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ORIGINAL RESEARCH PAPER

Combined Hydrolysis to Yield Increase of Cellulose and (CNCs), Starting from Agroindustrial Rice Husk Waste in Morelos, Mexico

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ABSTRACT

Rice husk (*Oryza sativa* L.) is considered an agroindustrial waste that requires alternative treatment to prevent it from becoming an environmental contaminant. Some recent reports have been able to produce Nanocellulose (CNCs) with this biomass, although with low yields, after using a complex of endo and exoenzymes. Work aimed to evaluate the previous effect of natural proteolytic enzymes (Bromelain/Papain) to degrade the fibrous structure of rice husk before obtaining Cellulose and for whitening, an alternative with hydrogen peroxide. In combination with subsequent enzymatic hydrolysis the new complex formed by four reagents: D-(+)-Cellobiose, endo-1,4-β-D glucanase, β-glucosidase, and Cellobiohydrolase, for the synthesis of Nanocrystals (CNCs). Satisfactory results indicated that it was possible to soften and modify the plant tissue structure, which allowed a final cellulose yield of 56.35%. The hydrolysis with the enzyme complex applied to Cellulose, achieved the obtaining of CNCs between 200-595 nm, after using the four enzymes in complex, with better results when used in a single phase, achieving a final yield of 1.8 -4.2% nanocellulose with process optimization.

Keywords: *Oryza Sativa L, Agroindustrial Waste, Husk, Enzymes, Hydrolysis, Cellulose, Bromelain, Papain, Proteins*

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INTRODUCTION

One of the main wastes from the industrialization of rice (*Oryza sativa* L.) is the husk that covers the grain, considered a biomass that accumulates as a result of threshing and becomes a waste that is difficult to manage. Rice husk in recent years has aroused great interest because it has been possible to obtain cellulose from this biomass with interesting results in yield, ranging from 29 to 41% [3, 18, 21, 33, 38, 43]. This residue is composed of 35% cellulose, 25% hemicellulose, 20% lignin, 17% ash, and mainly 94% silica [6]. Proteins are also found at 11.77% in polishing (pericarp and integument) from bleach [1, 37]. Rodríguez-Almarza [36] also found 12.09 to 10.66%, in three polishing fractions of different sizes. However, Valverde et al [42] mentioned 3.8% of husks. Precisely its elasticity and rigidity depend on many factors such as the type of polymer and its concentration. The concentration of salts and pH also influence, which accelerates the gelation of polymers such as pectins and some proteins [5]. It has been a common practice, to treatments with papain and bromelain, proteolytic enzymes extracted from plants [30], to tenderize meats [27]. In the case of papain, it has a broad specificity, that is, it digests the vast majority of proteins, and treated meats tend to become softer and softer. These proteases have also been

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used to coagulate milk, and form cheese, as well as in the beer clarification process. But, despite these applications in animal tissue, little has been known about its effect on plants.

Therefore, rice husk is used in a direct cooking process once it is fragmented and sieved following three stages, according to protocols described with alkaline chemical treatments (NaOH 5%) [19], blanching (NaClO) at 1% [22] and acid pretreatment (HCl) at 0.65% [20]. This method has some disadvantages, performing many washes to achieve adequate pH and decantation, which produces losses, at least when the particles are very fine.

If you work with the original husk and 10 and 30 mesh, you can achieve up to 41% cellulose yield. Long-grain rice and other sieves yield between 28 and 36% [17]. Several ways to obtain nanocellulose crystals (CNCs) have been used. Mechanical processes with refining and homogenization at high pressure, grinding, crushing at low temperatures (cryo-crushing), and ultrasound [1,13]. But one widely known method has been acid hydrolysis, which uses strong acids such as H_2SO_4 or HCl, at not-so-high temperatures [8,12]. Also, a new process has been proposed to obtain cellulose nanoparticles based on the reaction (TEMPO) oxidation of cellulose by applying 2,2,6,6-tetramethylpiperidine1-oxyl [39], better called nanometric chemical scissors to obtain cellulose microfibrillated [8].

However, an environmentally friendly biological way has been used in recent times to obtain nanocellulose from rice husk. Enzymatic hydrolysis is used for the production of nanoparticles with low energy consumption [32]. This procedure results in crystalline nanofibers linked to the amorphous phase [11]. The enzyme cocktail has the function of breaking down the cellulose polymer into smaller molecules [4, 7]. The added buffer ensures constant enzymatic activity [2, 23, 25, 26]. In comparison of both routes, enzymatic and chemical [18], it was found that evident results recognize the synthesis of nanocellulose (NC) by both routes, generating greater contaminants in the medium due to acid hydrolysis, being more feasible and faster with enzymatic hydrolysis using a couple of enzymes (D-(+) cellobiose and endo-1,4-β-D glucanase) with which there is less aggression to the environment and greater performance. Based on the above, these essays aimed to evaluate the combined effect of natural proteolytic enzymes, applied to rice husk, to obtain cellulose, and additionally verify the effectiveness of enzymatic hydrolysis made up used four enzymes: (D-(+)-Cellobiose, endo-1,4-β-D glucanase, β-glucosidase and Cellobiohydrolase), for synthesis of (CNCs) under different conditions.

EXPERIMENTAL

Materials:

Materials used in the present work: NaOH, CAS: 1310-73-2, Marque: Emsure, HCl, CAS: 7647-01-0, Marque: J.T.Baker, NaClO, Brand: Cloralex, Sodium acetate ($C_2H_3NaO_2$) (SIGMA-ALDRICH), Acetic acid (CH_3COOH) (SIGMA-ALDRICH), H_2O_2, Brand: Schwarzkopf C7252-10MG, D-(+)-Cellobiose, (Brand: SIGMA-ALDRICH), E2164-100UN endo-1,4-β-D-glucanase from Acidothermus cellulolyticus. (Brand: SIGMA-ALDRICH), E6412-100UN Cellobiohydrolase I from *Hypocrea jecorina*. (Brand: SIGMA-ALDRICH), 49290-250MG β-Glucosidase from almonds, Dialysis membrane, 14000 Dalton, Bromelain/Papain (enzymes) SKU:2377 (Caps. 450 mg).

Effect evaluation of Proteolytic Enzymes on rice husk

Before assessing the effect of proteases on rice hulls, the best conditions for their application were tested in comparison with different tissues, which offered references on the best conditions for applying the enzymes [27]. (Data not shown). A solution of 200 mL of natural enzymes Bromelain + Papain (Brom./Pap.) was prepared in a 50/50 w:p ratio, and applied to 10 g of rice husk, under constant stirring for 72 h.

Preliminary tests

A solution of 200 mL of natural enzymes Bromelain + Papain (Brom./Pap.) was prepared in a 50/50 w:p ratio, and applied to 10 g of rice husk, under constant stirring for 72 h.

The effect of proteolytic enzymes (Brom./Pap.) on the husk was verified through an analog Durometer (Shore A ASTM2240), with which the degree of hardness of the biomass was measured in the following treatments (Table 1):

Obtaining cellulose

Rice husk preparation

To obtain cellulose, rice husks from the Morelos A-2010 variety were used. This husk was

Table 1. Treatments and procedures applied to rice husk, to compare the proteolytic activity of natural enzymes (Brom./Pap.).

