# Optimization of the Autoclave-Assisted Alkaline Delignification of Cocoa (*Theobroma cacao*) Pod Husks Using KOH to Maximize Reducing Sugars

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Cocoa pod husks are a type of biomass that is still poorly explored. This work was carried out as part of the optimization of the delignification process for this residue with potassium hydroxide, to maximize the reducing sugars content. Screening for potentially influencing factors showed that the biomass to solvent ratio and the temperature had the greatest effect on the delignification process. Optimization of these factors using a composite central plan revealed that the quadratic model was the most suitable for predicting the rate of delignification. The predicted $R^2$ (0.815) was in good agreement with the adjusted $R^2$ (0.906). The correlation coefficient ($R^2$ = 0.945) between the predicted and experimental results confirmed the fit of the model. The optimal conditions were a biomass to solvent ratio of 9.14% and a temperature of 128 °C, which resulted in a maximum degree of delignification of 93.9%, with 80% of the solids recovered. This study found that the removal of extractables before the pretreatment considerably improved the delignification of cocoa pod husks and the production of reducing sugars, which increased from 3.15 ± 0.006 mg/mL to 5.33 ± 0.143 mg/mL. Scanning electron microscopy and X-ray diffraction confirmed physicochemical changes in the biomass after pretreatment.

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## INTRODUCTION

Due to growing concerns about the environment, climate change, and increasingly scarce natural resources, efforts have recently been made to produce chemicals and materials from renewable biomass (Lee *et al.* 2011). Lignocellulosic biomass, which is a renewable and abundant resource, can be envisioned as the only sustainable resource for the production of chemicals (Mansur *et al.* 2014). In this context, several studies have focused on the use of lignocellulosic residues as raw materials, thanks to their availability as organic residues and to the fact that they are not fully exploited in current agro-industrial production systems (Bensah and Mensah 2013; Sharma *et al.* 2013; Bensah and Mensah

2018; Ghazanfar *et al.* 2018; Júnior *et al.* 2020).

The cocoa pod husk (CPH) is a carbohydrate-rich lignocellulosic source (43.9% to 45.2%) (Samah *et al.* 2011). It is highly abundant and available in West African countries, *e.g.*, Côte d'Ivoire, which produced 2.15 million tons of cocoa beans in 2019, or 42% of the total world production (ICCO 2019; Statista 2019). At the same time, the production of CPH is estimated at 6.450 million tons, which constitutes more than 75% of the dry weight of the whole fruit (Cruz *et al.* 2012; Campos-Vega *et al.* 2018).

This residue is available and abundant in Côte d'Ivoire. In addition, its valorization potential has been demonstrated (Ouattara *et al.* 2021). However, the carbohydrates contained in this lignocellulosic biomass are found in a network consolidated by lignin. Therefore, delignification is needed to modify this structure in order to access these carbohydrates (Arenas-Cárdenas *et al.* 2017). In this context, the use of appropriate delignification strategies is necessary and crucial in order to reduce the recalcitrant nature of this biomass (Dahunsi *et al.* 2019a). As such, delignification is a key step in the process of converting lignocellulosic biomass into valuable products. It aims to destructure the lignins and disrupt the crystal structure of the cellulose and hemicellulose to increase the amorphous portion in order to increase the accessibility of the biomass to enzymatic saccharification, or acid hydrolysis, to generate fermentable monosaccharides (Thamsee *et al.* 2019; Dąbkowska-Susfał 2020).

Thus, the optimization of the delignification conditions prior to the lignocellulosic biomass development process should be one of the most important steps in the development of an efficient and economical method (Jung *et al.* 2017). Consequently, several delignification strategies have been explored, *e.g.*, physical, physico-chemical, chemical, biological, and combined delignification. However, the physicochemical and physical delignification processes are inefficient and biological delignification processes are expensive and can be time consuming. As such, chemical delignification processes are the most utilized type, and the alkaline delignification in particular has met with considerable success (Premkumari *et al.* 2019). Delignification using alkalis can efficiently and selectively remove lignins, thus increasing the digestibility of cellulose. In addition, they lead to less formation of inhibitory compounds and less solubilized hemicelluloses as well as being generally carried out at low temperatures (Jönsson and Martín 2016). The primary mechanisms of alkaline delignification include cell wall disruption, carbohydrate solubilization, and the removal of the inhibitors of cellulose accessibility, namely lignins (Júnior *et al.* 2020). Solvation and saponification are the first reactions (Ghazanfar *et al.* 2018).

Several studies have shown the effectiveness of alkaline delignification for the delignification of CPH. Thus, acid and alkaline delignification were performed on this residue, using sulfuric acid ($H_2SO_4$) and sodium hydroxide (NaOH), in order to determine their effects on the increase in the potential of biogas production *via* anaerobic digestion (Ward-Doria *et al.* 2016). The alkali delignification showed the best reductions in lignins, reaching a maximum value of 43.8%. In addition, studies by Dahunsi *et al.* (2019a,b) have produced biogas from the mono-fermentation of cocoa pods pretreated with sulfuric acid and alkaline hydrogen peroxide. The results of these studies further revealed that the use of low-cost mild alkali is more effective in solubilizing lignins and further improving biogas yield. However, the authors did not find any optimization studies that looked at the delignification of cocoa pod husk with potassium hydroxide (KOH), which is an alkali that could potentially pretreat cocoa pod husks. Potassium hydroxide has a very strong reactivity to carbon nanofibers and carbon nanostructures as well as a great capacity for

the deacetylation of biomass (Paixão *et al.* 2016). In addition, the resulting filtrate could be efficiently reused for further delignification and could also be used to produce potassium fertilizer (Xie *et al.* 2018). In this context, other biomasses have been pretreated using this alkali and evaluated to produce bioproducts. Thus, the delignification of sugarcane bagasse with potassium hydroxide has been performed in order to improve enzymatic hydrolysis (Paixão *et al.* 2016). A mild KOH and glycerol delignification was applied to four West African biomasses (bamboo, rubber, elephant grass, and Siam grass) (Bensah *et al.* 2019). In addition, a comparison of the NaOH and KOH delignification using rice straw has been made by some authors. They showed that at an equal hydrolytic enzymatic load, the KOH treatment led to a higher sugar content than the NaOH treatment (Ong *et al.* 2010).

Most studies on the pretreatment of lignocellulosic biomass have been less interested in the removal of extractables before delignification. However, He *et al.* (2008) showed that substrates lacking these substances after pretreatment were favorable in terms of the improvement of biodegradability and biogas production. This study focused primarily on the removal of these extractables before the delignification process, in order to dissolve waxes, fats, and other impurities. Subsequently, the factors likely to influence the pretreatment of this residue with potassium hydroxide were examined, followed by the optimization of the most influential factors using a central composite design (CCD) based on a response surface methodology (RSM). The optimal conditions from this study will be applied to produce reducing sugars by being used as inputs in the bioproducts production.

