RESEARCH / INVESTIGACIÓN

Porous Sponges from the Mesocarp of *Theobroma Cacao L.* Pod Shells for Potential Biomaterial Applications

Sandra Heredia[1], Diego Costa[2], Iván Choez[1], Ana Barragan[1], María F. Quijano[1], Washington Cárdenas[2], Patricia Manzano[1], Carol Reyes[3], Daynet Sosa[1], Jose F. Alvarez-Barreto[3*]

DOI. 10.21931/RB/2021.06.01.14

Abstract: Lignocellulosic materials have garnered significant attention in recent years to generate biomaterials, but nothing has been investigated with cacao residues of significant importance in Ecuador. This study's objective was to generate porous, three-dimensional sponges from cacao pod shell mesocarp with potential use in biomaterial application. Discs from the mesocarp of cacao pod shells were subjected to neutral, acid, and alkaline treatments, at 25oC and 100oC, followed by washing and lyophilization. Sponge composition was evaluated, with the alkaline treatment resulting in the highest cellulose content and the lowest percentage of lignin, with the removal of hemicellulose corroborated by FITR. The sponges presented high water absorption capacities, which increased with the treatment temperature; mainly, the alkaline generated structures had the largest capacity. The sponges' porosity also depended on the treatment, with the acid and alkaline treatments generating larger pores, which significantly grew with treatment temperature. Preliminary *in vitro* cytotoxicity tests were carried out using Wharton's jelly mesenchymal stem cells, according to ISO 10993.5.2009, with none of the materials being cytotoxic; however, those with greater lignin contents resulted in lower cell viability. In general, it is considered that the alkaline generated sponges presented the more significant potential for biomaterial applications, which could be further tested with *In vitro* cell proliferation and differentiation studies and possible *in vivo* assays.

Key words: Cacao, lignocellulosic biomass, waste, biomaterials, valorization.

Introduction

Lignocellulosic biomass has been considered for varied technologies within a framework of its valorization. Applications include environmental as an adsorbent for water purification, biotechnological, microorganism substrates for different bioprocess, and biomedical as biomaterials. Secondary lignocellulosic biomass, derived from agro waste, is increasingly prevalent worldwide, and the novel was for its valorization is the focus of extensive research. Ecuador supports its economy mainly on agriculture, and essential crops include corn, tubers, and cacao. Regarding the latter, this country is essential for being one of the largest producers of fine-aroma varieties[1].

Nonetheless, due to this variety being prone to diseases and its low plant productivity, a clone, CCN51, has gained more preponderance among farmers, despite having more insufficient organoleptic properties[2]. This last crop alone generates more than 200,000 tons of waste per year, with most of it being lignocellulosic material from the pod shell, which is mainly used for animal feed or as fertilizer in the same crop. However, these applications are inefficient since animals do not easily digest the material, and its potential as a fertilizer has not been demonstrated[3].

The two main components of this type of residues, cellulose and lignin, are different commercial interests. On the one hand, cellulose can be digested to generate reducing sugars, used by different microorganisms to produce various metabolites, such as bioethanol, biogas, and lactic acid. On the other hand, lignin can be processed into materials used in constructions, among other areas. Lignocellulosic biomass has also been used as absorbent and adsorbent materials for oil, heavy metals, and other essential water contaminants. Moreover, both lignin and cellulose possess different cyto and biocompatibility levels, making them suitable candidates for biomaterial applications, such as scaffolds for tissue engineering of controlled drug delivery systems[4,5].

Lignin and some of its derivatives have already been used in these applications, both alone or in combination with other materials, displaying high levels of cytocompatibility and the ability to support cell adhesion, proliferation, and differentiation[4,6,7]. However, it has been reported that they can reach cytotoxic levels, which could hinder the potential application of lignocellulose-based biomaterials[8]. On the other hand, cellulose has been more widely employed, with applications in thin films, composite hydrogels, and three-dimensional porous scaffolds, among others. This polysaccharide has been combined with biopolymers such as chitosan, collagen, and alginate[9,10], with actual results in skin, bone, cartilage, and even nerve tissue engineering[11–13]. However, they have been used separately and assembled in residual biomass for different biomedical applications. Scaffolds have been fabricated from lignocellulosic apple matrices through their chemical and enzymatic pretreatments. These constructs were efficient in supporting mesenchymal stem cell proliferation and differentiation[14].

As the agricultural residue represented by cacao pod shells is rich in these biopolymers, we believe that it can be used to generate porous structures that could potentially be used as biomaterials. Thus, they would represent feasible alternatives to other biopolymers, both natural or synthetic, more commonly used in the fabrication of such matrices, namely chitosan, poly-a hydroxy esters, and alginate, among others[15–24].

[1] Escuela Superior Politecnica del Litoral, ESPOL, Centro de Investigaciones Biotecnologicas del Ecuador, CIBE, Guayaquil, Ecuador.
[2] Laboratorio para Investigaciones Biomédicas, FCV, Escuela Superior Politécnica del Litoral, Guayaquil, Ecuador.
[3] Biomaterials Laboratory, Institute for the Development of Alternative Energies and Materials, IDEMA, Department of Chemical Engineering, College of Sciences and Engineering, Universidad San Francisco de Quito, Quito, Ecuador

Corresponding author: jalvarezb@usfq.edu.ec

The valorization of the residue mentioned above as a biomaterial has not been previously reported in the literature. As it has high contents of essential biopolymers (cellulose and lignin), we hypothesize that suitable structures for biomedical applications could be potentially generated. We believe that structures with a high cellulose and critical lignin composition levels could result in highly cytocompatible and stable structures. Thus, this study's objective was to establish an appropriate protocol for generating porous, three-dimensional sponges from cacao pod shell mesocarp and perform a preliminary assessment into their cytocompatibility properties that could translate into biomaterial applications. The mesocarp was chosen since other layers of the pod shells are either too woody and difficult to process or disintegrated quickly with any treatment[25].

Materials and methods

The general procedure used to generate the sponges and their subsequent characterization is shown in figure 1.

Mesocarp samples from cacao pod shells

CCN51 cacao pods were obtained from Guayas, Ecuador, always from the same location and similar maturation degree. The mesocarp was manually separated from the other layers of the shells, namely the endocarp and exocarp, and discs of 6.6 mm in diameter and 5.6 mm in thickness were obtained. Due to enzymatic darkening and accelerated oxidation, the samples were treated immediately.

Generation of porous sponges

The mesocarp samples were subjected to the treatments specified in Table 1. Briefly, a known mass of discs was suspended in the reagent solution (sodium hydroxide, acetic acid, or water) at a ratio of 1g per 15ml. Treatments were carried out at room temperature or 100ºC. Those at room temperature were performed for 72 h, with changes in solution at 24 and 48h, stirring at 50 rpm, while those at 100ºC were done in a reflux system for 3 h, with changes in solution at 1 and 2h. These times were based on previous laboratory trials. At room temperature, 3h did not result in significant changes in the structure, while longer times in the reflux system caused the samples' complete disintegration. After the treatment, the resulting samples were thoroughly rinsed with distilled water until neutralized, later frozen, and lyophilized.

