, the difference between the phenol concentration between the autoclave (0.483 g/L) and the optimal results (0.425 g/L) is negligible (10%) and therefore the autoclaving methodology was chosen as the best procedure to pre-treat the coffee husk with sulfuric acid.

Table 3. Results for sulfuric acid at 3 and 6% and citric acid at 6 and 12% under autoclaving conditions (60 min, 121 °C, and 15 psi).

Acid	DNS (g/L)	Glucose (g/L)	Phenol (g/L)
Sulfuric acid 3%	10.988	0.831	0.425
Sulfuric acid 6%	8.626	0.300	0.425
Citric acid 6%	5.368	0.065	0.291
Citric acid 12%	1.183	0.103	0.383

Table 4. Conditions required to achieve the highest concentration of the parameters evaluated under citric acid pretreatment.

Acid	Highest concentration (g/L)	Acid concentration (%)	Conditions
Glucose	0.306	12	Autoclave (60 min, 121 °C, and 15 psi).
Phenol	0.587	6	Methodology proposed (1440 min, 70 °C)
DNS	4.985	6	Autoclave (60 min, 121 °C, and 15 psi).

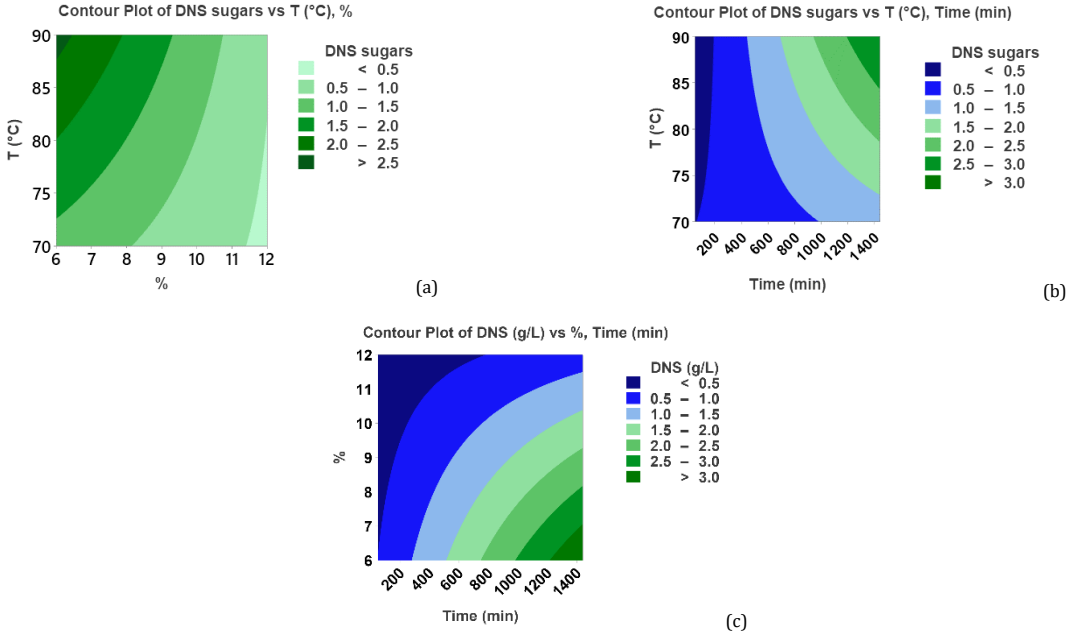

Figure 17. Contour plots for DNS reaction conditions (Temperature, concentration, and time) under citric acid. (a) Contour plots temperature *versus* concentration for DNS (reducing sugars) in citric acid pretreatment. This figure illustrates the ranges of DNS (reducing sugars) concentration (0.5-2.5 g/L) obtained and classified by colors. Dark green represents the higher DNS (reducing sugars) concentration in the temperature (°C) and acid concentration (%) relation. (b) Contour plots temperature *versus* time for DNS (reducing sugars) in citric acid. This figure illustrates the ranges of DNS (reducing sugars) concentration (0.5-3 g/L) obtained and classified by colors. Dark green represents the higher DNS (reducing sugars) concentration in the temperature (°C) and time (min) relation. (c) Contour plots concentration vs time for DNS (reducing sugars) in citric acid. This figure illustrates the ranges of DNS (reducing sugars) concentration (0.5-3 g/L) obtained and classified by colors. Dark green represents the higher DNS (reducing sugars) concentration in the acid concentration (%) and time (min) relation.

