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Generation of porous scaffolds from cacao mesocarp for biomedical applications using surface response methodology.

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Resumen

La ingeniería tisular es el campo donde se aplican células, en combinación con biomateriales que induzcan a la proliferación de estas, con el fin de regenerar distintos tipos de tejidos en un organismo. En la actualidad, distintos biomateriales citocompatibles han sido desarrollados para esta aplicación; sin embargo, en Ecuador no se producen este tipo de materiales. Una alternativa viable es el uso de material lignocelulósico, para la producción de este tipo de biomateriales, que puede ser encontrado en desechos agroindustriales, debido a la alta biocompatibilidad de la celulosa. En base a esto, el presente estudio busca el aprovechamiento de residuos provenientes de la mazorca de cacao tipo CCN-51, muy abundante en Ecuador, para la producción de andamios porosos mediante tratamientos alcalinos aplicando un diseño de superficie de respuesta (Central Composite Designs). Los andamios se produjeron mediante un ataque alcalino con NaOH, variando distintas condiciones de operación tales como la concentración del reactivo, concentración de biomasa, tiempo de operación, temperatura, y dimensiones del mesocarpio para generar un modelo de predicción a través de la implementación un diseño central compuesto (CCD). Se determinó que la temperatura y concentración de NaOH fueron las variables más influyentes del modelo. El análisis CCD permitió predecir parcialmente el comportamiento de cada variable de salida (contenido de lignina, celulosa, cenizas y rendimiento) con respecto a las variables de entrada antes mencionadas. Se obtuvieron valores máximos de rendimiento y contenido de celulosa de 7.91% y 63.77%, respectivamente. Un análisis fisicoquímico mediante microscopia electrónica de barrido (SEM), termogravimetría, y espectroscopia de transmisión de infrarrojo con transformada de Fourier (FTIR), demostró cambios morfológicos y estructurales importantes dependientes de la composición del andamio.

Palabras clave: lignocelulosa, cacao, andamio, CCN-51, diseño central compuesto.

Abstract

Tissue engineering is the area where cells are applied in combination with biomaterials that induce the proliferation of these cells, in order to regenerate different types of tissues in an organism. Different cytocompatible biomaterials have been developed for this application; however, there is no production of this type of materials in Ecuador. A viable alternative, to produce biomaterials that help to cellular regeneration, is the use of lignocellulosic material which can be found in agroindustrial waste, due to its high biocompatibility. Based on this, the present study evaluates the use of waste from the cacao shell type CCN-51, which is very abundant in Ecuador, for the production of porous scaffolds by alkaline treatments applying a response surface design (Central Composite Designs). Scaffolds were produced by means of an alkaline attack using NaOH, varying operating conditions such as reactant concentration, biomass concentration, operating time, temperature, and mesocarp dimensions to generate a prediction model through the implementation of a central composite design (CCD). It was found that temperature and NaOH concentration were the most influential variables in the model. The CCD analysis allowed to partially predict the behavior of each output variable (lignin content, cellulose, ash and yield) with respect to the input variables mentioned before, obtaining maximum values of yield and cellulose content of 7.91% and 63.77%, respectively. A physicochemical analysis by scanning electron microscopy (SEM), thermogravimetry, and infrared transmission spectroscopy with Fourier transform (FTIR) showed important morphological and structural changes depending on the composition of the scaffold.
Key words: lignocellulose, cocoa, scaffolding, CCN-51, composite central design.

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1 Introduction

Tissue engineering is the area where cells and an extracellular matrix secreted by them are applied in combination with biomaterials in order to regenerate different types of tissues in an organism [1]. A large number of people throughout the world require the supply of organ and tissue transplants. Over time, the demand for transplants will continue to grow as the population increases, encouraging tissue engineering to innovate and develop more ways to induce and conduct tissue regeneration to replace organ and tissue transplants [2].

Biomaterials are very important within the area of tissue engineering because they are used as scaffolding which serve as a basis for tissue growth. These can be modeled in 2 or 3 dimensions, according to the requirements [3], [4]. In order for biomaterials to be applied and give favorable results, they should possess some characteristics such as appropriate mechanical support, biocompatibility, and biodegradability [5]. Biomaterials can be classified according to their nature as synthetic (i.e. metals, ceramics, polymers and plastics), or natural biomaterials such as protein fibers and composite materials (i.e. lignocellulosic materials) [6].

Lignocellulose is the main component of plant cell walls; it is produced from the process of photosynthesis, which creates a source rich in carbon, consisting of three main components: cellulose, hemicellulose, and lignin [7]. This material is useful as a source of energy and production of new biodegradable materials. Cellulose and lignin content in this kind of material are the most abundant sources of carbon in nature since these structures contain many carbon-carbon and carbon-oxygen bonds which help improve the molecular interactions of different cellular components [8]. These materials show

morphological similarities to human tissues; due to this, some studies have already been carried out to produce structured compounds, at nano and larger scales, for their use in tissue regeneration of bones, cartilages [9], and cardiac muscles, or for production of structures for drug-delivery within the body [10]–[13]. lignocellulosic materials are widely available since these are often generated as by-products or waste in several production processes in the agroindustry. Furthermore, because of the depletion of petroleum-based resources, the use of renewable feedstock to produce materials has also been sought such as: carbon fibers from lignin, bioethanol from cellulose and hemicellulose, and the development of new biomaterials based on lignocellulosic structures [14].

Lignocellulosic biomass can represent up to 90 [%w/w] of the overall agro-industrial waste [15], and it is currently used in a wide range of applications following different types chemical, biological or physical treatments to improve material properties [16]. Some of the most widely studied sources lignocellulosic materials include wood [14], rice husk, sugarcane bagasse, banana peel, peel of citrus fruits, bagasse and cocoa shell [16].

Ecuador is one of the most important producers and exporters of fine aroma cacao in the world, with 327 000 hectares of cultivation throughout the country; this variety is highly valued in the international market for its unique organoleptic characteristics. However, this high-quality cacao has been gradually replaced by the production of cacao CCN-51 (“clon cacao nacional 51”), which is currently about double that of fine aroma cacao. Its production in Ecuador is in the range of 200-300 [kg/hectare] which represent a big production amount. CCN-51 cacao also generates wastes that represent approximately 2 million [tons/year] in the country [17], which are used as fertilizer or animal feed [18]. This lignocellulosic residue could be very useful as raw material for scaffolds production, because it is a new application with many advantages as biodegradable material, due to

the high levels of biocompatibility for the components present in its structure (hemicellulose, lignin and cellulose) [19].

Heredia et al (2014), carried out different studies with cacao mesocarp, applying different types of chemical treatments (acid, alkaline and neutral) to obtain porous structures that could serve as scaffolds. The results showed that alkaline treatment was the most favorable treatment for the delignification of the mesocarp because it reduced lignin content while maintaining high levels of cellulose in the structures. On the other hand her analysis by Scanning electron microscopy (SEM) also showed a favorable morphology in the scaffolds produced by alkaline treatment due to the greater degree of porosity. Furthermore, through mesenchymal stem cell cultures, it was demonstrated that the materials were cytocompatible [20]. However, the study did not evaluate the effect of varying the conditions of the alkaline treatment.

Based on this, the present study aims to use the mesocarp of the cacao pod shell to produce scaffolds by alkaline treatment through central composite design, varying different operating conditions such as reagent concentration, biomass concentration, treatment time, temperature, and mesocarp thickness size to it can be applied to medicine and at the same time give an added value to a material that until now is considered as waste.

2 Materials and methods

2.1 Preparation of the scaffolds

2.1.1 Gathering of cacao pod shell.

Healthy pod shells from cacao CCN-51 type cacao in the same state of maturation were used to produce the porous scaffolds. The shell is divided into three distinct sections, as seen in figure 1 these are:

- The endocarp, which is the internal section of the pod, formed by woody tissue [21].
- The mesocarp is the middle section which has a characteristic yellow, fleshy structure, and is formed by parenchyma tissue.
- The exocarp, the outer section of the shell, can be yellow, green, red or purple, and is composed by epidermal tissues [21].

Once the cacao pods for samples were selected, the shell was separated from the seeds, and then the mesocarp was manually separated from the other shell layers. Due to the lack of a defined maturation scale, this was estimated based on appearance of the pod such as color, texture and shells, without visible signs of disease or overmaturation [22].

Figure 1. Internal structure of the cacao pod.

2.1.2 Isolation of the mesocarp from cacao pod shells

The shell was cut in long sections (figure 2), and the mesocarp was separated from the endocarp and the exocarp. Samples were obtained cutting the mesocarp in uniform disks of 5 [mm] in diameter with a cork borer.

Figure 2. Generation of cocoa mesocarp samples.

2.1.3 Scaffold production by different types of chemical treatment

To corroborate the information provided by Heredia [20], scaffolds were obtained under different chemical treatments. In the case of the alkaline treatment, it was carried out 0.1M with sodium hydroxide (NaOH); acid treatment was carried out with 0.1M hydrochloric acid (HCl), and distilled water (H_2O) was used for the neutral treatment. All treatments were applied at 25℃ for 24[h].

2.1.4 Statistical analysis

In this statistical analysis, 2 initial hypotheses are proposed:

Null hypothesis, which describe that all the averages of factors are equal.

$$\mu_1 = \mu_2 = \mu_3 \ldots \quad (1)$$

Alternative hypothesis, which describes that at least some average is different.

Based on this, p is used as the probability value with a confidence level of 95% = 0.05. If $p \leq 0.05$, null hypothesis is rejected, and the alternative hypothesis is accepted, but if $p > 0.05$ null hypothesis cannot be rejected [23].

The scaffolds for each of the condition sets defined previously were analyzed. one-way analysis of variance (ANOVA) using the software MiniTab 17 was performed along with Tukey pair comparison with a 95% confidence level ($p<0.05$) to determine significant differences between samples treated with different conditions [24], [25].

2.1.5 Design of experiments

2.1.5.1 Central composite design (CCD)

The central composite design (CCD) is one of the most common experimental methods used for the design of a response surface, which generates a mathematical model that can be used to predict values of analyzed variables. The predicted values can then be used for process improvement and optimization.[26]. CCD uses statistical methods based on a multivariable nonlinear model [27]. It helps to minimize the number of experiments and maximize the production process of scaffolds with high cellulose content and low lignin content [28]. To carry out the experimental model and the design of the response surface of the system, JMP Pro® statistical analysis software was used. Table 1 shows the independent variables that were considered, and their defined limits used to generate the model:

- **Biomass concentration [%w/v]:** this variable represents the relationship between mass of mesocarp in 100 [ml] of (NaOH) solution.
- **NaOH concentration [M]:** this variable represents the concentration of the NaOH solution used for the alkaline treatment.
- **Treatment time [h]:** represents the duration of each treatment.
- **Treatment temperature [ºC]:** the temperature at which the alkaline treatment was applied.
- **Mesocarp sample thickness [mm]:** while diameter was kept constant at 5[mm], sample thickness was varied.

In table 1, Alphas (α) represent maximum and minimum limits of each input variable, that will be used to generate the different set of experiments (29 experiments). Central points are defined by column 0; in this case, the model will consist of 3 central points, and the variability on these points will represent the same variability throughout the entire surface.

Table 1. Description of different factors and limits established for the design of experiments,with 3 central points and no replicates,

Factor/Input Variable	Levels		
	-α	**0**	**α**
Biomass concentration [%w/v]	5	10	15
NaOH concentration [M]	0.1	0.55	1
Treatment time [h]	4	14	24
Treatment temperature [°C]	25	37.5	50
Sample thickness [mm]	3	6.5	10

Each treatment was carried out following similar procedures. A general procedure consists of the following steps:

1. Cutting the mesocarp samples with 5 [mm] diameter and a thickness between 3 and 10 [mm].
2. Weighing the samples to achieve the desired biomass concentration in a NaOH solution of desired concentration.
3. Applying heat at a defined temperature between 25-50, under stirring (100 rpm), for the corresponding time.
4. Washing thoroughly with distilled water until pH neutralizes.

