ENZYMATIC UPCYCLING OF BACTERIAL CELLULOSE FROM KOMBUCHA TO OBTAIN CELLOBIOSE

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Received November 24, 2022

The production of kombucha generates bacterial cellulose (BC) as a by-product, which is usually discarded. However, BC can be a source of cellobiose, a disaccharide with prebiotic benefits. In this study, the yield of cellobiose released from BC collected from a medium-sized kombucha producer was evaluated by enzymatic hydrolysis using the commercial cocktail Celluclast 1.5 L. The BC was hydrolyzed at solid contents of 2, 3 and 4% (m/v), enzyme dosage of 2.2 U/g cellulose, pH 5, 50 °C, and 150 rpm for 72 h. Industrial BC was characterized by FTIR and XRD to confirm the presence of common BC characteristics. The same analyses were performed after enzymatic hydrolysis, resulting in a change in crystallinity. The maximum cellobiose production (10-11 g/L) was obtained with 4% BC (w/v) in 48 h of hydrolysis; there was no significant difference when the time was extended to 72 h. The maximum glucose production under the same conditions was 3 g/L, showing that Celluclast 1.5 L has high cellobiose selectivity (78%). However, the cellobiose yield only ranged from 35 to 26%, indicating that cellobiose accumulation in the medium caused enzyme inhibition.

Keywords: SCOBY, enzymatic hydrolysis, biofilm, agro-industrial waste

INTRODUCTION

Kombucha, a tea-based fermented beverage, has gained great popularity in recent years. Tea fermentation takes place thanks to a symbiotic consortium of bacteria and yeast called SCOBY, responsible for the production of several compounds associated with health benefits, mainly anti-inflammatory, anticancer, antihypertensive, antidiabetic, hepatoprotective, and antimicrobial.[1–3]

Another common product of kombucha-like fermentation is the biofilm formed at the air-liquid interface, composed predominantly of bacterial cellulose (BC).[4,5] This product is not consumed in the beverage and, in large volumes of production, it becomes a residue of the process. However, it is composed of pure cellulose fibrils and has similar characteristics to BC produced in synthetic media, such as high water retention capacity, high crystallinity and thermostability.[6–8] Due to its properties, several applications of BC were reported in the literature, such as edible packaging, food ingredient, in textile industry and tissue engineering, as adsorbent of metal ions and in nanocomposites for electronics.[2,9] Some studies have focused on the enzymatic hydrolysis of BC to produce cellulose nanocrystals and glucose.[10–13]

In the enzymatic hydrolysis of cellulose, three main enzymes are needed to act simultaneously to obtain high sugar yields. According to Silveira *et al*.[14] and Rabinovich *et al*.,[15] endo-β-1,4-glucanases (EGs) are responsible for breaking down glycosidic bonds at the microfibril surface, primarily where cellulose chains are more loosely associated with one another, causing a subtle increase in the availability of both reducing and non-reducing chain ends. Exo-β-1,4-glucanases or cellobiohydrolase (CBHs) are responsible for the production of cellobiose from these recently formed chain ends, and β-1,4-glycosidases (βG) hydrolyze cellobiose into glucose.

In this study, the cellobiose was the product of interest. Cellobiose is an oligosaccharide

consisting of two glucose molecules linked through a β-1,4 bond, showing some applications as a substrate for biotechnological processes,[15–17] in food industry as texture and filler agent,[18] and also as a prebiotic compound[19–23] similar to fructooligosaccharides, galactooligosaccharides, and inulin. To favor the production of cellobiose, we chose the enzyme Celluclast 1.5 L, which has in its composition mainly endo- and exo-β-1,4-glucanases. However, it is known that cellobiose accumulation is a strong inhibitory product mainly for exo-β-1,4-glucanases. In this sense, the study was conducted to evaluate the potential for cellobiose production from kombucha BC and the maximum possible yield using Celluclast 1.5 L.