Urbaneja *et al.* [15] found the same results, where coffee husk was pretreated with sulfuric acid in an autoclave (121 °C, 60 min), and the best glucose result (6.310 g/L) was found at 2% of H_2SO_4, and Morales-Martinez *et al.* [16] found that a concentration of H_2SO_4 between 3.6 and 4.38% v/v and a reaction time between 40 and 51 min in autoclave were the optimal conditions for hemicellulose (53.62%).

Kefale *et a*l. [17] described that a high acid concentration in pretreatment using autoclave methodology gave less ethanol production and required shorter reaction times to avoid the degradation of reducing sugars into furfurals and leuvinic acid. Therefore, it can be concluded that the best concentration to pretreat the coffee husk with sulfuric acid is 3% under autoclaving conditions.

The results presented for citric acid show that, to have the best results for the acid pretreatment, different conditions must be taken under consideration because there is a low uniformity in the results (Table 4).

According to Bukhari *et al.* [18], the best performance for citric acid used to recover xylose (61.2%) was obtained at 5%, 120 °C and a reaction time of 60 min for the pretreatment of oil palm trunk biomass acid for the production of bioethanol. Sahu and Pramanik [19], analyzed cotton gin that was exposed to high concentrations of acetic acid, high temperature, and long reaction time (150 °C, 60 min, and 700 mM concentration) giving, as a decrease in sugar yield. Gomes *et al.* [20], evaluating acid pretreatment using only citric acid in sugarcane bagasse, found that the lowest yield values (83-84%) of acid pretreatment were obtained at the highest concentrations of citric acid (10%) and longer reaction times (> 60 min).

Following the results obtained and the results discussed above, it was decided to use citric acid at 6% in the autoclave as organic acid pretreatment. Differences in phenol results below 6 % (0.291 g/L) and 12 % (0.383 g/L) are not so significant (20%), However, the reducing sugars (DNS) are almost four times higher in 6% citric acid compared to 12% citric acid (Table 3).

3.3. Enzymatic hydrolysis

To compare which of the acids, 3% sulfuric or 6% citric acid, provides better conditions for enzymatic hydrolysis, the previously pretreated coffee husk was used in a reaction with commercially available *Trichoderma reesei* cellulase (Sigma-Aldrich). The preparation is a blend of cellulases well-known and most used enzymes for biofuel production due to its great performance in the degradation of lignocellulosic material [21]. However, one of the main drawbacks of this enzyme is the deficiencies in β-glucosidase and cellobiohydrolase.

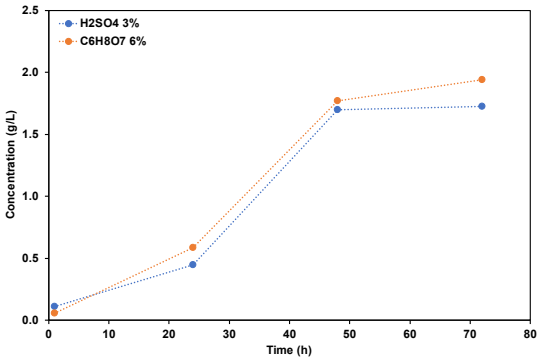

Figure 18. Glucose production during enzymatic hydrolysis of coffee husk pretreated with 3% of sulfuric acid or 6% of citric acid within 72 hours. The condition of enzymatic reaction of pretreated coffee husk was 50 °C, pH = 5.0.