2.1.6 Bleaching and lyophilization of scaffolds.

After each treatment, a whitening process was performed submerging samples in 1 [%v/v] sodium hypochlorite solution. Bleaching was done because the NaOH treated samples presented dark coloration, which could potentially prevent a good visualization of cell growth inside of the scaffolds [29], [30] that could hinder further studies, this component does not produce structural changes in the scaffolds, because it is used in low concentrations and the only effect that produce is the bleaching of antioxidants present in the scaffolds. Once the bleaching was completed, samples were thoroughly washed with

distilled water to remove the excess of sodium hypochlorite. Subsequently, the samples were frozen and lyophilized.

2.2 Physical and Chemical characterization.

2.2.1 Determination of yield process production (YPP)

To determine the yield process production, was used the standard AOAC 934.01 [31], first the mesocarp samples were weighed before applying the treatment and after lyophilization. (YPP) was calculated through equation 1.

$$Percentage\ of\ (YPP)\ =\ \frac{W_f\ [g]}{W_i\ [g]} \times 100\ \% \qquad (2)$$

Where, W_f represents the final weight after lyophilization and W_i represents the weight of the mesocarp samples prior treatment.

2.2.2 Total ashes

The total ash content represents the amount of inorganic material [20] present in the scaffolds. Ash content such as magnesium, potassium and calcium salts. To determinate ash content method AOAC 942.05 was used, where 1[g] of dry sample was weighed and put into a clean crucible. Then, the sample was calcinated in a muffle at 550 [°C] for 8 [h], and the final weight was registered [31]. Ash content was calculated based on equation 2.

$$Percentage\ of\ total\ ash = \frac{W_{cs}\ [g] - W_{ec}[g]}{W_{ds}[g] - W_{ec}[g]} \times 100\ \% \qquad (3)$$

Where W_{cs} represents the weight of calcinated sample, W_{ec} represents the weight of empty crucible and W_{ds} represents the weight of dry sample.

2.2.3 Lignin content

The lignin content was determinate using the standard AOAC 973.18, 1 [g] of dry sample was mixed with 15[ml] of 72 [%v/v] sulfuric acid (H_2SO_4) and subjected to constant stirring at room temperature for 2 [h]. The mixture was then heated in a reflux system at 100 [°C] with 125 [ml] of distilled water for 4 [h]. Afterwards, the sample was filtered, washed, and dried, to finally register the final weight of the sample [31]. Sulfuric acid solubilizes protein complexes by heating and hydrolyzes the cellulose present in the sample [32]. Equation 3 was used to calculate lignin content.

$$Percentage\ of\ \ lignin\ content = \frac{W_f\ [g]}{W_i\ \ [g]} \times 100\ \% \quad (4)$$

Where W_f and W_i represent the final and initial sample, respectively.

2.2.4 Cellulose content

The cellulose content was determined through the methodology proposed by Dominguez et al [33]. First, 1 [g] of dry sample was put into a volumetric balloon with 15 [ml] of acetic acid 80 [%v/v] concentration and 1.5 [ml] of nitric acid 68 [%v/v] concentration; then the mix was put in reflux during 20 [min]; the sample was later washed, filtered and dried at 105 [°C] for 24 [h]. A final calcination was carried out at 550 [°C] for 8[h] [33]. In this case the mixture of acetic acid and nitric acid solubilizes the proteins, lignin, lipids and hemicellulose leaving the cellulose content in the sample intact [34]. Equation 4 was used to calculate the cellulose content in the sample.

$$Percent\ of\ Cellulose\ content = \frac{(material\ A)[g] - (material\ B)\ [g]}{initial\ weight\ sample\ [g]} \times 100\ \% \quad (5)$$

Where the material A represents the dry sample mass and the material B represents the calcined sample mass.

2.2.5 Humidity

Humidity represent the amount of water or other liquids present in a sample [35]. To determine the percentage of humidity, atandard AOAC 934.01 was used. Briefly,1[g] of cocoa mesocarp was put in crucibles, to be dried in an oven at 105 [°C] until remains constant with a Δw% of 5% between each measurement. [31][36] . Humidity was determined by equation 5.

$$Percentage\ of\ Humidity = \frac{water\ content\ [g]}{Mi\ [g]} \times 100\% \quad (6)$$

Where the water content represents the difference between the initial and final mass of the sample and M_i represents the initial mass of the sample.

2.2.6 Scanning electron microscopy (SEM)

Different scaffolds were chosen for analysis of their morphology based on their contents of lignin and cellulose (3 central points and 3 random points). These were observed with a JEOL JSM-IT300 scanning electron microscope, using MP-96040EXCS External Control Software program. The conditions used for the analysis of the scaffolds were a pressure of 50[Pa] and a current of 5[kv].

2.2.7 Thermogravimetric analysis (TGA)

The thermogravimetric analysis is commonly used for the characterization of lignocellulosic biomass; over time, this technique has also been applied for the characterization of other types of materials as ceramics, plastics, crystals or metals to identify or measure the physical and chemical changes [37]. This analysis is based on the thermal decomposition of the sample along with temperature variations during a defined period of time and is used to know the thermal stability conditions of the material [38].

The scaffolds were analyzed to observe the degradation behavior of the main compounds in the lignocellulosic material (cellulose, lignin, hemicellulose) by the TGA-50 thermogravimetric analyzer in a temperature range between 19 and 500 [℃], on nitrogen atmosphere and temperature rate of 10 [ºC/min].

2.2.8 Fourier Transform Infrared Spectroscopy (FTIR)

Infrared spectra of chosen scaffolds were obtained in a wavenumber range between 600 and 4000 [cm^{-1}] using a Fourier Transform Infrared Spectrometer (FTIR) equipped with a Smart iTR module with Attenuated Total Reflectance (ATR), with the software Microlab PC.

3 Results and discussions

3.1 Physical-chemical characterization of raw material and basic, acid and neutral treatment.

First, the percentage of humidity of the raw material was measured, and it was measured that the loss of water with respect to the initial mass of the sample was (88.92± 0.28) [%w/w].

Furthermore, YPP for each treatment was obtained. As it can be seen in figure 3, the alkaline treatment had a YPP of 4.52% the acid treatment a value of 5.66%, and the neutral treatment a value of 5.13%.

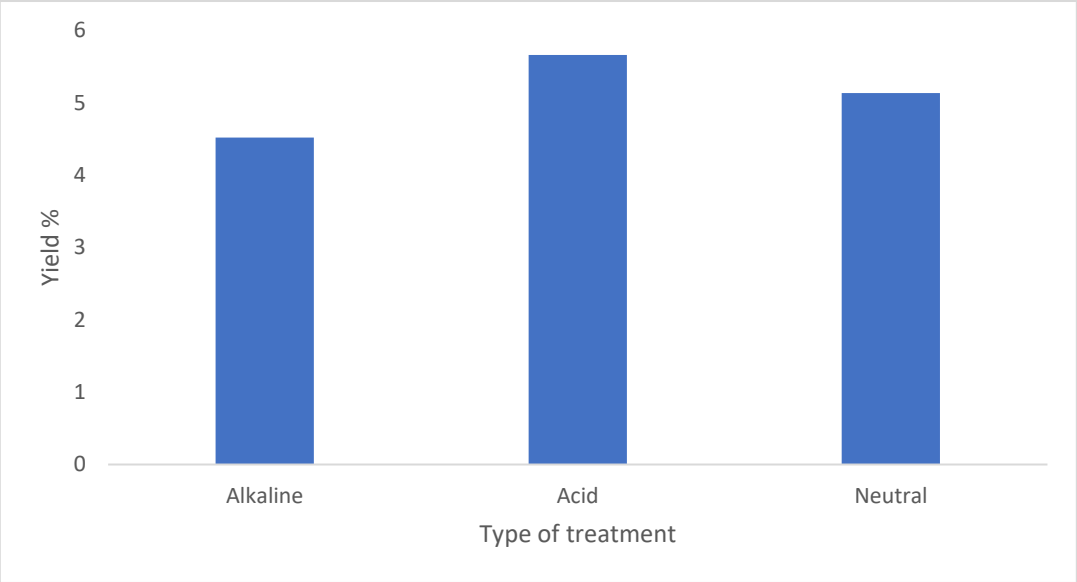


Figure 3. YPP of each treatment applied to the mesocarp, acid treatment presents the highest YPP (analysis without repetitions).

Alkaline treatment, presented the lowest YPP due to the greater amount of digestion and solubilization of lignin and hemicellulose of the structure of the scaffolds, as it has been reported for other types of lignocellulosic materials [33]. Figure 4 shows the levels of lignin content in the scaffolds produced with each treatment and the raw material, where the following results were obtained: the alkaline treatment presented the lowest lignin content, with a value of (3.79±1.45) [%w/w], on the other hand, acid treatment presented the highest lignin content, with a value of (29.18±5.03) [%w/w].

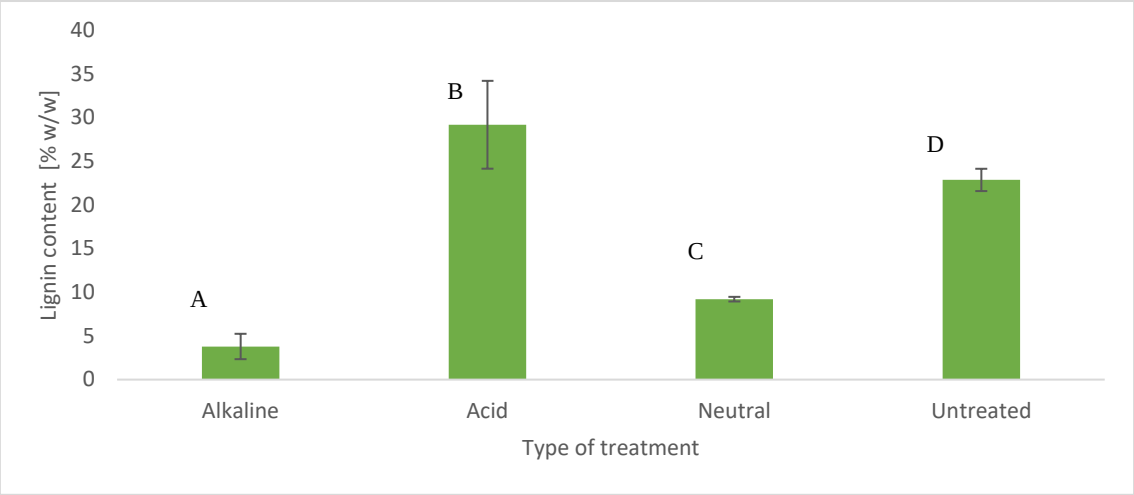


Figure 4. Effect of different treatments (alkaline, acid and neutral) on the content of lignin on scaffolds obtained from the mesocarp of cacao CCN-51 pod shell. ***Bars*** with the same letter represent the treatments with no significant differences, while different letters represent treatments that were significantly different ($p<0.05$).

In this analysis, it is shown that the alkaline and neutral treatments produced the samples with the lowest lignin content ((3.79±1.45) and (9.21±0.26) [%w/w], respectively). Lignin removal is very important in the production of scaffolds, because it presents a lower degree of cytocompatibility compared to cellulose [39], [40]. It is due to this fact that the alkaline and neutral treatments so far, were those that presented the best results to produce the porous structures. Especially for fibroblasts cells, the lignin content at high concentrations can sometimes produce cytotoxicity; however, it is not an advantage to completely eliminate this compound, since with the decrease of the amount of lignin the material loses traction resistance.

The results for the cellulose content in the different scaffolds and in the raw untreated material are shown in figure 5, It can be seen that the similar cellulose content between alkaline treatment, acid treatment and raw material, but in this case neutral treatment presented the lowest cellulose content, with a value of (50.90±2.77) [%w/w].