EXPERIMENTAL

Materials

The biofilm from kombucha was obtained from a local kombucha producer (Itajaí, SC, Brazil), which produces 2000 L of kombucha monthly and discards 15 L of biofilm. Figure 1A shows the fermentation tank from which the biofilm was collected. The biofilm was kept refrigerated until use. Commercial cocktail Celluclast® 1.5 L (Novozymes) was kindly provided by LNF (Bento Gonçalves, RS, Brazil).

Purification and drying of bacterial cellulose (BC)

The purification of BC was performed by first fractionating the biofilm into small pieces to facilitate the removal of impurities. Then, washing steps were performed in 0.1 mol/L NaOH at 50 °C. The NaOH solution (enough volume to cover the BC pieces) was changed every 24 h until a white color was reached (Fig. 1B). The purification step was completed in distilled water at 50 °C until neutral pH was achieved. The purified BC was dried in a vacuum oven at 60 °C during 3 days. The dried cellulose was ground in a domestic blender for further enzymatic hydrolysis.

Physicochemical characterization

The methodology used for BC characterization was described by Leonarski *et al.*[8] Fourier Transform Infrared Spectroscopy was recorded on an Agilent Cary 600 Series (Santa Clara, United States). The wavelength range from 4000 to 500 cm^{-1} was used, with a resolution of 4 cm^{-1} and accumulation of 16 scans in attenuated total reflectance (ATR) mode. X-ray diffractometry (XRD) was measured with Rigaku DRX MiniFlex600 equipment (Tokyo, Japan), using CuKα radiation, at a voltage of 40 kV and filament emission of 1.5 mA. The samples were scanned in the range of 5° to 50° 2θ, with a scan speed of 0.05°/step.

The crystallinity (%) was determined using peak-fitting of Gaussian functions and calculated according to Equation (1), described by Mohammadkazemi *et al.*:[24]

$$\text{Crystallinity (\%)} = \left(\frac{S_c}{S_t}\right) \times 100 \quad (1)$$

where S_c is the sum of the net area, and S_t is the sum of the total area.

Crystal allomorphs (cellulose Iα and Iβ) were analyzed by Equation (2) based on Z discriminant function:[25]

$$Z = 1693 \cdot d_1 - 902 \cdot d_2 - 549 \quad (2)$$

where d_1 is the d-spacing peak (100), and d_2 is the d-spacing peak (010). $Z < 0$ means that cellulose is rich in Iβ form, while $Z > 0$ signifies that Iα is the predominant form.

