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Lignin enrichment and enzyme deactivation as the root cause of enzymatic hydrolysis slowdown of steam pretreated sugarcane bagasse

Research Paper

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The enzymatic hydrolysis (EH) rate normally decreases during the hydrolysis, leaving unhydrolyzed material as residue. This phenomenon occurs during the hydrolysis of both cellulose (avicel) and lignocellulosic material, in nature or even pretreated. The progression of EH of steam pretreated sugarcane bagasse was associated with an initial (fast), intermediate (slower) and recalcitrant (slowest) phases, at glucan to glucose conversion yields of 61.7, 81.6 and 86%, respectively. Even though the EH of avicel as a simpler material than steam pretreated sugarcane bagasse, EH slowdown was present. The less thermo-stable endo-xylanase lost 58% of initial enzyme activity, followed by β-glucosidase that lost 16%, culminating in FPase activity loss of 30% in the first 24 hours. After 72 hours of EH the total loss of FPase activity was 40% compared to the initial activity. Analysis of the solid residue from EH showed that lignin content, phenolic compounds and ash increased while glucan decreased as hydrolysis progressed. During the initial fast phase of EH, the total solid residue surface area consisted predominantly of internal surface area. Thereafter, in the intermediate and recalcitrant phases of EH, the ratio of external:internal surface area increased. The proposed fiber damage and decrease in internal surface area, probably by EH action, was visualized by scanning electron microscopy imagery. The higher lignin/glucan ratio as EH progressed and enzyme deactivation by thermo instability were the main effects observed, respectively to substrate and enzyme.

Introduction

The use of lignocellulosic agricultural residues such as sugarcane bagasse for the production of second generation biofuels (2G bioethanol) represents an important development into renewable energy, and is receiving international interest. Bioethanol can be produced from lignocellulosic biomass, such as sugarcane bagasse, by pretreatment, enzymatic hydrolysis (EH) and sugar fermentation processes. However, the recalcitrance of the lignocellulosic biomass as well as the cost of pretreatment, enzymes production and enzymatic biomass conversion still represents major process bottlenecks for industrial application of the process [1].

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Biomass recalcitrance to enzymatic hydrolysis and subsequent bioconversion is due to several physical and chemical properties of the lignocellulose. The β-1,4 orientation of the glucosidic bonds within cellulose results in the formation of intra- and intermolecular hydrogen bonds between glucose monomers, making the microfibrils highly crystalline and resistant against biological and chemical breakdown [2]. Hemicellulose and lignin also limit the cellulose accessibility to cellulases. Lignin, which is a complex cross-linked polymer of phenyl propane units, plays a major role in the recalcitrance and structural rigidity of the substrate fibers [3]. Furthermore, lignin also encrusts the carbohydrate polymer matrix of cellulose and hemicellulose by forming covalent as well as hydrogen bonding in the lignin–carbohydrate complexes (LCCs) [2], thus limiting enzyme access for hydrolysis.

http://dx.doi.org/10.1016/j.nbt.2016.01.004

Pretreatment (chemical/mechanical/thermal) is critical to reduce the recalcitrance of heterogeneous lignocellulosic material and increase the accessibility of the substrate to EH [4]. However, the EH of pretreated lignocellulose still requires high dosages of enzyme and long incubation periods compared with other enzyme processes [5]. Depending on the pretreatment, after EH there is normally a fraction of the biomass carbohydrates that is not completely hydrolyzed [6]. The kinetic profile during EH of pretreated lignocellulose is characterized by a fast hydrolysis phase and a recalcitrant/slowdown phase. The factors that influence the overall performance during EH of lignocellulose can be placed into two categories: (1) those related to the substrate structure, which are heavily influenced by the feedstock chosen and the type of pretreatment and condition, (2) and those related to the mechanism and interactions of the cellulase enzymes with cellulose, which depend on the nature and source of the enzyme complex. There is no consensus as to which of these factors/categories have the most significant impact on the hydrolysis process. However, it is probably an interaction among the several effects related to both substrate and enzyme factors, which contribute to incomplete EH.