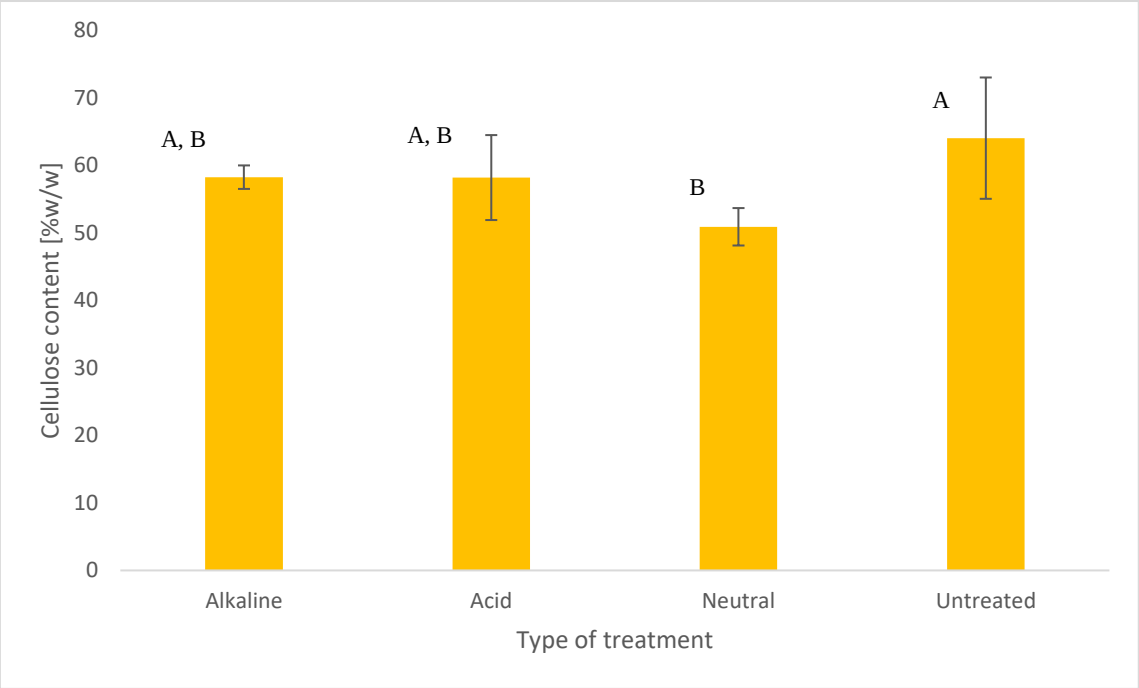


Figure 5. Effect of different treatments (alkaline, acid and neutral) on the content of cellulose on scaffolds obtained from the mesocarp of cacao CCN-51 pod shell. ***Bars*** with the same letter represent the treatments with no significant differences, while different letters represent treatments that were significantly different ($p<0.05$).

In this case, the alkaline, acid and neutral treatments were statistically equal, with values around 58 and 64%. However, the alkaline treatment a greater cellulose content compared to the neutral and the raw material. Thus, it can be said that the alkaline treatment continues to be the best option to produce the scaffolds.

Figure 6 shows the ash content after the different treatments applied, compared to the raw material.

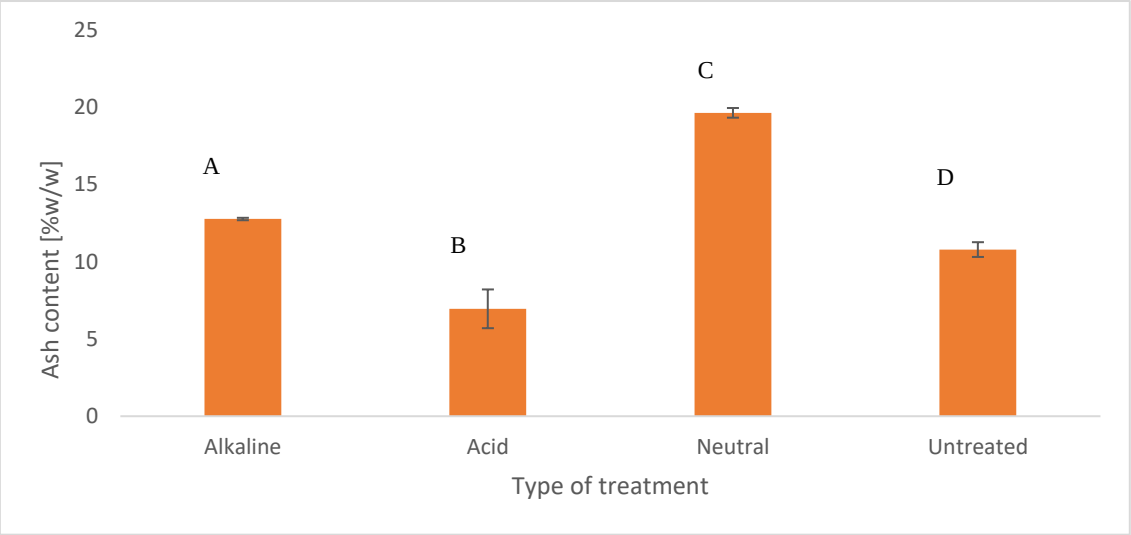


Figure 6. Effect of different treatments (alkaline, acid and neutral) on the ash content on scaffolds obtained from the mesocarp of cacao CCN-51 pod shell. ***Bars*** with the same letter represent the treatments with no significant differences, while different letters represent treatments that were significantly different ($p<0.05$).

The highest ash content was found with the neutral treatment with (19.65±031) [%w/w], and the lowest with the acid treatment with (6.95±12) [%w/w], which indicates that the neutral treatment yielded higher amounts of inorganic material in the form of carbonates, oxalates, silica and metals [41]. The organic and inorganic compounds present useful properties for the structuring of a new material that serves as a base of cell proliferation. Inorganic compounds is considered as a bioactive material, on this and many other cases, are not to be completely removed because they provides high profiles of degradation and mechanical support to the scaffold [42].

Based on the results, this treatment presented lower levels of lignin and high levels of cellulose [43] which could potentially improve scaffold biocompatibility the alkaline treatment was selected for a more detailed analysis. Although lignin has a lower degree of biocompatibility [44] as it was discussed before, it is not advisable to completely remove this compound from the scaffolding given that lignin provides support and stability to three dimensional structures [45].

3.2 Central composite design.

The results obtained for the different experiments generated by the JMP software, according to the limits established in table 1, are shown in table 2. This Face Centered (CCF) Central Composite design (DOE) was made up of 29 formulations non-replicated and 3 central points; this methodology assumes that the variation of the entire surface is the same as that of the central point.

Table 1. Results for the DOE used to study the production of lignocellulosic scaffolds through the alkaline treatment of cacao pod shell mesocarp.

#	Biomass Conc. [%w/v]	NaOH Conc. [M]	Time [h]	Temp. [°C]	Thickness [mm]	YPP [%]	Cellulose [% w/w]	Lignin [% w/w]	Ashes [% w/w]
1	15.00	1.00	4.00	50.00	10.00	5.48	54.75	33.73	17.46
2	15.00	1.00	4.00	25.00	3.00	8.92	55.63	32.99	16.99
3	10.00	0.55	24.00	37.50	6.50	6.21	49.82	24.38	22.54
4	10.00	0.55	14.00	37.50	10.00	4.52	49.30	20.92	24.26
5	5.00	0.10	4.00	25.00	3.00	10.74	45.78	22.02	16.34
6	10.00	0.55	14.00	37.50	3.00	7.22	54.16	27.93	21.66
7	5.00	1.00	4.00	50.00	3.00	4.81	47.97	24.07	20.84
8	5.00	1.00	24.00	50.00	10.00	1.22	42.76	15.22	19.51
9	15.00	0.10	24.00	25.00	3.00	5.27	47.98	26.46	20.63
10	15.00	0.10	4.00	50.00	3.00	3.82	56.30	21.80	12.39
11	15.00	0.55	14.00	37.50	6.50	6.87	55.56	25.19	20.48
12	10.00	0.55	14.00	37.50	6.50	4.01	58.97	13.47	20.47
13	15.00	0.10	4.00	25.00	10.00	6.19	59.24	18.62	20.29
14	10.00	0.10	14.00	37.50	6.50	5.85	52.83	15.74	20.87
15	5.00	0.55	14.00	37.50	6.50	5.98	51.53	16.05	22.55
16	15.00	0.10	24.00	50.00	10.00	3.12	41.89	15.12	23.00
17	5.00	0.10	4.00	50.00	10.00	4.79	48.33	13.49	23.50
18	5.00	1.00	24.00	25.00	3.00	5.77	62.16	18.86	25.64
19	5.00	1.00	4.00	25.00	10.00	5.33	49.88	14.43	26.45
20	10.00	0.55	14.00	25.00	6.50	5.08	49.98	9.94	23.66
21	10.00	0.55	14.00	37.50	6.50	4.01	57.63	14.73	20.72
22	10.00	0.55	14.00	50.00	6.50	1.96	48.85	18.83	9.14
23	5.00	0.10	24.00	25.00	10.00	3.31	64.32	16.40	10.78
24	10.00	0.55	14.00	37.50	6.50	3.59	60.11	15.25	20.81
25	10.00	1.00	14.00	37.50	6.50	4.75	59.95	21.91	6.93
26	15.00	1.00	24.00	50.00	3.00	4.22	48.45	16.55	17.19

27	10.00	0.55	4.00	37.50	6.50	3.98	58.74	21.58	22.43
28	15.00	1.00	24.00	25.00	10.00	5.09	64.57	15.69	7.06
29	5.00	0.10	24.00	50.00	3.00	2.25	46.82	8.23	15.95

Note: *Highlighted rows represent the CENTRAL POINTS of the design*

In figure 7, the gray area in each profile represents the variability of data. It could be due to the differences in the degrees of maturity of the cocoa pods used for the production of the scaffolds, because, in plants, the oxidation degree is produced by the enzyme polyphenol oxidase, which produces quinones, reactive chemical species that cause damage or cell death [46]. Cacaos were selected only by observing similar physical characteristics between them, but the exact level of maturity cannot be defined due to the lack of availability of a maturation scale in the literature, particularly for CCN-51.

Figure 8 shows the prediction profiles generated by the model, which correlates the output to the input variables. Regarding biomass concentration, as this parameter increases, so do lignin, cellulose and YPP, but the ash content decreases.

The reduction of treatment time increases the YPP, achieving a maximum value of 10.74 % at 4 [h], while cellulose, lignin and ash contents steadily increasing in the material. However, a big reduction in treatment time, would make no significant changes in the structure or physicochemical characteristics of the raw mesocarp material. For cellulose and lignin, max. values can be 64 [%w/w] for cellulose and 35 [%w/w] for lignin; similarly, ash content and YPP reached their maximum values of 15 [%w/w] and 8 % respectively approximately, at 37.5 [°C].

Nonetheless, from this point on, increasing the temperature cause a decrease in the value of every output variable. This is not desirable as there would be a destruction of the scaffolding, a phenomenon that was observed experimentally.

The increase in scaffold thickness shows a minimum and then a progressive increase in lignin and ash contents, but for the cellulose content, a maximum can be seen, followed by progressive decline with increased scaffold thickness. On the other hand, a minimum can be seen for yield, which seems to stabilize with the increase of thickness.

This variability could also be in part because of changes in the geographic origins of the samples, and it is known that climate conditions affect pod composition Alvarez-Barreto et al (2018). Additionally, there were limitations in the equipment used; for example, the temperature control of the stirring and heating plates showed fluctuations in this parameter, affecting specially treatment processes with longer times.