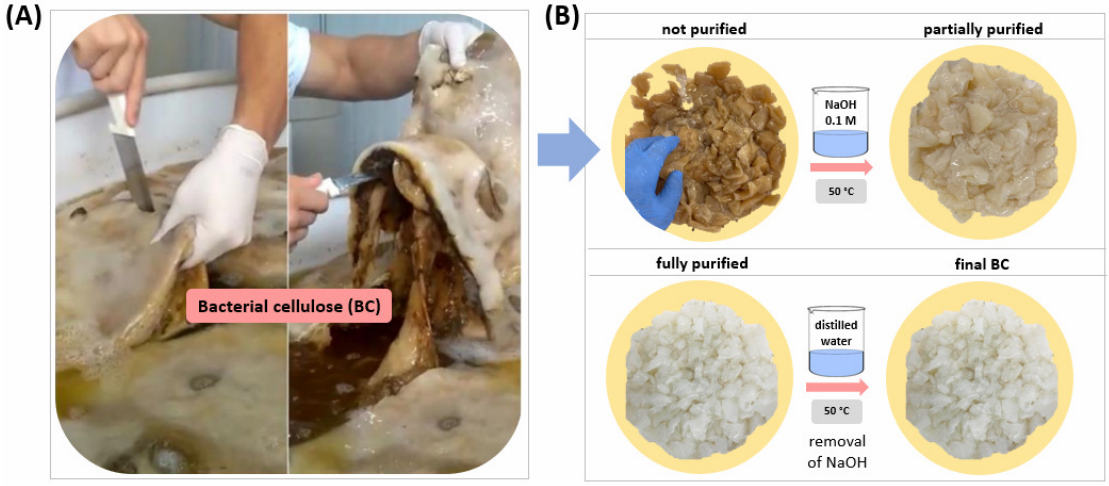

Figure 1: (A) Bacterial cellulose being removed from industrial tank, (B) Modification of visual appearance of bacterial cellulose (BC) during the purification protocol (soaking in 0.1 mol/L NaOH until white, followed by washing in distilled water until neutral pH)

Conditions of enzymatic hydrolysis

The commercial cocktail Celluclast® 1.5 L (37 FPU/mL) was diluted in 0.1 mol/L sodium acetate buffer, pH 5. The dosage used was 6% (w/w) based on dry mass of BC, corresponding to 2.2 U/g cellulose. The hydrolysis was carried out (in triplicate) in 50 mL of buffer, 50 °C and 150 rpm varying the BC content by 2, 3 and 4% (w/v). Aliquots were harvested at 24, 48 and 72 h, followed by enzyme inactivation at 90 °C for 10 min, centrifugation and filtration (0.2 µm).

Sugar analysis and yield calculation

Cellobiose and glucose concentrations were quantified by high performance liquid chromatography (HPLC, Accela TM, Thermo Scientific, Waltham, MA), with refractive index (RI) detector and HPX 87-H column (Bio-Rad ®). The mobile phase (eluent) was a 0.005 mol/L sulfuric acid solution (pH 2.6), at a flow rate of 0.6 mL/min at 45 °C. The calculation of glucose and cellobiose yields was performed using Equations (3) and (4):

$$Y_{glucose} = 100 * \left(\frac{C_{glu} * 0.9 * V}{M_{cel}}\right) \quad (3)$$

$$Y_{cellobiose} = 100 * \left(\frac{C_{cello} * 0.95 * V}{M_{cel}}\right) \quad (4)$$

where C_{glu} and C_{cello} are the glucose and cellobiose concentration (g/L); V is the reaction volume (L); and M_{cel} is the mass of cellulose used in the hydrolysis (g); 0.9 and 0.95 are the correction factors used for hexoses (glucose and cellobiose, respectively) to obtain the amount of sugars in the polymeric form.

The calculated yields were confirmed by the analysis of the dry mass of spent cellulose after hydrolysis. The solid content remaining at the end of the hydrolysis was oven-dried at 50 °C for 72 h and the dry mass was obtained.

Statistical analysis

Statistical analysis was conducted by Past software. The results were evaluated by analysis of variance (ANOVA), and the significant differences were determined using Tukey's test at a probability level of less than 5% ($p < 0.05$).

RESULTS AND DISCUSSION

Physicochemical properties of BC from kombucha

The results from FTIR and XRD analyses were used to confirm that the properties of BC produced during the kombucha fermentation were similar to BC produced from isolated bacteria and synthetic medium already described in the literature.[26] The FTIR spectra (Fig. 2A) show the typical absorption bands found in BC that at 3342 cm^{-1} attributed to OH- elongation, that at 2895 cm^{-1} – to the CH stretching of the CH_2 and CH_3 groups, and those at 1650 cm^{-1} and 1427 cm^{-1} corresponding to the glucose carbonyl group (C=O). The crystalline regions in the cellulose structure are demonstrated between the peaks at 1318 cm^{-1} and 1161 cm^{-1}. The peak at 1161 cm^{-1} indicates the presence of the C_1CO_4 group and those at 1033, 1054, and 1108 cm^{-1} correspond to the stretching vibrations of C_2O_2, C_3O_3, and C_6O_6, respectively.[8,12] Figure 2B shows the FTIR analysis for BC samples after enzymatic hydrolysis. All samples showed the same bands as BC before the hydrolysis, with an additional peak at 1552 cm^{-1}. This peak was also observed by other researchers that performed enzymatic hydrolysis of BC,[10,27] and was assigned to the acetate used as buffer in the hydrolysis.[28] Some studies also cited changes in the intensity of the peaks close to 1430 cm^{-1}, showing an increase or decrease in BC crystallinity.[29,30] In our study, the intensity of this peak was the same even after enzymatic treatment, and it was not possible to verify the change in crystallinity by FTIR analysis. However, this analysis was performed using XRD.

