**Combining biomass wet disk milling and endoglucanase/ β -glucosidase hydrolysis for
the production of cellulose nanocrystals**

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Abstract

Cellulose nanocrystals (CNCs), a biomaterial with high added value, were obtained from pure cellulose, *Eucalyptus* holocellulose, unbleached Kraft pulp, and sugarcane bagasse, by fibrillating these biomass substrates using wet disk milling (WDM) followed by enzymatic hydrolysis using endoglucanase/ β -glucosidase. The hydrolysis experiments were conducted using the commercial enzyme OptimashTMBG or a blend of *Pyrococcus horikoshii* endoglucanase and *Pyrococcus furiosus* β -glucosidase. The fibrillated materials and CNCs were analyzed by X-ray diffraction, atomic force microscopy, scanning electron microscopy, and the specific surface area (SSA) was measured. WDM resulted in the formation of long and twisted microfibers of 1000–5000 nm in length and 4–35 nm in diameter, which were hydrolyzed into shorter and straighter CNCs of 500–1500 nm in length and 4–12 nm in diameter, with high cellulose crystallinity. Therefore, the CNC's aspect ratio was successfully adjusted by endoglucanases under mild reaction conditions, relative to the reported acidic hydrolysis method.

Keywords: Cellulose nanocrystals; Cellulose microfibers; Wet disk milling; Enzymatic hydrolysis; Endoglucanases

1. Introduction

Natural fibers derived from forests and crops can be valuable sources of nanofibers as biomaterials with high added value, which have a large number of applications in nanotechnology. Nanofibers derived from pure cellulosic materials are known as microfibrillated celluloses (MFCs) and cellulose nanocrystals (CNCs). However, the chemical composition of nanofibers derived from lignocellulosic biomass depends on the biomass source employed and the treatment conditions applied. They are often called lignocellulosic nanofibers (LCNFs) and may contain considerable amounts of **lignin and hemicellulose, leading to a lower material viscosity due to the hemicellulose charges that reduce the interfibrillar interactions** (Paakko et al., 2007).

Because of their exceptional mechanical and strength properties, MFCs and CNCs are usually applied as reinforcement materials in nanocomposites (Xu et al., 2013). **The tensile strength of the crystal structure was assessed to be approximately 5 to 10 GPa (Hancock et al., 2000).** The geometric dimensions of MFCs generally measure 10–40 nm in diameter and more than 1 μm in length, yielding an aspect ratio of 100–150 (Siró & Plackett, 2010). CNCs generally measure 2–20 nm in diameter and exhibit a wide distribution of lengths, ranging from 100 to 600 nm (Siró & Plackett, 2010). Large CNCs can be obtained from tunicate and bacterial celluloses, whereas CNCs from wood are smaller in structure (Beck-Candanedo et al., 2005). CNCs exhibit a light weight, high crystallinity and high mechanical properties (Habibi et al., 2010).

Cellulosic nanofibers can be prepared by mechanical, chemical, or enzymatic treatments. Therefore, MFCs can generally be obtained by mechanical fibrillation methods. Wet disk milling (WDM) (Hiden et al., 2009) and high-pressure homogenization (Henriksson et al., 2007) are examples of mechanical processes that fibrillate the cell wall

to the nanoscale by applying shear force at a high speed, where the procedure is often repeated over several passes to increase the degree of fibrillation. WDM presented many advantages in comparison to other fibrillation methods, as it can be used in continuous mode and can be scaled up to process large amounts of sample (Hideno et al., 2009). On the other hand, CNCs can be obtained by chemical or enzymatic treatments in combination with the aforementioned mechanical processes (Henriksson et al., 2007; Zhu et al., 2011). Cellulose microfibrils of plant cell walls contain crystalline regions that consist of highly ordered cellulose chains, intercalated by amorphous regions that consist of less-ordered cellulose chains (Park et al., 2010). When subjected to acid hydrolysis, cellulose microfibrils undergo transverse cleavage along the amorphous regions, resulting in the formation of CNCs with a relatively low aspect ratio. CNCs are typically prepared by acid hydrolysis using HCl or H₂SO₄ (Habibi et al., 2010). Their geometric characteristics depend on the biomass source, as well as the processing conditions applied, such as the type and concentration of acid used, the duration of hydrolysis, and the temperature at which the reaction is carried out. Mild hydrolysis conditions are suitable for avoiding excessive cellulose molecular weight loss or even the complete hydrolysis of cellulose into glucose (Henriksson et al., 2007).