Treatments		Quantity (g) / Grain (Sieve Ø 30)	Washing	Stirring (h)
1.	Biomass treated with (Bromelain/Papain)	10	30 min	72
2.	Biomass Control (water)	10	30 min	72
3.	Biomass Control (Untreated)	10	-	-

washed with distilled water at 65°C temperature for 90 min, to constant agitation, to eliminate dirt and harmful fauna.

Later, it was placed on metal trays and dried in an oven at 45°C / 24 h. After drying, it was crushed in a conventional blender, turning it into a fine powder. It was weighed and sieved with the # 30, after which it was weighed again and separated into 250 mL, repetitions of 20 g for both treatments [18].

Alkaline treatment

The solution 5% NaOH cooking treatment was prepared to take a ratio of 1:10 husk/solution and added to the flask of each treatment, subsequently placed on a grill at 102 °C for 90 min with constant stirring, at At the end of cooking, they were allowed to rest for 20 min until they were completely cooled and continued with filtering and washing with distilled water, to achieve a neutral pH. At the end, the biomass was placed in properly labeled Petri dishes and placed in an oven at 80°C for 180 min, and once dry, its weight was recorded [19].

Bleaching treatment

In the tests, two solutions were tested a 1% solution of hydrogen peroxide (H_2O_2) for the first treatment (T1) and another solution with sodium hypochlorite (NaClO) 1%, treatment (T2). Then each solution was mixed with biomass, on constant stirring at 80°C temperature for 90 min. After time had passed, solutions were allowed to cool. Followed the filtering and washing, adding distilled water until reaching neutral pH.

To conclude this treatment, the samples were placed in Petri dishes and taken to a heating oven at 80°C for 180°C. min, after the time they were removed and allowed to cool and then the weight obtained was recorded [22].

Acid pretreatment

In this stage, a solution of hydrochloric acid (HCl) was used for both treatments at a concentration of 0.65% w/w, to eliminate macromolecular components such as hemicellulose.

Next, the biomass in each treatment was emptied into beakers, to immediately add the HCl solution.

These flasks were placed on a heating rack at 80°C during 90 min constant stirring. After a time, they were allowed to cool and then filtered and washed with distilled water to obtain a completely neutral pH.

Finally, the samples were placed in Petri dishes, for subsequent drying in an oven at 80°C for 180 min, allowed to cool and weigh each one [14].

Cellulose yield

The cellulose samples obtained after pretreatment were dried in an oven at 80°C for 180 min and then allowed to cool to room temperature.

Subsequently, they were weighed on an analytical balance, to record the weight obtained per stage [25]. The calculation of the pulp yield was done with the following formula:

C: Cellulose mass
CA: Rice husk mass.

Obtaining nanocellulose

The previously weighed and bleached cellulose was used in the enzymatic hydrolysis. Two tests were carried out (Test 1) with the use of all the enzymes together on the cellulose applied at the same time and (Test 2) with enzymes applied to the cellulose in pairs (exo and endoglucanases) in two different phases or times, following the methodology described [27].

The enzyme complex was formed by the following enzymes, D-(+)-cellobiose (SIGMA-ALDRICH), endo-1,4-β-D-glucanase from Acidothermus cellulolyticus (SIGMA-ALDRICH), β-glucosidase from almonds (SIGMA -ALDRICH) and Cellobiohydrolase from Hypocrea jecorina (SIGMA-ALDRICH).

Therefore, it began with the preparation of the stock solutions, taking into account the enzymatic activity indicated by the supplier of each of them (Sigma-Aldrich). Four amber bottles were sterilized and labeled with the name of each enzyme. To the first, 5 mg of D-(+)-cellobiose and 50 mL of deionized water that was previously sterilized were added, covered, and placed at 4 °C. To the second bottle, 1 mL of endo-1,4-β-D-glucanase and 50 mL of sterilized water were added, it was also covered and placed at 4 °C. Immediately, 3 mg of β-glucosidase in 50 mL of deionized and sterilized water was placed in the third bottle, then covered and refrigerated. To the last bottle, 5 mL of Cellobiohydrolase was added and then 50 mL of previously sterile deionized water was added and it was covered and then placed in the refrigerator for conservation and subsequent use. The enzymes D-(+)-cellobiose and endo-1,4-β-D-glucanase were previously used as a complex for the production of nanocellulose [18,26], which obtained good results in both yields and crystallization.

Enzymatic hydrolysis

In (Test 1) all the enzymes were used together at the same time with the cellulose. Deionized water (350 mL), was placed on a heating rack at a temperature 30°C. Subsequently, 5 g of Cellulose was added with 49 mL of sodium acetate and acetic acid buffer pH 4.65. Immediately add 1 mL of the stock enzyme solution: D-(+)-cellobiose, cellobiohydrolase, endo-1,4- β-D-glucanase, and β-glucosidase. After the covered bottle was placed under constant stirring for 72 h at 30°C. When the established time was up, the pH was checked and it was left stirring for 24 h at the same temperature [9].

In (Test 2) the enzymes were applied in pairs (exo and endoglucanases), in two different phases or times on the cellulose. 350 mL of deionized water was used and heated to 50°C, then 5 g of Cellulose plus 49 mL of sodium acetate and acetic acid buffer pH 4.65 were added. Immediately, 1 mL of stock solution containing (D-(+)-cellobiose and 1 mL of Cellobiohydrolase) was added and kept under constant stirring at a temperature of 50°C/72 h [2, 25]. Subsequently, it was allowed to cool to room temperature and the pH was verified in the range 4-5. Finally, it was left under constant stirring at the same temperature for 24 hours. After this stage, and with the indicated pH, it was continued with the addition of 1 mL of solution formed by: (endo-1,4-β-D-glucanase and β-glucosidase), it was left for 72 h at 30°C with constant stirring [9].

Dialysis of sample

At the end of the hydrolysis, the samples from both tests were taken to 14,000 Da membranes to perform dialysis. These bags were suspended in a 2000 mL beaker containing deionized water. Leaving it under constant agitation for 72 h/24 h and three water changes as indicated [26]. Once the dialysis was completed, the neutral pH of the solution was verified. As a final result of the dialysis, a supernatant and a precipitated sediment were obtained. The supernatant was taken with a pipette and dispensed into 15 mL tubes, which were centrifuged at 3000 rpm for 10 min [10]. Subsequently, the supernatant was dried in an oven at 80°C/24 h and then allowed to cool and weighed.

In the case of test 2, the supernatant was taken in 50 mL of water and placed in an ultrasonic bath for 30 min/28 Hertz [35]. Subsequently, the supernatant was taken and 15 mL was placed in centrifuge tubes to centrifuge at 3000 rpm for 10 min. At the end, the sample was dried in an oven at 80°C/24 h, after which time it was allowed to cool to room temperature and weighed.