Figure 2C and Table 1 show four diffraction peaks corresponding to type I cellulose: 2θ = 14.4°, 16.6°, 22.6° and 33.9°.[31,32] XRD analysis provides parameters (crystalline peak angle and interplanar distance variation) used to evaluate possible changes in the morphology of BC possibly caused by chemical and/or mechanical treatments.[6] The values of full width at half maximum (FWHM), interplanar distances (d-spacing), crystallite size, Z value, and degree of crystallinity are shown in Table 1.

The values found for FWHM are slightly lower than those reported by Grande *et al.*[33] and Lee *et al.*,[34] who presented values of 1.93° and 1.71° for the peaks at 14.5° and 22.6°, respectively. The crystallite size obtained was close to that reported by Ruan *et al.*[35] for the peaks at 14.6° and 22.6°, showing values equal to 5.6 nm and 6.5 nm, respectively. The d-spacing values were similar to those reported by several studies for BC produced using different cultivation media.[36–38]

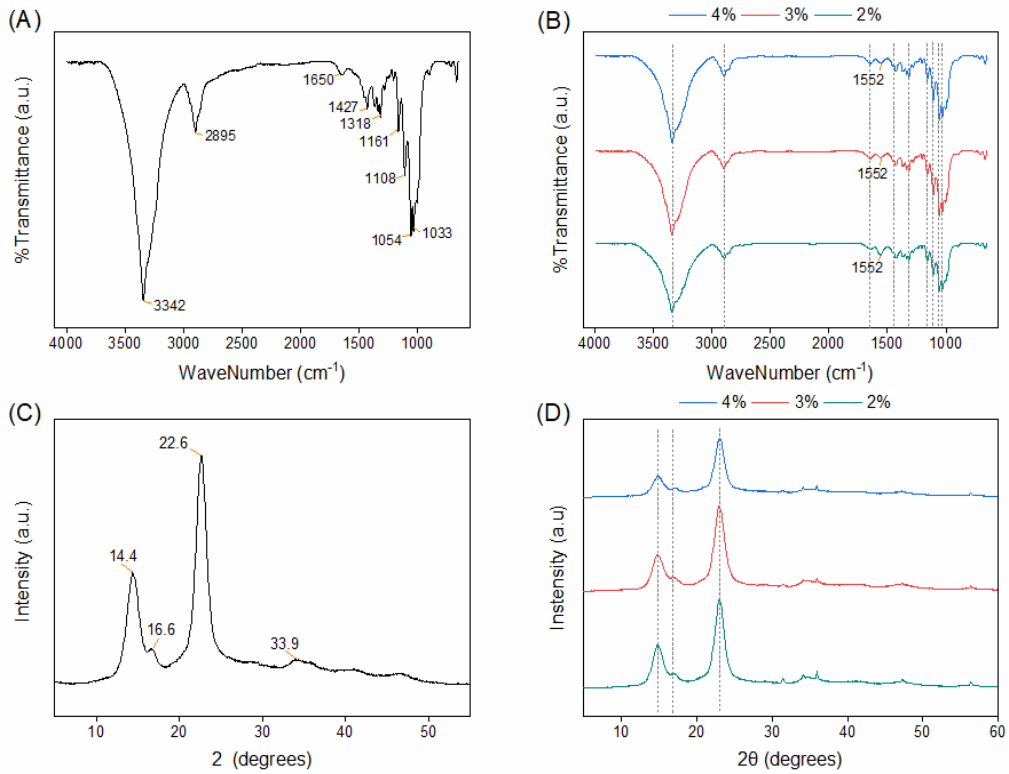

Figure 2: FTIR of BC from kombucha – samples before (A) and after (B) enzymatic hydrolysis; XRD for samples before (C) and after (D) enzymatic hydrolysis (2, 3 and 4% – BC content used in enzymatic hydrolysis)