Nevertheless, acidic hydrolysis has been the most studied procedure for obtaining CNCs, the use of endoglucanases (EC 3.2.1.4) allows for the advantageous adjustment of the CNC dimensions, the use of mild reaction conditions, and the protection of excessive cellulose degradation. CNCs derived from sulfuric acid hydrolysis will have negative surface charges, due to sulfur functional group (Beck-Candanedo et al., 2005), while CNCs resulted from an enzymatic hydrolysis process will not have surface charge. Furthermore, it has been reported that the CNCs prepared by the enzymatic method were associated with

better mechanical and thermal properties as compared to the nanocrystals obtained from sulfuric acid hydrolysis (George et al., 2011).

Endoglucanases have the ability to catalyze the hydrolysis of the 1,4- β -glycosidic linkages of the amorphous regions of cellulose. In nature, endoglucanases hydrolyze cellulose in synergy with cellobiohydrolases (EC 3.2.1.91, which act upon the reducing and non-reducing ends of cellulose chains) and β -glucosidases (EC 3.2.1.21, which catalyze the hydrolysis of cellobiose into glucose). Endoglucanases have also been reported to enhance cell wall swelling and, as a consequence, to facilitate the fibrillation when the biomass is subjected to hydrolysis before or during mechanical treatment (Henriksson et al., 2007). Furthermore, CNCs can be a valuable co-product of lignocellulosic biomass ethanol production, as it has been reported that the residues resulting from the enzymatic hydrolysis of lignocellulose biomass may contain significant amounts of CNCs (Duran et al., 2011, Zhu et al., 2011).

The objective of the present work was to obtain MFCs (through fibrillation using WDM) for subsequent enzymatic hydrolysis into CNCs, using biomass of different origins and compositions as feedstock (viz., pure cellulose, *Eucalyptus* holocellulose, unbleached Kraft wood pulp (UKWP), and sugarcane bagasse). Enzymatic hydrolysis was performed using a blend of *Pyrococcus horikoshii* endoglucanase and *Pyrococcus furiosus* β -glucosidase (cloned and expressed in *Escherichia coli*), or the commercial enzyme OptimashTMBG (Genencor International, USA), which exhibits high endoglucanase, β -glucosidase, and hemicellulases activities.

2. Material and Methods

2.1. Cellulosic substrates

Four types of cellulosic substrates were used: (i) commercial pure MFC (Celish[®] KY-100G; Daicel Chemical Industries, Japan) made from purified wood pulp; (ii) *Eucalyptus* holocellulose, delignified with the Wise method by using sodium chlorite and acetic acid (Wise et al. 1946); (iii) UKWP from Pine; and (iv) sugarcane bagasse, which was kindly supplied by the Usina Itarumã Sugar Mill (State of Goiás, Brazil). Bagasse samples were pre-ground in a cutter mill and fractionated through a sieve measuring 1.00 mm in size before wet disk milling. The chemical composition of each substrate was determined by acid hydrolysis according to Suitler et al. (2011).

2.2 Enzyme sources and activities

Hydrolysis experiments were performed using two enzyme preparations. The first preparation consisted of an enzyme blend of endoglucanase from *P. horikoshii* (EGPh) and β -glucosidase from *P. furiosus* (BGPf). Both hyperthermophilic enzymes were expressed in *E. coli* and purified according to Ando et al. (2002) and Kado et al. (2011), respectively. Each enzyme was diluted to 10 μ M in 50 mM sodium citrate buffer, pH 5.0, and mixed in a EGPh:BGPf ratio of 1:5 (Teixeira et al., 2013). The second preparation was the commercial enzyme Optimash[™]BG, diluted at 4% (v/v) in 50 mM sodium citrate buffer, pH 5.0. This preparation exhibited no cellobiohydrolase activity, and contained 223.9 IU/mL carboxymethyl cellulase (CMCase), 3.31 IU/mL β -glucosidase, 54.9 IU/mL xylanase, 1.4 IU/mL α -L-arabinofuranosidase, and 0.7 IU/mL β -xylosidase.