## EXPERIMENTAL

### Vegetal Material

The plant material exhibited in Fig. 1 consists primarily of cocoa pods husks from the cocoa harvest in the Soubré region (Southwest Ivory Coast).


**Fig. 1.** Cocoa pod husk: Fresh (A); dry (B); and powder (C)

### Chemicals and Solvents

The chemicals and solvents were of analytical grade.

### Methods

*Harvesting the material*

The ripe cocoa pods husks were harvested from a plantation in the village of *Balodougou* (Soubré). This plantation is a high productivity cocoa plantation, in the region of Nawa (South-West, Ivory Coast) between latitudes 5°47'22.14348″N and 5°50´1.1742″N and longitudes 6°32´50.60112″W and 6°35´38.16492″W. The selected fruits were sorted and then dry cleaned to remove impurities, *e.g.*, dust. Each pod was cut

into two parts to remove the pulpy seeds and mucilage. The cocoa pods husks were then washed thoroughly with water several times to remove the residual mucilage, then roughly chopped using a stainless-steel knife.

*Drying and grinding the material*

The fresh CPH were cut into small pieces, dried in the sun for 48 h, and then further dried at a temperature of 60 °C in a hot air dryer (an electric hot air food dryer) for 24 h. The cocoa pods husks were crushed into finer particles with a laboratory hammer mill (*AR 108B*) and then sieved through a screen fitted with a 250 µm sieve. Then, the CPH particles were packed in polyethylene containers to prevent moisture uptake before being stored at room temperature for later analysis.

*Extractables and lignocellulosic content*

The extractables were removed according to the procedure outlined by Poursat (2015). The amount of lignins insoluble in sulfuric acid was determined via gravimetry following the laboratory analysis procedure (LAP) adopted by the National Renewable Energy Laboratory (NREL) (Sluiter *et al.* 2012). The holocellulose content was analyzed *via* by the chlorite method (Boudjema 2016). The cellulose was isolated from holocellulose after the solubilization of hemicelluloses in a dilute hydroxide solution (Bourahli 2018). The hemicellulose content was obtained by subtraction between the content of celluloses and that of holocelluloses.

*Determination of Total Phenolic Content (TPC)*

To verify the degradation of lignin in the delignified sample, the total phenolic compounds in the filtrate were estimated by the Folin-Ciocalteu method.

To 1 mL of the filtrate resulting from the optimized delignification 9.14% (w/v), 9 mL of distilled water were added and then 30 µL were taken and placed in a test tube. To this test sample, 2.5 mL of Folin-Ciocalteu reagent was added. After mixing, it was left to stand for 10 min, and 2 mL of $Na_2CO_3$ solution (7.5%, w/v) was added for neutralization. The whole was heated for 15 min and then left to stand for 2 h at a temperature of 30 °C. Finally, the absorption at 765 nm was measured with a UV-vis spectrophotometer.

*Estimation of reducing sugars*

The amount of reducing sugars released were estimated using the dinitrosalicylic acid (3,5-DNS) method outlined in Miller (1959) using a UV-visible spectrophotometer (UV/VIS Jasco V-530).

*Scanning electron microscopy (SEM) analysis*

The micrographic study of the CPH was carried out using a SH-4000 M (Hirox, Tokyo, Japan) scanning electron microscope at the following conditions: 15 to 20 nm, a magnification of 30x to 60000x, and an acceleration voltage of 5 to 30 kV in 5 steps. In addition, energy-dispersive X-ray spectroscopy (EDS) was performed with a XFlash 6/30 detector (Brucker, Billerica, MA).

*X-ray diffraction and crystallinity*

X-ray diffraction allows an internal assessment of the cellulosic microstructure of a sample in terms of its crystal characteristics. The equipment was operated at a potential of 60 kV and a current of 80 mA at a power of 3 kW, and the samples were scanned over

an angular range of 10° to 90° using a XRD diffractometer (EMMA (enhanced multi-material analysis), IGBT, GBC Scientific Equipment, Braeside, Australia). The crystallinity index was calculated according to Eq. 1,

$$CrI = \frac{I_{002} - I_{am}}{I_{002}} \quad (1)$$

where CrI is the cellulose crystallinity index (%), $I_{002}$ is the intensity of the crystalline part of cellulose ($2\theta$ = 22.10° and 22.32°), and $I_{am}$ is the intensity of the amorphous part of cellulose ($2\theta$ of between 18.30° and 18.86°) (Segal *et al.* 1959).

## Optimization of the Thermochemical Pretreatment

The pretreatment of the cocoa pod husks was carried out with KOH, after removing the extractables in an alternating bath of ethanol and water.

*Factor screening plan*

Plackett and Burman screening experimental designs estimate the primary effects or 'weights' of k factors on a given property (response) to distinguish the truly influential factors. These designs use experimental orthogonal Hadamard matrices made up of low level (-1) and high level (+1) entries (Rais *et al.* 2011). The experiments number (*N*) that composes these matrices is always a multiple of 4. These matrices are used to separate the factors according to their influence on the response. Hadamard matrices allow the calculating of the effects of *k* factors, with *k* less than or equal to *N*-1. It is a fractional factorial design (Sivamani and Baskar 2014) based on a first order linear regression model as follows :

$$y = b_0 + \sum b_i x_i \quad (2)$$

The interception coefficient $b_0$ is the main effect and is calculated as follows :

$$b_0 = \frac{\sum_{i=1}^{N} Y_i}{N} \quad (3)$$

The effects ($b_i$) of the factors were determined following Eq. 4, and was analyzed using NemrodW (2000 version, NemrodW, Peypin, France).

$$b_i = \frac{(\sum Y^+ - \sum Y^-)}{N} \quad (4)$$

The symbols $Y^+$ and $Y^-$ represent responses of trials in which the independent factors are at high and low levels, respectively, and N is the number of trials. The positive coefficient increases the response while the response decreases for the negative coefficient. On the classical statistical significance tests (Student's t test), factors with a high coefficient value are considered significant whether they are positive or negative. Therefore, a Plackett and Burman type screening scheme was used to determine the influence of five factors, *i.e.*, the alkali concentration (%), contact time (*h*) at room temperature, biomass to solvent ratio (%), autoclaving time (min), and reaction temperature (°C).

*Delignification process*

The main factors involved in the delignification process and their domains of variation were chosen on the basis of previous studies (Nazir *et al.* 2016; Ghazanfar *et al.* 2018; Premkumari *et al.* 2019). A mass of cocoa pod husks was placed in a 250 mL flask, containing 100 mL of KOH with a concentration of between 1 and 5% w/v and according to the biomass to solvent ratio varying from 5 to 10% w/v, given by the design of the experimental design. The mixture was subjected to room temperature for a period of between 4 h and 8 h, then at 30 °C in a water bath or at 130 °C in an autoclave (Digital

vertical autoclave LX-C, *HEFEI Huatai medical equipment co. LTD),* for a time ranging from 15 to 60 min. Tables 1 and 2 exhibits the experimental field and the experiment matrix, respectively.

