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Generation of Porous Scaffolds from Cacao Mesocarp for Biomedical Applications using Surface Response Methodology

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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 cellular regeneration is the use of lignocellulosic material which can be found in agroindustrial waste, due to its high biocompatibility. Therefore, the present study evaluates the use of waste from the pod shell of cacao type CCN-51, which is 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 Fourier transformed infrared spectroscopy (FTIR) showed important morphological and structural changes depending on the composition of the scaffold.

1. Introduction

There different types of biomaterials, such as ceramics, magnetic structures, and metal alloys, among others, that can be used in biomedical applications (Adamaki et al., 2016; Savvidou et al., 2019). Particularly, biopolymers are of crucial importance in tissue engineering because they are used to generate scaffolding, which serves as a basis for tissue growth (Toscano et al., 2018). They can be classified according to their nature as synthetic or natural biomaterials, with lignocellulosic biomass becoming an important source. Lignocellulose is the main component of plant cell walls; it is produced through photosynthesis, consisting of three main components: cellulose, hemicellulose, and lignin (Zhang et al., 2019). Frequently, these materials show morphological similarities to human tissues; due to this, some studies have already been carried out to produce structured matrices for their use in tissue regeneration (Dutta et al., 2019; Rivero et al., 2018). Lignocellulosic materials are widely available since these are generated as by-products, or waste, in several production processes in agroindustry (Acosta et al., 2018). Ecuador is one of the most important producers and exporters of fine aroma cacao in the world; particularly, production of cacao clone CCN-51 (“clon cacao nacional 51”), generating agri-wastes that represent approximately 2 million tons/year (Mogro et al., 2016). This lignocellulosic residue could be useful as raw material for the generation of scaffolds, due to the high

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levels of biocompatibility of the components present in its structure (hemicellulose, lignin and cellulose) (Camacho et al., 2015). Heredia et al (2014) studied scaffold generation from the mesocarp of caco pod shells, 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 for the delignification of the mesocarp, while maintaining high levels of cellulose in the structures. Scanning electron microscopy (SEM) also showed a high degree of porosity. Furthermore, through mesenchymal stem cell cultures, it was demonstrated that the materials were cytocompatible (Heredia, 2014). 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 shells 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. In this way, an added value would be given to a material that, until now, is considered as waste.

2. Materials and methods

2.1 Preparation of the scaffolds

Healthy pod shells from CCN-51 type cacao in the same state of maturation were used to produce porous scaffolds. The shell was separated from the seeds, and then the mesocarp was manually isolated from the other layers. A general procedure for scaffold generation consisted of the following steps: 1) Cutting the mesocarp in disks 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; 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; and 5) freezing and lyophilization. A Face Centered Central Composite Design (CCD) was applied. Table 1 shows the independent variables that were considered, and their defined limits used to generate the model. Alphas (α) represent maximum and minimum limits of each input variable that will be used to generate the different set of experiments (29 treatments). Central points are defined by column “0”; in this case, the model consisted of 3 central points, and the variability on these points represent that of 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 (℃)	25	37.5	50
Sample thickness (mm)	3	6.5	10

2.2 Physical and Chemical characterization.

To determine the process yield (YYP), the standard AOAC 934.01 was used (International, 1990). Ash content was estimated through standard AOAC 973.18 (International, 1990), while lignin was determined through AOAC 973.18, and cellulose content was quantified following the methodology proposed by Dominguez et al (Domínguez et al., 2012). Further characterization was carried out by scanning electron microscopy (SEM), using a JEOL JSM-IT300, at 50Pa and 5kv. Thermogravimetric analysis (TGA) was performed in a TGA-50 thermogravimetric analyzer, in a temperature range of 19- 500 ℃, under a nitrogen atmosphere and temperature rate of 10 ºC/min. Infrared spectra of chosen scaffolds were obtained using a Fourier Transform Infrared Spectrometer (FTIR) equipped with a Smart iTR module with Attenuated Total Reflectance (ATR).

