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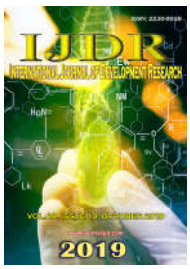

RESEARCH ARTICLE OPEN ACCESS

EXTRACTION OF EUCALYPTUS PULP NANOFIBERS BY ENZYMATIC HYDROLYSIS WITH AND WITHOUT ACID PRE-TREATMENT

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ABSTRACT

Cellulose nanofibers are used as reinforcement in polymeric matrices, as they allow to improve properties of these materials. Cellulose nanofibers are commonly prepared by conventional acid hydrolysis using strong acids (H_2SO_4 / HCl) that pollute the environment, corrode reactors unlike enzymatic hydrolysis with fungal cellulases whose products are biodegradable, non-polluting. This study aimed to extract nanofibers obtained by enzymatic hydrolysis with and without pretreatment with dilute acid. Nanofibre suspensions were characterized by molecular weight determination by chromatography, Transmission Electron Microscopy (MET), Infrared Spectroscopy (FTIR), X-ray Diffraction (XRD). Molecular masses, molecular mass distribution of hydrolysates are less than 100.000 Da, regardless of hydrolysis condition tested. Nanofibers produced by enzymatic hydrolysis showed better crystallinity indices when compared to produced using acid pretreatment. Nanofiber obtained using 90% of cellulase enzymes for 240 minutes produced nanofibers with 75.58% critalinity index, higher than produced by concentrated sulfuric acid, diameters of 27.5 ± 8 nm. Although, production of nanofibers using cellulases showed good results, pretreatment of enzymes would further increase nanofiber crital indexes.

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INTRODUCTION

In recent years there has been growth in development of biodegradable products using technological processes with renewable, fossil fuel reduction (Ramesh and Radhakrishnan, 2019; Thambiraj and Shankaran, 2017; Fazeli; Keley and Biazar, 2018; Zhu: Romain and Williams, 2016). In this context has been interest in replacing biotechnological procedures, conventional chemical processes, due to prevalence of environmental policies. High availability of lignocelulósicas fibers, coupled with need for renewable source for production of polymers, opens great opportunity for technological advances that agre delegate value to products of agribusiness, at same time, act on carbon fixation in nature. Lignocelulósicas fibres are excellent raw materials for production of polymers, composites, this is demonstrated by high number of searches use of these fibers, deposits of national, international patents, high number of products already marketed ((Eichhorn *et al.,* 2010; Ortega; Baillie, 2011; Sam *et al.,* 2015; Staroszczyk *et al.,* 2017; Silva *et al.,* 2012). Main components of vegetable fibers are cellulose, hemicelluloses, lignin.

Cellulose is linear glucose, homopolymer is formed by amorphous regions that appear as imperfections in microfibrills, crystalline regions Fernandes *et al.,* 2011; Eichhorn *et al.,* 2010). Microfibrills of pulp are extracted from cell walls of cellulosic fiber, its diameter, generally speaking, is approximately 2 to 20 nm with a length of few microns depending on species of plant from which it was extracted. Cellulose nanocrystals are usually isolated from lignocelulosicas fibers through acid hydrolysis are characterized by their nanometric range where its regions grow under controlled conditions, which allows formation of individual crystals of high purity. When isolated from sources of wood, hydrolysed cellulose particles have dimensions of 100-300 nm in length, 3-10 nm wide. Use of cellulose microfibrills has been much studied recently, being renewable source material due to its mechanical properties such high area specifies, low coefficient of thermal expansion (Chen *et al.,* 2011; Haafiz *et al.,* 2013). These materials, however, differ from nanofibers, they tend to agglomerate, this interferes with efficiency of their use in films, requiring research on methods that allow their individualization. Major problem of acid hydrolysis is care that will be required its disposal to avoid