Nanocellulose yield

The yield was determined by drying the test samples (Test 1) with the use of all the enzymes together on the cellulose, applied at the same time, and (Test 2) with enzymes applied to the Cellulose in pairs of exo and endoglucanases in two phases or different times, until reaching a constant mass in the nanocellulose of both tests, in an oven at 80°C for a period of 24 h. At the end of the drying process, the samples were allowed to cool to room temperature and then weighed on an analytical balance, to subsequently record the weight obtained for each test [25].

Characterization and functional groups

Once the products were obtained, a sample of the tests was taken (Test 1) using all the enzymes together on the cellulose, applied at the same time. (Test 2) with enzymes applied to cellulose in pairs of exo and endoglucanases in two different phases or times, to which an FTIR, SEM, and DSC analysis was carried out, which are mentioned below.

Infrared spectroscopy with Fourier transform (FTIR)

In this analysis, a Perkin Elmer spectrophotometer (Model Spectrum Two) was used, in which the structure of the sample from Test 1 and Test 2 was determined after being subjected to the enzymatic hydrolysis process. This allowed us to know the characteristics of the synthesized nanocellulose, compared with a commercial microcrystalline cellulose sample. The completely dry samples were scanned in a 16-scan reading grid in a range of 4000 to 500 cm^{-1} described by Zhang *et al.* [44].

Scanning Electron Microscopy (SEM)

Morphological analysis was performed using scanning electron microscopy (SEM) with a team Jeol brand model JSM-6010 LA, in which the samples were prepared in a vacuum and coated with gold to avoid static load operated at 20kV [34].

Differential Scanning Calorimetry (DSC)

A thermal analysis (DSC) was carried out in collaboration with Dr. A. Vargas Torres from the UAEH Tulancingo Hidalgo campus. The crystallinity and stability degree of nanocellulose obtained by both tests were verified. Approximately 5 mg samples were prepared in standard Tzero aluminum pans, equilibrated at 20 °C, and held isothermal for 5 min [40]. The heat change associated with the denaturation of the cellulose molecule was measured under heating at a constant rate in 40 mL aluminum trays, which was carried out from -40 to 300 °C for 5 °C /min. [18].

Statistical analysis

The data obtained from the response variables were processed with Anova statistics accompanied by a /Tukey (HSD) test (before verification of the assumptions), with a confidence level of 95%. Statistical differences between the three treatments were determined using the statistical software SAS ver. 6.

RESULTS AND DISCUSSION

Effect of proteolytic enzymes (Brom/Pap) on biomass

Table 2 shows the values observed in the rice husk (biomass), once both enzymes (Brom/Pap) were applied. The final values (9.87 Kgf) of the treatment where the natural enzymes were applied, showed a notable loss in the hardness of the husk, that is, the plant tissue softened, due to the denaturation of structural proteins of the cell wall. In the untreated or water-treated controls, they did not have a significant variation at the beginning of the test, with final values between 40 and 15.1 Kgf respectively.

It has been a common practice, to treatments with papain and bromelain, proteolytic enzymes extracted from plants [30], to tenderize meats. These proteases have also been used to coagulate milk, and form cheese, as well as in the beer clarification process. [27]. In this case, it was considered that the protein content of the husk is low, compared to animal tissue. However, these tests demonstrated that they were also useful in achieving a macerating effect on the husk before its conversion into Cellulose, without prior experience in plant tissue.

Cellulose yield from previously treated husk

Table 3 provides data about the different stages of obtaining Cellulose from biomass previously treated with natural enzymes (Brom/Pap). Higher percentage values ranging from cooking with NaOH (77%) to the acid pre-treatment (HCl) (49.1- 56.3%), a result higher than obtained by some researchers [18], who started from 60.5% yield, until finishing the pretreatment (28.6%). Therefore, it can be inferred that a prior treatment with proteolytic enzymes to the husk, favors greater use of the fiber that is converted into cellulose.

Effect of Hydrogen Peroxide and Sodium Hypochlorite on Cellulose yield

Table 3 shows the weight and yield of the product obtained in stages, where (NaClO) and (H_2O_2) were previously treated to obtain cellulose. In this table, it was observed that hypochlorite has a higher performance in the NaOH cooking and blanching stages with 77-76.9% compared

Table 2. Hardness values were recorded with the texturometer (Kgf), after the husk was treated with proteolytic enzymes (Brom/Pap), compared to the controls treated with water and untreated. Statistical treatment with Anova/Tukey (HSD) (p=0.05).

Treatment	Initial average (Kgf)	Final average (Kgf)	Signif *
[1] Treated biomass (Brom/Pap)	15.13±0.05	9.87 ±0.11	c
[2] Biomass Control (water)	20.16±0.11	15.13±0.05	b
[3] Biomass Control (Untreated)	40.0±0.01	40.0± 0.001	a
M.S.E.	0.003	0.007	-
C.V	0.230	0.375	-

* Different letters in the same column are significantly different for (α =0.05). M.S.E (Mean Square Error) and C.V. (Coefficient of

Table 3. Process stages and performance achieved in obtaining cellulose from biomass previously treated with proteases (Brom/Pap).

Starting weight	Treatment (NaClO)		Treatment (H_2O_2)		Referencia Hernández et al. [18]	
	Weight (g)	Yield (%)	Weight (g)	Yield (%)	Weight (g)	Yield (%)
Alkaline treatment.	15.40	77	14.56	38.8	12.1	60.5
Bleaching	15.38	76.9	12.14	28.6	7.76	38.8
Acid Pretreatment	9.82	49.1	11.3	56.3	6	28.6

to peroxide, which had between 32.8-28.6% and in the last pretreatment stage a difference was observed. of 7.2%, surpassing (H_2O_2) over (NaClO).

So evidently hypochlorite can be a sustainable alternative to the use of commercial chlorine, with less water in the washes. The above corroborates the previous results of García et al. [15], who obtained good results with commercial chlorine at 0.5%: Although it is worth mentioning that this same one says that its performance is lower in cellulose, if the concentration increases to 10%. Other authors [18], when comparing yields by stages, obtained lower final yields (28.6%), although in general it has been reported between 28-36%, up to 41%. This study offered better results in performance between 49-56% which may be due to better handling between washes. For their part, Martelli et al. [25], by bleaching the fiber obtained from soybean straw, with hydrogen peroxide and sodium

hypochlorite, using a higher concentration (3.3%), offered differences in the coloration of the cellulose.