3. Results and discussions

3.1 Central composite design.

Figure 1 shows the prediction profiles from the model generated by the analysis of the CCD data. As biomass concentration increases, so do lignin, cellulose and YPP, but the ash content decreases. The reduction of treatment time increases YPP, achieving a maximum value of 10.74 % at 4 h, while cellulose, lignin and ash contents steadily increase in the material. However, a large reduction in treatment time, would make no significant changes in the structure. For cellulose and lignin, maximum values were 64 and 35 %w/w,

respectively. Similarly, ash content and YPP reached their highest values of 15 and 8 %, respectively, at around 37.5 ℃. The increase in scaffold thickness shows a minimum for both lignin and ash contents, and a maximum for cellulose. On the other hand, a minimum can be seen for YYP, which seems to stabilize with the increase of thickness. In figure 1, the gray area in each profile represents data variability, which could be a result of differences in the degrees of maturity of the cocoa pods, as well as changes in the geographic origins of the samples (Alvarez-Barreto et al., 2018).

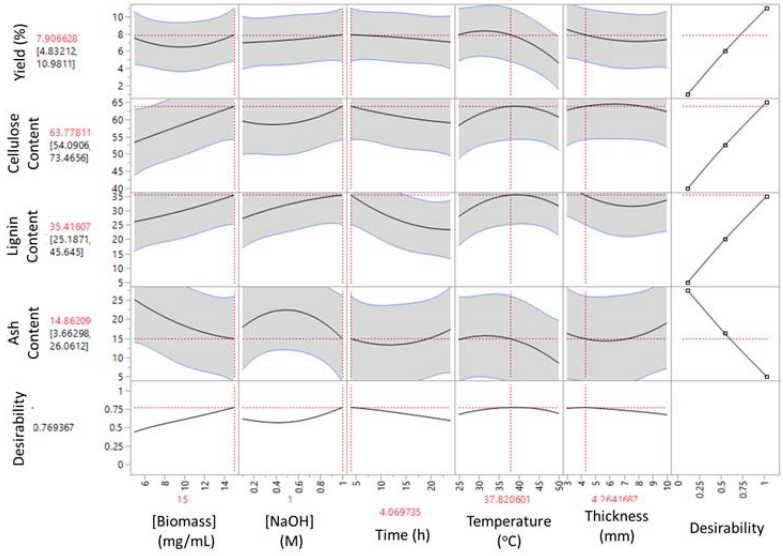

Figure 1: CCD prediction profiles of output variables as functions of input variables. Vertical dotted red lines show the values of each input variable that optimize the desirable output variables.

Table 2 shows the overall level of influence of each input variable on the experimental model, as well as for each output variable. Overall, the most influential variables were temperature, followed by treatment time and NaOH concentration. These results are in accordance with those found in the literature regarding alkaline treatments applied to other types of lignocellulosic materials (Cardona et al., 2013). Particularly, an increase in temperature decreases cellulose crystallinity and increases the speed of delignification, which produces greater access to the cellulose present in mesocarp (Redding et al., 2011). Heredia et al. (2014) also reported that an increase of NaOH concentration generates a decrease in the cellulose content.

Table 2: Level of influence of each input variable in the experiment model.

Effect	Input Variable				
	Temperature (ºC)	**Time (h)**	**NaOH Conc. (M)**	**Thickness (mm)**	**Biomass Conc. (mg/mL)**
Overall	0.425	0.218	0.198	0.193	0.184
Yield (wt%)	0.537	0.207	0.177	0.144	0.029
Cellulose Content (wt%)	0.690	0.225	0.159	0.157	0.106
Lignin Content (wt%)	0.284	0.243	0.240	0.229	0.151
Ash Content (wt%)	0.452	0.243	0.205	0.198	0.188