Crude Fiber

An adaptation from the norm AOAC 989.03 was used[26]. Briefly, 2 grams of defatted sample (previously treated in a Soxhlet system, using hexane) were treated with 1.25% sulfuric acid and 1.25% NaOH in reflux for 1h. Then, the sample was washed with distilled water and dried at 105ºC for 2h. After weighing the dried sample, calcination followed at 500ºC. Crude fiber (CF) content was determined through equation (1).

$$\%CF = \frac{(WT - WC)}{WS} * 100 \quad (1)$$

WT is the weight of the treated sample, WC is the weight after calcination, while WS represents the defatted sample.

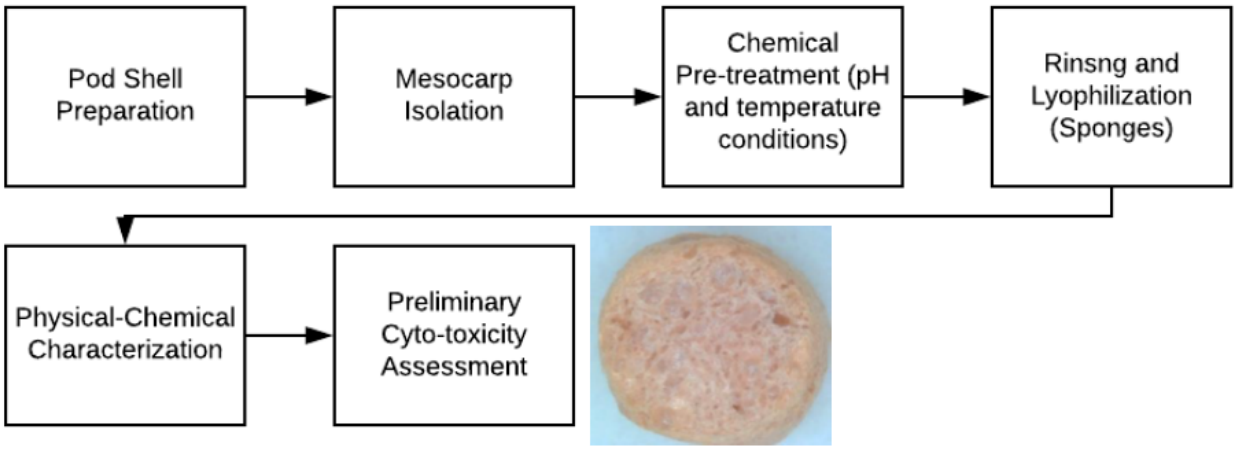


Figure 1. General experimental procedure for the generation and characterization of cacao mesocarp sponges. The stereoscopic picture presents an example of a generated sponge.

Treatment Type	Reagent	Concentration	Temperature	Time
Neutral	H_2O	-	25ºC	72h
Neutral	H_2O	-	100ºC	3h
Acid	CH_3COOH	1N	25ºC	72h
Acid	CH_3COOH	1N	100ºC	3h
Alkaline	NaOH	1N	25ºC	72h
Alkaline	NaOH	1N	100ºC	3h

Table 1. Experimental conditions of the different pretreatments applied to cacao mesocarp samples to obtain porous sponges

Ash Content

Norm AOAC 932.01 was followed. One gram of sample was calcinated at 700ºC for 2h. Total ash content was determined through equation (2). Where WS and WC are the weights of the sample and after calcination, respectively.

$$\%TA = \frac{WC}{WS} * 100 \quad (2)$$

Cellulose Content

One gram of dried sample was digested in 15 mL of 80% acetic acid and 1.5mL of concentrated nitric acid, under reflux, for 20 min. The digested sample was then filtered and washed with ethanol, followed by drying at 105ºC. Calcination followed at 540ºC. Cellulose content was calculated through equation (3).

$$\%CC = \frac{(WT - WC)}{WS} * 100 \quad (3)$$

WT is the sample's weight after reflux, WC is the weight after calcination, while WS represents de defatted sample.

Lignin Content

According to AOAC 973.18, one gram of defatted sample was digested in 15 ml of 72% sulfuric acid, under moderate agitation for 2h, at room temperature. Then, the digested sample was taken to a reflux system with water for 4h, followed by filtration, thorough rinsing with water, and drying at 105ºC for 3h. Equation (4) was used to determine the percentage of lignin in the sample.

$$\%LC = \frac{WF}{WI} * 100 \quad (4)$$

Where, WI and WF are the initial and final sample weights, respectively.

Water Absorption

To determine the water absorption capacity of the sponges, swelling assays were conducted. Lyophilized samples were placed in 24-well plates, incubated in phosphate-buffered saline (PBS, pH 7.2), at 37oC. Sample weight was monitored over time, and the swelling degree was determined through equation (5).

$$\%SD = \frac{(W_t - W_0)}{W_0} * 100 \quad (5)$$

Where, W_0 is the initial weight, and W_t is the weight at a specific time, t.

Scanning Electron Microscopy, SEM

Foam morphology was analyzed in a Scanning Electron Microscope FEI, model INSPECT. For this, samples were sputter-coated with platinum and observed at 50 Pa and 7.5 KV. Pore size analyses were carried out using the software ImageJ[27,28].

Fourier Transform Infrared Spectroscopy, FTIR

Samples were analyzed with a Cary 630 FTIR Spectrometer from Agilent Technologies, with an ATR module, with an interval of 650cm^{-1} to 4000 cm^{-1} in wavelength.

Cytocompatibility Analyses

Cell viability was used as a preliminary test to assess the material's cytocompatibility; ISO 10993.5.2009 (Biological evaluation of medical devices -- Part 5: Tests for *in vitro* cytotoxicity) was used for this purpose. Mesenchymal stem cells were isolated from the Wharton's Jelly of human umbilical cord after approval from the Bioethics Committee at Hospital Luis Vernaza, Guayaquil (Document number HLV-DOF-CEI-007, with date February 27, 2015). Cells from the third passage were plated in 96-well plates at $5x10^3$ cells/cm^2, and cultured over 24 h with Dulbecco's modified essential media (DMEM, supplemented with 10% fetal bovine serum, FBS and 1% penicillin-streptomycin). Sponge samples were disintegrated in non-supplemented DMEM at a 1mg/mL concentration and sterilized in an autoclave. The wells were then washed with phosphate buffer saline (PBS, pH 7.2), and processed sample suspensions were added. Control was used with only DMEM (without material). These were incubated, for 72h, in a humid atmosphere with 5% CO_2 at 37oC. After that, wells were rinsed with PBS, and an MTT (Thiazolyl blue tetrazolium bromide) solution with a concentration of 5 mg/ml, in PBS, was added, followed by incubation under culture conditions, for 3h. After that, dimethyl sulfoxide was added, and optical density was obtained in a spectrophotometer at 590nm. Cell viability was calculated through equation 6.

$$Cell\ viability = \frac{ODs}{ODc} * 100 \quad (6)$$

Where ODs and ODc are the optical densities of the sample and control, respectively.

Statistical Analyses

Results are reported as the average ± standard deviation. Four replicates were used unless otherwise specified. For crude fiber, ashes, cellulose, and lignin, averages from four different pods were reported. Statistically significant differences were determined through ANOVA, with multiple comparisons with Tukey, with a confidence level of 95%.