Cellulose characterization

Fourier transform infrared spectroscopy (FTIR)

Fig. 1 shows the comparison of cellulose extracted analysis from rice husk from the bleaching treatments with hydrogen peroxide (T1) and sodium hypochlorite (T2). As well as the control sample (T0), using Fourier transform infrared spectroscopy (FTIR). The functional groups of cellulose were identified, where the O-H

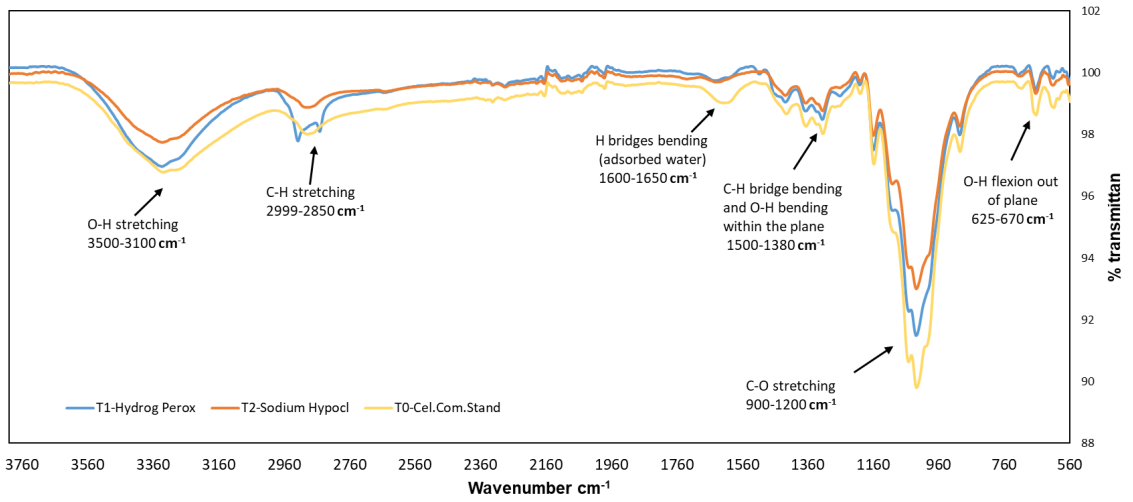

Fig. 1. Analysis comparing cellulose samples extracted from rice husk using Fourier transform infrared spectroscopy (FTIR). Commercial cellulose standard (T0) and cellulose samples were processed with Hydrogen peroxide (H_2O_2) (T1) and Sodium hypochlorite(NAClO) (T2). Source: Author himself.

stretching is appreciated in the range of 3500 to 3100 cm^{-1} and in the range of 2999 to 2850 cm^{-1}, with the presence of the aliphatic section of the C-H bonds.

The above coincides with Han et al [16], who observed a broad band of stretching vibrations of the O-H groups within the region of 3600 to 3100 cm^{-1}, which represents the main functional groups found in lignocellulosic materials, less marked in the samples.

Subsequently, the bending of H bridges is shown in the range of 1650 to 1600 cm^{-1}, which refers to the water absorbed. At the range of 1420 cm^{-1}, it was possible to see the deformation of CH_2. At 1500 to 1380 cm^{-1}, in-plane OH bending appears along with C-H bridge bending.

Finally, the C-O stretching signal was obtained between 900 and 1200 cm^{-1} and the out-of-plane O-H bending between 600 and 660 cm^{-1}. According to the spectral data, the similarity is evident in the groups that characterize both samples and (control) cellulose commercial standard.

Evident that the peaks above 1730 cm^{-1}, corresponding to the C=O groups, are not present in the samples, which indicates the elimination of hemicellulose and lignin in the process. These results coincide with the cellulose spectra obtained by other authors [15], who used similar processes that included both reagents.

Scanning Electron Microscopy (SEM) of Cellulose

Morphological analysis was performed for two samples and a control as shown in the micrographs in Fig. 2. For the untreated sample (T0), it shows free microfibrils, i.e. defibrillation. In the case of Hydrogen peroxide (T1), a packing of the fibers is observed as well as an initial unraveling, similar to a filamentary structure. While the one treated with Hydrogen peroxide (T2) shows a separation of individual fibers with an apparent decrease in diameter. This indicates the removal of non-cellulosic components of the hull, macromolecules that favor the microfiber structure, which facilitates their separation. This agrees, with observations by Johar et al [21]. On the other hand, it has been explained [9] that the analysis carried out on the inner epidermis of the husk without any pretreatment shows a completely smooth, waxy, and shiny surface.

Synthesis of (CNCs) and characterization of nanocellulose under different conditions.
Fourier Transform Infrared Spectroscopy (FTIR).

The FTIR spectrum (Fig. 3) corresponds to the nanocellulose obtained after the enzymatic hydrolysis applied in the two tests: (Test 1) all enzymes were used together, at the same time over the cellulose. In (Test 2), enzymes (exo and endoglucanases) were applied in couples at two different phases or times on the cellulose.

The graph shows the range from 3600 to 3100

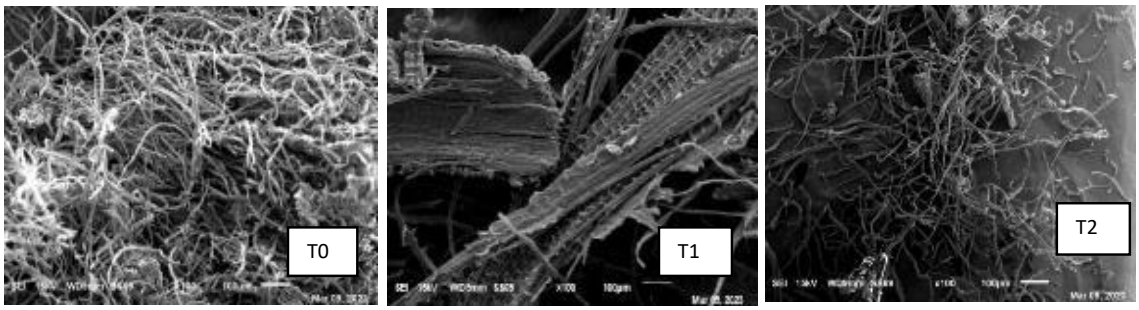

Fig. 2. Micrographs showing the morphological analysis of cellulose with different treatments: Hydrogen peroxide (T1), Sodium hypochlorite (T2), and control sample (T0).

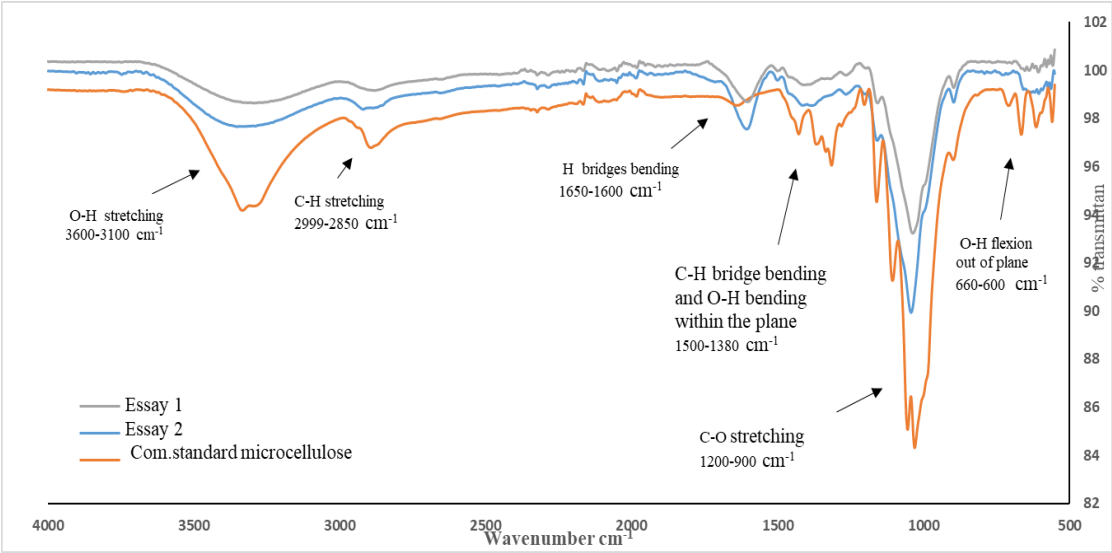

Fig. 3. Analysis comparing nanocellulose samples extracted from rice husk, using Fourier transform infrared spectroscopy (FTIR). It shows Commercial microcellulose standard (Control) and nanocellulose samples obtained from both assays 1 and 2.