**Table 1.** Experimental Area of the Screening Plan

| Variables | Level (-1) | Level (+1) |
|---|---|---|
| Concentration (KOH) (%) (A) | 1 | 5 |
| Biomass to solvent ratio (%) (B) | 5 | 10 |
| Contact time (h) (C) | 4 | 7 |
| Heating time (min) (D) | 15 | 60 |
| Temperature (C) (A) | 30 | 130 |

**Table 2.** Experimental Matrix of the Screening Plan

| Assay | KOH Concentration (%) | Biomass to Solvent Ratio (%) | Contact Time (h) | Heating Time (min) | Temperature (°C) |
|---|---|---|---|---|---|
| 1 | 1 | 1 | -1 | -1 | -1 |
| 2 | -1 | 1 | 1 | 1 | -1 |
| 3 | -1 | -1 | 1 | -1 | 1 |
| 4 | 1 | -1 | 1 | 1 | -1 |
| 5 | -1 | 1 | -1 | 1 | 1 |
| 6 | 1 | -1 | -1 | 1 | 1 |
| 7 | 1 | 1 | 1 | -1 | 1 |
| 8 | -1 | -1 | -1 | -1 | -1 |

After this operation, the delignified solid substrate was separated from the hydrolysate obtained by filtration through Whatman No.1 paper. This substrate was washed with distilled water until the pH became 7. It was then dried in an oven at a temperature of 105 °C for 6 h (Nazir *et al.* 2016). This substrate was then analyzed to assess the degree of delignification.

*Degree of delignification and solid recovery*

The degree of delignification was defined as the weight fraction of lignins removed during the pretreatment and was calculated according to Eq. 5,

$$\mathrm{D}(\%) = \left(\frac{1 - L \times m_{\mathrm{pre}}}{L_0 \times m_{\mathrm{s}}}\right) \times 100 \quad (5)$$

where $L$ is the lignin content in the pretreated biomass (%), $L_0$ is the lignin content in the raw biomass (%), $m_{\mathrm{pre}}$ is the dry mass of biomass after pretreatment, and $m_{\mathrm{s}}$ is the dry mass of the raw (untreated) biomass used (Dąbkowska-Susfał 2020). Factors considered to be influential as a result of the screening test were subjected to optimization using a central composite design based on the response surface methodology (RSM).

The solid recovery was obtained using alternate ethanol-water extraction (Premkumari *et al.* 2019).

*Central composite design (CCD)*

After the screening step, the influential factors were subjected to a central composite design (CCD) based on the response surface methodology (RSM) to determine the optimal conditions for delignification. Design Expert software (DEMO version 11,

Stat-Ease, Minneapolis, MN), which is an experimental design tool, was used for this purpose. This methodology induces the formulation of a second order polynomial, which describes the process, as shown in Eq. 6,

$$Y = b_0 + \sum b_i X_i + \sum\sum b_{ij} X_i X_j + \sum b_{ii} X_i^2 \quad (6)$$

where $b_0$ is the means effect, $b_i$ is the main effects, $b_{ij}$ is the interaction effects of order 2, $b_{ii}$ is the quadratic effects, and $X_i$ and $X_j$ are the coded variables. This equation was shown to be effective in experimental models and will help to study the linear, quadratic, cubic, and interaction effects of factors on the degree of delignification.

*Model fit and statistical analysis*

Design Expert software was used to generate the experimental data and develop the regression model. The goodness of fit of the regression model was expressed by the regression coefficients ($R^2$), and statistical significance was determined by the Fisher's, F-value, *p*-value, and t-test (ANOVA). The response area and the contour lines were both evaluated to estimate the model as well as to determine the optimal levels to fit the second order polynomial equation. Central composite design (CCD) was used to optimize the delignification of cocoa pod husks.

*Model validation*

Model validation was based on the analysis of the calculated errors and the coefficient of determination ($R^2$) between the results predicted by the model and those obtained experimentally.

*Choice of acid for hydrolysis*

Concentrated HCl and $H_2SO_4$ acids are able to swell and dissolve cellulose (Samah *et al.* 2011), and the acid hydrolysis is much faster than the enzymatic method (Sharmada *et al.* 2016). The problem with enzymatic hydrolysis is the poor accessibility of cellulose, due to the rigid association of cellulose with lignin (Mosier *et al.* 2005; Muharja *et al.* 2018). Indeed, 90% of the yield of monosaccharides could be obtained by using concentrated acid pretreatment of the biomass (Palmqvist and Hahn-Hägerdal 2000).

*Acid hydrolysis process*

3 g of the solid fraction obtained after delignification was subjected to acid hydrolysis (Nazir *et al.* 2016), by placing the sample in a 250 mL Erlenmeyer flask and adding a solution of sulfuric acid ($H_2SO_4$) to it at a level of 3% with the ratio of biomass to solvent (1:10) for 2 h in an autoclave at 110 °C. After this operation, the hydrolysates were filtered through Whatman No.1 paper and then neutralized with a slow addition of NaOH for the determination of the reducing sugars.

## RESULTS AND DISCUSSIONS

### Initial Lignocellulosic Composition of the Cocoa Pod Husks (CPH)

Table 3 exbibits the lignocellulosic composition of CPH. The CPH in the present study were high in carbohydrates and could be alternative candidates to produce platform biomolecules. However, comparing the results of this study with those observed in various studies (Table 3) showed several disparities. These observed differences could be linked to

geographical factors, the location of the materials collected, different analysis methods, the variety of biomass, differences in the solvents used, or different collection periods as well as to different climatic and storage conditions (Ouattara *et al.* 2021). In addition, the maturation of a living plant material tends to increase the lignin and lignocellulose contents (Thamsee *et al.* 2019). The values obtained for all lignocellulosic fibers during this study were close to those of Sandesh *et al.* (2020). However, these values are much higher than those obtained in other studies (Marsiglia *et al.* 2016). The cellulose values were close to those generally reported by various literatures (Titiloye *et al.* 2013; Sandesh *et al.* 2020). However, they are lower than those reported in a recent study by Nazir *et al.* (2016). The CPH in the area of this study therefore have enormous potential for recovery. However, due to their relatively high lignin content (25.62% ± 4.32%), adequate pretreatment is necessary for the release of fermentable sugars.

**Table 3.** Lignocellulosic Composition of the Cocoa Pod Husks (CPH) in Addition to the Results of Several Studies

| Lignin (%) | Cellulose (%) | Hemicellulose (%) | Extractables (%) | Reference |
|---|---|---|---|---|
| 24.16 | 28.25 | 16.75 | - | (Sandesh *et al.* 2020) |
| 33.96 | 30.41 | 11.97 | 23.66 ± 1.77 | (Titiloye *et al.* 2013) |
| 18.19 | 23.04 | 38.08 | - | (Asiedu *et al.* 2019) |
| 34.82 | 44.69 | 11.15 | - | (Nazir *et al.* 2016) |
| 25.62 ± 4.32 | 31.68 ± 2.07 | 16.97 ± 2.07 | 21.57 ± 1.04 | This work |

## Optimization of Cocoa Pod Husk (CPH) Delignification

*The screening of factors and estimation and the statistics of the coefficients relating to delignification*

Tables 4 and 5 show the experimental design with the results obtained from the screening plan and the estimation of the factor coefficients, using the NemrodW software, respectively.