The incomplete cellulose to glucose conversion yield and decreased cellulose to glucose conversion rate during EH caused by lignocellulosic substrate changes (category 1) could be explained by: changes in the heterogeneous substrate composition [7] cellulose crystallinity [8], degree of polymerization [9] and surface accessibility [6]. On the other hand, the slowdown caused by the enzymes (category 2) could be explained by: thermal- and mechanical deactivation [10], unproductive binding to lignin [11], end-product inhibition [12], steric hindrances (by lignin and/or hemicellulose) and loss of synergism between the individual cellulase enzymes [8].

Various studies have indicated the effects that changing individual parameters have on efficient EH. However, it is most probably that a combination of factors will contribute to the observed cellulose to glucose conversion rate and yield during EH. In this context, the aim of this work was to study the correlation of hydrolysis rate with changes in physicochemical properties of the steam pretreated sugarcane bagasse that occurs during EH. Un-catalyzed steam explosion was used to reduce the recalcitrance of industrial sugarcane bagasse to EH as it has been widely used and has shown to be effective for herbaceous materials without the need of adding catalyst [13]. The chemical composition, crystallinity, degree of polymerization and accessibility of the substrate were also evaluated. Complementary, the enzyme activities (FPase, CMCase, β-glucosidase and endo-xylanase) under EH conditions as well as the cellulase adsorption pattern during EH were monitored for 72 hours. Elucidating the substrate characteristics that contribute to slowdown the cellulose to glucose conversion rate and incomplete conversion could contribute to the implementation of successful enzyme and substrate feeding and enzyme recovery strategies for optimal EH procedures.

Materials and methods

Material

Sugarcane bagasse (SCB) from an industrial plant in Malelane, Mpumalanga, South Africa was supplied by TSB Sugar. The samples were packed in zipped plastic bags and stored in a temperature and moisture controlled room (20°C and 65% humidity) until used for chemical composition and pretreatment. Steam explosion pretreatment was conducted in a pilot plant located in installations of CIEMAT biomass unit (CIEMAT – Renewable Energies Department, Madrid, Spain) [14]. The material was pretreated at 210°C for 5 min. The resulting pretreated material (slurry) was hand pressed in a piston with 20 ton weight to obtain a separate liquid and solid fraction. The solid fraction was washed 10-fold (10 g water/g pretreated solid) and the resulting water insoluble solids (WIS) were used in the EH experiments.

Chemical composition analysis

Carbohydrate, lignin and ash content of the raw material and steam pretreated solid residue from EH were determined according to the National Renewable Energy Laboratory (NREL, Golden, CO, USA) methods [15].

Enzymes, enzyme activities and protein determinations

The commercial enzyme preparations used for the study were Spezyme CP and Novozym® 188, kindly donated by Genencor (Genencor International, Rochester, NY, USA) and Novozymes A/S (Bagsværd, Denmark), respectively. Spezyme CP is a cellulase produced by *Trichoderma reesei* with an activity of 60 FPU/mL, and protein concentration of 188 mg/mL. Spezyme CP also has endo-xylanase activity of 5263 IU/mL. Novozym® 188 is a β-glucosidase produced by *Aspergillus niger* with an activity of 700 IU/mL and protein concentration of 114 mg/mL.

The level of enzyme activities of the enzyme mixture used for EH was monitored during an incubation period of 24, 48 and 72 hours at 50°C with an agitation of 100 rpm. The filter paper activity and β-glucosidase activity of the enzyme preparations were determined using filter paper Whatman No. 1 and cellobiose solution as substrates, respectively, with assays conditions as previously described [16]. In the case of β-glucosidase activity, the glucose was detected using Glucose Oxidase/Peroxidase (GOPOD, Megazyme) reagent. 0.05 mL of the β-glucosidase reaction was mixed with 1.5 mL GOPOD and incubated for 20 min. The absorbance was read at 510 nm. The CMCase and xylanase activities were carried out using 0.5% CMC and 1% birchwood xylan as substrate (Sigma), respectively. The assays were conducted by adding 0.05 mL of enzyme dilution to 0.450 mL substrate and incubated for 5 min at 50°C. The reaction was stopped by adding 0.750 mL DNS and boiled for 15 min, and absorbance reading at 540 nm [17].