environmental problems. An interesting alternative, in keeping with environmental preservation Efforts is enzymatic hydrolysis using fungal cellulases produced semi-solid fermentation. cellulase don't attack other pulp substitutes, reflecting high specificity of molecule compared to those of endoglucanases that, making celobiohidrolases enzymes of greater affinity with cellulose. This enzyme is also able to hydrolyze cellulose crystalline molecules with, approximately 80% of degradation (Zhang, 2006). *Aspergillus niger* cellulase source is intended for food use, *Trichoderma viride* is used for non-food applications, although enzymes of both fungi can fulfill many tasks. According to Gokhale. (1986) *Aspergillus niger* can be considered, sometimes, than other fungi, admittedly good producers of celulolíticos, hemicelulolíticos complexes, *Trichoderma viride*. Aim of this work was to produce eucalyptus pulp nanofibers obtained by enzymatic hydrolysis with, without acid pretreatment.

MATERIALS AND METHODS

Materials

Samples: A-cellulose pulp of eucalyptus (96-98%) were ceded by Bahia pulp Company (Brazil), cassava starch used was donated by Cargill Agrícola S.A. Sucrose, invert sugar (60% of inversion) commercials were donated by Guarani S.A.

Methods

Nanofibers by acid hydrolysis (control): Cellulose nanofibers of eucalyptus pulp (control) were prepared by acid hydrolysis (64%) of H_2SO_4. Total of 12 mL. g $^{-1}$ cellulose pulp was subjected to constant stirring (Ika stirrer mod. C-MAGH57) during 15 minutes, temperature of 50 °C. After treatment, samples were filtered, centrifuged for 10 minutes at 4400 xg at temperature of 10 °C, to separate crystals from solution by centrifugation. Then suspensions were subjected to dialysis (pH 5-7) (Silva *et al.,* 2012).

Nanofibers by enzymatic hydrolysis: Cellulose pulp was crushed in mill (Mod. Te-645, tecnal), 0.5 g was transferred to bottles of 250 mL Erlenmeyer flasks. Was added phosphate buffer at pH optimum of enzyme (pH = 6.0), enzymatic extract of *A. niger*. Erlenmeyer flasks were kept under stirring in refrigerated incubator Tecnal (mod. You to 150 rpm) 39812, 30 °C for predetermined times (Table 1). Programmed time has elapsed, Erlenmeyer flasks were dipped in boiling bath (100 °C/5 min) for inactivation of enzymes, cooled to 4 °C. Material was centrifuged for 10 minutes at 4400 xg, washed with distilled water. This operation was repeated several times, until constant pH (5-7). Supernatant was stored in refrigerator for quantitative determinations. Yield was determined by gravimetry after drying an oven (Mod. 394/2 YOU, Tecnal) from an aliquot of 10 mL of suspension.

Nanofibers pre-treated with dilute sulphuric acid: Cellulose pulp was crushed in mill (model TE-645, Tecnal) by 2 minutes, 0.5 g was transferred to bottles of 250 mL Erlenmeyer flasks, carried out pre-treatment with dilute sulphuric acid in two stages to reduce time of enzymatic hydrolysis. Were added 100 mL of sulphuric acid solution at concentration of 0.5, 1.0% to vial Erlemeyer containing eucalyptus pulp, medium was autoclaved at 121°C for 15 days, 30 minutes (Table 2). Contents of vials was bottles Erlemeyers solution transfer to membrane in refrigerated incubator with shaking (Model TE-39812, Tecnal) with successive exchanges of water for 3 days until reaching constant pH (5.0 -7.0) indicative also of absence of acid waste. Water present in dialysis membranes was discarded, content was placed in Erlemeyers bottles of 250 mL where 40 mL of enzyme were added, 10 mL of acetate buffer (pH = 5.0).

Characterization of nanofibers

Fourier transform infrared spectrophotometry analysis (FTIR): For structural analysis of cellulose nanofibers technique was used with infrared spectroscopy (FTIR) Fourier transform through equipment Spectrum 100 (Perkin Elmer), with computerized registration system, operating at resolution of 4 cm^{-1} in region of 4000-400 cm^{-1}. Samples were prepared in KBr pellets (1 mg to 250 mg of KBr in nanofiber media).