Results and Discussion

In the generation of porous structures, it was essential to assess which methods could result in more significant cellulose contents and lower lignin compositions. Breaking down lignin structure could provide greater porosity to the structure, an important architectural feature for biomaterial applications. Moreover, cellulose, for instance, is a highly cytocompatible polymer[20], while lignin, also cytocompatible[4], albeit to a lesser degree[8], has limited biodegradability[29]. It is important to remember that this study aimed not to perform a thorough biological evaluation of the generated materials but to establish a methodology to obtain porous structures composed of biopolymers previously reported as cyto- and bio-compatible.

Being a lignocellulosic material, cacao mesocarp could represent a local alternative (in Ecuador and Latin America in general) for the generation of new biomaterials. Different treatments would cause composition changes, translating into different yields and altering the dimensions concerning the original sample, as shown in Table 2. Neutral treatments, independent of the temperature, had the most significant yields and the lowest reduction on sample dimensions. On the other hand, alkaline treatments resulted in the lowest yields and

largest reduction in dimensions: diameter, thickness, and volume. For all cases, these effects were more pronounced at 100oC, which consistently resulted in lower yields and more considerable volume reduction for 25oC. This could sign greater levels of removal of specific components of the mesocarp, organic, such as fiber, and inorganic in the form of ashes.

Crude fiber is primarily composed of cellulose, lignin, hemicellulose, and pectic compounds[30]; however, it is merely used to assess their changes and not on the actual values of their contents. As seen in Figure 2, crude fiber content significantly increased with temperature. At 25oC, the neutral treatment produced the lowest amount of crude fiber, comparable to the untreated samples, while there were no significant differences between the acid and alkaline treatments, despite them having larger crude fiber content. At 100oC, on the other hand, it was the alkaline treatment that resulted in the largest percentage of crude fiber ($p<0.05$).

Analyzing fiber components, lignin content for all different treatments is shown in Figure 3. The highest content of lignin, at both temperatures, was found in the acid treatment, reaching a maximum of approximately 50%. The lowest values were obtained with the alkaline treatment, reaching a minimum of about 20%, comparable to that of the untreated sample. In different lignocellulosic biomass types, it has been reported that alkaline conditions, especially with sodium hydroxide, resulting in lower lignin content.

Regarding cellulose content (Figure 4), there were significant increases in this parameter concerning temperature for all the applied treatments. At 25oC, water resulted in a cellulose content similar to that of the untreated mesocarp (approximately 29%), significantly lower than those of the acid and alkaline treatments, with the latter yielding the highest content. At 100oC, however, the acid treatment resulted in the lowest cellulose content, while the alkaline still produced the highest value of (48.88 ± 1.67) %.

The acid's primary purpose is the solubilization of hemicellulose, not quantified here, weakening the interactions between lignin and cellulose[31]. However, in this case, under the acid condition, the composition of lignin is significantly greater than that of the cellulose, which could be due to acid hydrolysis of the latter. On the other hand, the alkaline treatment is more directly related to lignin removal by inducing biomass swelling, decreasing crystallinity, and, consequently, rupturing its structure[32]. Water could have a similar effect, albeit smaller. This explains the differences in fiber content between treatments, but not when compared to the untreated mesocarp. As previously mentioned, this study did not quantify other biomass components, and some of them are present in significant amounts, such as hemicellulose, pectin, and reducing sugars. Their removal could alter compositions by any of the treatments, causing an increase in the percentage content of crude fiber, cellulose, and lignin.

Treatment Conditions	Yield (%)	Diameter (mm)	Thickness (mm)	Volume Reduction (%)
Neutral at 25°C	72.26±2.31	6.54±0.17	5.49±0.55	3.74±0.15
Neutral at 100°C	49.19±1.67	6.51±0.16	5.01±0.54	12.95±0.14
Acid at 25°C	35.15±1.82	6.36±0.17	5.00±0.79	17.09±0.18
Acid at 100°C	35.72±2.73	6.12±0.33	4.20±0.63	36.74±0.17
Alkaline at 25°C	28.53±0.34	6.15±0.29	4.97±0.72	22.94±0.18
Alkaline at 100°C	17.73±1.40	5.86±0.51	4.23±0.52	40.45±0.18

Table 2. Yield of treated mesocarp from cacao pod shell after different treatment conditions to produce porous sponges, along with their average dimensions.

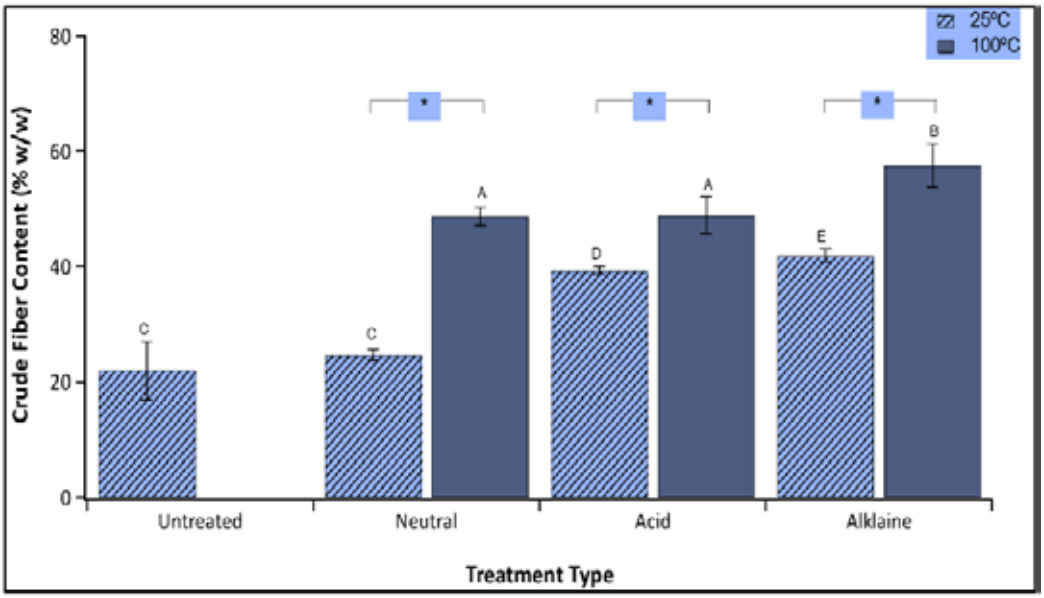


Figure 2. Crude fiber content of cacao mesocarp porous sponges generated by treatments with different reagents at different temperatures. * represents significant differences between the two temperatures. The same letters indicate that the values are statistically the same, while different letters denote differences ($p<0.05$).