cm^{-1} where the O-H stretching is found, indicating the vibration in the nanocellulose molecules, formed by a wide band in both samples, due to a greater number of O-H groups exposed by the treatment in obtaining nanocellulose. This result coincides with what was reported by Han et al. [16], which obtained nanocrystals through acid hydrolysis from wood waste. On the other hand, the control sample of commercial microcrystalline cellulose presented a more pronounced peak in the range of 3600 to 3100 cm^{-1}, which makes it different from the two tests where nanocellulose was obtained.

The presence of the aliphatic section of C-H bonds was also observed in the range of 2999 to 2850 cm^{-1} and a peak in the range of 1650-1600 cm^{-1} that belongs to the bending of H bonds of the adsorbed water. Some reports infer that from 1500 to 1380 cm^{-1} there is in-plane O-H bending along with C-H bridge bending [14].

Next, a signal characteristic of the nanocellulose was observed in the range of 900 to 1200 cm^{-1} representing C-O stretching. Finally, out-of-plane O-H bending was obtained from 660 to 600 cm^{-1}. The spectra obtained in these tests coincide with what was previously reported for these same functional groups when obtaining nanocellulose, highlighting the waves between 1600-1632 cm^{-1} with bending of the OH groups, with evident adsorbed water (hydrogen bonds), which corroborates the results with enzymatic hydrolysis, using D-(+) cellobiose and endo-1,4-β-D-glucanase reported [26]. As well as what was reported by other authors when comparing acid

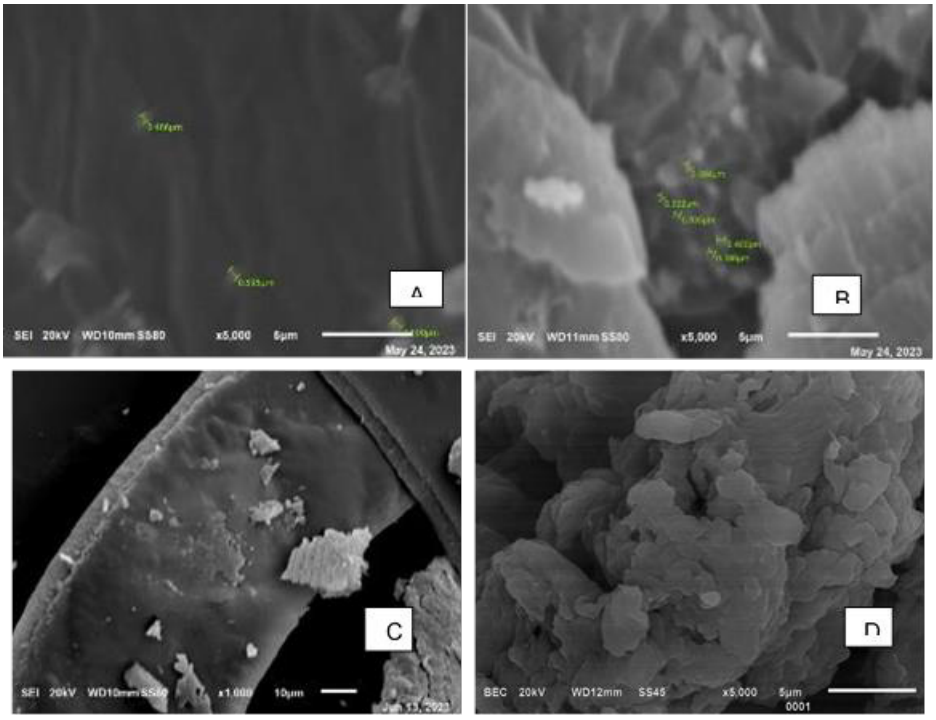

Fig. 4. Micrographs obtained by SEM, with nanocellulose derived in the experiments: (Test 1) with the use of all the enzymes together on the cellulose, applied at the same time. (Test 2) with enzymes applied to cellulose in pairs (Exo and Endoglucanases), in two different phases or times. A and B) Measurements of some isolated particles after dialysis. Test 2. C and D) Nanocellulose particles adhered to the surface of crystallized material.

hydrolysis with enzymatic hydrolysis applied in two phases, to obtain nanocellulose from rice husk [18]. Such reports agree that this treatment eliminates lignin, as the C=O groups disappear at 1732 cm^{-1}, in addition to the elimination of hemicellulose macromolecules, changes attributed to ester groups reported for uronic and acetyl hemicellulose or carboxylic groups [17].

Scanning electron microscopy (SEM).

Fig. 4 shows nanocellulose obtained in both tests. When all the enzymes were applied together on the cellulose at once (Test 1), nanoparticles between 200 - 595 nm were observed, dispersed on the surface. The nanoparticles' size averages around 200 nm, although focusing with this technique does not allow delving into areas with greater dispersion. Similar results had been reported [18, 34], that hydrolysis induces the release of cellulose nanowhiskers on the smooth surface, as observed under TEM and AFM.

The particles showed different lengths from 178-778 nm. When enzymes in pairs of exo and endoglucanases were applied to cellulose at two different times (Test 2), aggregates or lumps were visualized, which evidenced the degradation activity in enzymatic hydrolysis. Despite the ultrasonic treatment, it was not possible to sufficiently separate the nanoparticles, in this case, visualized up to magnitudes of 5000 X (line 5 µm). The above does not coincide with the results previously obtained by Hernández et al. and others [18, 26]. They worked with transmission microscopy, to better disperse the nanoparticles in the shape of straight rods, which are joined by electrostatic forces to form conglomerates that are visible with smaller magnitudes (SEM).

Differential scanning calorimetry (DSC).

The analysis shows the energy consumption of nanocellulose (NC) in both tests, in a range of approximately 38°C and 398°C.

Fig. 5 reflects the melting point of the treated samples after enzymatic hydrolysis. Fig. (5a) shows how the temperature increased above 200°C, with the formation of an endothermic peak at 209°C, a temperature where the crystallization of nanocellulose (NC) in the Test is reflected. 1.