X-ray diffraction analysis: Samples were analyzed by x-ray diffraction (Shimadzu, mod. XRD-6000), operating CuKα radiation (λ = 1.5433 Å), 40 kV voltage, current of 30 mA, sweep between 8, 80 degrees, results were used to calculate indices of crystallinity of cellulose pulp (Tang *et al.,* 2013), nanofiber media of eucalyptus pulp, shown in equation [1]:

$$IC: [(I_{002}-I_{am})/I_{002}] \times 100 \quad \text{eq. (1)}$$

Transmission electron microscopy (TEM): Cellulose nanofibers solution obtained from eucalyptus pulp was analyzed by Transmission Electron Microscopy (TEM) in order to determine the diameter of fibers, to indicate State of aggregation of crystals. Nanofiber solution was mixed in equal volumes with dicarbonate acetate aqueous 2% (AU), 10 mL of mixture UA-nanocelulose was dismissed grid of copper mesh 400, left to stand for 30-60 seconds. Grid was dry, viewed scanning electron microscope CM12-transmission (STEM) operating in field of course 80 kV. Lengths, widths were measured directly from electronic micrographs of transmission using Image Tool 6.3 (Media Cybernetics, Inc., Bethesda, MD) with 30 measurements to determine values of mean values, standard deviation.

Molecular mass determination by efficiency liquid chromatography: Determination of molecular mass (MM) of enzymatic hydrolysates of eucalyptus pulp was performed by high efficiency Liquid Chromatography GPC with Refractive Index detection (GPC-IR, HPLC Model 200 series, Mark Perkin Elmer), using columns SB 803, 804, 805, 806 series, having mobile phase aqueous solution of sodium nitrate (0.5% w/v) at flow rate of 1.0 mL/min were injected 80 µL of hydrolysates of aqueous solutions (0.5% w/v). Dextran standards (American Polymer Standards) of different molecular weights (102000, 207200, 431800, 655200, 759400, 2025000, 1360000, 2800000, 34500000, 5900000) have also been injected under same conditions. Values of molecular weights were obtained from calibration curve (Molecular Weight x log retention time) dextranas patterns.

RESULTS AND DISCUSSION

Fourier transform infrared spectrophotometry analysis (FTIR): Investigation chemical structure of nanofibers eucalyptus pulp after acid treatment, enzymatic (with, without acid pretreatment) occurred by infrared spectroscopy is depicted Figure 1. Infrared spectroscopy reveals similarities all spectra with slight wavelength variations, indicating that all