The protein concentration of the enzyme preparations as well as the supernatant resulting from EH was determined by Bicinchoninic acid (BCA) assay (Thermo Scientific Pierce BCA Protein Assay) using bovine serum albumin as protein standard.

Enzymatic hydrolysis of WIS

EH was performed by transferring 2 g (2%, m/v) of WIS to a 250 mL Erlenmeyer flask containing 100 mL of sodium-citrate buffer (0.05 M), pH 5. Mixing was provided by orbital shaker set at 100 rpm and temperature at 50°C. Cellulase was added to ensure an activity of 15 FPU/g WIS (44 mg/g) and β-glucosidase activity of 15 U/g WIS (2.44 mg/g). Samples were taken at 0, 3, 6, 9, 12, 18, 24, 48 and 72 hours for analysis of glucose, xylose and cellobiose by HPLC as described below and to determine changes in the

solid fraction as described in section of 'Chemical composition analysis'. The glucan conversion rate (g/L hour) was determined using the slope function of the change in glucose released over time. The glucan to glucose (and eventually cellobiose) conversion yield (%, theoretical glucose in pretreated material) was calculated according to Eqn 1:

$$\text{Glucan conversion}\,(\%) = \frac{[\text{Glucose}] + 1.053[\text{Cellobiose}]}{1.111\,f\,[\text{Biomass}]} \times 100\% \quad (1)$$

where [Glucose] is the glucose concentration (g/L) released during enzymatic hydrolysis; [Cellobiose] is the cellobiose concentration (g/L) released during enzymatic hydrolysis; [Biomass] is the dry biomass concentration at the beginning of the enzymatic hydrolysis (g/L); f is the glucan fraction in dry biomass (g/g); 1.053 is the correction factor of cellobiose to equivalents of glucose; 1.111 is the conversion factor of glucan to equivalents of glucose.

Phenolic content in the solid residue

For determination of the phenolic content of the solid residues from EH, samples (50 mg) were treated with HCl in butylated hydroxytoluene as previous reported [18]. The samples were diluted with dioxane and filtered through a 0.22 μm nylon membrane syringe filter and the phenolic acids and aldehydes (ferulic acid, vanillin, vanilic acid, syringic acid, syringaldehyde and *p*-coumaric acid) were determined by HPLC analysis using a Luna C18 column.

FT-IR analysis of solid residue from enzymatic hydrolysis

Fourier Transform – Attenuated Total Reflectance Spectroscopy (FT-ART) analysis was performed with samples (1 mg) of the solid residue from EH as well as unhydrolyzed pretreated materials (control), which were dried and milled passing through a 100 mesh-screen and stocked with phosphorous pentoxide (P_2O_5). The samples were analyzed by Perkin Elmer Spectrum GX, where infrared spectra were obtained by using 32 scans in the range of 400–4000 cm^{-1}. FT-IR spectra were baseline corrected at 1850, 1190 and 790 cm^{-1}.

Crystallinity of the solid residue from enzymatic hydrolysis

Oven dried (45°C, 24 hours) samples of the solid residue from EH were defragmented into a powder with a mortar and pestle. X-ray diffraction data (XRD) was obtained with a LabX-XRD-6000 Shimadzu (X-ray Diffractometer), accelerating voltage of 40 kV and current of 30 mA. The scan was done with a 2θ range between 8° and 28° with a step of 0.05° and a scan rate of 2°/min. The crystallinity index (CrI) was determined as the percentage of crystalline material in the biomass, according to Eqn 2:

$$\text{CrI} = \frac{I_{002} - I_{\text{am}}}{I_{002}} \times 100 \quad (2)$$

where CrI is the relative degree of crystallinity; I_{002} is the intensity of the diffraction from the 002 plane at $2\theta = 22.5°$; I_{am} is the intensity of the background scatter at $2\theta = 18.7°$.