Endoglucanase activity measurements were performed against carboxymethyl cellulose (CMC; CMCase activity) according to the method of Ghose (1987), but adapted according to Teixeira et al. (2013), using a 3,5-dinitrosalicylic acid reagent without phenol.

The β -glucosidase, xylanase, α -L-arabinofuranosidase, and β -xylosidase activities were determined as described by Silva et al. (2013).

2.3 Wet disk milling

The cellulosic substrates were suspended in water, maintained for at least 24h, and then subjected to WDM to prepare MFCs. Water suspensions containing 2.0 wt% bagasse or Celish[®] KY, 1.7 wt% *Eucalyptus* holocellulose, or 1.5 wt% UKWP were disk milled using a Supermasscolloider MKCA6-2 (Masuko Sangyo Co., Ltd., Saitama, Japan) with a disk clearance of 10 μ m at a rotational speed of 1800 rpm. The WDM treatment durations for the various materials were 3.32 min/g of Celish[®] KY, 3.31 min/g of *Eucalyptus* holocellulose, 4.24 min/g of UKWP, and 1.50 min/g of sugarcane bagasse.

2.4 Enzymatic saccharification

Using the EGPh/BGPf blend, the enzymatic hydrolysis of 1 wt% (0.350 g, dry basis) WDM-treated products was carried out for 72 h at 85 °C and 600 rpm, with stirring on a 12 Plus Carousel hotplate (Radleys, UK). Hydrolysis using the commercial enzyme Optimash[™]BG was carried out at 45 °C and 230 rpm in a rotary shaker. Both hydrolysis experiments were performed with a CMCase activity load of 345 UI/g of substrate. The enzymatic hydrolysis assays were done in duplicate, and 300 μ L aliquots were withdrawn at 3, 6, 9, 24, 48, and 72 h for sugar content analysis. Samples taken at 72 h were diluted and directly used for atomic force microscopy (AFM) imaging.

2.5 High-performance liquid chromatography analysis

Monosaccharides and cellobiose were quantified using a high-performance liquid chromatography system equipped with a refraction index detector (RI-2031 Plus; Jasco Co., Japan), an Aminex HPX-87P column (7.8 mm I.D. x 30 cm; Bio-Rad, USA), and a de-ashing system and Carbon-P micro-guard cartridges (Bio-Rad, USA). Degassed deionized water was used as the mobile phase and the products were eluted at a flow rate of 0.7 mL/min at 80 °C.

2.6 Measurements

Disk-milled samples were washed once with distilled water and ethanol and then carefully washed with t-butyl alcohol several times to exchange the water content in the samples. The samples were then freeze-dried to maintain a morphology as similar as possible to that of the original samples. These freeze-dried samples were subjected to X-ray diffraction, field emission scanning electron microscopy (FE-SEM), and specific surface area (SSA) analyses.

Wide-angle X-ray diffraction patterns were obtained using a Rigaku RINT-TTR III X-ray diffractometer (Tokyo, Japan), applying nickel-filtered Cu K α radiation ($\lambda = 0.1542$ nm) at 50 kV and 300 mA. Disk pellets (measuring 1 cm in diameter, 0.8 mm in thickness, and weighing 0.1 g) were prepared by compacting the freeze-dried samples under a 2 ton load with a press (KBr-disk apparatus). The diffraction intensity was detected over the 2θ range of 2°–60°, at intervals of 0.02° and a scan rate of 2°/min. The crystallinity index was calculated according to Segal et al.(1959).

The SSAs of the samples were obtained using a BELSORP-Max system (Bel Japan Inc., Japan). Samples were initially degassed at 105 °C for 6 h. The SSA values were determined on the basis of a multipoint analysis of nitrogen adsorption isotherms at liquid

nitrogen temperature ($-196.15\text{ }^{\circ}\text{C}$), using the Brunauer–Emmett–Teller method (Brunauer et al., 1938).

The morphology of samples after WDM treatment and after enzymatic hydrolysis was observed by FE-SEM (S-4800N; Hitachi High-Tech. Co., Japan). The freeze-dried biomass samples were coated as a 1-nm-thick layer by an osmium plasma coater (NEOC-AN; Meiwa Fosis, Japan). Images were captured at an accelerating voltage of 1.5 kV.