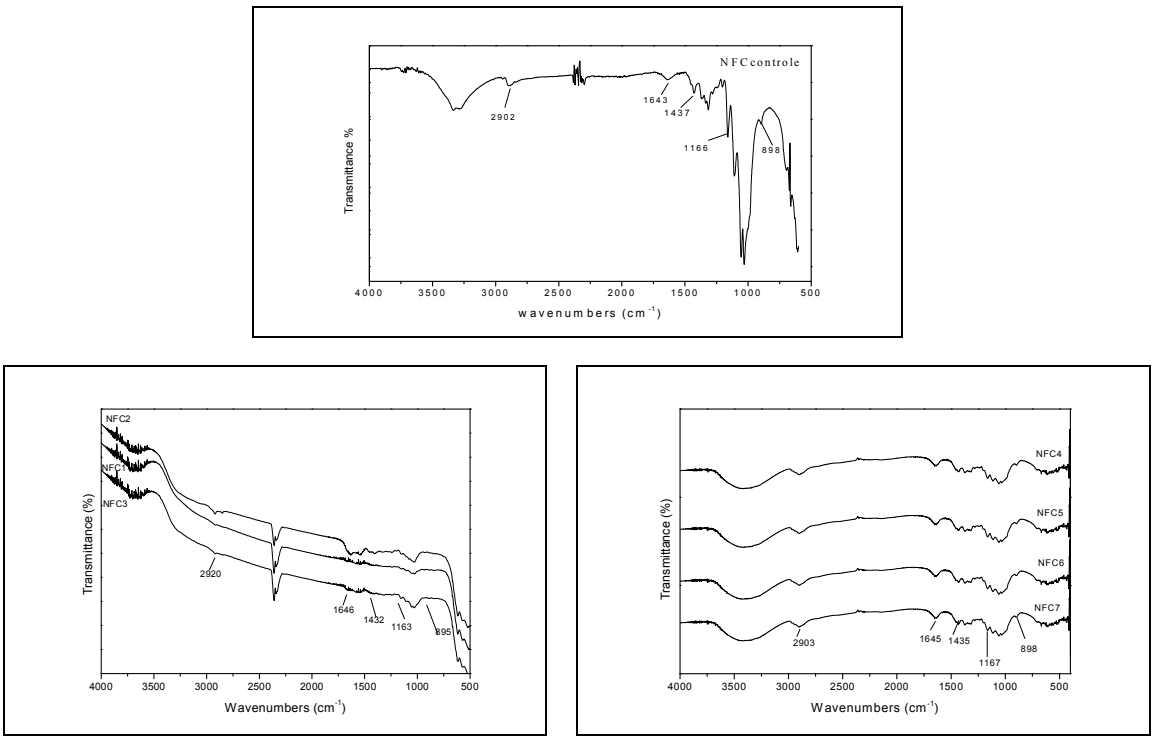

Figure 1. FT-IR spectra of eucalyptus pulp nanofiber produced by conventional acid hydrolysis (NFC), under different conditions of enzymatic hydrolysis (NFC1- NFC3) enzyme, acid pre-hidrolysis (NFC4- NFC7).