The standard deviation of the degree of delignification estimated by the NemrodW software was 1.075. This value validated the chosen experimental field. It therefore appears that the delignification was strongly influenced by the variation of these two factors.

**Table 4.** Experimental Design and Results of the Screening Plan

| Assay | A (%) | B (%) | C (h) | D (min) | E (°C) | Degree of Delignification (*Y*) (%) |
|---|---|---|---|---|---|---|
| 1 | 5 | 10 | 4 | 15 | 30 | 89.87 |
| 2 | 1 | 10 | 7 | 60 | 30 | 90.15 |
| 3 | 1 | 5 | 7 | 15 | 130 | 85.49 |
| 4 | 5 | 5 | 7 | 60 | 30 | 75.75 |
| 5 | 1 | 10 | 4 | 60 | 130 | 93.00 |
| 6 | 5 | 5 | 4 | 60 | 130 | 86.80 |
| 7 | 5 | 10 | 7 | 15 | 130 | 91.35 |
| 8 | 1 | 5 | 4 | 15 | 30 | 81.43 |

The statistical analysis of the coefficients illustrated in Table 5 shows that the coefficients $b_2$ and $b_5$ had sufficient significance to be considered in the optimization process. They reflect the effects of the biomass to solvent ratio and the temperature, respectively.

**Table 5.** Estimated Coefficients for the Screening Plan

| Name | Coefficient | Standard Deviation | Significance |
|---|---|---|---|
| b0 | 86.730 | 1.075 | Significant |
| b1 | -0.788 | 1.075 | No significant |
| b2 | 4.362 | 1.075 | Significant |
| b3 | -1.045 | 1.075 | No significant |
| b4 | -0.305 | 1.075 | No significant |
| b5 | 2.430 | 1.075 | Significant |

These results agreed with the results in Shet *et al.* (2016), which showed that in the optimization of the pretreatment of CPH with sodium carbonate ($Na_2CO_3$), the most significant factor was the biomass to solvent ratio. Given this, this ratio is important in the process of pretreating lignocellulose, since it maintains thermal equilibrium and adjusts the concentration of chemicals and therefore strongly impacts the cost of pretreatment (Xie *et al.* 2018). The graphic representation of the effects of the factors is illustrated by the diagram of the effects indicated in Fig. 2. By considering the confidence interval of the values of the coefficients (delimited by the two vertical dotted lines), one can affirm that at most two factors are active on the degree of delignification of the cocoa pod husk. These are the biomass to solvent ratio and the temperature.

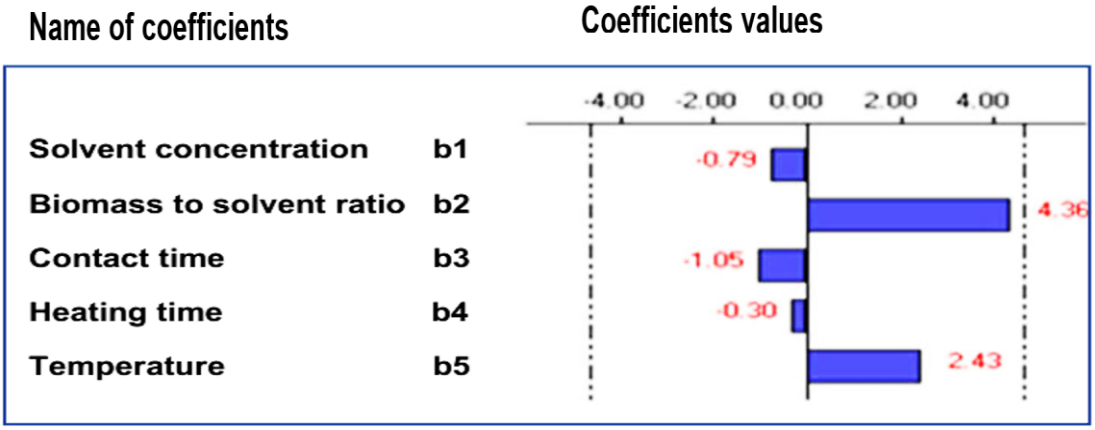


**Fig. 2.** Representation of the effects of factors on the degree of delignification

The observations in this study are consistent with those reported in a recent study by Júnior *et al.* (2020); it was shown that the reaction time has a negative effect on the pretreatment of waste yerba mate (*Ilex paraguariensis*) with KOH. This observation revealed that the change of this variable from the lower level to the higher level contributed to a decrease in the response. Consequently, it was appropriate to study these factors in the region of the lowest level (Júnior *et al.* 2020).

*Analysis of the influence of different factors on the degree of delignification*

In addition, the analysis of the coefficients (Table 5) showed that the delignification was significantly increased if a solution with a biomass to solvent ratio of 10% was treated with 1% KOH for 4 h at room temperature, followed by 15 min of heating to a temperature of 130 °C.

*Experimental design for the optimization of the delignification process*

The most significant factors from the screening plan were subjected to optimization in order to intensify the delignification process. For this purpose, the biomass to solvent ratio and the temperature were considered. These two factors were subjected to optimization in order to develop a correlation related to the degree of delignification of the CPH. This design made it possible to experiment with different combinations of biomass to solvent ratios and temperatures. Table 6 shows the coded and actual values of the variables studied in this design.

**Table 6.** Coded Variables and Actual Levels Used in This Design

| Variables | Symbol Coded | Levels Coded | | | | |
|---|---|---|---|---|---|---|
| | | $-\alpha$ | -1 | 0 | 1 | $+\alpha$ |
| Biomass to solvent ratio (% p/v) | $X_1$ | 3.96 | 5 | 7.5 | 10 | 11.04 |
| Temperature (°C) | $X_2$ | 9.29 | 30 | 80 | 130 | 151.71 |

*Mechanism of delignification and the recovery of solids after the pretreatment of CPH*

Table 7 shows that the degree of delignification ranged from 74.9% to 93.9%, and it continuously increased. While analyzing the results from Table 7, the values obtained were well above the majority of those reported in the literature for various residues, with a maximum value of 67.5% that was obtained for elephant grass with a 1% KOH pretreatment at a temperature of 121 °C for 1 h (Bensah *et al.* 2019). Likewise, values between 49.7% and 87.7% were found for corn straw pretreated with 2% KOH at a temperature of 50 °C (Dąbkowska-Susfał 2020). Table 7 shows that an increase in temperature led to greater delignification and therefore lower solids recovery. This confirms the hypothesis that the alkaline delignification of lignocellulosic residues at high temperatures leads to the solubilization of lignocellulose by breaking the covalent and ester bonds between the constituents. Pretreatments using alkalis at high temperatures causes chemical swelling in the cellulose and induces a saponification reaction, which promotes the cleavage of the ester bonds in the lignocellulosic matrix (Woiciechowski *et al.* 2020). This leads to an increase in the internal surface area, a decrease in the degree of polymerization and crystallinity, as well as a breakdown of the chemical bonds between the lignins and carbohydrates. This results in the destruction of the lignin structure and also leads to an increase in the porosity of the biomass and its accessibility to subsequent hydrolysis (Woiciechowski *et al.* 2020).