Internal and external surface of solid residue from enzymatic hydrolysis

The internal and external surface area of the solid residue from EH was determined by the modified Simon's staining (SS) pore size method [19]. The amount of dye adsorbed onto the fiber was determined as the difference in the initial concentration dye minus the residual dye concentration in the supernatant. For all samples, the concentrations of direct orange (DO) and direct blue (DB) in the dye-stripping solution as well as in the supernatant were determined using the Beer–Lambert law for binary mixtures that were solved simultaneously:

$$A_{455\text{nm}} = \epsilon_{\text{DO}/455}\,\text{LC}_{\text{DO}} + \epsilon_{\text{DB}/455}\,\text{LC}_{\text{DB}} \quad (3)$$

$$A_{624\text{nm}} = \epsilon_{\text{DO}/624}\,\text{LC}_{\text{DO}} + \epsilon_{\text{DB}/624}\,\text{LC}_{\text{DB}} \quad (4)$$

where A is the absorption of mixture at 455 or 624 nm, ε is the extinction coefficient of each component at the respective wavelength (ε was calculated by preparing standard curves of each dye and measuring the slope of their absorbance at 455 and 624 nm). In this study the values were $\varepsilon_{\text{DO}/455} = 25.67$; $\varepsilon_{\text{DB}/455} = 3.09$; $\varepsilon_{\text{DO}/624} = 0.86$; $\varepsilon_{\text{DB}/624} = 16.45$ g/cm, L is the path length of the cuvette (1 cm).

Degree of polymerization of glucan in the solid residue from enzymatic hydrolysis

The degree of polymerization of glucan in the solid residue from EH was determined by incubation of the 0.5 g of the solid in beakers containing 20 mL deionized water, 0.188 g sodium chlorite, and 63 μL glacial acetic acid in a water bath at 70°C. Three more doses of sodium chlorite (0.188 g) and glacial acetic acid (63 μL) were added after 2, 3 and 4 hours, as described somewhere [20]. The α-cellulose viscosities were measured using the TAPPI test method where 0.125 g α-cellulose was placed in a test tube with 12.5 mL distilled water for 1 hour. Thereafter, 12.5 mL cupriethylenediamine solution was added and stirred for 5 min and left for 2 hours at room temperature until solubilization of glucan was complete. The soluble glucan pulp viscosity was measured using a Brookefield viscometer with 0.5% glucan solution, using 0.5 M Cupriethylenediamine as a solvent. Pulp viscosities determined as centipoise (cp) were converted to degree of polymerization (DP) according to Eqn 5:

$$\text{DP}^{0.905} = 0.75\,[954\log(X) - 325] \quad (5)$$

where X is the viscosity in centipoises.

Scanning electron microscopy of solid residue from enzymatic hydrolysis

To analyze the samples of WIS and solid residue from EH by scanning electron microscopy, samples were washed with deionized water and dried at 45°C for 24 hours. The samples were mounted on a 10 mm stub using double-coated tape. They were then coated with gold in an Edwards Pirani 10S150A sputter coater and finally examined at different magnifications in the scanning electron microscope, with accelerating voltage of 7 kV and working distance of 10 mm.

HPLC analysis

Glucose, xylose, arabinose and cellobiose and present in the liquid fraction from EH were analyzed by HPLC. The samples were analyzed on an Aminex HPX-87H Column equipped with a Cation-H Micro-Guard Cartridge (Bio-Rad, Johannesburg, South Africa) with column temperature set to 65°C with a mobile phase of 5 mM sulphuric acid and a flow rate of 0.6 mL/min.