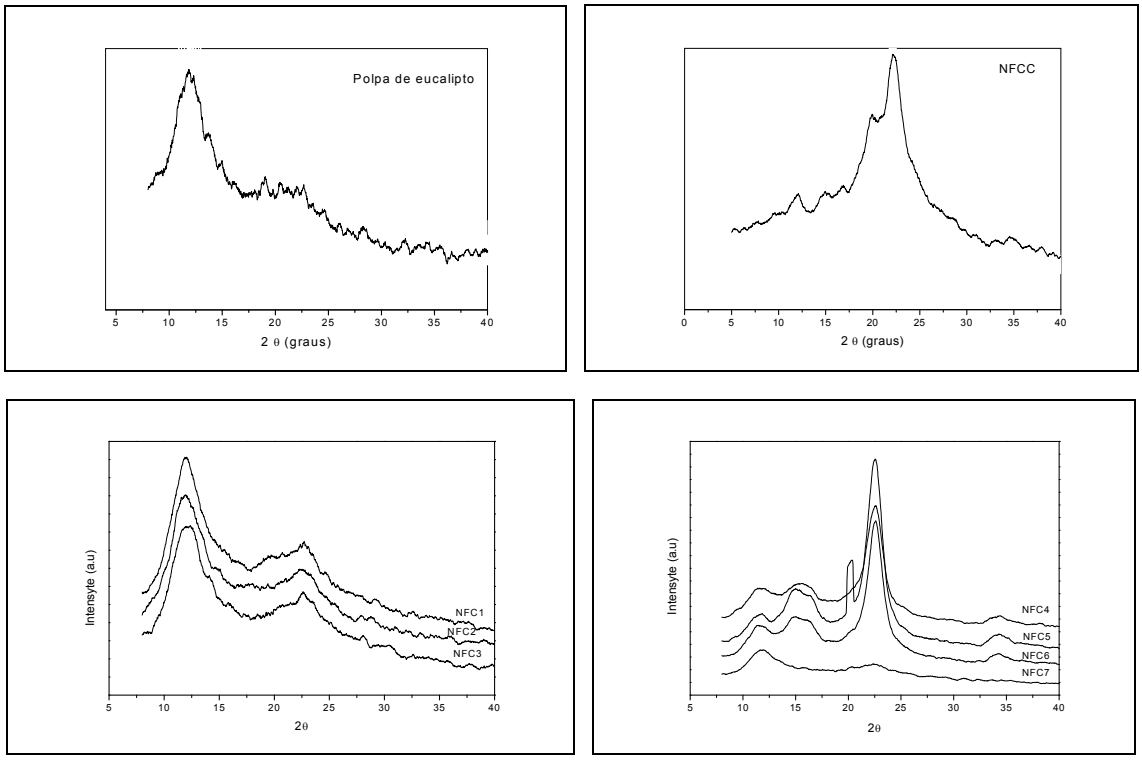

Figure 2. X-ray Difratogramas of eucalyptus pulp, control (NFCC), nanofibers obtained by enzymatic hydrolysis (NFC1- NFC3), enzymatic hydrolysis with acid pré-hydrolysis (NFC4-NFC7)

samples have similar chemical compositions. Bands around 2900, 3400, 1430, 1370, 890 cm^{-1} present all spectra are associated with characteristics of native cellulose type I (Tang *et al.,* 2013). Large absorptions bands that extend between 3400-3600 cm^{-1}, are referring to stretch of links –OH, absorption in 2900 cm^{-1} is asymmetric stretch links related CH_2 of cellulose. Appearance of peak around 1645 cm^{-1} is indicative of absorption of water by cellulose (Rosa *et al.,* 2012). Stretch of connection-CO cellulose appears in region between 1160-1165 cm^{-1}. Band in 895 cm^{-1} can be related to presence of amorphous cellulose material, 1248 cm^{-1} is band on hemicellulose present fiber. Specter of cellulose pulp obtained via acid hydrolysis (NFCcontrol) is compliance with literature, shows no differences between chemical groups present on enzymatic hydrolysis although there are differences inflections of groups (Alemdar; Sain, 2008).

X-ray diffraction analysis: X-ray diffraction analysis of high angle allows characterization of microstructure of crystalline materials. Figure 2 are represented diffractograms of eucalyptus

Table 1. Hydrolysis conditions of eucalyptus pulp with cellulase enzyme extracted from *Aspergillus niger*.

Hydrolysis conditions	Essay 1	Essay 2	Essay 3
Enzyme (mL)	10	90	90
Acetate buffer pH 5,0 (mL)	90	10	10
Time (min)	90	120	240
Code	NFC1	NFC2	NFC3

*NFC= Nanofiber

Table 2. Conditions of hydrolysis with sulfuric acid pretreatment

Concentration acid (%)	Pré-trat (min)	Enzyme (mL)	Temperature (°C)	While enzymatic hydrolysis (min)	Code
0,5	15	40	30	30	NFC4
0,5	30	40	30	30	NFC5
1,0	15	40	30	30	NFC6
1,0	30	40	30	30	NFC7

Table 3. Crystallinity index (CI) of nanofiber cloth produced by acid hydrolysis, enzymatic

Samples	Hydrolysis		Ic (%)
	Acid	Enzymatic	
Eucalyptus pulp	-	-	60.00
NFC control	H_2SO_4 64%/17min	-	70.00
NFC1	-	10% Enz/90 min	63.95
NFC2	-	90% Enz/120 min	75.58
NFC3	-	90% Enz/240 min	54.12
NFC4	0.5% ac/15 min	40% Enz/30 min	54.60
NFC5	0.5% ac/30 min	40% Enz/30 min	61.93
NFC6	1.0% ac/15 min	40% Enz/30 min	60.60
NFC7	1.0% ac/30 min	40% Enz/30 min	45.48

Table 4. Average values (± standard deviation) analyses of characterization of nanocompósitos

Samples	Morphology	Average diameter
NFC1	Undefined form	0
NFC2	Around circular	27.5 ± 8 nm
NFC3	Around circular	110. 2 ± 20 nm
NFC4	Around circular	30.10 ± 5 nm
NFC5	Undefined form	0
NFC6	Undefined form	0
NFC7	Undefined form	0

Table 5. Molecular weight (MM) molecular weight distribution limits (LDMM) of eucalyptus pulp cellulose hydrolysates obtained different conditions