Topographic images of samples that had been enzymatically hydrolyzed for 72 h were analyzed by AFM (JEOL SPM-5200S, Japan). **At least 5 samples derived from the enzymatic hydrolysis of each of the studied material were analyzed.** Approximately 100 μL of a diluted suspension (final concentration of approximately 0.01 wt%) was loaded on a freshly cleaved mica surface (Digital Instruments, USA) that had been previously treated for 15 min with 0.01% polyethyleneimine (PEI). After spending 1 min on the PEI-coated mica surface, the sample was spin-coated for 1 min at 3000 rpm using a MS-A100 spin coater (Mikasa Co. Ltd., Japan). All images were captured in the tapping mode at a scan rate of 1 Hz, using a point probe (measuring 4 μm in thickness, 125 μm in length, and 30 μm in width) with a silicon SPM sensor, a resonance frequency of 320 kHz, and a force constant of 42 N/m. The frequencies of the fiber diameters and lengths were estimated by measuring at least 100 fibers from a total of four topographic images obtained from each coated mica surface.

3 Results and Discussion

3.1 Biomass characterization

The chemical composition of each material is summarized in **Table 1**. Celish[®] KY can be characterized as being practically pure cellulose with a very low xylan content.

UKWP, *Eucalyptus* holocellulose, and sugarcane bagasse contained, respectively, 73.3%, 49.4%, and 41.9% cellulose; 13.4%, 21.3%, and 26.7% hemicellulose; and 8.1%, 6.7%, and 28.0% lignin. As the physical pretreatment based on grinding via the WDM method does not modify the chemical composition of the biomass (Silva et al., 2010), the microfibrillated materials were characterized before or after WDM.

3.2 Wet disk milling and enzymatic hydrolysis

In the WDM process, the samples were subjected to a large pressure drop by the shearing and impact forces. These conditions led to a high degree of fibrillation, forming long and twisted fibers from all materials with diameters ranging from 4 to 35 nm, as indicated by SEM imaging (**Figure 1**). With the aim being to obtain CNCs, the microfibrillated materials prepared using WDM were hydrolyzed by a blend of purified EGPh and BGPf, both expressed in *E. coli*, or by the commercial enzyme OptimashTMBG. The profiles of the enzymatic hydrolysis of the WDM-treated materials are shown in **Figure 2**. Hydrolysis using the EGPh/BGPf blend resulted in glucose concentrations and cellulose-to-glucose conversion yields of 1.0–7.4 mg/mL and 17.0–67.0%, respectively, depending on the biomass type. The use of OptimashTMBG resulted in glucose concentrations and cellulose conversion yields of 0.8–2.4 g/L and 15–29%, respectively, depending on the biomass type.

Depending on the structural carbohydrates and lignin content of the biomass sources and the enzyme blend used for their hydrolysis, the microfibrillated materials, which were further tested for the presence of CNCs, were not composed exclusively of cellulose as they also contained hemicellulose and lignin. The EGPh/BGPf blend acts on the cellulose component during enzymatic hydrolysis. EGPh hydrolyzes the amorphous cellulose regions

and is able to release cellobiose to some extent after an initial endo-type attack (Kim & Ishikawa, 2010), whereas BGPf is able to hydrolyze cellobiose and short cellooligosaccharides at high temperatures, releasing glucose (Kado et al., 2011). CNCs obtained using the EGPh/BGPf blend may thus exhibit distinct properties, such as a relatively weak surface charge and low hardness loss, compared with those prepared using sulfuric acid hydrolysis. On the other hand, OptimashTMBG with its high endoglucanase, β -glucosidase, and hemicellulases activities may be more effective in the hydrolysis of UKWP, *Eucalyptus* holocellulose, and sugarcane bagasse, which (besides cellulose) also contain hemicellulose or the hemicellulose-lignin complex. In fact, the hydrolysis of UKWP, *Eucalyptus* holocellulose, and sugarcane bagasse by OptimashTMBG allowed for the partial hydrolysis of hemicellulose, as 0.4, 1.7, and 1.1 mg/mL of xylose were detected in the hydrolysates after 72 h, respectively.