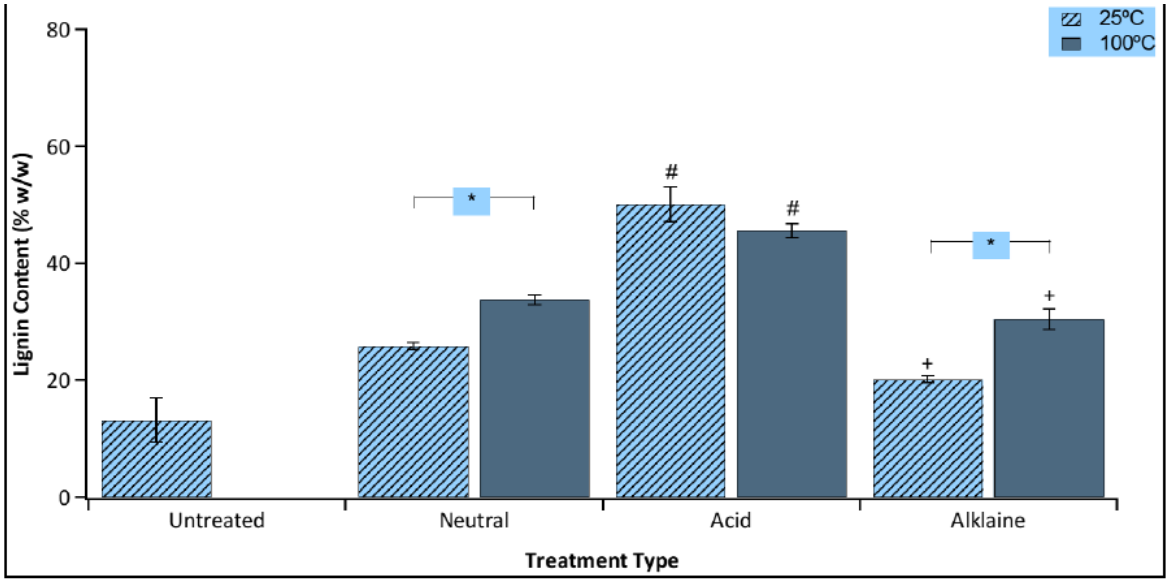


Figure 3. Lignin content of cacao mesocarp porous sponges generated by treatments with different reagents at different temperatures. * represents significant differences between the two temperatures. + And # the treatment type that resulted in the lowest and highest cellulose content, respectively, at a given temperature.

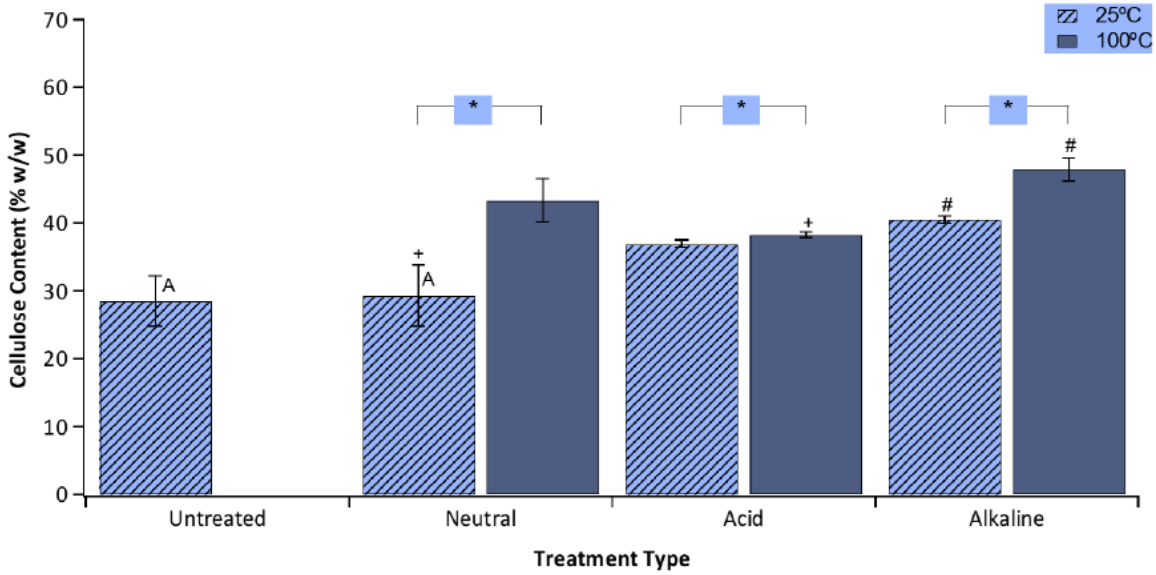


Figure 4. Cellulose content of cacao mesocarp porous sponges generated by treatments with different reagents at different temperatures. * represents significant differences between the two temperatures. The same letters indicate that the values are statistically the same. + And # the treatment type that resulted in the lowest and highest cellulose content, respectively, at a given temperature.

Another essential component of the biomass is represented by inorganic material in the form of ashes, presented in Figure 5 for the present study. The acid treatment resulted in the most efficient method to remove ashes, with up to 95% removal. Acetic acid is particularly efficient at this task, creating soluble acetate salts of calcium, magnesium, and potassium (among other cations)[33]. On the other hand, the alkaline treatment could create insoluble hydroxides that could remain in the structure, even after washes.

FTIR analyses were carried out to confirm structural and compositional changes in the sponges, as shown in Figure 6. Characteristic hemicellulose peaks are observed at 1057 and 1090 cm^{-1} [34], but the signal at 1270 cm^{-1} is significantly diminished in the sponges resulting from the alkaline treatment. Cellulose peaks are also found in all samples, especially those at 1154 and 898 cm^{-1}. The untreated samples displayed all the characteristic peaks for lignin, including 1605 y 1517 cm^{-1}, corresponding to phenyl ring skeletal vibrations, and adsorption of aromatic methoxy groups at 2850 cm^{-1} [35]. These peaks were observed in the treated samples' spectra; however, the vibration of aromatic rings at 850 cm^{-1} disappeared with the alkaline treatment. A similar effect is observed with the absorbance of aldehyde/ketone groups at 1730 cm^{-1}. These results corroborate that the alkaline treatment is efficient at removing not only lignin but also hemicellulose.

Through changes in the composition, where lignin and cellulose levels varied significantly, it was essential to evaluate the sponges' water absorption capacity. This parameter

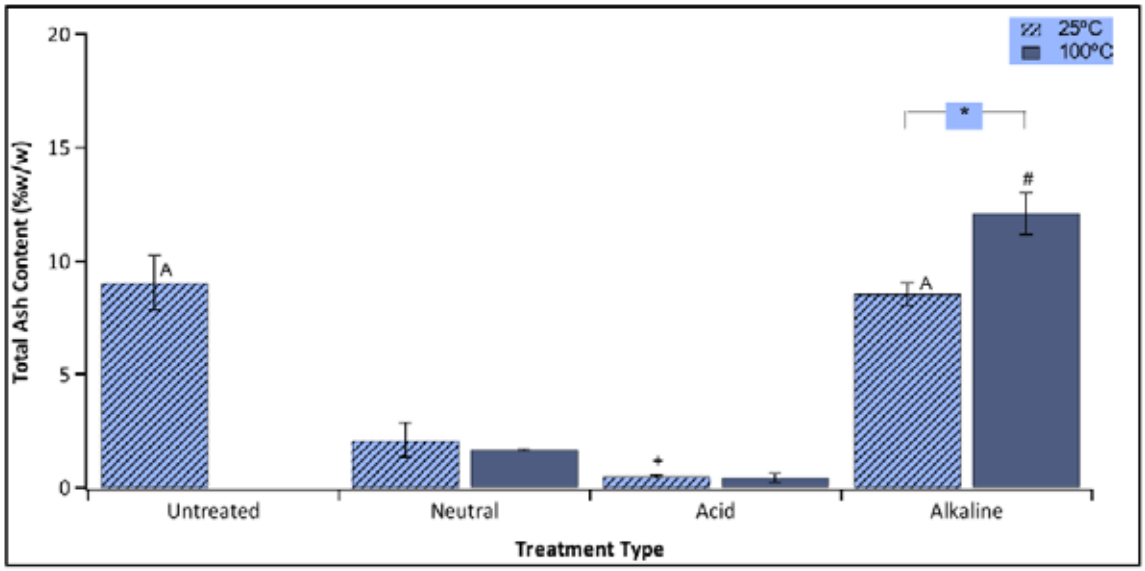


Figure 5. Ash content of cacao mesocarp porous sponges generated by treatments with different reagents at different temperatures. * represents significant differences between the two temperatures. The same letters indicate that the values are statistically the same. + And # the treatment type that resulted in the lowest and highest cellulose content, respectively, at a given temperature.