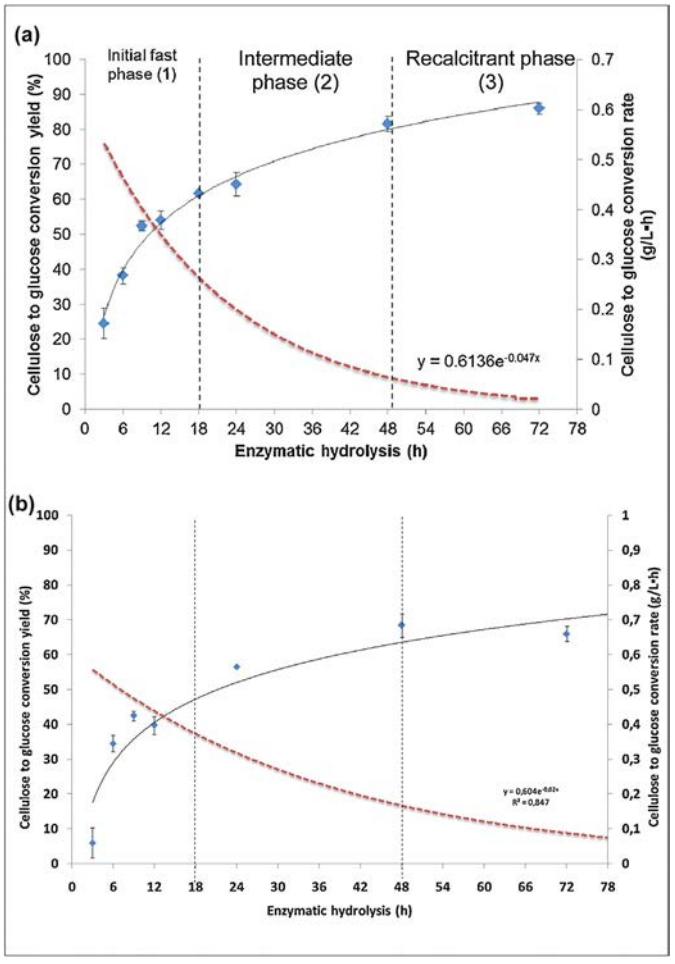

FIGURE 1

Kinetic profile of EH of (a) steam pretreated SCB and (b) avicel showing the cellulose conversion to glucose % (◇) and glucose release rate (g/L hour). Striped lines indicate the three distinctive phases of EH. Hydrolysis conditions: substrate loading 2% (w/v), enzyme dosage 15 FPU/g WIS, 50°C, pH 5.0, 100 rpm.

The concentrations of compounds were measured with a RI detector (Shodex, RI-101) operated at 5°C.

The phenolic acids and aldehydes (ferulic acid, vanillin, vanilic acid, syringic acid, syringaldehyde and p-coumaric acid) were analyzed on a Phenomenex Luna C18 reversed phase column (4.6 mm × 150 mm, 5 μm particle size) equipped with a Phenomenex Cation-H Cartridge Luna C18 pre-column. Column temperature was set to 25°C and flow rate to 0.7 mL/min. The mobile phases used for elution were 5 mM trifluoroacetic acid in water and 5 mM trifluoroacetic acid in acetonitrile. The phenolic acids and aldehydes peaks were detected with a Dionex Ultimate 3000 diode array detector at 280 nm.

Results

Kinetic profile of enzymatic hydrolysis

The glucan to glucose conversion yield (%) and glucose rate (g/L hour) during EH of steam pretreated SCB and crystalline cellulose, avicel, was carried out under identical hydrolysis conditions (Fig. 1a and b). The kinetic profile followed a conventional batch EH process characterized by a fast initial hydrolysis rate, a moderate hydrolysis rate and a slow or recalcitrant phase. The initial fast phase (1) until 18 hours was characterized by a high glucan conversion of 60.6% and 55% for steam pretreated SCB and avicel, respectively. The initial phase (1) was followed by an intermediate phase (2) where a 20% or 13% of glucan was further hydrolyzed (82% and 68% total) until 48 hours for steam pretreated SCB and avicel, respectively. The last 24 hours corresponded to the recalcitrant phase (3), where only about 4.3% additional glucan was hydrolyzed in both substrates (86% and 73% total for steam pretreated SCB and avicel, respectively).