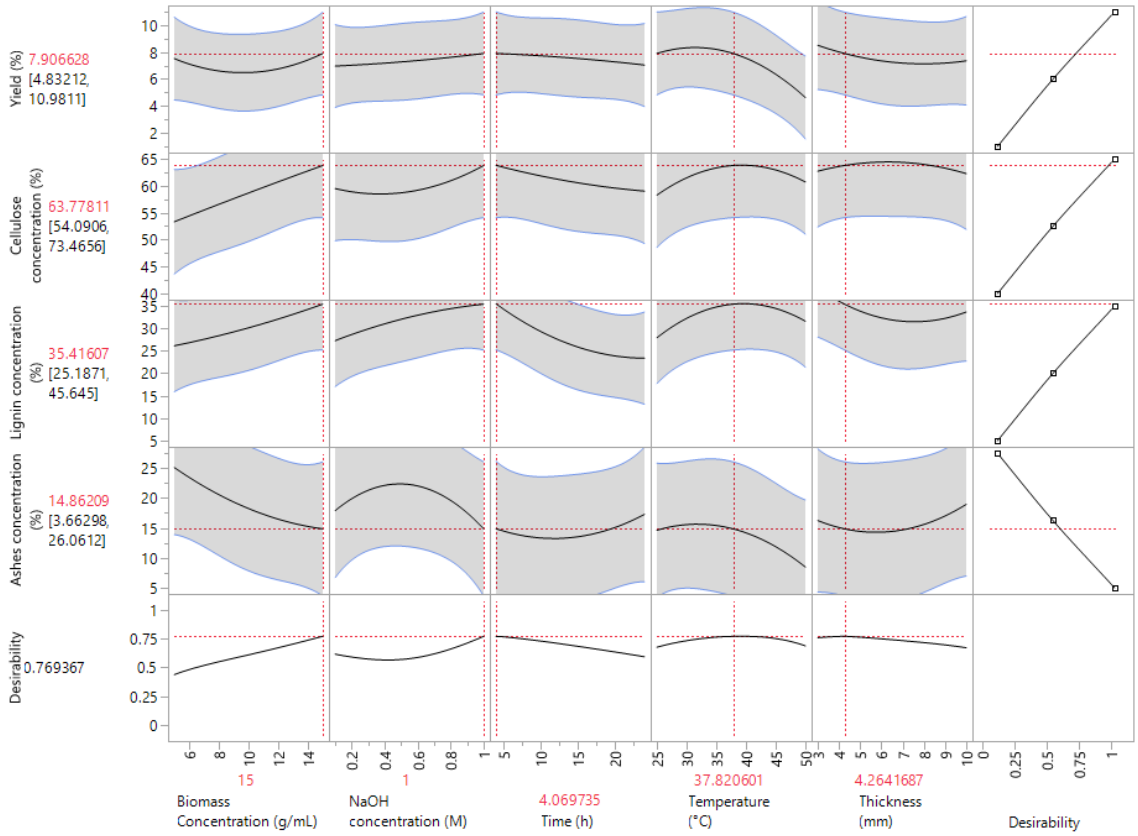


Figure 7. DOE prediction profiles of output variables as functions of input variables, according to experimental data. Vertical dotted red lines show the values of each input variable that optimize the desirable output variables.

Data prediction and the variability in experimental model are presented in figure 8, where the pink area shows a great variability in the data, as also depicted by the gray area shown before in figure 7. Each of the analyzed parameters shows a value of p> 0.1 it means that null hypothesis, cannot be reject, showing that data do not present a good adjustment in the models, , possibly due to variability in the methodology and/or feedstock.

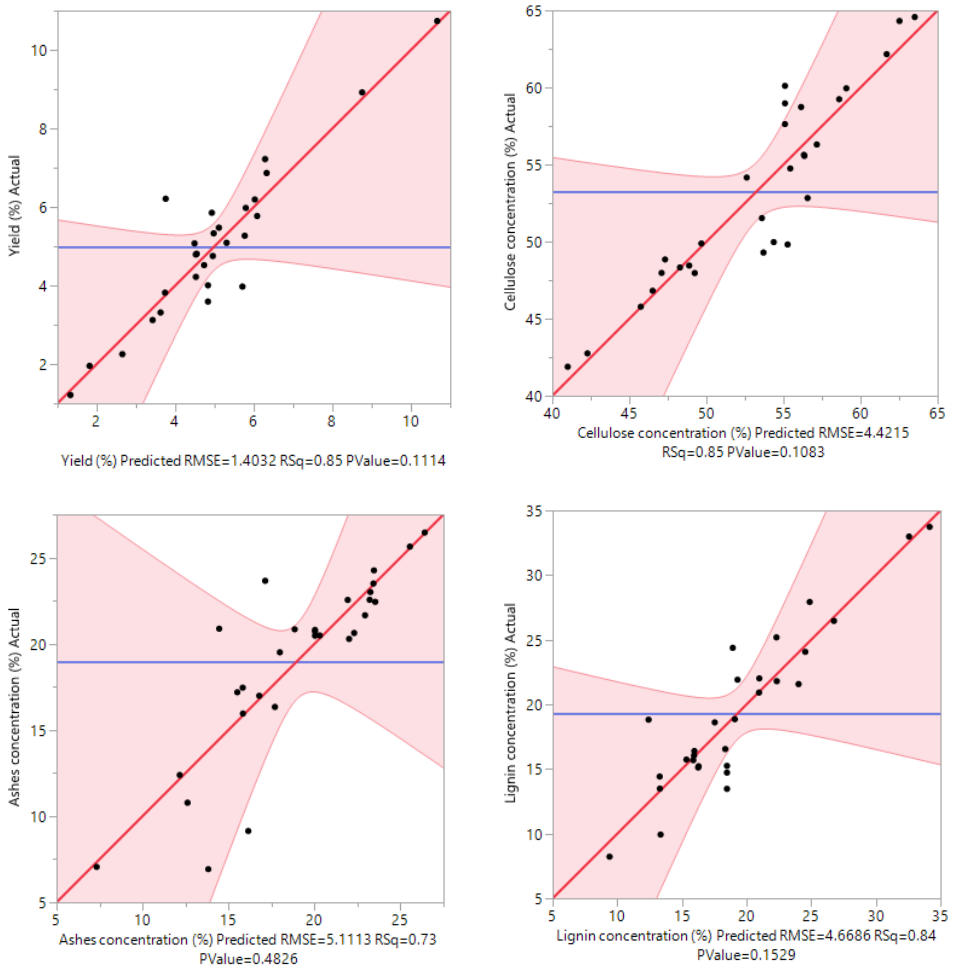


Figure 8. Variability of the responses according to output variables (lignin, cellulose, ashes and yield) black dots represent the data for the model.

Figure 9 shows the overall level of influence of each input variable on the experimental model, as well as for each output variable. In the case of yield and cellulose content, temperature was the most important variable; on the other hand, all input variables had similar levels of influence on lignin content. For the ash content, the most influential

variable was NaOH concentration because it modified the amorphous inside structure of lignin. This process of depolymerization facilitated the separation of lignin from the cellulose in lignocellulosic materials.[41].

Overall, the most influential variables were temperature, followed by treatment time and NaOH concentration. These results are in accordance with those found the literature regarding alkaline treatments applied to other types of lignocellulosic materials [47]. Particularly, an increase in temperature decreases cellulose crystallinity and increases the speed of delignification, which produces greater access to the cellulose present in mesocarp [48].

Based on the analysis of data in Table 2, increasing NaOH concentration helps to obtain more cellulose with respect to lignin content in the structure of the scaffold, as long as extent treatment time, temperature and the thickness of the scaffolds are low.

These results have some similarities with those obtained by Heredia et al. (2014) where it is mentioned that the increase of NaOH concentration generates a decrease in the cellulose content. Nonetheless, an exact comparison is not possible due to other variables that were not analyzed in the previous investigation, such as the thickness of the scaffolding and the concentration of biomass in the production process.

In this case, treatment time was also compared with that reported by other authors [20], [49]; increments in time in the hydrolysis process increases cellulose content, which is constant with some of the experiments of our model. This could be due to other variables that also influenced the results (temperature, NaOH concentration, biomass concentration and thickness) that were not evaluated by these authors.

Regarding these results, it can be said that thickness of the scaffolds has a big influence in cellulose content, as expected, the small size of scaffolds, produces lower

concentrations of cellulose, and if the scaffolds are bigger, the cellulose content decrease. This may be due to the difficult interaction between the NaOH solution with the internal section of the sample, when the thickness increases.

Summary Report

Overall

Column	Main Effect	Total Effect
Temperature (°C)	0.31	0.425
Time (h)	0.106	0.218
NaOH concentration (M)	0.125	0.198
Thickness (mm)	0.106	0.193
Biomass Concentration (g/mL)	0.106	0.184

Yield (%)

Column	Main Effect	Total Effect
Temperature (°C)	0.488	0.537
Time (h)	0.168	0.207
Thickness (mm)	0.127	0.177
Biomass Concentration (g/mL)	0.088	0.144
NaOH concentration (M)	0.008	0.029

Cellulose concentration (%)

Column	Main Effect	Total Effect
Temperature (°C)	0.477	0.69
Time (h)	0.011	0.225
NaOH concentration (M)	0.085	0.159
Biomass Concentration (g/mL)	0.045	0.157
Thickness (mm)	0.028	0.108

Lignin concentration (%)

Column	Main Effect	Total Effect
Temperature (°C)	0.174	0.284
Thickness (mm)	0.18	0.243
Time (h)	0.174	0.24
Biomass Concentration (g/mL)	0.201	0.229
NaOH concentration (M)	0.082	0.151

Ashes concentration (%)

Column	Main Effect	Total Effect
NaOH concentration (M)	0.325	0.452
Thickness (mm)	0.088	0.243
Biomass Concentration (g/mL)	0.088	0.205
Time (h)	0.073	0.198
Temperature (°C)	0.104	0.188

Figure 9. Level of influence of each input variable (Temperature, time, NaOH concentration, thickness, biomass concentration) in the experiment model.

Some analysis was carried out to validate the generated prediction model, where scaffolds were produced with the predicted conditions that produced a maximum cellulose level. The selected conditions are pointed by red lines in figure 8, and the values of the input variables were the following:

- NaOH concentration: 1 [M]
- Biomass concentration: 15 [%w/v]
- Temperature: 37.5 [℃]
- Time: 4 [h]
- Thickness: 4.5 [mm]

The analysis was carried out for 3 different cacao shells with similar degrees of maturity which are shown in table 3.

Table 2. Validation analysis of the experimental model, using the treatment to maximize the cellulose content in the samples. Study made with 3 replicas using different cacao pods with similar degree of maturity.

Output variable	**Predicted Value**	**Experimental Values**			**Experimental average**	**Deviation from predicted value [%]**
		Cacao 1	**Cacao 2**	**Cacao 3**		
YPP [%]	7.91	6.17	6.49	6.85	6.50 ± 0.28	17.78
Cellulose Content [%w/w]	63.77	56.57±1.55	51.16±1.99	56.67±0.34	54.80 ± 2.57	14.01
Lignin Content [%w/w]	35.42	33.86±0.57	37.00±0.35	25.78±0.97	32.21±5.78	9.03
Ash Content [%w/w]	14.86	21.21±0.26	23.74±1.66	23.43±0.34	22.79±1.38	53.39

In general, there is a considerable variability in the results, perhaps related to differences in the cacao pod shells used. Despite trying to use pods with similar degrees of maturity, the structural content of each one can vary significantly, thus affecting the resulting data shown in table 3.

The YPP from each shell was very similar, but there was a deviation of about 14% when compared to the model. For cellulose, the theoretical value of the model was 63.77 [%w/w], while the experimental content was (54.80±2.57) [%w/w], with a deviation from

predicted value of 17.78%. Meanwhile, the predicted lignin content was 35.42 [%w/w], and the experimental value was (32.21±5.78) [%w/w], with a 9.03% deviation. Ash content presented the greatest deviation from the predicted content, with a value of 53.39%. It can be said that for all the variables, except ashes, the model predicts their levels within a 20% deviation which indicates a good prediction of the output variables. However, variability in the original sample is still a concern, due to it is difficult to use one cacao pod for a single experiment due to the low YPP that it presents as it can be seen in table 2, because of approximately 120 [g] of mesocarp of cacao obtained from a single pod generate an approximate production of 4-5 [g] of treated samples, and because of this the variability of the data can keep constant.

3.3 Thermogravimetric analysis (TGA)

TGA analyses were performed to determine the degree of material thermal stability in function of its composition.