Hydrolysy	Condition		Tr Médio (min.)	MM médio (Da)	Tr (min.)		LDMM (Da)	
	Acid	Enzyme			mínimo	máximo	mínimo	máximo
NFC Control	H_2SO_4 64%/17min	-	32.80	48.66312 <100.000	32.24	34.89	71.73977 <100.000	11.43167 <100.000
NFC1	-	10% Enz/90 min	43.19	<< 100.000	47.17	42.56	<< 100.000	<< 100.000
NFC2	-	90% Enz/120 min	43.19	<< 100.000	45.93	42.45	<< 100.000	<< 100.000
NFC3	-	90%Enz/240 min	43.20	<< 100.000	46.14	42.56	<< 100.000	<< 100.000
NFC4	0.5% ác/15 min	40% Enz/30 min	43.18	<< 100.000	45.93	42.46	<< 100.000	<< 100.000
NFC5	0.5% ác/30 min	40% Enz/30 min	43.18	<< 100.000	45.93	42.56	<< 100.000	<< 100.000
NFC6	1.0% ác/15 min	40% Enz/30 min	43.19	<< 100.000	46.04	42.56	<< 100.000	<< 100.000
NFC7	1.0% ác./30 min	40% Enz/30 min	43.20	<< 100.000	45.93	42.56	<< 100.000	<< 100.000

pulp from cellulose nanofibers obtained by acid hydrolysis (control), enzymatic hydrolysis (NFC1- NFC3), enzymatic hydrolysis with acid pretreatment (NFC4- NFC7). All samples after submitted to enzymatic treatment (with and without acid treatment) presented main reflections approximately 2 θ = 22°, giving rise to typical pulp type I standard, composed of 2 alomorfos, called cellulose I α, triclinic structure-, l-β, monoclinic structure, which has better mechanical properties, caused by presence of hydroxyl groups, with their respective hydrogen bonds (Rosa *et al.,* 2012). Control, little reflection on 2θ= 16° for amorphous cellulose residual fraction as lignin, hemicellulose, responsible for covering up for one of peaks relating to cellulose. Nanofibers obtained by enzymatic hydrolysis, this peak appears region of 2θ= 12°, could not be identified. All specimens, regardless of treatment employed (acid or enzymatic) differed from diffractogram of eucalyptus pulp, probably due to partial removal of amorphous material, decreased crystallinity.

Decrease of crystallinity after acid treatment was also noted by Tang *et al*. (2013). Index values crystallinity of fibres were measured by integration of amorphous, crystalline areas of each sample, calculated using equation of Buschle-Diller and Zeronian (1) are presented in Table 3. Results showed that enzyme treatment for an intermediate time was more suitable for removal of part of amorphous material constituent of eucalyptus pulp fiber than enzymatic treatment with dilute acid pretreatment. Despite concentrated acid hydrolysis occur in 15 minutes, enzymatic hydrolysis, minutes 120- 90, effluents generated in chemical hydrolysis are more toxic, producing sulphurous lignin, corrosion equipment, affecting entire chain of production. Therefore, enzymatic hydrolysis represents alternative clean technology for production of cellulose nanofibers. Combination of dilute acid pretreatment followed by acid hydrolysis enzyme resulted decrease Cis regarding celulose pulp, indicating that pretreatment of cellulose with acid can result adsorption of conjugate base on fiber surface,