3.3. Morphological change after enzymatic hydrolysis

AFM analysis allowed the visualization and measurement of MFCs and of CNCs obtained after enzymatic hydrolysis. A topographic image of Celish[®]KY after WDM pretreatment and EGPh/BGPf hydrolysis is shown in **Figure 3**, and is representative of the hydrolysis of MFCs to CNCs that occurred to different extents for all the biomass sources and enzymes used. **Figure 4** shows the dimensional change of MFCs by enzymatic hydrolysis with the EGPh/BGPf blend and OptimashTM BG. It was possible to visualize the formation of CNCs for all of the cellulosic materials used (Supplementary material 1). However, the number of CNCs formed after sugarcane bagasse hydrolysis with the EGPh/BGPf blend (only 14 CNCs) did not reach the minimum recommended number of CNCs per AFM image, hindering a representative estimation of its dimensions.

Nonetheless, it is noteworthy that the composition of sugarcane bagasse was more complex than that of the other biomass sources studied and that reaction conditions could be further optimized to increase the number of CNCs formation. A higher amount of CNCs derived from sugarcane bagasse was observed from the use of Optimash™ BG (79 CNCs) than from using the EGPh/BGPf blend (Supplementary material 1). Optimash™ BG contains several xylan-degrading enzymatic activities, which hydrolyze the hemicellulose part, making cellulose more accessible to endoglucanases. This result corroborates the findings of other studies, that the residual material remaining after the enzymatic hydrolysis of cellulosic biomass to ethanol contains a considerable amount of CNCs (Duran et al, 2011; Zhu et al., 2011). CNCs have been obtained from complex lignocellulosic agro-industrial residues, such as those from coconut husk fibers (CNCs with an average diameter of 5 nm) (Rosa et al., 2010), and soy hulls (CNCs with a length of 122 nm and a diameter of 2.7 nm) (Neto et al., 2013), using sulfuric acid hydrolysis. Nonetheless, sugarcane bagasse is a promising biomass source for obtaining CNCs, using pretreatments that can alter the sugarcane bagasse composition and/or suitable enzyme blends containing accessory enzyme activities (e.g., xylan-degrading enzymes and feruloyl esterases (EC 3.1.1.73)) that would remove the unwanted hemicellulose-lignin complex coating of the cellulose microfibril. In the present study, we used enzymes with β -glucosidase activity to avoid cellobiose accumulation and to allow for the co-production of CNCs and glucose syrups. CNCs from cellulosic biomass show great potential for the dedicated production or co-production of CNCs and monosaccharides, which may increase the viability of producing ethanol from cellulosic biomass (Duran et al, 2011; Zhu et al., 2011).

CNCs with a relatively high aspect ratio were obtained by the effective adjustment of their length and diameter via enzymatic hydrolysis (Supplementary material 2). A

homogeneous distribution of fibers with nanoscale lengths were observed for Celish® KY, *Eucalyptus* holocellulose, UKWP, and sugarcane bagasse, whose high frequency decreased from 3000 to 600 nm, from 2000 to 500 nm, from 3000 to 750 nm, and from 1400 to 500 nm, respectively (**Figure 4**). The aforementioned result is in accordance to Filson et al. (2009) that reported a length distribution of 100 nm to 1.8 μ m using transmission electron microscope, for CNCs produced by enzymatic hydrolysis of recycled pulp. The diameter was also reduced, and a higher frequency of nanofibers with diameters ranging from 4 to 12 nm was observed for all the materials and enzymes tested. Because the diameter of cellulose microfibrils is approximately 5 nm and the diameter of microfibril bundles varies from 5 to 20 nm (Paakko et al., 2007), it can be suggested that the combination of the WDM treatment and enzymatic hydrolysis enables the production of the crystalline cellulose structures (CNCs) as they exist in plant cell walls. In accord with the aforementioned results, in this study, enzymatic hydrolysis also increased the cellulose crystallinity (**Table 2**) and SSA (**Table 3**), in agreement with the morphological analysis performed by AFM.

The well-known distribution of amorphous regions along cellulose microfibrils (Habibi et al., 2010) allowed the adjustment of the CNC length via the action of endoglucanase. However, in this study, in addition to the length adjustment, an adjustment to the diameter of the CNCs was also observed, suggesting that the effects of endoglucanase are exerted between the microfibrils. Nevertheless, there is still no consensus on the distribution of crystalline and amorphous regions within the cellulose microfibrils. The cellulose microfibril model proposed by Wickholm (1998) describes the crystalline regions as being present only in the inner core of microfibrils, with a non-crystalline ordered structure existing between the core and the surface. Moreover, in

305 concordance with the findings of this work, Henriksson et al. (1999 & 2007) suggested the
306 presence of less-ordered cellulose chains on the surface of and between cellulose crystalline
307 microfibrils that play a role in maintaining the whole microfiber structure. In addition, the
308 enzymatic hydrolysis of the amorphous regions along and between the crystalline
309 microfibrils can cause a swelling effect by exposing the internal chains to the water phase,
310 thus releasing the crystalline structures (Josefsson et al., 2008).