Some experiments were selected for analysis, three of them corresponded to the central points (experiments developed under the same operating conditions), and the other 3 experiments, under different operating conditions as it can be seen in table 4, they were selected based on their different of lignin and cellulose contents, to observe how these affect the thermal stability of the material.

Table 3. Experiments under different operating conditions selected.

#	Biomass Conc. [%w/v]	NaOH Conc. [M]	Time [h]	Temp. [°C]	Thickness [mm]	YPP [%]	Cellulose [% w/w]	Lignin [% w/w]	Ashes [% w/w]
5	5.00	0.10	4.00	25.00	3.00	10.74	45.78	22.02	16.34
25	10.00	1.00	14.00	37.50	6.50	4.75	59.95	21.91	6.93
28	15.00	1.00	24.00	25.00	10.00	5.09	64.57	15.69	7.06

According to some authors, who made an approximation of decomposition of lignocellulosic biomass components, this process is divided into 4 stages: first, elimination of humidity with temperatures >100 [℃], decomposition of hemicellulose between 150 and 310 [℃], degradation of lignin and cellulose between 310 and 500 [℃] and decomposition of lignin with temperatures >450 [℃] [37], [50]–[52].

Figure 10 A shows the TGA curves for the different samples at central point of the DOE, where material degradation along with temperature increase can be seen. The similar shapes of the curves suggest that there was a similar degradation process in each sample, including superficial and internal water loss, cellulose, hemicellulose and lignin decomposition. [53].

On the other hand, figure 10 B shows (differential thermogravimetric) DTG curves for the same samples, where peaks for each curve are the same and represent temperatures of maximum material degradation speed. A first degradation at 67℃, corresponds to superficial water loss [52], as already mentioned; then between 200 and 430 [℃] it can be seen the temperature range where the most loss of mass occurs, approximately 35% with respect to the initial mass of the sample, the peaks at 270, 307 and 309 [℃] show the highest rate of degradation, which can represent the degradation of a continuous reaction or the progressive degradation of different compounds present in the samples as cellulose, hemicellulose and [54] [55].

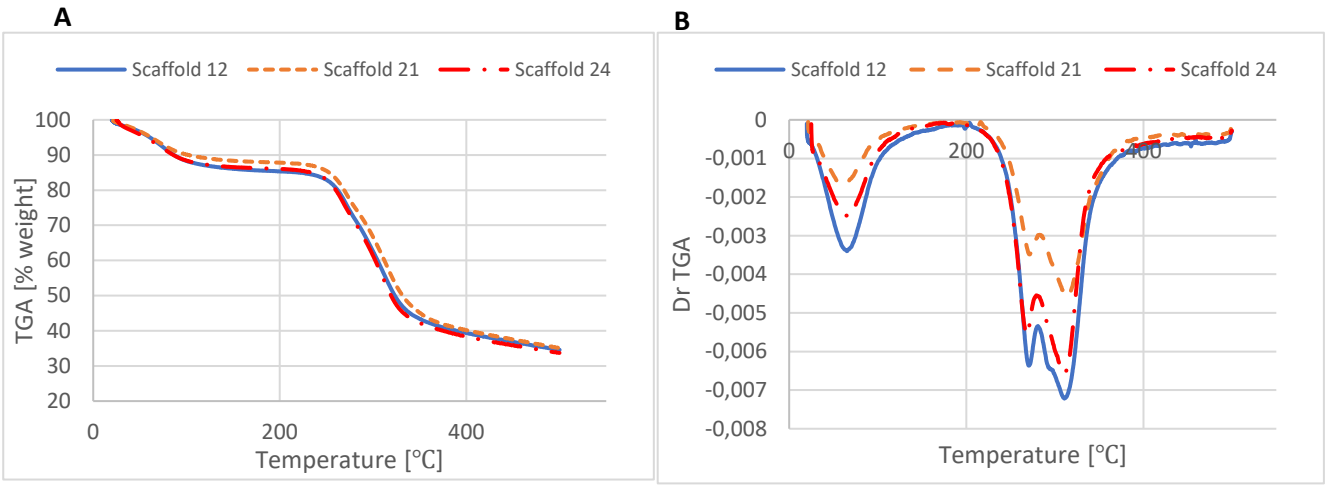


Figure 10. Thermal analysis of central points of experimental model (scaffolds 12, 21 and 24 with cellulose content: 58.90 [%w/w]; lignin content:14.48 [%w/w]) A) Thermogravimetric (TGA) curve, B) Differential thermogravimetric (DTG).

Figure 11 A shows TGA curves for selected formulations, and the differences are due to the different cellulose and lignin contents. However, each one shows similar trends, first with respect to superficial water loss between 60 and 100 [°C], and the internal water loss between 100 and 150 [°C], the samples number 25 and 28 had greater mass loss, it could be happened because the samples 25 y 28 had greater structural mass (thickness of 6.5 y 10 [mm] respectively), and retain more water inside them, compared to sample 5 that had a thickness of 3[mm].

The degradation of the most amount of material degradation for these 3 samples occurs within a temperature between 260 and 430 [°C], with approximately 55% of the total weight in each sample that as in the previous analysis can represent the degradation of a continuous reaction or the progressive degradation of different compounds. The loss of superficial and internal water were produced in similar ranges of temperature mentioned before, hemicellulose degradation could started before or after 284 [°C], at 284 [°C] it was the highest degradation rate, for sample 5, 267 [°C] for sample 25, and 273 [°C] for

sample 28, then the cellulose degradation at 320 [°C] for sample 5, 308 [°C] for sample 25, and 287 [°C] for sample 28, as can be seen in Figure 11B.

Lignin is the last material to degrade. This compound is characterized by having a high thermal stability, with temperatures >450 [°C], as it was mentioned previously. In this case, sample 5 had the highest amount of mass loss, followed by samples 25 and 28 respectively, which can be influenced by the cellulose content in each sample, because sample 5 has the lowest cellulose content (45.78 ± 0.94) [%w/w] and sample 28 has the highest cellulose content (64.57 ± 1.32) [%w/w].

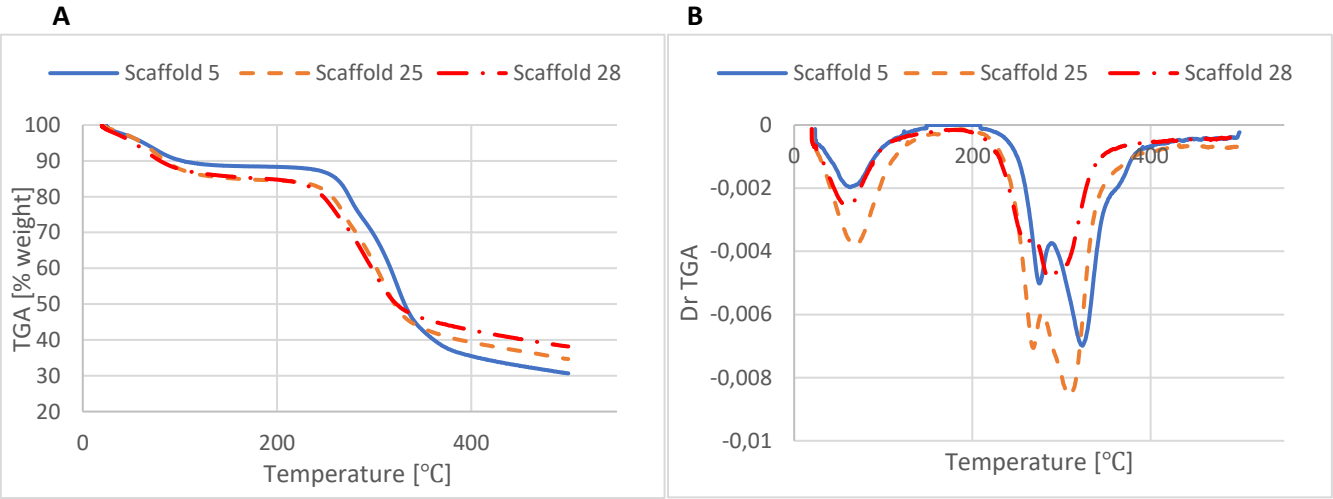


Figure 11. Thermal analysis of scaffold 5 (45.78 [%w/w] cellulose, 22.01 [%w/w] lignin); scaffold 25 (59.94 [%w/w] cellulose, 21.91 [%w/w] lignin); scaffold 28 (64.57 [%w/w] cellulose, 15.68 [%w/w] lignin). A) Thermogravimetric (TGA) curve, B) Differential thermogravimetric (DTG).

3.4 Fourier Transform Infrared Spectroscopy (FT-IR)

The FT-IR analysis allowed to verify the information obtained about the presence of cellulose and lignin in the structure of the porous scaffolds.

Figure 12 shows the spectrum of one central point of the model, compared to the spectrum of mesocarp without treatment. Red bands represent the characteristic peaks of the bonds

O-H, C-H, C=O and C-O-C which correspond to the presence of cellulose [56], [57]. Both in the spectrum of cocoa without treatment and in the spectrum of the scaffold, these peaks can be observed, confirming the presence of cellulose in the structure. On the other hand, the black bands represent the C=C, phenol-hydroxyl and aryl-ether bonds characteristic of the lignin structure[58]. As observed, between the spectrum of cacao without treatment with that of the scaffold, the intensity of the characteristic peak of lignin at 1190 [cm^{-1}] decreases, which shows a percentage of removal of lignin in the structure of the scaffold.

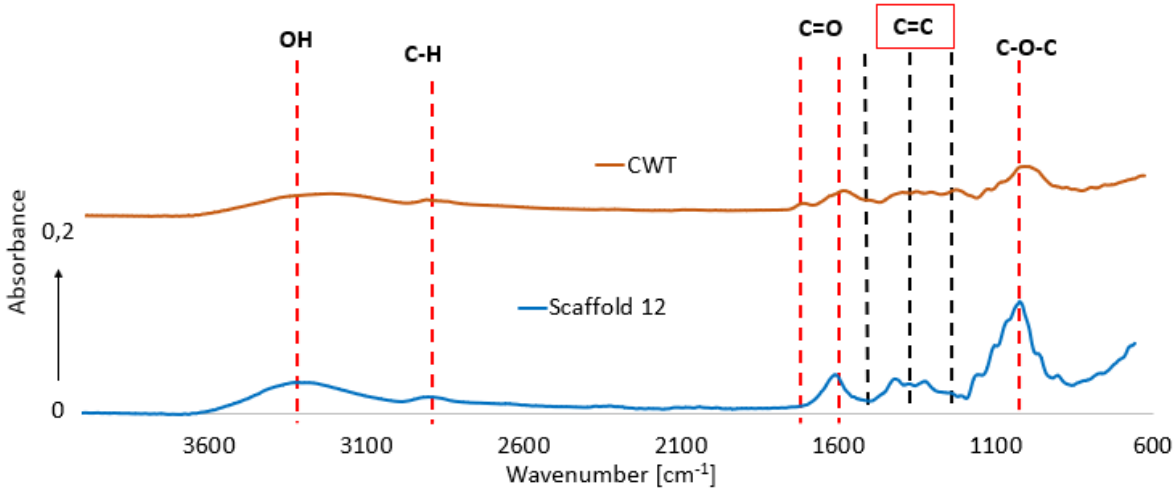


Figure 12. FT-IR spectrum of one central point of the model, cellulose content: 58.90 [%w/w]; lignin content:14.48 [%w/w], compared with the spectrum of the cacao without treatment (CWT), red bands represent characteristic peaks of cellulose and black bands represent the characteristic peaks of lignin.

A comparison was also made between the same samples before analyzed in TGA (scaffolds 5, 25 and 28), with the sample of cacao without treatment in figure 13 in order to see the effect of the different content of lignin and cellulose in each of them. In figure 12, the intensity of characteristic peaks of cellulose is almost the same between the scaffolds samples and it can be seen an increment in the intensity of these peaks to compared with the sample without treatment, but the scaffold 25 has some little variation,

where it can be the decrease of the peak at a wavenumber of 2842 [cm^{-1}] characteristic of C-H bond, as well as the increase of a peak at 1712 [cm^{-1}], which may represent some kind of contaminant on the scaffold.