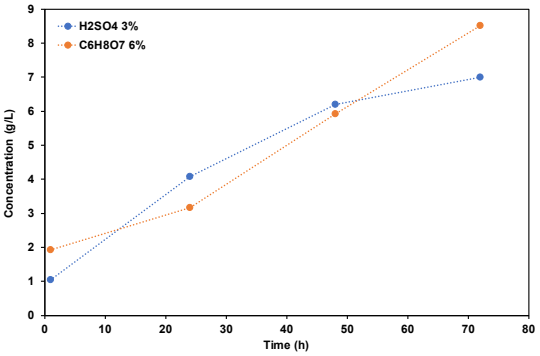

Figure 19. Reducing sugars production during enzymatic hydrolysis of coffee husk pre-treated with 3% of sulfuric acid or 6% of citric acid within 72 hours. The condition of the enzymatic reaction of pretreated coffee husk was 50 °C, pH = 5.0.

For the enzyme, cellobiohydrolase liberates cellobiose molecules from the reducing and nonreducing ends of a cellulose chain, and the *β*-glucosidase hydrolyzes cellobiose into glucose [22].

A glucose test and DNS procedure were applied to quantify the concentration of glucose and reducing sugars obtained during 72 hours of enzymatic hydrolysis. The reaction conditions were 2 g of coffee husk, 40 mL of 0.1 M citrate buffer, and 1 mL of *Trichoderma reesei* cellulase.

Figures 18 and 19 present the glucose and reduced sugar production against time. The citric acid pretreatment presents a higher concentration in both parameters and, as a result, leads to a higher yield for enzymatic digestion and reducing sugars. In Figure 18, it can be analyzed that both acids show the same behavior over time; however, after 10 hours, the citric acid surpasses the sulfuric acid in glucose production, and this difference increases at approximately 47 hours, which is the breaking point. In Figure 19, it can be observed that the breaking point between the acids is at 50 hours where the production of reducing sugars increases for citric acid and passes through sulfuric acid, which shows a more stable behaviour since this point with no increase.

As was mentioned before, citric acid is presented as a viable option for further investigation due to the advantages it presents (lower toxicity and more environmentally friendly compared to sulfuric acid), and because, as was proven with the results, it presents better performance than sulfuric acid. As was exposed by Gomes *et al.* [20], production costs can be reduced by increasing the percentage of citric acid and decreasing the reaction time.

In this investigation, the highest glucose yield (3.89 %) was found for pretreated coffee husk with 6% citric acid for 72 h (Figure 18). MacAskill *et al.* [23] found a similar result for softwood steam pretreatment using citric acid as a catalyzer at 215 °C for 2 min, finding a glucose yield of 2.60% after enzymatic hydrolysis (CTec2, 50 °C and 72 h).

However, the result is low compared to the results found by Sahu and Pramanik [19], where 84% glucose production was found for enzyme hydrolysis (Enzyme cocktail, 50 °C, 150 rpm, 40 h) of cotton gin using 500 mM maleic acid at 150 °C for 45 minutes as a pretreatment; by Bukhari *et al.* [18] where 34.8% glucose production was found for enzymatic hydrolysis (Cellic CTec2-Novozymes, 155 rpm, 50 °C, 48 h) of oil palm trunk pretreated with 5% citric acid for 120 min at 120 °C; and by Morales-Martinez *et al.* [16] where coffee husk was pretreated with 5.68% sulfuric acid for 45 min at 121 °C and peroxide-alkaline using 8% H_2O_2 for 35.75 h and 9.62:1 v/w obtaining 69.35% of glucose production after enzymatic hydrolysis (Cellic CTec3-Novozymes, 200 rpm, 50 °C, 72 h).

Sahu and Pramanik [19] used a *β*-glucosidase enzyme obtained from *Aspergillus niger* and *Trichoderma reesei* cellulase, solving the problem of the enzyme with the deficiency in *β*-glucosidase, therefore the low performance with regard to enzyme digestibility in this research could be due to the lack of this compound that must be considered in future research. In the case of Morales-Martinez *et al.* [16] and Bukhari *et al.* [18], they both used Cellic Novozymes, which might be a better option for biofuel production requiring less preparation to have a better performance than only using *Trichoderma reesei* for enzymatic hydrolysis.