311 The presence of native less-ordered cellulose chains between the cellulose
312 crystalline microfibril structures is counterintuitive. If the distribution of amorphous
313 regions occurs only along the cellulose microfibrils, then the swelling effect caused by
314 endoglucanases might play a key role in separating the cellulose crystalline microfibrils and
315 disassembling the microfibril bundles. A non-catalytic effect of the carbohydrate-binding
316 domains (CBDs) of endoglucanases has been described (Rabinovich, 2002). These CBDs
317 are suggested to bind to amorphous regions and disrupt hydrogen bonding, resulting in
318 swelling and thus spreading the microfibril bundles of cellulose. The hypothesis of a non-
319 catalytic assistant effect of the CBD was successfully confirmed by studying individual
320 bacterial CBDs of glycosyl hydrolase family 2 (Kilburn et al., 1993). The EGPh used in this
321 work was previously crystallized and classified as belonging to family 5, subfamily 1 (Kim
322 et al., 2008; Kim & Ishikawa, 2010), enzymes of which are frequently multimodular and
323 possess CBDs (Aspeborg et al., 2012). However, no CDB structure was identified as part of
324 the EGPh structure, therefore invalidating the aforementioned hypothesis regarding the
325 non-catalytic effect of CBDs.

326 It has been reported that the swelling effect caused by the endoglucanase
327 pretreatment of wood pulp followed by mechanical treatment facilitates the fibrillation of
328 wood pulp into MFCs (Henriksson et al., 2007). Indeed, this procedure allows for the

production of MFCs with a desirably higher aspect ratio than that obtained by acid pretreatment (Paakko et al., 2007). Enzymatic pretreatment was also studied by Zhu et al. (2011), who reported the formation of CNCs (with a diameter of 20 nm and length of 500 nm) from Kraft *Eucalyptus* pulp, using a commercial endoglucanase (Novozym 476) followed by mechanical treatment in a micro fluidizer. Filson and Dawson-Andoh (2009) used endoglucanase for the enzymatic hydrolysis of recycled pulp as a feedstock to obtain CNCs, whereby the reaction mixture was subjected to microwave heating. This procedure allowed for the formation of CNCs with widths of 30–80 nm and lengths of 100 nm to 1.8 mm, higher than those obtained in the present work. In the present study, the materials were processed by a simpler mechanical pretreatment (viz., WDM) that allowed for the formation of MFCs that could be enzymatically hydrolyzed to successfully form CNCs. It is also noteworthy that the thermostable EGPh/BGPf blend can allow for the use of advantageously higher temperatures in the hydrolysis step, thus hindering bacterial contamination.

The morphological characteristics (e.g., the aspect ratio and crystallinity) of the CNCs produced in this work make the structures suitable as potential nanomaterials for the reinforcement of polymeric matrices. Moreover, the development of a process for obtaining CNCs from cellulosic materials via a biotechnological route rather than an acidic one could economically benefit a biorefinery platform, owing to the production of a biomaterial with high added value.

4. Conclusions

The enzymatic hydrolysis of wet-disk milled nanofibers showed the potential to integrate CNCs and monosaccharides production not only from pure cellulose but also from

more complex and less-expensive materials such as UKWP and *Eucalyptus* holocellulose. The dimensions of the CNCs were successfully adjusted by microfibrillation, followed by enzymatic hydrolysis performed using a thermophilic endoglucanase and β -glucosidase blend or the commercial enzyme preparation OptimashTMBG. The obtained CNCs had different characteristics such as nanoscopic morphology, SSA, and crystallinity from microfibrillated cellulose. The morphological characteristics of the CNCs obtained make them suitable as potential nanomaterials for the reinforcement of polymeric matrices.

Acknowledgements

This work was financed by FINEP from the Brazilian Ministry of Science, Technology, and Innovation (grant number 01.09.0566.001421/08), by the Japan International Cooperation Agency (JICA), and by the Japan Science and Technology Agency (JST). R. S. S. Teixeira is grateful to BIOMM S/A (Montes Claros, Brazil) for a postdoctoral fellowship.