On the other hand there is a decrease in the characteristic peak of lignin to 1190 [cm^{-1}] in all the scaffolds compared to the peak of the cocoa spectrum without treatment which also shows a percentage of removal of this compound in scaffolding, but in the scaffolding 15 it can be seen that the decrease of the peak is greater than the others, because this scaffold has the lowest lignin content (15.68 ± 0.49) [%w/w], unlike the scaffolding 5 that has the highest lignin content (22.01 ± 0.32) [%w/w].

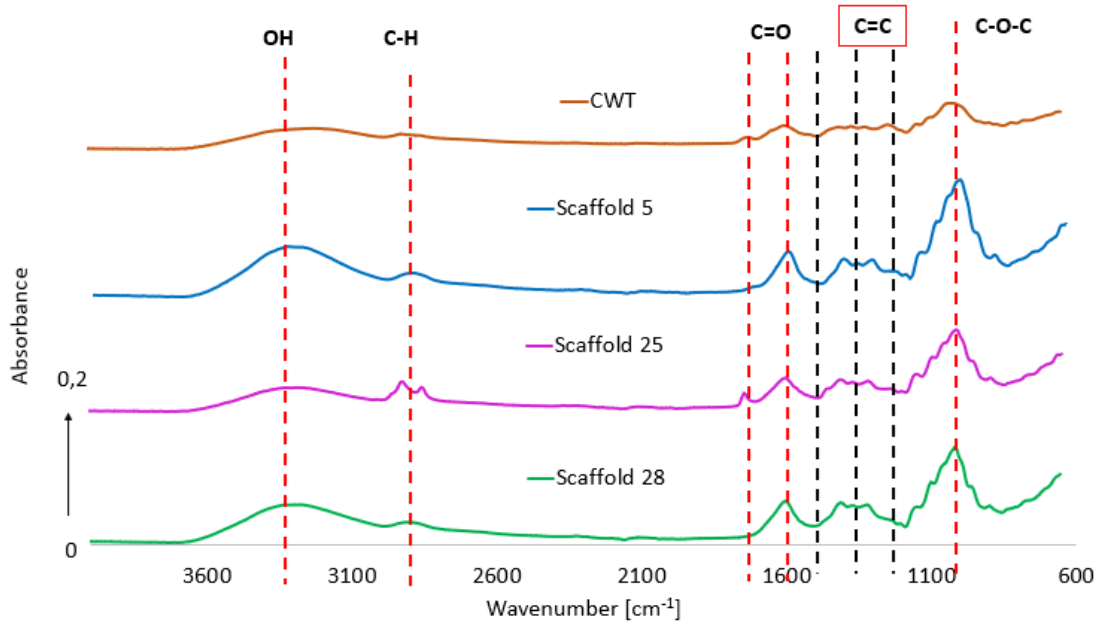
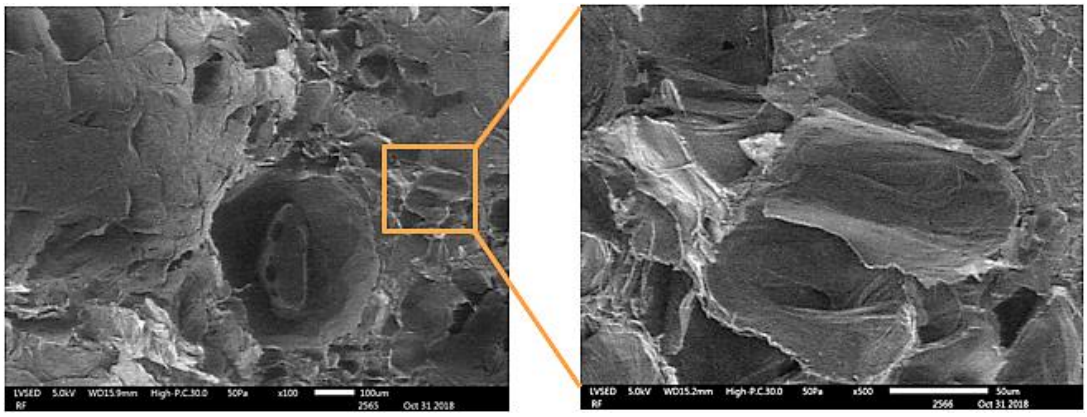


Figure 13. FT-IR spectra of scaffold 5 (45.78 [%w/w] cellulose, 22.01 [%w/w] lignin); scaffold 25 (59.94 [%w/w] cellulose, 21.91 [%w/w] lignin); scaffold 28 (64.57 [%w/w] cellulose, 15.68 [%w/w] lignin), compared with the spectrum of the cocoa without treatment (CWT), red bands represent characteristic peaks of cellulose and black bands represent the characteristic peaks of lignin.

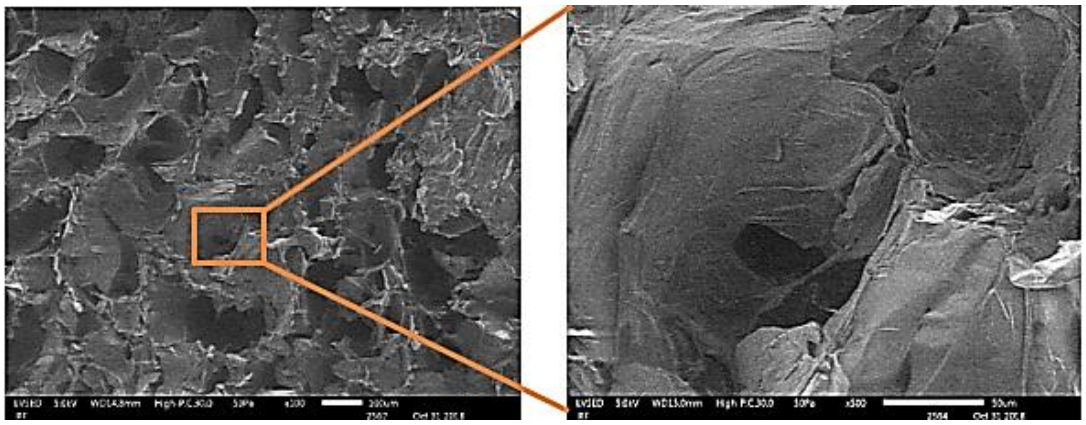
3.5 Scanning electron microscopy (SEM)

The morphology of the scaffolds was analyzed by scanning electron microscopy (SEM). Figures 14 A through 14 D, show the structures of one of the scaffolds at the conditions of DOE's central point (scaffolding 12), and other formulations with different levels of lignin and cellulose.

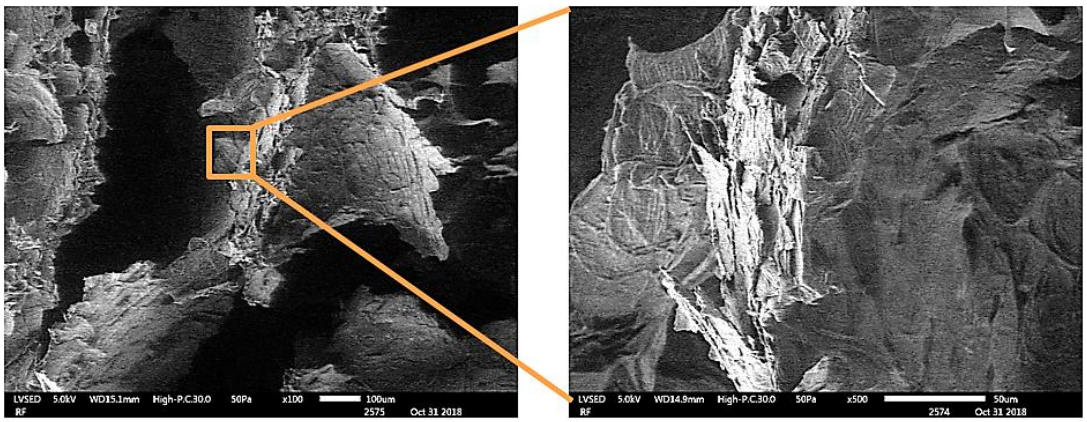
A



B



C



D

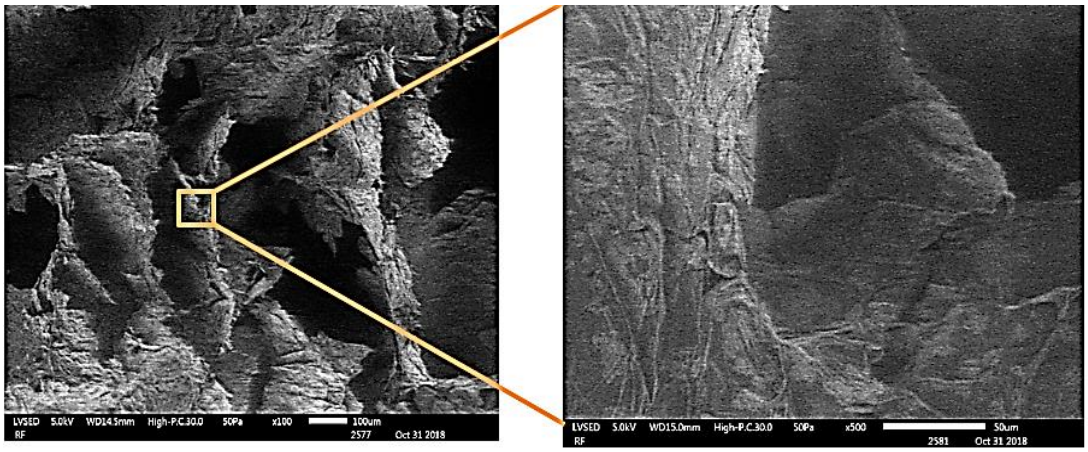


Figure 14. Scanning electron micrographs of different cacao-mesocarp scaffolds. The formulations chosen from the DOE were: A) central point (58.90 [%w/w] cellulose, 14.48 [%w/w] lignin);B) Scaffold 5 (45.78 [%w/w] cellulose, 22.01 [%w/w] lignin); C) Scaffold 25 (59.94 [%w/w] cellulose, 21.91 [%w/w] lignin); D) Scaffold 28 (64.57 [%w/w] cellulose, 15.68 [%w/w] lignin). The images were taken at 100X (left) and 500X (right).

Figure 14 A shows the structure of scaffold 12 (central point), where high levels of non-uniform porosity were observed, due to apparent variations in pose size, a magnification of 500X shows the existence of areas with high levels of roughness and shallow pores in the structure of the scaffolds.

Figures 14 B, C and D, show the morphology of scaffolds 5, 25 and 28, respectively. In this case, it is observed that scaffolds 25 and 28 have very high porosity levels, in comparison with the scaffold 12, due to the high degree of digestion of lignin in its structure, with a removal of approximately 15 [%w/w], unlike scaffolding 5, which has smaller pore size and less deep, but more uniform, for the lower degree of material removal (approximately 10 [%w/w] lignin content). As the internal section of the scaffolding is not uniform in all cases it can be seen areas where the sample has lower levels of roughness as seen in figures 14 B and D, and areas with greater roughness as in Figure 14 C.

In general, the scaffolds of central points show an irregular morphology with different pore sizes, and different levels of roughness in different areas. This great removal of lignin, because the average content of lignin of these samples are (14.48±0.91) [%w/w]. The scaffolds 25 and 28 have larger, deeper and irregular pore sizes due to these have the lower content of lignin, unlike the scaffold 5 that shows more material that was not removed during hydrolysis process in its structure, due to it present the highest content of lignin (22.01±0.32) [%w/w].