Table 1
Full width at half maximum (FWHM), interplanar distances (d-spacing), crystallite size and crystallinity degree of bacterial cellulose (BC) from kombucha

2θ	FWHM	Crystallite size (nm)	d-spacing (nm)	Z value	Crystallinity (%)
BC before enzymatic hydrolysis					
14.4	1.34	5.99	0.61	10.2	88.3
16.6	0.48	16.58	0.53		
22.6	1.30	6.22	0.39		
33.9	0.81	10.19	0.26		
BC after enzymatic hydrolysis 2%					
14.9	1.61	4.97	0.59	13.9	60.4
16.9	1.28	6.29	0.52		
23.1	1.61	5.03	0.39		
BC after enzymatic hydrolysis 3%					
14.9	1.69	4.74	0.59	13.7	56.7
16.9	1.27	6.30	0.52		
23.0	1.70	4.77	0.39		
BC after enzymatic hydrolysis 4%					
14.9	1.60	5.01	0.59	13.9	51.3
17.0	1.25	6.42	0.52		
23.1	1.62	4.99	0.39		

The Z value discriminates whether BC is enriched by triclinic structure Iα (contains three angles not equal to 90°) or monoclinic structure Iβ (the cell contains one chain).[39,40] When Z is higher than zero, it indicates that BC is Iα-rich, as obtained in this study. Figure 2D shows the XRD

values for the BC after hydrolysis. The crystallite size was calculated for the three main peaks: at 2θ = 14.5°, 16.6°, and 22.6°, showing values between 4.74-5.01 nm, 6.29-6.42 nm, and 4.77-5.03, respectively (Table 1). These values are lower when compared to BC before hydrolysis, agreeing with the results presented in the literature,[41,42] which describe a decrease in the crystallite size and thinning of the cellulose microfibrils by the action of the enzymatic hydrolysis. According to Klafe *et al.*,[41] Iα-type cellulose (observed by the Z value > 0) is more susceptible to enzymatic hydrolysis than Iβ-type. In Table 1, it can be observed that the structure of BC became Iβ (Z value close to -13.8 for all the samples) after hydrolysis, which may be one of the reasons for the decrease in the hydrolysis yield after 48 h (discussed below).

The BC crystallinity was 88.3%, agreeing with the values found in other studies – between 80 to 90% for BC purified with NaOH.[6,8] Alkaline treatment is responsible for removing several compounds (microorganisms, proteins, amino acids, melanoidins, and other fermentation residues) and, depending on the time and temperature used, can result in greater crystallinity.[8] As the enzymes act on the cellulose fibrils, the amorphous and crystalline regions are being hydrolyzed, which reflected in the crystallinity. After hydrolysis, the crystallinity reached 51.3 to 60.4%. Kafle *et al.*[41] reported a decrease in BC crystallinity after 72 h of hydrolysis, according to the results presented in this study.

Cellobiose and glucose released during enzymatic hydrolysis

Figure 3 shows the sugar concentration (cellobiose and glucose) obtained after the enzymatic hydrolysis of BC using solid contents of 2, 3 and 4% (w/v). It can be seen that, as the BC content added in the hydrolysis was increased, the production of glucose and cellobiose increased as well. However, the cellobiose concentration was higher in all the experiments. At 2% and 3% (m/v), the maximum glucose production was 1.3 and 2.0 g/L, respectively, with no significant variation ($p < 0.05$) during the 72 h of hydrolysis. On the other hand, at 4% (w/v) of BC, the maximum glucose production was 3.5 g/L at 72 h.