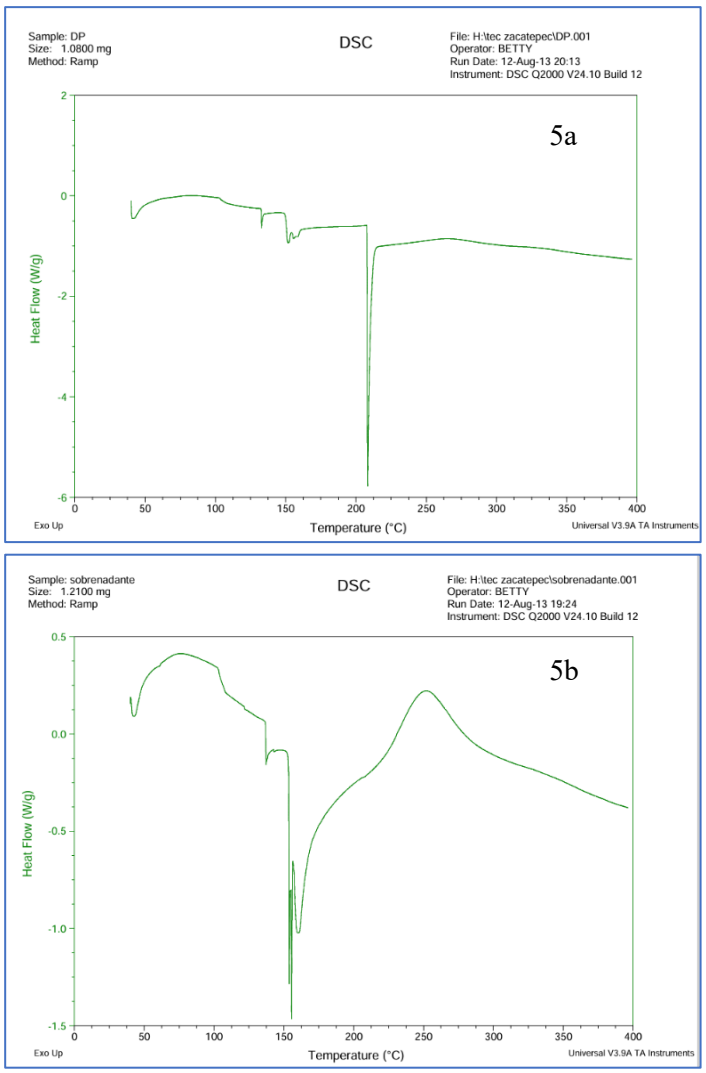

Fig. 5. Differential scanning calorimetry (DSC) analysis of temperature range from 38- 398°C, from nanocellulose obtained in both tests. Image 5a) nanocellulose obtained using all the enzymes together on cellulose. Imag5 b) nanocellulose enzymes applied in pairs of exo and endoglucanases, in two phases

In addition, three peaks are indicated at 125°C, 149°C and 150°C, which are attributed to the evaporation of the water bound in the sample. This result coincides with what was reported by Rashid and Dutta [34], who obtained peaks below 150°C when characterizing nanocellulose extracted from rice husk. Notably, [29] inferred that commercial cellulose shows an endothermic peak at 330°C, corresponding to the fusion of crystals.

Fig. (5b) corresponds to Test 2, where the beginning crystallization at 155°C was shown, corroborating with what some researchers have proposed [26].

In this case, endothermic peaks of 140°C and 160°C were observed, which were attributed to the evaporation of moisture from the cellulose and volatile materials loss, as previously reported by Manals et al. [24]. In this sample, a less homogeneous behavior was also observed, indicative of polymorphism or recrystallization by fusion, which coincides with what was previously reported by Sulliva et al. [40].

The crystallization peaks in both tests do not coincide with what was reported by [44], who mentioned greater crystallinity and thermal stability at 277°C when they obtained nanocellulose from sugarcane bagasse pulp.

Table 4. Yield obtained of nanocellulose in the different tests when applying hydrolysis with the enzyme complex.

Treatment	Initial weight (g)	Final weight (g)	Yield (%)
Essay 1	5	0.09	1.8
Essay 2	5	0.21	4.2

Nanocellulose production.

Table 4 shows the weight and percentage yield obtained from nanocellulose in the tests. A lower nanocellulose percentage (1.8%), was obtained in comparison with Test 1, surpassed by the test when we applied the complex at different times Test 2 4.2%), which marks a difference of 2.4%. In this regard, Michelin et al. [28] mentioned that the composition of the cellulose complexes, when hydrolysis is applied to lignocellulosic biomass, plays an important role in the final yield obtained, which was corroborated in these tests.

A lower percentage of nanocellulose was present in assay 1 (1.8%) compared to assay 2, which had (4.2%), a difference of 2.4%. It could be defined that it is more efficient when working with four enzymes (Exo and Endonucleases), applied in two different phases or times, compared to when a single pair of enzymes, is explained in other studies [26].

Some authors have explained, that endoglucanase activity (EG) cleaves random internal β-1,4-glycosidic bonds of cellulose chains, typically in amorphous regions, creating new cellulose chain ends. Cellobiohydrolases (CBH), or exoglucanases, act processively on the different ends of cellulose chains forming cellobiose units. A final class of cellulase refers to β-glucosidases, which hydrolyze cellobiose into glucose [45]. However, we agree that it is about the approach that you want to obtain CNF or CNC.

Considered, that even the performance obtained experimentally is far from what is desired, for an industrial phase, especially due to the increase in the number of enzymes in the complex, which causes an increase in production costs, without a justification in yield.

CONCLUSIONS

The plant tissue of the rice husk was modified by applying Bromelain and Papain as natural proteolytic enzymes, which favored the synthesis process and a higher cellulose yield.

To whiten fiber, hydrogen peroxide (H_2O_2) could be used as a substitute for sodium hypochlorite (NaClO), which generates less pollution and less water consumption in washing.

The nanocellulose synthesis using four enzymes together, had a greater optimization of time, less water consumption in washing, and nanoparticles between 200-595 nm, with adequate thermal stability, but lower yield. While, when the enzymes applied in pairs (exo and endoglucanases), were used at different times, a higher yield was achieved, with longer duration of process and polymorphism or recrystallization stages.