4 Conclusions

The process of scaffolds production started with the obtaining of mesocarp samples, these samples were subjected under alkaline attack at different operating conditions, using as input variables, temperature, time, NaOH concentration, thickness and biomass concentration and as output variables YPP, cellulose content, lignin content and ash content. The scaffolds obtained by alkaline treatment presented a higher amount of cellulose in relation to the amount of lignin in its structure, which can improve the degree of cytocompatibility of the scaffolds. The experimental design showed great variation, causing a lack of adjustment of the data to the mathematical model obtained, Nevertheless, the model could predict a behavior of an output variable with respect to input variables, with the possibility to predict certain of values, specifically those of cellulose, lignin and YPP within a 20% deviation. Temperature and NaOH concentration were determinated to be the most important input variables in the production of scaffolds. However, sample variability was an important factor that significantly affected the accuracy of the predictions of the model. Nonetheless, this model is useful to predict the behavior of the process and the involved variables and allowed the observation of changes in scaffold thermal stability and morphology according to different operational conditions and resulting scaffold compositions. The production of scaffolds rich in cellulose from cacao pod shells is thereby feasible and could be further studied in terms of the cellular activity on their surface with the cultivation of cells inside the scaffolds to observe the level of cellular proliferation and the viability of the material.

5 References

[1] S. Bose, M. Roy, and A. Bandyopadhyay, “Recent advances in bone tissue engineering scaffolds,” *Trends Biotechnol.*, vol. 30, no. 10, pp. 546–554, 2012.

[2] Y. SHOUFENG, L. KAH-FAI, D. ZHAOHUI, and C. Chua, “The Design of Scaffolds for Use in Tissue Engineering. Part I. Traditional Factors,” vol. 7, no. 6, pp. 679–689, 2001.

[3] J. Hubbell, “Biomaterials in Tissue Engineering,” *Nat. Biotechnol.*, vol. 13, pp. 565–576, 1995.

[4] S. J. Hollister, “Porous scaffold design for tissue engineering,” *Nat. Mater.*, vol. 4, no. July, 2005.

[5] R. Rosales, K. Alvarado, and F. Ojeda, “Ingeniería Tisular en Odontología.,” *ADM*, vol. LXIX, no. 4, pp. 164–167, 2012.

[6] L. Grenier and R. González, “Sistemas biomaterial-droga para la liberación controlada de antibióticos,” *Biomédica*, vol. 14, pp. 30–38, 1994.

[7] T. Ishii, “Structure and functions of feruloylated polysaccharides,” *Plant Sci.*, vol. 127, pp. 111–127, 1997.

[8] T. Ishii and T. Nishijima, “Inhibition of Gibberellin-Induced Elongation Growth of Rice by Feruloyl Oligosaccharides,” vol. 36, no. 8, pp. 1447–1451, 1995.

[9] C. Eugenia, P. Bonilla, J. E. Perilla, C. Eugenia, P. Bonilla, and J. E. Perilla, “The past , present and near future of materials for use in biodegra - dable orthopaedic implants.,” *Ing. e Investig.*, vol. 31, no. 2, pp. 124–133, 2011.

[10] E. Entcheva, H. Bien, L. Yin, C. Chung, M. Farrell, and Y. Kostov, “Functional cardiac cell constructs on cellulose-based scaffolding.,” *Biomaterials*, vol. 25, pp. 5753–5762, 2004.

[11] B. M. Darder, P. Aranda, and E. Ruiz-hitzky, “Bionanocomposites : A New Concept of Ecological , Bioinspired , and Functional Hybrid Materials.,” *Adv. Mater.*, vol. 10, pp. 1309–1319, 2007.

[12] S. Kamel, “Nanotechnology and its applications in lignocellulosic composites , a mini review.,” *Express Polim. Lett.*, vol. 1, no. 9, pp. 546–575, 2007.

[13] A. Rahmani *et al.*, “Recent advances on biomedical applications of scaffolds in wound healing and dermal tissue engineering,” *Artif. Cells, Nanomedicine, Biotechnol.*, vol. 0, no. 0, pp. 1–15, 2017.

[14] A. Castillo *et al.*, “Aprovechamiento integral de los materiales lignocelulósicos,” *Iberoam Polim*, vol. 13, no. 4, pp. 140–150, 2012.

[15] D. Abarca, R. Martínez, J. Muñoz, M. Torres, and G. Vargas, “Residuos de café , cacao y cladodio de tuna: fuentes promisorias de fibra dietaria,” *Rev. Tecnológica ESPOL*, vol. 23, pp. 63–69, 2010.

[16] M. Riaño, I. Gutiérrez, J. Muñoz, and C. Rivera, “Bioethanol Production from agroindustrial lignocellulosic byproducts.,” *Tumbaga*, vol. 15, pp. 61–91, 2010.

[17] C. Mogro, V. Andrade, and D. Pesántes, “Positioning and efficiency of bananas , cocoa and flowers in the global market.,” *Cienc. UNEMI*, vol. 9, pp. 48–53, 2016.

[18] A. L. Chafla, R. Boucourt, and V. Torres, “Bromatological characterization of cocoa shell (Theobroma cacao), from seven cantons of the Amazonia , Ecuador.,” *Agric. Sci.*, vol.

50, no. 2, pp. 245–252, 2016.

[19] M. Camacho, S. Hernández, G. Martínez, D. Solís, and E. Vigueras, *Temas selectos en ciencia de materiales I*, 1st ed. México, 2015.

[20] S. Heredia, "'Estudio del efecto de las condiciones de los tratamientos químicos en el proceso de obtención de andamios porosos para aplicaciones biomédicas,'" ESCUELA SUPERIOR POLITÉCNICA DEL LITORAL, 2014.

[21] E. De la Cruz and I. Pereira, "Aportes para el estudio y comprensión de los aspectos biológicos y físico-geográficos del cacao (Theobroma cacao l.) en la subregión de Barlovento, estado Miranda.," *Sapiens*, vol. 13, pp. 9–222, 2012.

[22] F. Sánchez, J. Zambrano, J. Vera, R. Ramos, F. Gárces, and G. Vásconez, "Productividad de clones de cacao tipo nacional en una zona del bosque húmedo tropical de la provincia de Los Ríos, Ecuador," *Rev. Cienc. y Tecnol.*, vol. 7, pp. 33–41, 2014.

[23] O. J. Dunn, "Applied Statistics: Analysis of Variance and Regression," *Educ. Stat.*, vol. 15, pp. 175–178, 1987.

[24] I. K. W. Lai and F. K. S. Lam, "Perception of various performance criteria by stakeholders in the construction sector in Hong Kong," *Constr. Manag. Econ.*, vol. 28, no. 4, pp. 377–391, 2010.

[25] W. Zębala and R. Kowalczyk, "Estimating the effect of cutting data on surface roughness and cutting force during WC-Co turning with PCD tool using Taguchi design and ANOVA analysis," *Int. J. Adv. Manuf. Technol.*, vol. 77, no. 9–12, pp. 2241–2256, 2015.

[26] M. Muthukumar, D. Sargunamani, N. Selvakumar, and J. V. Rao, "Optimisation of ozone treatment for colour and COD removal of acid dye effluent using central composite design experiment," *Dye. Pigment.*, vol. 63, pp. 127–134, 2004.

[27] P. Taylor, S. Chattoraj, B. Sadhukhan, and N. K. Mondal, "Predictability by Box-Behnken model for carbaryl adsorption by soils of Indian origin.," *Environ. Sci. Heal.*, no. January 2015, pp. 37–41, 2013.

[28] C. J. Ellens, "Design , optimization and evaluation of a free-fall biomass fast pyrolysis reactor and its products," Iowa State University, 2009.

[29] M. Dom, P. Torre, B. Aliakbarian, B. Rivas, and A. Converti, "Release of ferulic acid from corn cobs by alkaline hydrolysis," *Biochem. Eng.*, vol. 40, pp. 500–506, 2008.

[30] M. J. Taherzadeh and K. Karimi, "Pretreatment of Lignocellulosic Wastes to Improve Ethanol and Biogas Production : A Review," *Mol. Sci.*, vol. 9, pp. 1621–1651, 2008.

[31] A. International, *AOAC: Official Methods of Analysis (Volume 1)*, 15th ed., vol. 1, no. Volume 1. New York: Journal of the Association of Official Agricultural Chemists, 1990.

[32] J. Mller, "Gravimetric determination of acid detergent fiber and lignin in feed: interlaboratory study.," *AOAC Int.*, vol. 92, pp. 74–90, 2008.

[33] M. Domínguez, A. Álvarez, M. Granados, and F. Hernández, "ESTUDIO DE LA CINÉTICA DEL PRETRATAMIENTO E HIDRÓLISIS ÁCIDA DEL BAGAZO DE CAÑA DE AZÚCAR," *Iberoam. Polímeros*, vol. 13, no. 4, pp. 200–211, 2012.

[34] H. Sloneker, "Determination of Cellulose and Apparent Hemicellulose In Plant Tissue by Gas-Liquid Chromatography," *Anal. Biochem.*, vol. 43, no. October, pp. 539–546, 1971.

[35] F. Nogués, *Energía de la Biomasa*, 1st ed. Zaragoza: Universidad de Zaragoza, 2010.

[36] M. Milver, E. Leonario, and R. Vargas, "DETERMINATION OF MOISTURE

CONTENT IN DRY COFFEE BEANS BY MEANS OF DYNAMIC SPECKLE," *Biotecnol. en el Sect. Agropecu. y Agroindustrial*, vol. 14, no. 2, pp. 84–91, 2016.

[37] E. Manals, M. Penedo, and G. Ortega, "THERMOGRAVIMETRIC AND THERMAL ANALYSIS DIFFERENTIAL DIFFERENT VEGETABLE BIOMASSES," *Chem. Technol.*, vol. 31, pp. 180–190, 2011.

[38] E. Villegas, "Caracterización de polímeros aplicando el método termogravimétrico," *Metod. y Mater.*, vol. 2, pp. 25–32, 2012.

[39] K. Nakasone and T. Kobayashi, "Cytocompatible cellulose hydrogels containing trace lignin," *Mater. Sci. Eng. C*, vol. 64, pp. 269–277, 2016.

[40] M. A. Kostiainen, "Functionalization of carboxylated lignin nanoparticles for targeted and pH-responsive delivery of anticancer drugs," *Nanomedicine*, vol. 12, pp. 2581–2596, 2017.

[41] R. Acosta, L. Garriga, and E. González, "'Effect of chemical pretreatment of saw- dust from Pinus sp . as feedstock in the production of ethanol.,' Cent. Azúcar, vol. 38, no. 4, pp. 67–71, 2011.Effect of chemical pretreatment of saw- dust from Pinus sp," *Cent. Azúcar*, vol. 38, no. 4, pp. 67–71, 2011.

[42] M. Stevens, "Biomaterials for bone tissue engineering.," *Materialstoday*, vol. 11, no. 2, pp. 18–25, 2008.

[43] J. Holguin, J. Peña, L. Ríos, and M. Vázquez, "Bagazo de sorgo dulce : una alternativa para la producción de etanol de segunda generación en Colombia (Parte I)," *Inf. Técnico*, vol. 81, no. 2, pp. 131–141, 2017.

[44] M. Martinez, A. Pacheco, and M. Vargas, "HISTOLOGICAL EVALUATION OF THE BIOCOMPATIBILITY AND BIOCONDUCTION OF A HYDROXYAPATITE-LIGNIN COMPOUND INSERTED IN RABBITS' SHINBONES," *MVZ*, vol. 14, no. 1624–1632, pp. 1624–1632, 2009.

[45] L. Palomar, G. Bautista, G. Montero, L. Díaz, R. Ramos, and E. Romero, "Determinación simplificada del contenido de lignina en paja de trigo por método volumétrico y su caracterización química.," *Cienc. y Tecnol.*, vol. 15, no. 1850–0870 115, pp. 113–124, 2015.

[46] Á. Azofeifa, "Problemas de oxidación y oscurecimiento de explantes cultivados in vitro.," *Agron. Mesoam.*, vol. 20, no. 1, pp. 153–175, 2009.