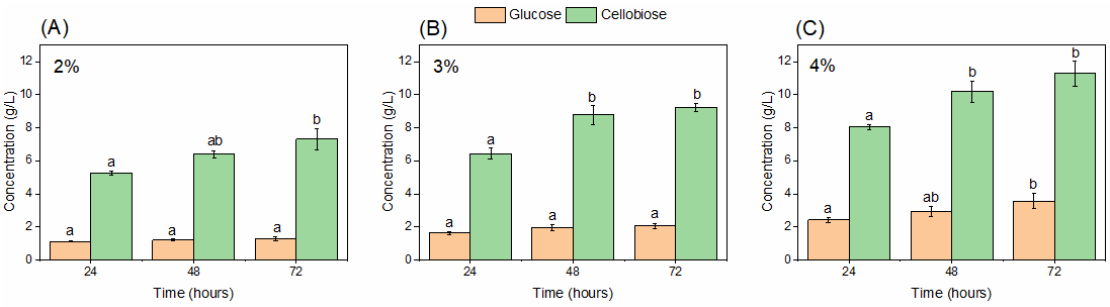

Figure 3: Cellobiose and glucose concentrations obtained by enzymatic hydrolysis of bacterial cellulose at different solid contents (w/v): (A) 2%, (B) 3% and (C) 4%

Cellobiose concentrations showed a significant increase in all the assays between 24 and 48 h. However, between 48 and 72 h, there was no significant difference ($p < 0.05$) in cellobiose concentrations, indicating that, after 48 h, the enzymatic action is negligible. The maximum concentration of this compound was 7.3, 9.2, and 11.3 g/L for the experiments containing 2%, 3%, and 4% (m/v) of BC, respectively. Kashcheyeva *et al.*,[12] using a combination of enzymes to improve the hydrolysis (CelloLux-A and BrewZyme), reached concentrations of reducing sugars of 11.1 g/L and 29.8 g/L for solid contents of 1 and 3% (w/v) of BC, respectively. However, the authors did not measure the cellobiose concentrations released, the result was presented based on glucose, according to colorimetric methodology. Brandes *et al.*[13] used Celluclast 1.5 L to hydrolyze BC and produce nanocrystals, not reporting the concentrations of sugar released.

Table 2
Sugar yield (%) and cellobiose selectivity obtained by enzymatic hydrolysis of bacterial cellulose at different solid contents (w/v): 2, 3 and 4%

BC	Time (h)	Yield (%)			Cellobiose selectivity (%)
		Glucose	Cellobiose	Total	
2% (w/v)	24	5.0 ± 0.1^{aA}	24.9 ± 0.6^{aA}	29.9	83.2
	48	5.4 ± 0.2^{aA}	30.4 ± 1.0^{abA}	35.8	84.9
	72	5.7 ± 0.6^{aA}	34.7 ± 3.1^{bA}	40.4	85.9
3% (w/v)	24	4.8 ± 0.2^{aA}	20.4 ± 1.0^{aB}	25.2	80.9
	48	5.8 ± 0.5^{abA}	27.7 ± 1.7^{bAB}	33.6	82.4
	72	6.2 ± 0.5^{bA}	29.2 ± 0.8^{bAB}	35.4	82.5
4% (w/v)	24	5.4 ± 0.4^{aA}	19.1 ± 0.3^{aB}	24.5	77.9
	48	6.6 ± 0.7^{abA}	24.2 ± 1.5^{bB}	30.8	78.6
	72	8.0 ± 1.0^{aA}	26.8 ± 1.8^{bB}	34.8	77.0

Different small letters indicate significant differences by Tukey's test ($p<0.05$) among the times for the same BC content; different capital letters indicate significant differences by Tukey's test ($p<0.05$) among the BC content for the same times; values are expressed as mean ± standard deviation of three replicates