i.e. cellulose fibers can carry sulfates groups adsorbed surface, preventing action of enzyme due to electrostatic repulsion or snaps into place active, being this effect proportional to concentration of acid, hydrolysis time (Bommarius *et al.,* 2008). Pretreatment with acid hydrolysis may also have altered highly ordered cellulose structure, resulting reduction Ic, loss of crystallinity due to change crystalline form from cellulose I to cellulose II without causing marked hydrolysis of cellulose chains. In literature, several crystallinity values are found for nanofibres obtained from different lignocellulosic sources: 68% for nanofibres from acidic straw hydrolysis (Abe; Yano, 2009), 86.21% for potato peel (Ramesh and Radhakrishnan, 2019), 77% of pineapple leaf nanofibers (Lakshmipriya; Sreekala and Sabu, 2019), initial crystallinity index of 45%, 54%, 72% for nanofifibrils of cellulose obtained by enzymatic hydrolysis (Chávez-Guerrero *et al.,* 2019). Higher crystallinity is key factor that determines better reinforcement with polymer matrix. Cellulose crystallinity can be attributed to bond between -OH groups. In case of raw cellulose fibers is involved by non-cellulosic domains, which is why crystallinity is lower. Increased crystallinity after hydrolysis is clear indication of removal of lignin, pectin, hemicelluloses (Lakshmipriya; Sreekala and Sabu, 2019), allowing us to conclude that extraction process employed this study was efficient.

Transmission electron microscopy (TEM): Structures resulting from hydrolysis of eucalyptus pulp that is used to characterize cellulose nanofibers were then investigated by Transmission Electron Microscopy (TEM). Analyses on suspensions show notable changes morphology of fibres after acidic, enzymatic treatments. Difratograma obtained from sample obtained by enzymatic hydrolysis (NFC1, NFC2, NFC3) it was observed that best crystallinity indices were obtained by sample NFC2 with $2\theta = 22$, $2\theta = 12$, with superior to those obtained by Ic nanofiber media obtained by acid hydrolysis, obtained by eucalyptus pulp (Ic = 75.58%) indicating partial removal of amorphous regions more not perfect dispersion of fibres confirmed by Transmission Electron Microscopy Analysis where fibres agglomerated with average diameter of 27.5 ± 8 nm (Table 4). From range of 18.2 to 75.4, fibers are range of nanocelulose that have great potential to be used as reinforcement in nanocomposites or nanobiocompósitos (Rosa *et al.,* 2010). According to Siró and Placket. (2010), cellulose nanofibrils are understood as aggregate of celluose microfibrils ranging diameter from 10-40 nm, length greater than 1000 nm, with aspect ratio values between 100-150 nm.

Molecular mass determination by efficiency liquid chromatography: Molecular weight, molecular weight distribution limits of cellulosic hydrolysates of eucalyptus pulp obtained under different conditions, listed in Table 2. However, on basis of average retention times of nanofibers obtained with concentrated acid (32.80 min) have been smaller than those obtained for resulting enzyme hydrolyze Microfiber (43.16 to 43.20), (Table 5), can estimate that first has increased molecular weight nanofiber media compared nanofibers. Same assumption can be performed for molecular weight distributions of nanofibers obtained by acid hydrolysis compared to enzymes. Obtained results showed that process of marbling led to a degradation of cellulosic chains. This means that glycosidic chain bonds may have broken, leading to decrease average molar masses of cellulosic molecules. Another fact that may be related to decrease molecular mass corresponds to presence of hemicelluloses, which part is eliminated during treatment. Other researchers agree that hydrolysis causes decrease fiber molecular mass, this parameter is used as indication of degree of hydrolysis efficiency, although literature does not express numerically ((Klungness; Caulfield, 1982; Erhardt, 1990).

Conclusion

Nanofibers produced by enzymatic hydrolysis of eucalyptus pulp showed increase degree of crystallinity (NFC2 = 75.58%) when compared to control (NFCC= 70.00%). other treatments reduced crystallinity of samples, which may suggest that they reduced both amorphous, crystalline material. Molecular masses, molecular mass distribution of hydrolysates are less than 100.000 Da, regardless condition of hydrolysis tested. FT-IR spectra of nanofiber-containing films showed dislocation -OH bending bands, C-O stretching vibrations. Values obtained for crystallinity indices of nanofibers isolated by enzymatic hydrolysis under different conditions allow us to conclude that process of extracting cellulose nanofibers using was efficient. However other parameters can be tested to increase crystallinity such as purifying enzyme extract or using commercial enzyme of high purit.

Conflict of Interest -Authors declare that they have no conflict of interest, this article does not containany studies with human participants or animals.

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