[47] E. M. Cardona, J. A. Rios, and J. D. Peña, "Alkaline Pretreatment of Elephant Grass (Pennisetum Sp) and King Grass (Pennisetum Hybridum) Cultured in Colombia for Ethanol Production," *Inf. Tecnológica*, vol. 24, no. 5, pp. 69–80, 2013.

[48] A. P. Redding, Z. Wang, D. R. Keshwani, and J. J. Cheng, "Bioresource Technology High temperature dilute acid pretreatment of coastal Bermuda grass for enzymatic hydrolysis," *Bioresour. Technol.*, vol. 102, no. 2, pp. 1415–1424, 2011.

[49] J. Benalcázar, "Evaluation of different chemical pretreatments to the biomass of cacao shell for alcoholic fermentation processes," Universidad San Francisco de Quito, 2018.

[50] J. Villaseñor, P. Sánchez, and J. L. Valverde, "Bioresource Technology Thermogravimetric – mass spectrometric analysis of lignocellulosic and marine biomass pyrolysis," *Bioresour. Technol.*, vol. 109, pp. 163–172, 2012.

[51] M. Carrier, A. Loppinet-serani, D. Denux, and J. Lasnier, "Thermogravimetric analysis as a new method to determine the lignocellulosic composition of biomass," *Biomaas and Bioenergy*, vol. 5, pp. 1–10, 2010.

[52] T. Ingram *et al.*, “Comparison of different pretreatment methods for lignocellulosic materials. Part II: Influence of pretreatment on the properties of rye straw lignin.,” *Bioresour. Technol.*, vol. 102, no. 8, pp. 5221–5228, 2011.

[53] T. Damartzis, D. Vamvuka, S. Sfakiotakis, and A. Zabaniotou, “Thermal degradation studies and kinetic modeling of cardoon (Cynara cardunculus) pyrolysis using thermogravimetric analysis (TGA),” *Bioresour. Technol.*, vol. 102, no. 10, pp. 6230–6238, 2011.

[54] A. M. Adel, Z. H. Abd El-Wahab, A. A. Ibrahim, and M. T. Al-Shemy, “Characterization of microcrystalline cellulose prepared from lignocellulosic materials. Part II: Physicochemical properties,” *Carbohydr. Polym.*, vol. 83, no. 2, pp. 676–687, 2011.

[55] Z. Shuping, W. Yulong, Y. Mingde, L. Chun, and T. Junmao, “Pyrolysis characteristics and kinetics of the marine microalgae Dunaliella tertiolecta using thermogravimetric analyzer,” *Bioresour. Technol.*, vol. 101, no. 1, pp. 359–365, 2010.

[56] L. Hernao, I. Rojas, and G. Giraldo, “CUANTIFICACIÓN DE CELULOSA PROVENIENTE DE RESIDUOS DE LA INDUSTRIA DEL MUEBLE,” *Redalyc*, vol. 8, pp. 23–28, 2009.

[57] C. N. Tejada and Z. Montiel, “Aprovechamiento de Cáscaras de Yuca y Ñame para el Tratamiento de Aguas Residuales Contaminadas con Pb (II),” *Inf. Tecnológica*, vol. 27, no. 1, pp. 9–20, 2016.

[58] R. Rumana, R. Langenfeld-Heyser, F. Reiner, and A. Polle, “FTIR spectroscopy, chemical and histochemical characterisation of wood and lignin of five tropical timber wood species of the family of Dipterocarpaceae,” *Wood Sci Technol*, vol. 44, pp. 225–242, 2010.

6 Annexes

6.1 Statistical analysis

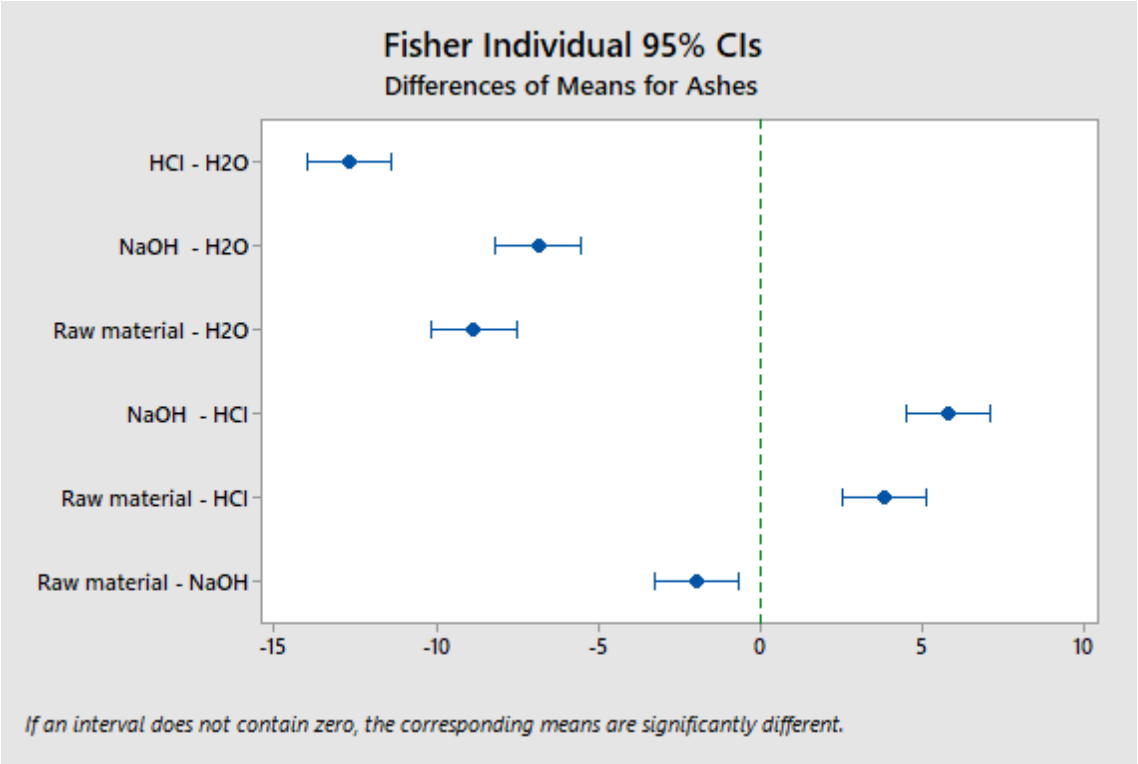


Figure 19. Fisher plot with 95% of confidence interval. Analysis to determine the differences of means for Ashes in each treatment.

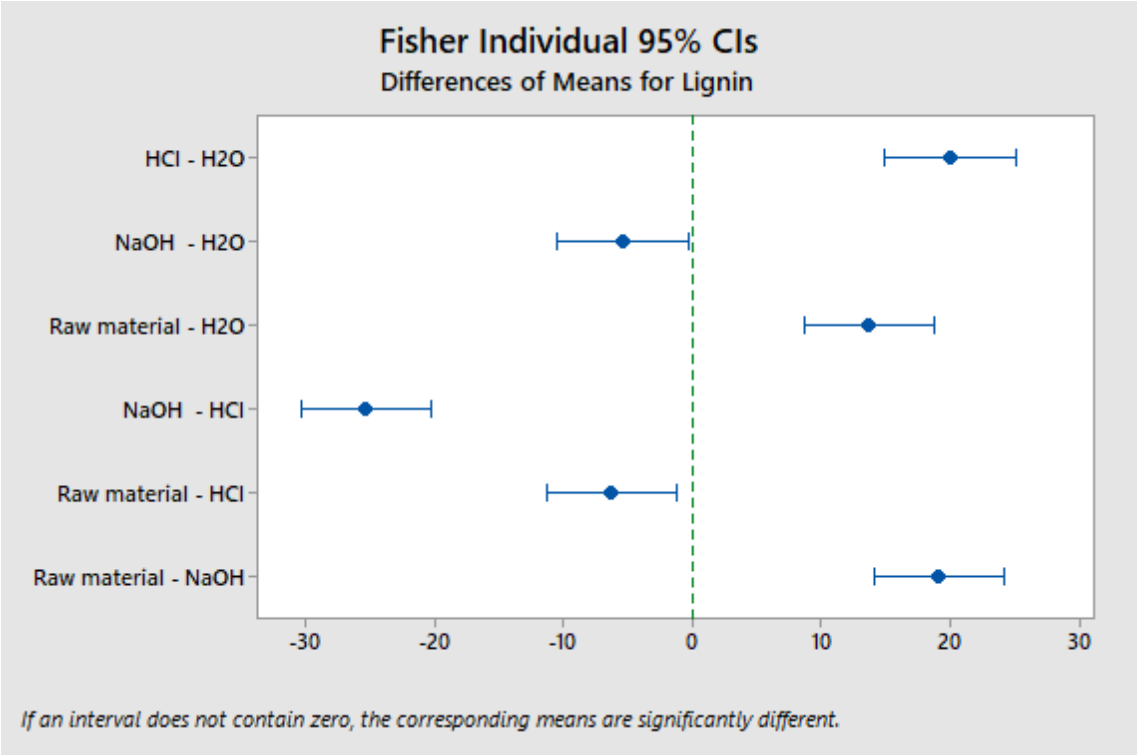


Figure 20. Fisher plot with 95% of confidence interval. Analysis to determine the differences of means for Lignin in each treatment.

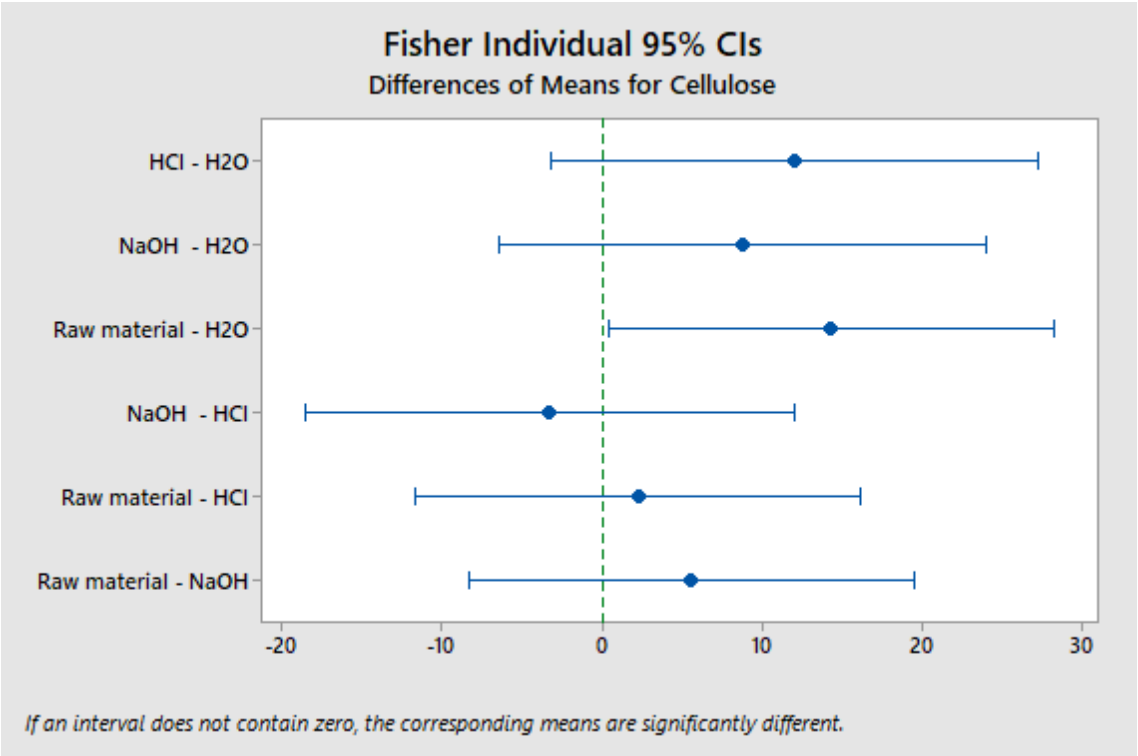


Figure 21. Fisher plot with 95% of confidence interval. Analysis to determine the differences of means for Cellulose in each treatment.