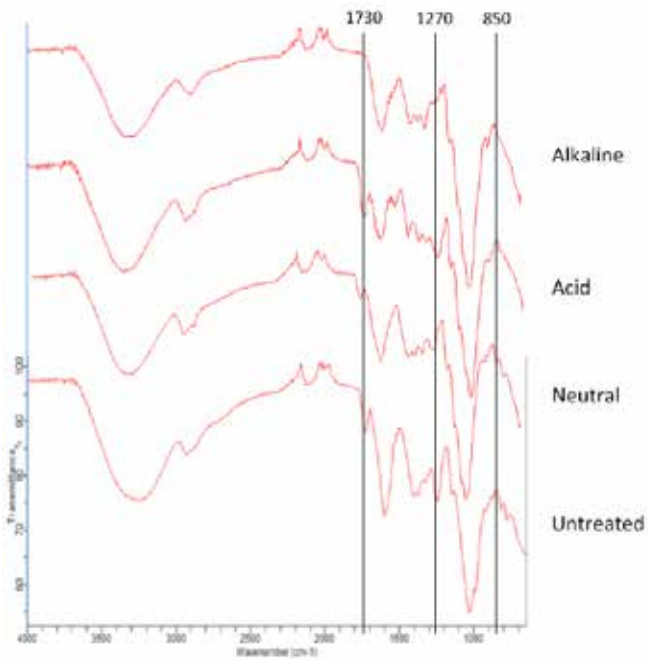


Figure 6. Fourier transform infrared spectra of the untreated samples and the sponges generated by neutral, acid and alkaline treatments.

is of upmost significance given that through the diffusion of nutrients in aqueous media (culture medium or blood), in potential tissue engineering applications, cell survival could be guaranteed[36]. Figure 7 shows the water uptake capacity for the sponges generated through different treatments. At 25oC (Fig. 7A) and 100oC (Fig. 7B), the sponges resulting from the alkaline treatment presented the largest water absorption capacity, followed by those generated through acidic treatments, and those obtained by neutral treatment had the lowest capacities. Nevertheless, all the samples' swelling was remarkably high, more significant than that of the untreated mesocarp, which could give an idea of the sponges' large porosity. Over 80% of the water absorption occurred at 1h for all the studied formulations.

Additionally, the stabilization of the absorption, which was observed even after 72h, indicates that the structures are stable under physiological conditions. However, the materials without treatment presented an already high capacity to absorb water, and, at 25oC, there were no differences between this condition and those treated with water. However, for all treatments, water absorption significantly increased at 100oC, which was more pronounced for the scaffold resulting from the acid and water treatments.

The highest absorption capacity of the sponges obtained through alkaline treatment could be explained by their lowest lignin content, which is the component that gives rigidity to the biomass[37]. The presence of hemicellulose is also an essential factor in lignocellulosic biomass stability and rigidity. Its pre-

sence was corroborated through FITR spectra; thus, this could be responsible for the lower values of water uptake levels presented by the scaffolds generated via water treatment. However, as seen in the spectra, hemicellulose levels seemed lower in the alkaline treated samples, translating into more flexibility of the structure and consequent higher water absorption capacity. As the temperature increases, the removal of hemicellulose and lignin becomes more effective, thereby providing less rigidity to the structure and allowing more significant water absorption levels. However, this large water absorption capacity could be an interesting characteristic for other applications, such as in environmental processes or microorganism immobilization for bioprocesses[38].

Close examination of sponge morphology could provide insights into alterations in composition and water absorption capacity. Changes in morphology can be seen in Figure 8, where untreated samples (Control) show micro-porosity, with apparent fibrous structures on its surface. The application of a neutral treatment, at 25oC, already induces a more generous definition of the micropores, but no significant changes in the structure are appreciable. This is corroborated through table 3, where average pore diameter and pore area distribution are shown. At 100oC, the porous structures become more extensive, with more excellent distribution. When treated with acetic acid, changes on the surface are more prominent as it appears more irregular, and larger pores become more frequent, particularly at the higher temperature, when the average pore diameter is more extensive than those obtained at room temperature.

Nonetheless, when looking at the distribution, most pores have similar sizes at both temperatures. On the other hand, even larger pores are observed after the alkaline treatment, and the differences on pore size between room temperature and 100oC are significant this time. It is essential to highlight that there is more excellent uniformity in pore shape as they tend to be more grounded with the alkaline treatment at 100oC. The larger porosity observed with the acid and alkaline conditions can be related to the greater extent of mass and volume loss after the treatment, particularly for the latter; water absorption capacity is also related to this feature, as it is shown in the swelling studies. Similarities in morphology between the untreated and the sponges resulting from neutral conditions corroborate that this treatment did not change the material's structure. The average pore size is related to the change in sample dimensions after the treatment (Table 2). A more extensive loss of mass, which would translate into a more considerable volume reduction, could also be related to more excellent pores; thus, in the case of the treatments at