The highest yield of reducing sugars of 17.02% is found at 72 h and 6% citric acid (Figure 19). This result is better than the results obtained by Woiciechowski *et al.* [24] where the coffee husk was hydrolysed with 1 N HCl at 120 °C for 60 min giving 11.24% reducing sugars; by Nava-Valente *et al.* [25] where the coffee husk was pretreated with 9% acetic acid for 60 min at 25 °C and hydrolysed using an enzyme cocktail at 50 °C, 41 min, liquid-solid relation of 5 mL/g giving as a result 10.2% reducing

sugars yield; and the results encountered by Castro Ferro [9] using subcritical hydrolysis at 250 °C, 30 min and 2000 psi finding a reducing sugars yield of 9.15%.

However, the rate is low compared to the results found by Silva *et al*. [26] where the coffee husk was exposed to liquid hot water pretreatment and hydrolysed using an enzyme cocktail (Cellic CTec2® and Cellic HTec2®) that gives 69.1% reducing sugar yield; and the results found by Menezes *et al*. [27] where the coffee husk was pretreated with calcium hydroxide at 121 °C for 30 min and hydrolysed with Celluclast 1.5L (Novozymes, Brazil) giving 38.43% reducing sugar yield.

The highest result found for sugar reduction (17.02%) in this research is compared with the highest yield obtained in the literature (69.01%) for the hydrolysis of coffee husk using citric acid in the pretreatment and shows a difference of 40%. Better conditions must be investigated to give as a result a higher amount of reducing sugars for the fermentation process and increase the amount of ethanol produced. However, the results show an improvement compared to the previous results found by the author of this article (9.15%), which confirms that more studies must be carried out until a higher conversion is achieved and determine the factors that could be used in the design of a pilot plant; one of this study could focus on a careful analysis of the enzyme *Trichoderma reesei* used and ways to improve enzyme performance.

4. Conclusion

The factorial design evaluated the concentration of reducing sugar (DNS), glucose, and phenol in the pretreatment of the coffee husk. Optimal conditions for citric acid pretreatment were obtained at 6%, 121 °C, 15 psi, and 60 min, and for sulfuric acid pretreatment at 3%, 121 °C, 15 psi and 60 min. The latter enzymatic hydrolysis was carried out in a batch process at 72 h at 50 °C and 150 rpm, using 1 mL of enzyme for the suspension of coffee husk 5% w/v in the 0.1 M citrate buffer relationship. The best result obtained was 17.017% for reducing sugars and 3.882% for glucose yield in pretreated coffee husk with 6% citric acid. These results show that alternative methods can be used for acid hydrolysis, avoiding the use of corrosive and dangerous acids such as sulfuric acid and reducing the cost of pretreatment (neutralisation and disposal), which is one of the biggest problems in second-generation biofuels.

The improvement in reducing sugar yield could be achieved by using an enzyme cocktail mixing *Trichoderma reesei* with another enzyme that could be able to cover deficiencies in *β*-glucosidase or by testing another type of enzyme available on the market.

Second-generation biofuels are presented as a viable opportunity for the replacement of conventional fuels, diminishhing the environmental impact produced by the extraction and giving extra value to the crop wastes, contributing to the circular economy.

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Ethical approval: All ethical guidelines have been adhered to.
Sample availability: Samples of the compounds are available from the author.

CRediT authorship contribution statement

Conceptualization: Nataly Alejandra Castro-Ferro, Halina Maniak; Methodology: Nataly Alejandra Castro-Ferro, Halina Maniak; Software: Nataly Alejandra Castro-Ferro Validation: Nataly Alejandra Castro-Ferro, Halina Maniak; Formal Analysis: Nataly Alejandra Castro-Ferro; Investigation: Nataly Alejandra Castro-Ferro; Resources: Nataly Alejandra Castro-Ferro, Halina Maniak; Data Curation: Nataly Alejandra Castro-Ferro; Writing - Original Draft: Nataly Alejandra Castro-Ferro; Writing - Review and Editing: Nataly Alejandra Castro-Ferro, Halina Maniak; Visualization: Nataly Alejandra Castro-Ferro; Funding acquisition: Nataly Alejandra Castro-Ferro, Halina Maniak; Supervision: Halina Maniak; Project Administration Nataly Alejandra Castro-Ferro, Halina Maniak.

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