Data Availability

All data have been included in the manuscript.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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REFERENCES

1. H.P.S. Abdul Khalil, A.H. Bhat, A.F. Ireana Yusra, 2012. Green composites from sustainable cellulose nanofibrils:

Review. Carbohydrate Polymers, 87 (2): 963-979. https://doi.org/10.1016/j.carbpol.2011.08.078.
2. Y. Albernas, G. Corsano, L. Mesa, R. Santos, & E. González, 2014. Estudio de la cinética de la hidrólisis enzimática del bagazo pretratado. Afinidad *LXXII,* 570 (83): 1-6. https://ri.conicet.gov.ar/handle/11336/125128.
3. V. N, An, H. T. C. Nhan, T. D. Tap, T. T. T. Van and P. Van Viet, 2020. Extraction of high crystalline nanocellulose from biorenewable sources of vietnamese agricultural wastes. Journal Polymers and Environment, 28: 465–1474. https://doi. org/10.1007/s10924-020-01695-x
4. C. A. Barcelos, V. A. Rocha, C. Groposo, A.M. Castro, A. M., Pereira, N. Jr, 2015. Enzymes and accessory proteins involved in the hydrolysis of lignocellulosic biomass for bioethanol production. Mycolgy: Current and Future. Developments, 1: 23-56, 2015. DOI:10.2174/9781681080741115010005.
5. S. Badui, 1999. Química de los alimentos. Longman S.A. México. 648 p. https://vdocuments.net/quimica-de-los-alimentos-baduipdf.html?page=1
6. D. Bhardwaj, M. Ansari, S. Kumar, & N.Tujeda, 2014. Biofertilizers function as key player in sustainable agriculture by improving soil fertility, plant tolerance and crop productivity. Microbial Cell Factories, 13, 66. https://doi.org/10.1186/1475-2859-13-66.
7. M. K. Bhat, S. Bhat, 1997. Cellulose degrading enzymes and their potential industrial applications. *Biotechnology Advances*, 15 (3-4): 583-620. DOI: 10.1016/s0734-9750(97)00006-2 Research Reviews.
8. L. Brinchi, F. Cotana, E. Fortunati, and J. Kenny, 2013. Production of nanocrystalline cellulose from lignocellulosic biomass: technology and applications. Carbohydrate Polym., 94 (1): 154-169. https://doi.org/10.1016/j.carbpol.2013.01.033
9. Y. C astillo, J. Juscamaita, P. Jorge, y L. Visitación, 2019. Pretratamiento e hidrólisis enzimática de la cascarilla de arroz. *SciELO Perú,* 85 (4): 476-488, http://dx.doi.org/10.37761/rsqp.v85i4.261
10. M. Cheng, Z. Qin, Y. Chen, S. Hu, Z. Ren, M. Zhu, 2017. Efficient extraction of cellulose nanocrystals through hydrochloric acid hydrolysis catalyzed by inorganic chlorides under hydrothermal conditions. ACS *ACS* Sustainable Chemistry & Engineering, 5 (6): 4656-4664. https://doi.org/10.1021/acssuschemeng.6b03194
11. De Campos, A. C. Correa, D. Cannella, E de M. Teixeira, J. M. Marconcini, A. Dufresne, H. C. Luiz, P.C. Mattoso y A.R. Sanadi, 2013. Obtaining nanofibers from curauá and sugarcane bagasse fibers using enzymatic hydrolysis followed by sonication. Cellulose, 20: 1491-1500. https://doi.org/10.1007/ s10570-013-9909-3.
12. W. P. Flauzino Neto, H. A. Silvério, N. O Dantas, D. Pasquini, 2013. Extraction and characterization of cellulose nanocrystals from agro-industrial residue–soy hulls. Industrial Crops and Products, 42: 480-488. https://doi.org/10.1016/j. indcrop.2012.06.041
13. N., Frone, D. M. Panaitescu, D. Donescu, 2011. Some aspects concerning the isolation of cellulose micro-and nano-fibers". UPB Scientific Bulletin, Series B: Chemistry and Materials Science , 73 (2): 133-52. https://www.scientificbulletin.upb. ro/rev_docs/arhiva/rez77363.pdf
14. García, M. 2009. Síntesis y carcaterización de nanopartículas a partir de celulosa de eucalyptus saligna y de hojas de maíz. Master's Thesis. Universidad Michoacana de San Nicolás de Hidalgo. http://bibliotecavirtual.dgb.umich.mx:8083/xmlui/handle/DGB_UMICH/166
15. S. K. García-Alcocer, S., S.Salgado-García, J. Córdova-Sánchez, A. Rincón-Ramírez, G. I. Bolio-López, R. Castañeda Ceja, A. R. Saucedo-Corona, R. Falconi-Calderón & C. Valerio-Cárdenas, 2019. Blanqueo de la fibra de celulosa de paja de caña de azúcar (*Saccharum* spp.) con peróxido de hidrógeno. Agro Productividad, 12 (7): 11-17. https://doi.org/10.32854/agrop.v0i0.1434
16. J. Han, Ch. Zhou, Y. Wu, F. Liu, Q. Wu, 2013. Self-assembling behavior of cellulose nanoparticles during freeze-drying: effect of suspension concentration, particle size, crystal structure, and surface charge. Biomacromolecules, 14 (5): 1529-40. https://doi.org/10.1021/bm4001734
17. R. P. Hernández A. Olarte Paredes, R. Salgado Delgado and A. M. Salgado Delgado. 2021. "Rice husk Var. 'Morelos A-2010' as an eco-friendly alternative for the waste management converting them cellulose and nanocellulose, International Journal of Environmental Analytical Chemistry, 101 (19):7571-7586 . https://doi.org/10.1080/03067319.2021.1972991
18. R.P. Hernández, R. D. Salgado, A. O. Paredes, A., Salgado, A., García, E., Medrano, A., & Martínez, F. 2022. Comparing acid and enzymatic hidrolysis methods for cellulose nanocrystales (CNCs) obtention from agroindustrial rice husk waste. Journal of Nanotechnology, *I*D 5882113, 1-11. https://doi.org/10.1155/2022/5882113
19. M. I. Hossain, H. Zaman, and T. Rahman, 2018. Derivation of nanocellulose from native rice husk". Chemical Engineering Research Bulletin , 20 (1):19-22. https://doi.org/10.3329/cerb.v20i1.36926
20. L. Jiang, E. Morelius, J. Zhang, M. Wolcott, J. Holbery, 2008.Study of the poly (3-hydroxybutyrate-co-3-hydroxyvalerate)/cellulose nanowhisker composites prepared by solution casting y melt processing, *Journal of Composite Materials* , 42 (24): 2629-2645. https://doi.org/10.1177%2F0021998308096327
21. N. Johar, I. Ahmad, A. Dufresne, 2012.Extraction, preparation y characterization of cellulose fibres y nanocrystals from rice husk. Industrial Crops and Products, 37 (1): 93-99. https://doi.org/10.1016/j.indcrop.2011.12.016
22. M. Kaur, S. Kumari, and P. Sharma 2018. Chemically modified nanocellulose from rice husk: synthesis and characterization. Advances in Research, 13 (3): 1-11. https://doi.org/10.9734/AIR/2018/38934
23. H. V. Lee, S. B. A. Hamid, S. K. Zain, 2014. Conversion of Lignocellulosic Biomass to Nanocellulose: Structure and Chemical Process, The Scientific World Journal, Article ID 631013, 20. https://doi.org/10.1155/2014/631013
24. E. Manals-Cutiño, M. Penedo-Medina, & G. Giralt, 2011. Análisis termogravimetrico y térmico diferencial de diferentes biomasas vegetales. Tecnología Química, 31 (2):180-190. http://scielo.sld.cu/pdf/rtq/v31n2/rtq05211.pdf
25. M. Martelli, M. Silva, M. Alice, O. Benedito, & D. Tapia, 2016. Uso de enzimas comerciales para producir nanofibras de celulosa a partir de paja de soja. Journal of Nanomaterials, Article ID 8106814, 1-10. http://dx.doi.org/10.1155/2016/8106814
26. 26.R.P. Hernández, C. F. R. Martínez, R. D. Salgado, A. O. Paredes, A., Salgado, A., García, 2023. Hidrólisis enzimática para la obtención de nanocristales (CNCS) a

partir de residuos agroindustriales de cáscara de arroz". Revista Cietifica Agroecosistemas. 11 (2): 54-61. http://aes.ucf.edu.cu/index.php/aes