Usually, pretreatment causes the solubilization of biomass components in the liquid phase, resulting in a loss of solid phase material. However, the extent of the loss depends on the pretreatment conditions (Dąbkowska-Susfał 2020). The solids recovery ranged from 55.6% to 80.2%, corresponding to tests 8 and 1, respectively. Similar values were obtained by Premkumari *et al.* (2019) with cotton swab. They found solids recovery values between 51.7% and 82.9% for 2% KOH pretreatments at temperatures between 50 and 120 °C.

Lower values were generally associated with higher temperatures under severe pretreatment conditions. This could be attributed to the partial or total solubilization of the lignins. In addition, the degradation of hemicelluloses can also occur due to its amorphous, heterogeneous, and branched structure with little resistance. This makes it more sensitive to solubilization at higher temperatures than semi-crystalline cellulose under alkaline conditions (Xu and Cheng 2011).

**Table 7.** Experimental Matrix and the Delignification Results

| Assay | Biomass to Solvent Ratio (%) ($X1$) | Temperature (°C) ($X_2$) | Degree of Delignification (%) | Solid Recovery (%) |
|---|---|---|---|---|
| 1 | -1 | -1 | 75.6225 | 80.179 |
| 2 | 1 | -1 | 87.9662 | 77.381 |
| 3 | -1 | 1 | 85.7900 | 75.299 |
| 4 | 1 | 1 | 93.8675 | 77.135 |
| 5 | -1.41421 | 0 | 74.9004 | 66.530 |
| 6 | 1.41421 | 0 | 91.1809 | 63.814 |
| 7 | 0 | -1.41421 | 86.2565 | 79.716 |
| 8 | 0 | 1.41421 | 92.5554 | 55.572 |
| 9 | 0 | 0 | 86.2325 | 73.837 |
| 10 | 0 | 0 | 89.8481 | 70.852 |
| 11 | 0 | 0 | 90.7439 | 78.672 |
| 12 | 0 | 0 | 90.7825 | 78.344 |
| 13 | 0 | 0 | 90.7792 | 79.955 |

The results of the delignification process obtained at the end of the pretreatment study were subjected to an analysis of variance (ANOVA) using Design Expert software.

*Choice of the model for optimizing the delignification process*

Table 8 shows that the quadratic model would be the most appropriate for predicting the delignification process of CPH. The predicted $R^2$ of this model (0.815) is in agreement with the adjusted $R^2$ (0.907). This means that more than 90.66% of the observed values could be explained by the model and the rest is the residual. Therefore, this model bodes well for providing a good prediction of the degree of delignification of these residues.

**Table 8.** Summary Statistics of the Model

| Model | Std. Dev. | $R^2$ | Adjusted $R^2$ | Predicted $R^2$ | Df | PRESS | F-value | *p*-value | Suggestion |
|---|---|---|---|---|---|---|---|---|---|
| Linear | 3.310 | 0.742 | 0.690 | 0.529 | 6 | 199.15 | 4.05 | 0.0987 | |
| 2FI | 3.410 | 0.752 | 0.669 | 0.441 | 5 | 236.56 | 4.62 | 0.0817 | |
| **Quadratic** | **1.820** | **0.946** | **0.907** | **0.815** | **3** | **78.18** | **0.655** | **0.621** | **Suggested** |
| Cubic | 1.780 | 0.963 | 0.910 | 0.892 | 1 | 45.88 | 0.088 | 0.782 | Aliased |

*Suggested model equation*

Regression equation generated by the Design Expert software translates, degree of delignification ($Y$) as a function of independent variables ($X_1$ and $X_2$), which correspond to the biomass to solvent ratio and the temperature, respectively and their linear and quadratic interactions, expressed by a second order polynomial, shown in Eq. 7,

$$Y = 31.86872 + 11.06579X_1 + 0.141704X_2 - 0.547396X_{11} \quad (7)$$

*Analysis of variance (ANOVA) for the suggested quadratic model*

According to Table 9, the *p*-value of the model is 0.0003, which is less than 0.0500, with a high F-value (24.29).

In addition, the F-value (0.6547) of the lack of fit implies that it is insignificant. However, a good model must have a significant regression and an insignificant lack of fit (Júnior *et al.* 2020). Moreover, the *p*-values of the terms $X_1$, $X_2$, and $X_{12}$ of the model are all less than 0.0500, also showing their significance at 95%.

**Table 9.** Statistical Analysis of the Suggested Quadratic Model

| Source of Variation | Sum of Squares | Df | Mean Square | F-value | *p*-value | Significance |
|---|---|---|---|---|---|---|
| Model | 400.20 | 5 | 80.04 | 24.29 | 0.0003 | Significant |
| Biomass to solvent ratio ($X_1$) | 235.94 | 1 | 235.94 | 71.60 | 6.35844E-05 | |
| Temperature ($X_2$) | 77.98 | 1 | 77.98 | 23.67 | 0.0018 | |
| $X_1X_2$ | 4.55 | 1 | 4.55 | 1.38 | 0.2784 | |
| $X_1^2$ | 81.42 | 1 | 81.42 | 24.71 | 0.0016 | |
| $X_2^2$ | 0.3959 | 1 | 0.3959 | 0.1202 | 0.7390 | |
| Residual | 23.06 | 7 | 3.29 | - | - | |
| Lack of Fit | 7.60 | 3 | 2.53 | 0.6547 | 0.6209 | Not significant |
| Pure Error | 15.47 | 4 | 3.87 | - | - | |
| Cor Total | 423.26 | 12 | - | - | - | |

*Influence of the independent variables on the degree of delignification*

Response surface methodology can be described as a graphical representation of the equations from the data analysis (Rachmawaty *et al.* 2019). It is very useful for assessing the importance of several factors, especially those that involve complex interactions (Wasli *et al.* 2009). In this context, the optimal levels of the variables were determined by plotting the three-dimensional and two-dimensional surface plots (Fig. 3), which were based on Eq. 7.