For data prediction profiles (data not shown), each of the analyzed parameters showed a value of p> 0.1, implying that the null hypothesis cannot be rejected. Thus, some limitations exist for predicting output variables, given a set of initial conditions. Table 3 shows the results of model validation, comparing predicted and experimental values, from the set of input variables: 1M NaOH, 15% in biomass concentration, 37.5ºC, 4h, and 4.5 mm in thickness. The analysis was carried out for 3 different cacao shells with similar degrees of maturity. The closest approximation from the model was for lignin content, with 9% deviation of the predicted value from the actual content (experimental). For YPP and Cellulose, the prediction was below the 20% deviation mark, but ash content, which also showed the greatest data variability in the model, could not be predicted, as the deviation surpassed 50%. Therefore, it is possible to say that the model can be used as an

indication of tendencies between the input and output variables, but not for prediction of all the output variables considered.

Table 3: Validation analysis of the experimental model, using conditions to maximize the cellulose.

Output variable	Predicted Value	Experimental	Deviation from predicted value (%)
YPP (%)	7.91	6.50 ± 0.28	17.78
Cellulose Content (%w/w)	63.77	54.80 ± 2.57	14.01
Lignin Content (%w/w)	35.42	32.21±5.78	9.03
Ash Content (%w/w)	14.86	22.79±1.38	53.39

3.2 Thermogravimetric analysis (TGA)

TGA analyses were performed to determine the degree of material thermal stability as a function of its composition. Samples were chosen according to their compositions, with varied degrees of cellulose and lignin content, as shown in Table 4.

Table 4: TGA results under different experimental conditions.

#	Biomass Conc. (%w/v)	NaOH Conc. (M)	Time (h)	Temp. (℃)	Thickness (mm)	Cellulose (% w/w)	Lignin (% w/w)	Temp. Max. Degradation (ºC)	Residue (wt%)
5	5	0.1	4	25	3.0	45.78	22.02	320	31.0
25	10	1.0	14	37	6.5	59.95	21.91	306	38.3
28	15	1.0	24	25	10.0	64.57	15.69	285	34.8

Figure 2A shows TGA curves for selected formulations (shown in Table 4). They presented similar trends, with superficial water loss between 60 and 100 ℃, and the internal water loss between 100 and 150 ℃. Samples 25 and 28 had greater mass loss, perhaps due to their greater dimensions, that could make them retain more water, compared to scaffold 5, with a thickness of 3 mm. The residual mass after TGA was directly dependent on cellulose content (Figure 2A), while temperature of maximum degradation, on the other hand, increased at largest lignin contents (Figure 2B). In lignocellulosic materials, lignin is the last component to degrade, since this compound is characterized by having a high thermal stability, with degradation temperatures >450 ℃ (Manals et al., 2011). Similar results have been observed by other authors, regarding lignin and cellulose composition on the thermal behavior of biomaterials (Tanase-Opedal et al., 2019; Xu et al., 2018).

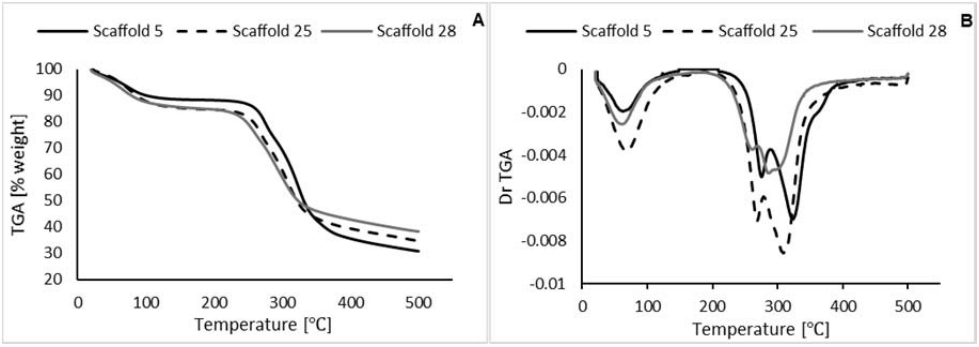

Figure 2: Thermal analysis of scaffolds with different compositions (Table 3) A) Thermogravimetric (TGA) curve, B) Differential thermogravimetric (DTG).