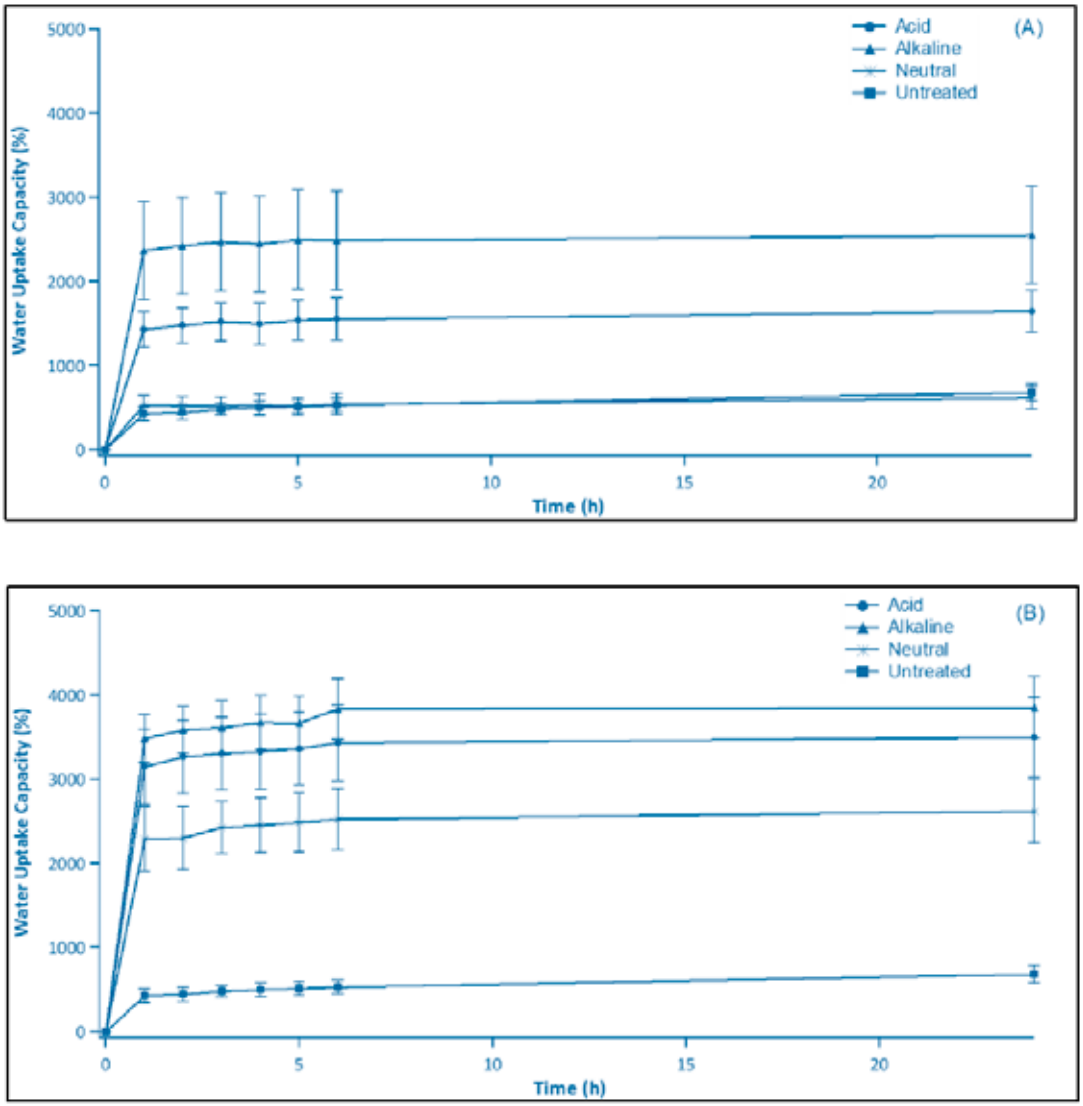


Figure 7. Water absorption kinetics of the sponges generated through different treatments at (A) 25°C and (B) 100°C.

100oC for acid and alkaline treatments, where pore areas are more extensive than in the other experimental conditions, there is also significantly greater volume reduction percentages (around 37 and 40%, respectively). On the other hand, it can be seen that, as there are no critical differences in pore size after neutral treatment at 25oC, the volume reduction is minimum (< 4%).

Porosity and pore size are essential parameters for biomaterial applications. For example, in the case of tissue engineering, the porous structure will be responsible for allowing cell migration and proliferation into the interior of the scaffold[39]. Should cells be unable to penetrate the porous network, tissue regeneration would not be homogeneous, hindering the process's success. Many authors report that pore size should be in the range of 150-300 µm (approximately 17000-70000 µm^2) to permit efficient cell proliferation and migration and synthesis of extracellular matrix and the subsequent formation of new tissue[40]. Nutrient transport would be guaranteed in any of the sponges due to their large water absorption capacity, but not all of them would be suitable for cell activity. From these results, it is apparent that the scaffolds obtained via alkaline treatment would be more appropriate for this purpose.

Table 4 shows the preliminary analysis of different sponges' cytocompatibility; in this case, only those at 100oC were chosen as they were considered the ones with the most significant potential for the proposed application. Only those formulations that yielded cell viability values greater than 70% would be considered potentially cytocompatible[41]. It is essential to point out that no changes in culture media were observed, indicating no pH variations due to the samples. Sponges after alkaline and neutral treatment resulted cytocompatible. However, the untreated samples displayed viability greater than 100%, possibly due to the presence of compounds that could interfere with the MTT protocol and the formation of the purple formazan salts. The acid-produced sponges were not cytotoxic; however, the values were close to the limit established by the norm, and thereby they would not be recommended for further cell studies.

Material's cytotoxicity is directly related to its composition, and, in this case, some trends arise. As previously mentioned, lignin content can reach cytotoxic levels, partially explaining why the acid-generated sponges yielded the lowest cell viability. Additionally, those samples that resulted from the alkaline treatment and those from the neutral and untreated sample had significantly lower lignin contents and, at the same time, higher cell viability percentages. Regarding cellulose, however, the values in its content, despite being significantly different, are not large enough to mark a trend related to cytotoxicity. One interesting point is ash content; in this study, the untreated samples and those treated under alkaline conditions presented the highest ash percentages, but that was not the case with those from the neutral treatment. Thus, there is no clear trend between ash content and cell viability, nor is there a relation between crude fiber and this parameter. Consequently, it may be implied that lignin content will have a greater effect on cell viability when analyzing the scaffold composition. As this is a preliminary test, to determine the potential biomedical applications of these materials, such as tissue engineering, further analyses are recommended in future studies, including cell proliferation and differentiation.