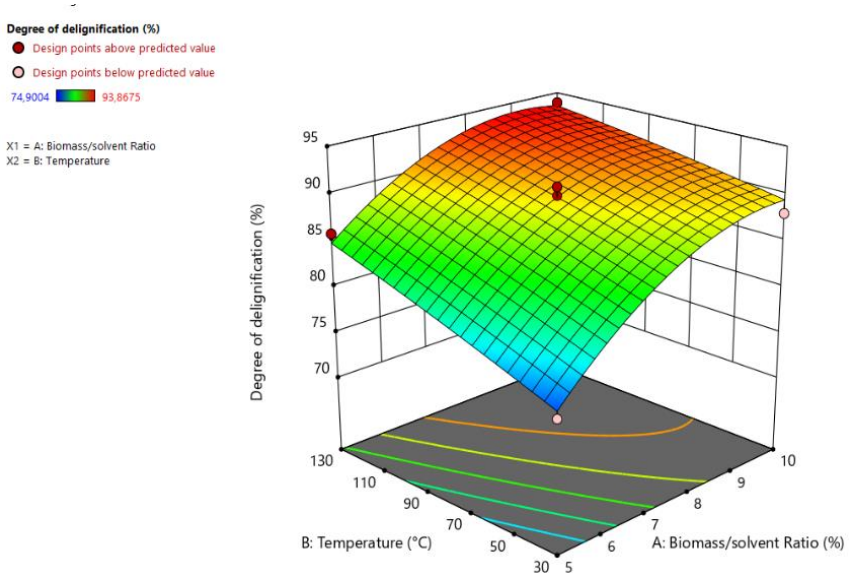


**Fig. 3.** Surface (3D) plot showing the effect of the temperature and biomass to solvent ratio on the degree of delignification of CPH

The degree of delignification shows a quadratic dependence of the biomass to solvent ratio with a high effect of the term $X_1^2$ (p-value less than 0.05). These results were consistent with the results of Alexander *et al.* (2020), who demonstrated the importance of the volume of the solvent. The increase in the degree of delignification as the biomass to solvent ratio increased could be explained by the availability of KOH for pretreatment. Since the KOH concentration in this study was set at 1% with a working volume of 50 mL for all samples, increasing this ratio would thus promote greater delignification until saturation of the substrate-solvent system. This leads to a considerable reduction in the mass transfer rate (Alexander *et al.* 2020). In addition, a similar trend was observed with CPH for production (Shet *et al.* 2018).

An increase in temperature from the range of 30 to 130 °C results in a continual increase in the degree of delignification from 74.9% to 93.87%. This observation was in accordance with the results of Bensah *et al.* (2019) and Souza *et al.* (2020), which have shown that a high temperature leads to greater delignification under alkaline conditions. However, a weak interaction effect between the biomass to solvent ratio and the temperature was observed, with a high *p*-value (0.2784 is greater than 0.05), showing that a simultaneous increase in these two variables would lead to a decrease in the degree of delignification. This was confirmed by the antagonistic effect of the negative coefficient for the $X_1X_2$ interaction term on the response and could be attributed to a limiting effect of the pretreatment reagent relative to the biomass in the reactor.

*Optimal delignification conditions*

In order to optimize responses, a useful approach is the desirability function methodology (Derringer and Suich 1980). This approach is used to optimize responses by inspecting the response model and adjusting the observed responses using a second order equation based on the levels of the independent variables (Shet *et al.* 2018). In this approach, the response $Y_i$ is converted into an individual desirability function ($d_i$). This function varies in the range of 0 to 1, with 0 being a totally undesirable response value and 1 being a perfectly desirable or ideal response value. Based on this, with the constraints

that the pretreatment parameters were within range and that the delignification achieved was maximal, the optimized conditions were a biomass to solvent ratio of 9.14%, with a 1% KOH pretreatment for 4 h at a temperature of 30 °C and autoclaved at a temperature of 128.3 °C for 15 min, with 93.9% of delignification. Furthermore, optimal conditions for CPH delignification, using 5% (w/v) NaOH and a biomass to solvent ratio of 1:10 coupled with microwave heating have been reported (Ahmad *et al.* 2021), reaching 86.6% of delignification. Likewise, the CPH was pretreated with a biomass to solvent ratio of 10% in an aqueous solution of 0.75% w/v NaOH and autoclaved at a temperature of 121°C for 20 min, reaching 43.8% of delignification (Ward-Doria *et al.* 2016). A study by Akhtar *et al.* (2014) showed that a large amount of 71.9% of lignin was removed by microwave assisted alkaline/acid pretreatment compared to conventional heating (34.6%). In a recent study, the orange peel was exposed to a conventional alkaline and ultrasonically assisted pretreatment at room temperature reaching 86% delignification and 92% delignification in 4 h respectively, with a maximum reducing sugars of 1.30 g/L in 6 h of treatment after enzymatic hydrolysis; compared to the conventional method where the sugar concentration was 0.814 g/L in 24 h (Utekar *et al.* 2021). Likewise, the effect of KOH was evaluated on other substrates, for cotton swab (120 °C for 1 h with 3% KOH, giving 68.9%) of delignification (Premkumari *et al.* 2019), corn straw (With conventional heating at 50°C for 24 h with 2% KOH, with 87.7%) of delignification (Dąbkowska-Susfał 2020).

*Validation of the model using the analysis of the residuals and the coefficient* $R^2$

Figure 4A shows the degree of delignification observed ($Y_{exp}$) compared to the predicted value ($Y_{Pred}$). The data predicted by the model were close to those observed. This was shown by the points grouped around the regression line. Moreover, the predicted value ($R^2 = 0.8153$) was in reasonable agreement with the adjusted $R^2$ (0.906).

Figure 4B shows a random distribution of the residual plots of the model without any trend. This indicated good predictions of the maximum response and the fit of the model (Qi *et al.* 2009). It can be deduced that the degree of delignification of CPH using KOH could be obtained thanks to the punctual prediction capacity of the software.

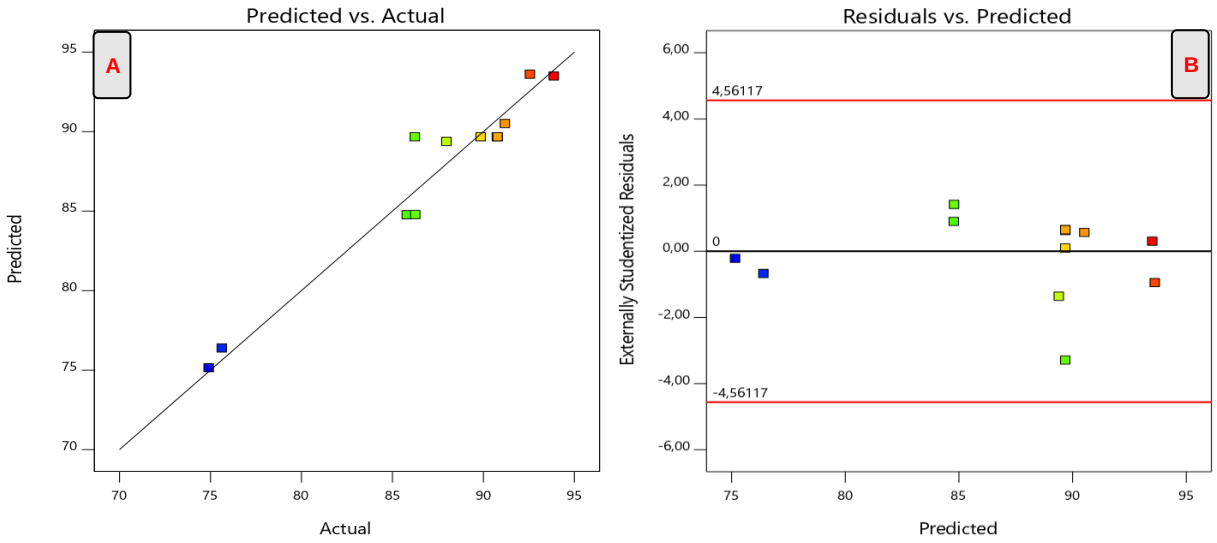


**Fig. 4.** The degree of delignification observed and predicted by the model (A) and the residuals as a function of the predicted response (B)

This hypothesis seems to be confirmed by the calculation of the residual error as well as by the percentage error compared for five points carried out under the optimized conditions (Table 10).