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Figure captions

Figure 1. Field emission scanning electron microscopy of biomass after pretreatment by wet disk milling. (A) Celish[®] KY; (B) *Eucalyptus* holocellulose; (C) unbleached Kraft wood pulp; (D) sugarcane bagasse. Magnification $\times 60,000$; the scale bar represents 500 nm.

Figure 2. Hydrolysis time course for wet-disk-milled Celish[®] KY ($\blacklozenge$, $\blacktriangle$), *Eucalyptus* holocellulose ($\blacksquare$, $\square$), unbleached Kraft wood pulp($\blacktriangle$, $\triangle$), and sugarcane bagasse ($\bullet$, $\circ$), showing the glucose concentrations and cellulose-to-glucose conversion yields resulting from enzymatic hydrolysis using the *Pyrococcus horikoshii* endoglucanase/*Pyrococcus furiosus* β -glucosidase blend (closed symbols – A and C) and Optimash[™] BG (open symbols – B and D).

Figura 3. Atomic force microscopy of Celish[®] KY pretreated by wet disk milling (A) and after enzymatic hydrolysis with the *Pyrococcus horikoshii* endoglucanase/*Pyrococcus furiosus* β -glucosidase blend (B). The scale bar represents 500 nm.

Figure 4. Dimensional change of microfibrillated fibers from Celish[®] KY (A), *Eucalyptus* holocellulose(B), unbleached Kraft wood pulp (UKWP) (C), and sugarcane bagasse (D) after wet disk milling (—) and after 72 h of enzymatic hydrolysis using the *Pyrococcus horikoshii* endoglucanase/*Pyrococcus furiosus* β -glucosidase (EGPh/BGPf) blend (— —) or Optimash[™]BG (.....). * Less than 100 cellulose nanocrystals (CNCs) were counted after enzymatic hydrolysis of the microfibrillated sugarcane bagasse with the EGPh/BGPf blend (14 CNCs) and Optimash[™] BG (79 CNCs).

Table 1. Chemical composition of the microfibrillated celluloses obtained by wet disk milling.

Biomass	Glucan (%)	Xylan (%)	Arabinan (%)	Mannan (%)	Acid-insoluble lignin plus ash (%)	Acid-soluble lignin (%)
Celish® KY	100.9	3.2	-	-	-	ND
<i>Eucalyptus</i> holocellulose	49.4	20.2	1.1	-	6.7	ND
UKWP	73.3	6.7	-	6.7	6.3	1.9
Sugarcane bagasse	41.9	25.0	1.7	-	22.7	5.3

Abbreviations: ND, not detected; UKWP, unbleached Kraft wood pulp from Pine.

Table 2. Crystallinity index of the studied materials after wet disk milling and after 72 h of enzymatic hydrolysis with the EGPh/BGPf blend or OptimashTMBG.

Biomass	Crystallinity index		
	WDM	EGPh/BGPf blend	Optimash TM BG
Celish [®] KY	72.76	81.02	79.04
<i>Eucalyptus</i> holocellulose	58.42	65.94	65.56
UKWP	59.33	68.48	65.71
Sugarcane bagasse	59.60	63.95	61.59

Abbreviations: BGPf, *Pyrococcus furiosus* β -glucosidase; EGPh, *Pyrococcus horikoshii* endoglucanase; UKWP, unbleached Kraft wood pulp; WDM, wet disk milling.

Table 3. Specific surface area of the studied materials after wet disk milling and after 72 h of enzymatic hydrolysis with the EGPh/BGPf blend or Optimash™BG.

Biomass	Specific surface area (m ² /g)		
	WDM	EGPh/BGPf blend	Optimash™ BG
Celish® KY	162.18	147.35	171.37
<i>Eucalyptus</i> holocellulose	82.15	131.29	91.63
UKWP	193.04	228.02	231.40
Sugarcane bagasse	39.57	35.30	43.89

Abbreviations: BGPf, *Pyrococcus furiosus* β-glucosidase; EGPh, *Pyrococcus horikoshii* endoglucanase; UKWP, unbleached Kraft wood pulp; WDM, wet disk milling.

Figure 1

FIGURES

Figure 2

Figure 3

Figure 4