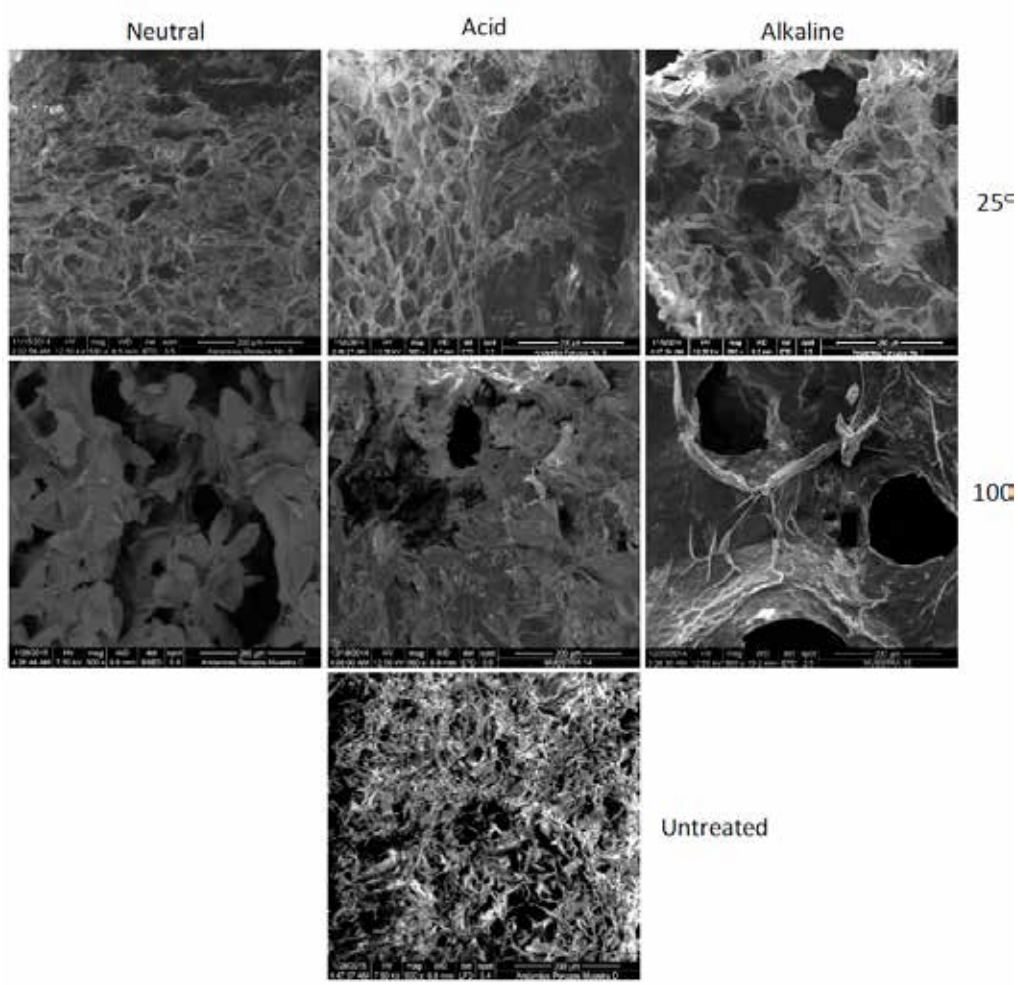


Figure 8. Scanning electron micrographs of mesocarp sponges from cacao pod shell, generated through different treatments. Magnification: 500X, Scale bar: 200 µm.

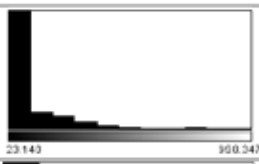
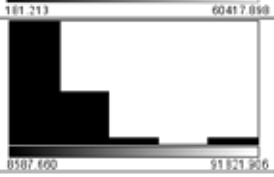
Treatment	Pore Size	
	Average Pore Diameter (µm)	Pore Area (µm²) Distribution
Untreated	13	
Neutral at 25°C	14	
Neutral at 100°C	18	
Acid at 25°C	49	
Acid at 100°C	131	
Alkaline at 25°C	52	
Alkaline at 100°C	169	

Table 3. Pore size analysis of cacao mesocarp sponges obtained through different treatments

Treatment Condition	Cell Viability (%)
Untreated	106.64 ± 9.19
Neutral*	94.72 ± 9.45
Acid*	76.79 ± 5.13
Alkaline*	90.96 ± 3.54
* Treatment at 100°C.	

Table 4. Cytocompatibility analysis of cacao mesocarp sponges using human umbilical cord-derived mesenchymal stem cell cultures

Conclusions

The present study tries to give an alternative to residual biomass valorization by generating highly specialized products that could potentially have an unusual application for this type of material. The results herein presented show that porous sponges, potentially cytocompatible, can be easily obtained through the chemical processing of the mesocarp from cacao pod shells. Of the tested methods, the alkaline treatment shows more significant promise for this application due to the structural changes that result in greater porosity, water absorption capacity, and an indication of low cytotoxicity levels. Nevertheless, in future studies, it would be advisable to optimize the sponge generation process through alkaline treatments, where different variables can be assessed, such as reagent concentration, temperature, and time, among others. Further *in vitro* studies for cell attachment, proliferation and differentiation would also be necessary.

Acknowledgments

We would like to thank the Cell and Organ Transplant Unit at Hospital Luis Vernaza, Guayaquil, Ecuador, for their generous support in gathering the umbilical cords for mesenchymal cell isolation.

Funding Information

This study was funded by the National Secretary of Science and Technology (SENESCYT, Ecuador) via its PROMETEO Program. Additional funding was possible through the Collaboration Grant Program at Universidad San Francisco de Quito.

Conflict of Interests

The authors declare that they have no conflicts of interest regarding this work.