**Table 10.** Results of the Validation Tests

| Assay | Biomass to Solvent Ratio (%) | Temperature (°C) | Experimental Value (%) | Predicted Value (%) | Residual | Error (%) |
|---|---|---|---|---|---|---|
| 1 | 9.143 | 128.300 | 94.262 | 93.936 | 0.326 | 0.346 |
| 2 | 8.939 | 129.200 | 95.017 | 93.884 | 1.133 | 1.192 |
| 3 | 9.281 | 128.750 | 94.866 | 93.906 | 0.960 | 1.012 |
| 4 | 9.258 | 129.000 | 94.902 | 93.899 | 1.003 | 1.057 |
| 5 | 9.357 | 129.200 | 93.916 | 93.886 | 0.030 | 0.032 |

The error percentage ranged from 0.032% to 1.192%, which were all less than 5%. Therefore, the model appears accurate, and the analysis of the response surface methodology can be a useful technique for predicting and optimizing the delignification.

## Estimation of the Composition of the CPH After Delignification Using KOH

Figure 5 shows the changes caused in the lignocellulosic composition of CPH under the impact of KOH. This figure shows a dramatic reduction in lignin content, indicating that KOH had a major effect on breaking down the lignin structure.

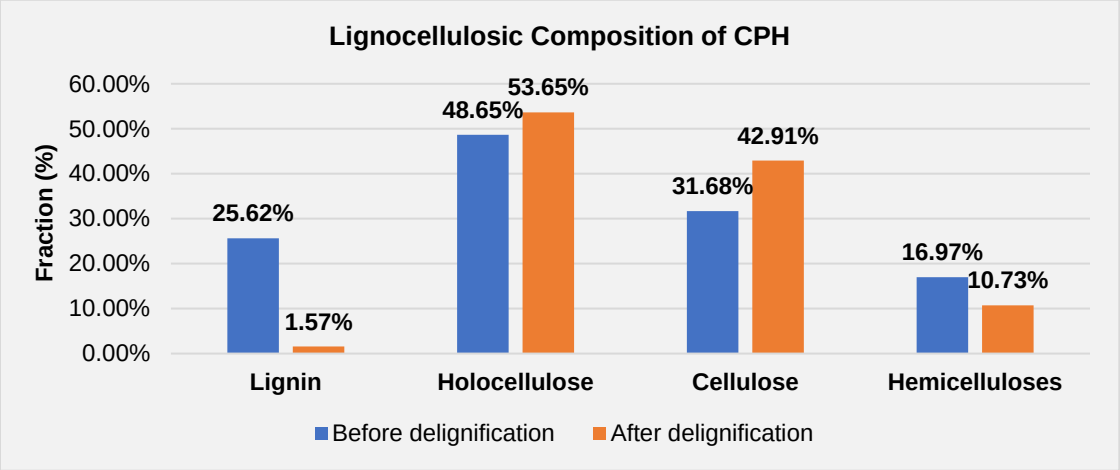


**Fig. 5.** Lignocellulosic composition of CPH before and after delignification

Likewise, an increase in the cellulosic fraction was observed to the detriment of that of hemicelluloses. This could be explained by the fact that at elevated temperature and in an alkaline environment, there is increased dissolution of lignin, hemicelluloses, extractables (Bensah *et al.*2019).

## Estimation of Total Phenolic Content and Reducing Sugars from CPH

Figure 6 shows the contents of total phenolic compounds resulting from delignification, and the contents of reducing sugars obtained after acid hydrolysis (3%, v/v, $H_2SO_4$, autoclave, 110 °C, 2h), of the raw and extracted samples. A significant reduction (55.6%) in the levels of total phenolics was observed (116.55 ± 0.01 to 51.75 ± 0.007 mg/mL).

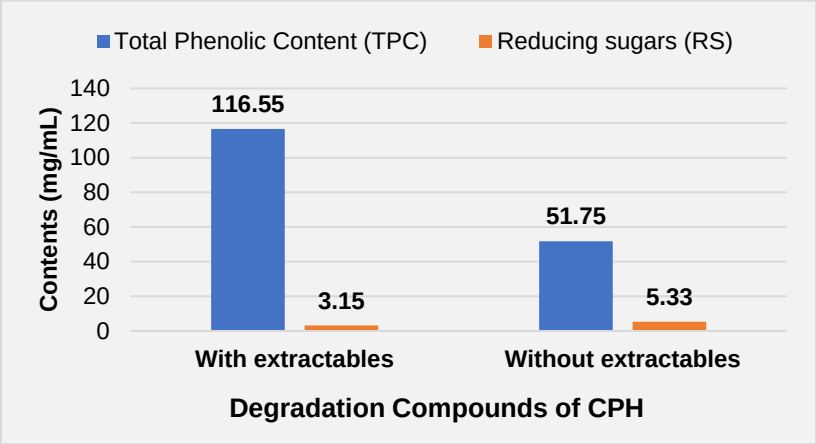


**Fig. 6.** Effect of extractables on the degradation products of CPH

These values suggest that some of the lignins may have been solubilized into phenolic degradation components (Asghar 2017) by the water-ethanol mixture during the removal of extractables. Therefore, a strong effect of KOH and an improvement in the subsequent acid hydrolysis was found. This improved the content of reducing sugars to 5.33 ± 0.143 mg / mL. Furthermore, Shet *et al.* (2018) reported lower values (4.09 mg/mL) of reducing sugars from 8.36% w/v CPH hydrolyzate, using 3.6 N HCl at room temperature.

## Effect of the KOH Pretreatment on the Morphology and Crystallinity of CPH

*Morphological study of cocoa pod husk (CPH)*

Micrographs of the non-pretreated (B) and pretreated (P) CPH are shown in Fig. 7.