Rovera *et al.*[11,43] evaluated the ratio enzyme/BC (w/w) (cellulase from *Trichoderma reesei* ATCC26921) on cellobiose and glucose release. The authors showed that the higher the cellulase dosage, the higher the glucose concentration. However, even at the minimum enzyme dosage tested (25% or ratio 0.25/1 – dry mass), low concentrations of cellobiose and glucose (1.54 and 2.25 g/L) were obtained after 166 h of hydrolysis using a solid content of 1.8% (w/v). In the present study, the enzyme dosage was 6% (w/w – based on BC dry mass). Enzyme cocktails have different enzymes with different mechanisms of action. While the cellulase used in the aforementioned study favored higher release of glucose, Celluclast 1.5 L favored higher release of cellobiose. Therefore, we can assume that the activity of β-glucosidases was very low, which resulted in high selectivity of the enzyme cocktail for cellobiose release (Table 2).

Table 2 also shows the cellobiose and glucose yields obtained. During the enzymatic hydrolysis, it can be observed that there was an increase in the hydrolysis yield for both sugars. At 4% BC, there was an increase in the glucose yield, reaching its maximum in 72 h. On the other hand, it was observed that the smaller the content of BC, the higher the cellobiose yield obtained (40.4% at 2% BC). Cellobiose selectivity also decreased with increasing BC content, from approximately 85% using 2% BC to 78% using 4% BC. It is worth mentioning that BC has high water absorption capacity. At 4% BC, we observe higher saturation of the medium due to the BC swelling, which could reduce the mass transfer phenomenon and the access of the enzyme to the substrate.

Brandes *et al.*[13] reached a maximum yield of 25.5% in the hydrolysis using 2 g of BC hydrogel (wet basis) using the same enzyme used in the present study. Kashcheyeva *et al.*[12] achieved 99.5% and 89.6% yield using 1% and 3% BC using the enzyme Celluclast 1.5 L. As previously mentioned, these high values may result from the enzyme cocktail used by the authors. A common factor found in both studies was that as the BC content decreased, the yield of reducing sugars increased. Due to the high crystallinity of bacterial cellulose, there are many hydrogen bonds between cellulose fibers, which are responsible for negatively affecting the access to enzymes and, consequently, slowing down the process.[13] Also, high selectivity of the enzyme was observed for the production of cellobiose (Table 2), which is in agreement with the literature.[44]

CONCLUSION

In the production of kombucha, large amounts of BC are generated, most of which are discarded. In this study, we evaluated the production of cellobiose as a way to upcycling the BC. The enzyme cocktail Celluclast 1.5 L is easily obtained commercially and showed promising results for cellobiose production by hydrolysis. However, because of the inhibition of endo- and exo-β-1,4-glucanases by cellobiose, the hydrolysis yield was between 35 and 40%. This indicates that, to increase the yield and make the process more attractive, it is necessary to adopt some strategy that avoids the phenomenon of

inhibition. However, at the end of hydrolysis, around 50% of the cellulose mass still remains. Based on the results and discussions presented, other hydrolysis strategies can be studied to increase the yield and viability of obtaining cellobiose, as well as oligosaccharides with a higher degree of polymerization. Subsequently, a purification step can be tested to separate glucose from cellobiose and other oligosaccharides to finally have greater precision in prebiotic activity assays.

In the literature, limited research exists on the hydrolysis of bacterial cellulose due to its intended use in specific applications. Existing studies focus mainly on the production of nanocrystals. In this study, however, cellulose derived from kombucha was used, an underexplored area of investigation. Additionally, the hydrolysis for cellobiose production remains quite recent even for lignocellulosic biomass. Therefore, the results indicate an interesting approach from the point of view of upcycling this material.

ACKNOWLEDGMENTS: The authors are grateful to CAPES-PRINT (project number 88887.310560/2018-00) and National Council for Scientific and Technological Development – CNPq (project number 403675/2021) for financial support.

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