Bibliographic references

1. Vargas Jentzsch , P., Ciobotă , V., Salinas , W., Kampe , B., Aponte , P.M., Rösch , P. et al. (2016). Distinction of Ecuadorian varieties of fermented cocoa beans using Raman spectroscopy. Food Chem, 211: 274–280.
2. Chetschik , I., Kneubühl , M., Chatelain , K., Schlüter , A., Bernath , K., Hühn , T. (2018). Investigations on the Aroma of Cocoa Pulp (Theobroma cacao L.) and Its Influence on the Odor of Fermented Cocoa Beans. J Agric Food Chem, 66(10): 2467–2472.
3. Jiménez Ramírez , O.A., Mantilla Badillo , C.L. (2016). Aprovechamiento de la cascara de mazorca de cacao En la elaboración de carbono activo Para el tratamiento de aguas residuales. http://repositorio.uts.edu.co:8080/xmlui/handle/123456789/2691 (accessed 14 Dec2018).
4. Farhat , W., Venditti , R., Mignard , N., Taha , M., Becquart , F., Ayoub , A. (2017). Polysaccharides and lignin based hydrogels with potential pharmaceutical use as a drug delivery system produced by a reactive extrusion process. Int J Biol Macromol, 104(Pt A): 564–575.
5. Fekete , T., Borsa , J., Takács , E., Wojnárovits , L. (2017). Synthesis of carboxymethylcellulose/starch superabsorbent hydrogels by gamma-irradiation. Chem Cent J, 11(1): 46.
6. Thakur , V.K., Thakur , M.K. (2015). Recent advances in green hydrogels from lignin: a review. Int J Biol Macromol, 72: 834–847.
7. Jiang , P., Sheng , X., Yu , S., Li , H., Lu , J., Zhou , J. et al. (2018). Preparation and characterization of thermo-sensitive gel with phenolated alkali lignin. Sci Rep, 8(1): 14450.
8. Nakasone , K., Kobayashi , T. (2016). Cytocompatible cellulose hydrogels containing trace lignin. Mater Sci Eng C doi:10.1016/j.msec.2016.03.108.
9. de Araújo Júnior , A.M., Braido , G., Saska , S., Barud , H.S., Franchi , L.P., Assunção , R.M.N. et al. (2016). Regenerated cellulose scaffolds: Preparation, characterization and toxicological evaluation. Carbohydr Polym, 136: 892–898.
10. Shaheen , T.I., Montaser , A.S., Li , S. (2019). Effect of cellulose nanocrystals on scaffolds comprising chitosan, alginate and hydroxyapatite for bone tissue engineering. Int J Biol Macromol, 121: 814–821.
11. Chicone , G., de Carvalho , V.F., Paggiaro , A.O. (2018). Use of Oxidized Regenerated Cellulose/Collagen Matrix in Chronic Diabetic Foot Ulcers. Adv Skin Wound Care, 31(2): 66–71.
12. Isobe , N., Komamiya , T., Kimura , S., Kim , U.-J., Wada , M. (2018). Cellulose hydrogel with tunable shape and mechanical properties: From rigid cylinder to soft scaffold. Int J Biol Macromol, 117: 625–631.
13. He , X., Xiao , Q., Lu , C., Wang , Y., Zhang , X., Zhao , J. et al. (2014). Uniaxially Aligned Electrospun All-Cellulose Nanocomposite Nanofibers Reinforced with Cellulose Nanocrystals: Scaffold for Tissue Engineering. Biomacromolecules, 15(2): 618–627.
14. Modulevsky , D.J., Lefebvre , C., Haase , K., Al-Rekabi , Z., Pelling , A.E. (2014). Apple Derived Cellulose Scaffolds for 3D Mammalian Cell Culture. PLoS One, 9(5): e97835.
15. Diban , N., Gómez-Ruiz , B., Lázaro-Díez , M., Ramos-Vivas , J., Ortiz , I., Urtiaga , A. (2018). Factors Affecting Mass Transport Properties of Poly(- -caprolactone) Membranes for Tissue Engineering Bioreactors. Membranes (Basel), 8(3): 51.
16. Alvarez-Barreto , J.F., Shreve , M.C., Deangelis , P.L., Sikavitsas , V.I. (2007). Preparation of a functionally flexible, three-dimensional, biomimetic poly(L-lactic acid) scaffold with improved cell adhesion. Tissue Eng, 13(6). doi:10.1089/ten.2006.0330.
17. Wang , X., Salick , M.R., Gao , Y., Jiang , J., Li , X., Liu , F. et al. (2018). Interconnected porous poly(- -caprolactone) tissue engineering scaffolds fabricated by microcellular injection molding. J Cell Plast, 54(2): 379–397.
18. Carfì Pavia , F., Ciappa , M., Lepedda , A., Fiorentino , S., Rigogliuso , S., Brucato , V. et al. (2017). A poly-L-lactic acid/ collagen/glycosaminoglycan matrix for tissue engineering applications. J Cell Plast, 53(5): 537–549.
19. Sabourian , P., Frounchi , M., Dadbin , S. (2017). Polyvinyl alcohol and polyvinyl alcohol/ polyvinyl pyrrolidone biomedical foams crosslinked by gamma irradiation. J Cell Plast, 53(4): 359–372.
20. Naseri-Nosar , M., Ziora , Z.M. (2018). Wound dressings from naturally-occurring polymers: A review on homopolysaccharide-based composites. Carbohydr Polym, 189: 379–398.
21. Skopinska-Wisniewska , J., Wegrzynowska-Drzymalska , K., Bajek , A., Maj , M., Sionkowska , A. (2016). Is dialdehyde starch a valuable cross-linking agent for collagen/elastin based materials? J Mater Sci Mater Med, 27(4): 67.
22. Kamoun , E.A. (2016). N-succinyl chitosan–dialdehyde starch hybrid hydrogels for biomedical applications. J Adv Res, 7(1): 69–77.
23. Zhao , S., Liu , Z.-X., Gao , H., Wu , Y., Fang , Y., Wu , S.-S. et al. (2015). A three-dimensional culture system using alginate hydrogel prolongs hatched cattle embryo development in vitro. Theriogenology, 84(2): 184–192.
24. Sívoli , L., Pérez , E., Caraballo , D., Rodríguez , J., Rodríguez , D., Moret , J. et al. (2013). Cytocompatibility of a matrix of methylated cassava starch and chitosan. J Cell Plast, 49(6): 507–520.
25. Forero Nuñez , C.A., Jochum , J., Sierra-Vargas , F.E., Sierra-Vargas , F.E. (2015). Effect of particle size and addition of cocoa pod husk on the properties of sawdust and coal pellets. Ing e Investig, 35(1): 17–23.
26. Barton , F.E., Windham , W.R. Determination of acid-detergent fiber and crude protein in forages by near-infrared reflectance spectroscopy: collaborative study. J Assoc Off Anal Chem, 71(6): 1162–7.
27. Salerno , A., Zeppetelli , S., Maio , E. Di, Iannace , S., Netti , P.A. (2010). Novel 3D porous multi-phase composite scaffolds based on PCL, thermoplastic zein and ha prepared via supercritical CO2 foaming for bone regeneration. Compos Sci Technol, 70(13): 1838–1846.
28. Trujillo-de Santiago , G., Portales-Cabrera , C.G., Portillo-Lara , R., Araiz-Hernández , D., Del Barone , M.C., García-López , E. et al. (2015). Supercritical CO2 foaming of thermoplastic materials derived from maize: proof-of-concept use in mammalian cell culture applications. PLoS One, 10(4): e0122489.
29. Garrison , T.F., Murawski , A., Quirino , R.L. (2016). Bio-Based Polymers with Potential for Biodegradability. Polymers (Basel), 8(7). doi:10.3390/polym8070262.
30. Norman , A.G. (1935). The Composition of Crude Fibre. J Agric Sci, 25(04): 529.
31. Bušić , A., Marđetko , N., Kundas , S., Morzak , G., Belskaya , H., Ivančić Šantek , M. et al. (2018). Bioethanol Production from Renewable Raw Materials and Its Separation and Purification: A Review. Food Technol Biotechnol, 56(3): 289–311.
32. Menezes , E.G.T., do Carmo , J.R., Alves , J.G.L.F., Menezes , A.G.T., Guimarães , I.C., Queiroz , F. et al. (2014). Optimization of alkaline pretreatment of coffee pulp for production of bioethanol. Biotechnol Prog, 30(2): 451–462.
33. Torget , R., Walter , P., Himmel , M., Grohmann , K. (1991). Dilute-Acid Pretreatment of Corn Residues and Short-Rotation Woody Crops. Appl Biochem Biotechnol, 28–29(1): 75–86.
34. Yang , H., Yan , R., Chen , H., Lee , D.H., Zheng , C. (2007). Characteristics of hemicellulose, cellulose and lignin pyrolysis. Fuel, 86(12–13): 1781–1788.

35. Rana , R., Langenfeld-Heyser , R., Finkeldey , R., Polle , A. (2010). FTIR spectroscopy, chemical and histochemical characterisation of wood and lignin of five tropical timber wood species of the family of Dipterocarpaceae. Wood Sci Technol, 44(2): 225–242.
36. Leddy , H.A., Awad , H.A., Guilak , F. (2004). Molecular diffusion in tissue-engineered cartilage constructs: Effects of scaffold material, time, and culture conditions. J Biomed Mater Res, 70B(2): 397–406.
37. LISPERGUER , J., PEREZ , P., URIZAR , S. (2009). structure and thermal properties of lignins: characterization by infrared spectroscopy and differential scanning calorimetry. J Chil Chem Soc, 54(4): 460–463.
38. Liu , S., Xu , Y., Zhao , Y., Zou , L., Lu , W. (2019). Hydrothermal modification of lignocellulosic waste as microbial immobilization carriers for ethanol production. Biochem Eng J, 142: 27–33.
39. Alvarez-Barreto , J.F., Sikavitsas , V.I. (2007). Improved mesenchymal stem cell seeding on RGD-modified poly(L-lactic acid) scaffolds using flow perfusion. Macromol Biosci, 7(5). doi:10.1002/mabi.200600280.
40. Wang , Y., Tomlins , P.E., Coombes , A.G.A., Rides , M. (2010). On the Determination of Darcy Permeability Coefficients for a Microporous Tissue Scaffold. Tissue Eng Part C Methods, 16(2): 281–289.
41. Hartje , L.F., Bui , H.T., Andales , D.A., James , S.P., Huber , T.R., Snow , C.D. (2018). Characterizing the Cytocompatibility of Various Cross-Linking Chemistries for the Production of Biostable Large-Pore Protein Crystal Materials. ACS Biomater Sci Eng, 4(3): 826–831.

Received: 25 November 2020
Accepted: 15 January 2021