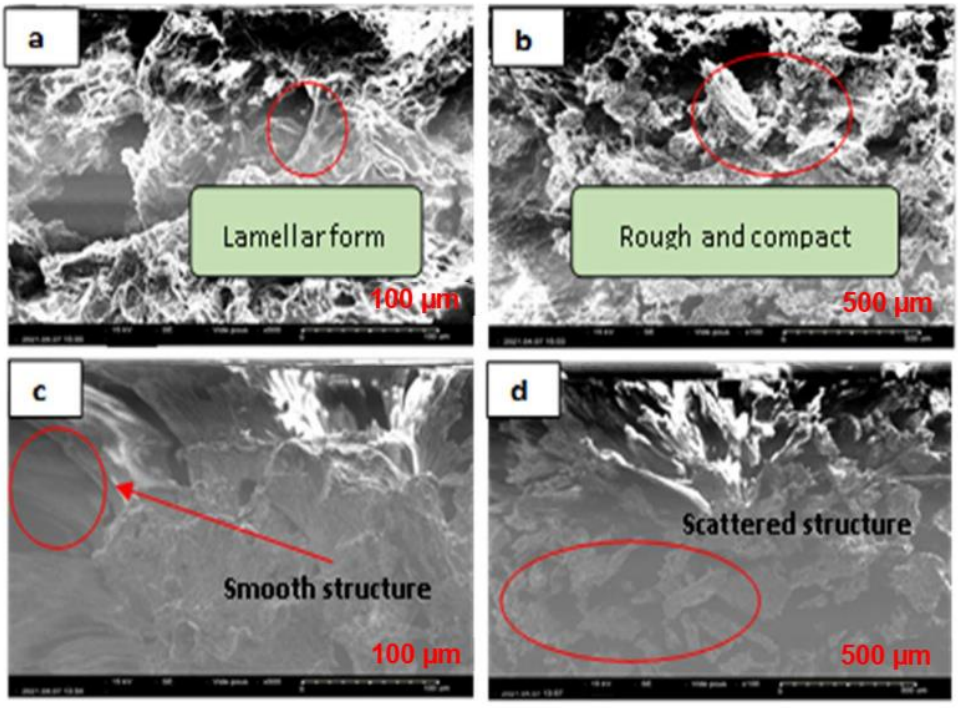


**Fig. 7.** Electron micrograph of the raw (a and b) and pretreated (c and d) CPH

Figure 7a and 7b present a rough and compact structure, composed of particles in lamellar form. The individual lignocellulosic structure of the crude CPH is not visible. This could be due to the presence of extractables, *e.g.*, waxes, fats, pectins, polyphenols, and other impurities (Daud *et al.* 2013). However, the removal of these extractables (21.57 ± 1.04 %) using the alternate ethanol-water treatment allowed better exposure of the lignins to alkaline attack. Thus, the pretreated CPH (Fig. 6c and 6d) appears smoother and has a dispersed and irregular structure, showing that the KOH has broken several bonds within the components; this was confirmed by the presence of several visible cavities.

*X-ray diffraction (XRD) analysis and crystallinity of the cocoa pod husk (CPH)*

Figure 8 shows the XRD diffractograms of pretreated and non-pretreated CPH. Additionally, Fig. 8 shows almost similar XRD shapes for untreated and processed samples, producing specific peaks at $2\theta$ = 22.10° and 22.32°. The amorphous background was characterized by a low intensity of diffraction at $2\theta$ of between 18.30° and 18.86°. These peaks confirm the presence of a highly controlled crystalline and amorphous cellulosic structure, respectively (Memon and Memon 2020).

The peak at $2\theta$ = 22.32° in the diffractogram of the pretreated CPH is narrower and more intense than the peak in the untreated CPH. This could be due to the dissolution of amorphous hemicelluloses and lignin components in CPH.

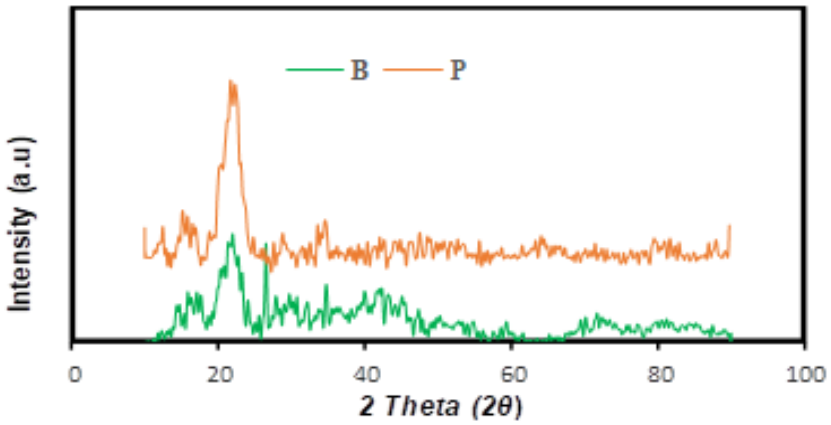


**Fig. 8.** XRD spectrum of the untreated (B) and treated (P) CPH

Table 11 shows the crystallinity index of untreated and pretreated CPH calculated from X-ray diffraction profiles. The degree of crystallinity obtained (65.2%) after delignification is close to that of crystalline cellulose obtained in other work (Akinjokun *et al.* 2021). But the value was lower than that obtained by (Lubis *et al.* 2018), which is 74%. A decrease in crystallinity after KOH treatment under optimized conditions was also observed. This finding has already been made in the studies of He *et al.* (2008) and Rambat *et al.* (2015) and could be due to the fact that optimized delignification with KOH not only removed lignins and some amorphous hemicelluloses, but also caused the destruction of some cellulosic crystalline areas of CPH by breaking cellulose chains during of the pretreatment process (Muharja *et al.* 2018).

**Table 11.** Crystallinity Index of the Crude and Pretreated CPH

| Samples | $I_{002}$ ($2\theta$ = 22.10 to 22.32°) | $I_{am}$ ($2\theta$ = 18.30 to 18.86°) | CrI (%) |
|---|---|---|---|
| CPH untreated (B) | 22.01 | 04.55 | 79.33 |
| CPH treated (P) | 56.06 | 19.50 | 65.22 |

In addition, this delignification resulted in an enlargement of the ratio of pores and internal surfaces (He *et al.* 2008). This will have the advantage of promoting access to sulfuric acid for subsequent hydrolysis into fermentable sugars, used for fermentation into bioproducts.

## CONCLUSIONS

1. The alkaline pretreatment under autoclave heating showed an improvement of the delignification process, which allowed for a reduced time. The optimized conditions were a biomass to solvent ratio of 9.14 %, with a 1% KOH pretreatment for 4 h at a temperature of 30 °C and autoclaved at a temperature of 128.3°C for 15 min. The maximum degree of delignification was 93.9%. This delignification was characterized by a decrease in the content of lignin and hemicellulose, but there was a slight increase in the cellulose.
2. This study, which could be the first of its kind, has shown that the extraction of extractables (total phenolic compounds) at around 55.6%, before delignification using KOH, is an effective strategy to facilitate the delignification of lignocellulosic residues, such as cocoa pod husks and improve their saccharification.
3. A value of 5.33 g/L ± 0.14 g/L in reducing sugars, three times greater than the value before delignification, shows that the results of this study could serve as a basis for the fermentative production of bioproducts.

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