27. O.A. Martínez-Jaime, R. Hernández-Hernández, J.M. Mendoza-Carrillo, C.M. Bucio-Villalobos, M.D. Salas-Araiza, D.V. Orozco Gutiérrez, 2016. Efecto del cloruro de calcio y enzimas proteolíticas sobre la blandura de la carne de res. Invest. y Desarrollo en Ciencia y Tecnología de Alimentos, 1 (2): 506-511. http://www.fcb.uanl.mx/IDCyTA/files/volume1/2/7/88.pdf
28. M. Michelín, D.G. Gomes, A. Romani, Ma.L. Polizeli, & J.A. Teixeira, 2020. Nanocellulose Production: Exploring the Enzymatic Route and Residues of Pulp and Paper Industry, Molecules, 25 (15):1-36 . https://doi.org/10.3390/molecules25153411
29. J. I. Morán, V. A. Alvarez, V. A. Cyras, and A. Vázquez, 2008. Extraction of cellulose and preparation of nanocellulose from sisal fibers. Cellulose, 15, 149-159. https://doi.org/10.1007/s10570-007-9145-9
30. B.M. Naveena, S.K. Mendiratta, A.S.R. Anjaneyulu, 2004. Tenderization of buffalo meat using plant proteases from *Cucumis trigonus* Roxb and *Zingiber officinale* Roscoe (Ginger Rhizome). Meat Science, 68 (3):363-369. https://doi.org/10.1016/j.meatsci.2004.04.004
31. A. N. Nakagaito and H. Yano, 2004. The effect of morphological changes from pulp fiber towards nano-scale fibrillated cellulose on the mechanical properties of high-strength plant fiber based composites. Applied Physics *A*, 78(4): 547-552. https://doi.org/10.1007/s00339-003-2453-5
32. M. Pääkkö, V. Ankerfors, H. Kosonen, A. Nykänen, S. Ahola, V. Österberg, J. Ruokolainen, V. Laine, P.T. Larsson, O. Ikkala, and T. Lindström, 2007. Enzymatic Hydrolysis Combined with Mechanical Shearing and High-Pressure Homogenization for Nanoscale Cellulose Fibrils and Strong Gels, Biomacromolecules, 8 (6):1934-1941, 2007. https://pubs.acs.org/doi/10.1021/bm061215p
33. L.V. Peñaranda-González, S.P. Montenegro-Gómez, P.A.Giraldo-Abad, 2017. Aprovechamiento de residuos agroindustriales en Colombia, Revista de Investigación Agraria y Ambiental, 8 (2):141-150. https://doi.org/10.22490/21456453.2040
34. S. Rashid and H. Dutta, 2020. Characterization of nanocellulose extracted from short, medium and long grain rice husks, **Industrial Crops and Products,** 154, 112627. doi:10.1016/j.indcrop.2020.112627
35. R. Rezanezhad, N. Noorddin, & A. Ghasem, 2013.Isolation of nanocellulose from rice waste via ultrasonication "nanocellulose from straw". Lignocellulose. 2 (1): 282-291. http://lignocellulose.sbu.ac.ir/Issue%2004/Ligno74_Rezanezhad_2013_anocellulose%20from%20Rice%20Waste_282_291_MAH_FDV.pdf
36. M.B. Rodríguez-Almarza, 2007. Determinación de la Composición Química y Propiedades Físicas y Químicas del Pulido de Arroz (*Oryza sativa* L.), Bachelor Thesis. Universidad Austral de Chile. Facultad de Ciencias Agrarias, 1-44. http://cybertesis.uach.cl/tesis/uach/2007/far696d/sources/far696d.pdf
37. 37. H. M. Samalvides. 2005. Incorporación de pulido de arroz como fuente de compuestos bioactivos en el desarrollo de galletas". Master's Thesis , Pontífice Univ. De Valparaiso (PUCV), Chile, 99. http://repositorio.ucv.cl/handle/10.4151/77518
38. J.J. Santos-Lema & C.A. Silva-Arroyo, 2019. Obtención de nanocelulosa a partir de la cascarilla de arroz mediante hidrólisis ácida. Doctoral Dissertation, Ing. Química. Universidad de Guayaquil. Ecuador. pp.146. http://repositorio.ug.edu.ec/bitstream/redug/40057/1/401-1370%20-%20obtenc%20nanocelulosa%20a%20partir%20cascarilla%20arroz%20mediante%20hidr%c3%b3lisis%20%c3%a1cida.pdf
39. G. Siqueira, J. Bras and A. Dufresne, 2010. Cellulosic bionanocomposites: a review of preparation, properties and applications. Polymers, 2(4): 728-765. https://doi.org/10.3390/polym2040728
40. E. Sullivan, R. Moon, & K. Kalaitzidou, 2015. Processing and Characterization of Cellulose Nanocrystals/Polylactic Acid Nanocomposite Films. Materials, 8(12): 1-11. https://doi.org/10.3390/ma8125447
41. G, Tiang, F.X. Kober, U. Lewandrowski, A. Sickmann, W.J. Lennarz, H. Schindelin, 2008. The catalytic activity of protein-disulfide isomerase requires a conformationally flexible molecule. Journal Biologycal Chemical, *283* (48): 33630-40. https://doi.org/10.1074/jbc.M806026200
42. A. Valverde, B. Sarria, & J.P. Monteagudo, 2007. Análisis comparativo de las características fisicoquímicas de la cascarilla de arroz". Scientia et technica, UTP, Colombia, vol. XIII, 37, 255-260. http://www.redalyc.org/articulo.oa?id=84903743J.
43. J. Vargas, P. Alvarado, J. Vega, y M. Porras, 2013. Caracterización del subproducto cascarillas de arroz en búsqueda de posibles aplicaciones como materia prima en procesos". Revista Científica, *Fac. Ciencias Químicas y Farmacia. Univ. de San Carlos. Guatemala,* 23 (1):1-16. DOI:10.54495/Rev.Cientifica.v23i1.115
44. K. Zhang, P Sun, H. Liu, S. Shang, J. Song, D. Wang, 2016. Extraction and comparison of carboxylated cell.ulose nanocrystals from bleached sugarcane bagasse pulp using two different oxidation methods. Carbohydr Polym. 15 (138): 237-43. doi: 10.1016/j.carbpol.2015.11.038.
45. F. Segato, A.R.L Damásio, R.C. de Lucas, F.M. Squina, R.A. Prade, 2014. Genomics review of holocellulose deconstruction by aspergilli, Microbiology and Molecular Biology Reviews, 78 (4): 588-613. https://doi.org/10.1128/mmbr.00019-14