3.3 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. A comparison was made between the scaffolds chosen for TGA, along with untreated mesocarp. In Figure 3, the intensity of characteristic peaks of cellulose, O-H, C-H, C=O and C-O-C (Sangian et al., 2018) was similar in all scaffold samples, but an increment peak intensity can be seen in comparison to untreated biomass. On the other hand, there was a decrease in the peak at 1190 cm-1 in all the scaffolds compared to CWT, corresponding to phenol-hydroxyl and aryl-ether bonds, characteristic of the lignin structure (Dávila et al., 2018). Thus, this corroborates lignin removal in the process, as observed in previous studies on biomass changes after alkaline treatments (Nargotra et al., 2018; Shahabazuddin et al., 2018).

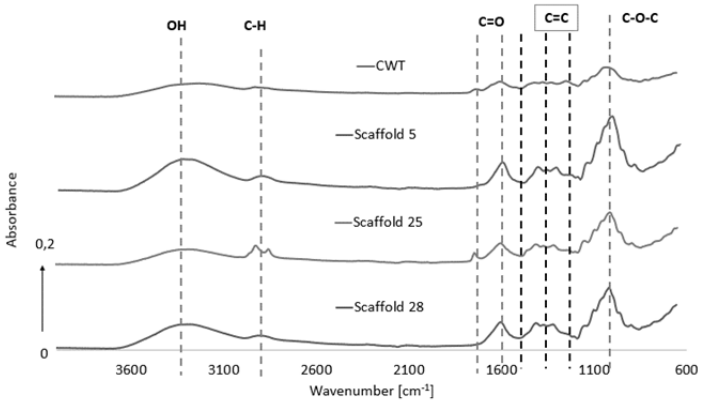

Figure 3: FT-IR spectra of scaffolds with different compositions (Table 4), and cacao without treatment (CWT).

3.4 Scanning electron microscopy (SEM)

Biomass structure is generally altered by alkaline treatments (Nargotra et al., 2018; Shahabazuddin et al., 2018).The morphology of the scaffolds is presented in Figure 4. Figure 4A shows the structure of scaffold 12 (central point), where high levels of non-uniform porosity were observed, due to apparent variations in pose size. Figures 4C-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 appears to have smaller pore size due to the lower degree of material removal (approximately 10 %w/w lignin content).

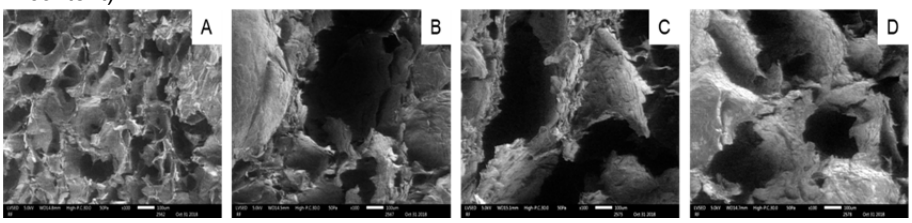

Figure 4: SEM of cacao-mesocarp scaffolds, according to Table 4. A) central point, ;B) Scaffold 5; C) Scaffold 25; D) Scaffold 28. Magnification: 100X. Calibration bar: 100µm.

4. Conclusions

It is possible to generate porous structures from the mesocarp of cacao pod shells as scaffolds for potential biomedical applications. Temperature and NaOH concentration were the most important input variables However, sample variability was an important factor that significantly affected model prediction. 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 *in vitro* cytocompatibility, and proliferation and differentiation.

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