The decrease in the glucose rate (g/L hour) mirrored the trend observed in the glucan conversion to glucose yield (%) at different time points during the hydrolysis. Similar pattern was observed for both substrates, steam pretreated SCB and avicel, suggesting that the slowdown is not only due to specific properties of the substrate. The rate decreased as expressed by exponential equation: $y = y_0 m^{-ax}$; where y is the rate, x is the variable time, y_0 is the value of y when $x = 0$, a is a constant and m is the base. The magnitude of the negative exponent indicated the rate at which the glucan conversion to glucose rate was decaying. The following functions were determined for steam pretreated SCB and avicel, $y = 0.6136e^{-0.047x}$ and $y = 0.604e^{-0.02x}$, respectively (Fig. 1). Although the transition from fast to slow hydrolysis rate took place for both substrates, the degree of reduction of hydrolysis rate seemed to occur at faster pace for the steam exploded SCB compared to that of avicel. The average glucose rate for the initial phase (1) was 0.49–0.55 g/L hour, decreasing to an average of 0.13–0.34 g/L hour for the intermediate phase (2) and finally 0.06–0.14 g/L hour in the recalcitrant phase (3), for steam pretreated SCB and avicel, respectively. After 50% of glucan conversion, the hydrolysis rate decrease 32 and 41%, respectively to SCB and avicel.

Effect of incubation conditions on enzyme activity and adsorption

One of the possible factors influencing the continuous slowdown of the glucan conversion is the enzyme deactivation due to temperature and agitation [10]. To determine the impact of EH conditions (50°C, 100 rpm) on the enzyme stability, several assays were conducted to monitor different enzyme activities FPase, CMCase, β-glucosidase and endo-xylanase (Fig. 2). The less thermo-stable activity was the endo-xylanase that kept 42% and 36% of the original activity after 24 and 72 hours, respectively. The endo-glucanase (CMCase) activity only decreased by 10% during the 72 hours incubation, with the critical decrease after the first 24 hours. The β-glucosidase activity, a crucial activity to prevent cellobiose inhibition, was reduced by 16% in the first 24 hours, and it further reduced 20% of the initial activity after 72 hours. The FPase activity reduced 30% after 24 hours.

Another enzyme-related factor that could promote slowdown of the glucan conversion is end-product inhibition by oligomers (gluco- and xylo-oligomers), cellobiose, glucose and xylose). Although EH was performed at 2% (m/v) solid loading that alleviates the effect of end-product inhibition [21], concentrations of glucose as low as 15 g/L has been proven to reduce the cellulase activity [22]. However, in the present study, a maximum glucose concentration of 11 g/L was only reached after 72 hours of enzymatic hydrolysis, at which stage the reaction had already passed into the slowdown phase. In terms of other end-products, low

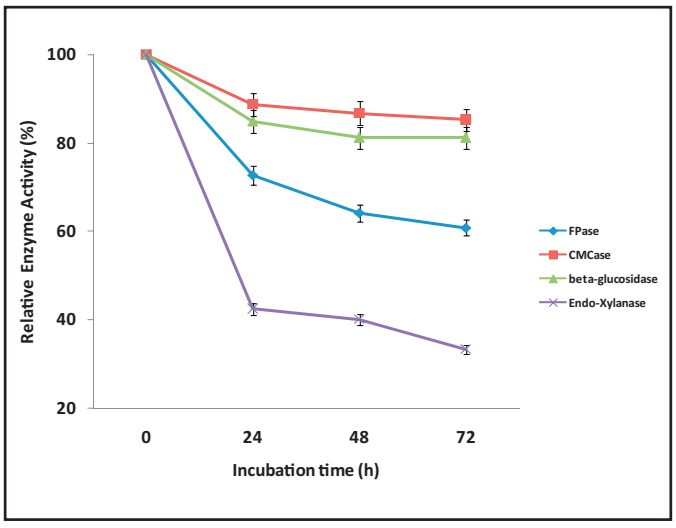

FIGURE 2

Enzyme stability of the FPase (filter paper activity), CMCase (endoglucanase), β-glucosidase and endoxaylanase activity over time at 50°C. The experiment was carried out at the same condition of EH, and the residual activity measured.

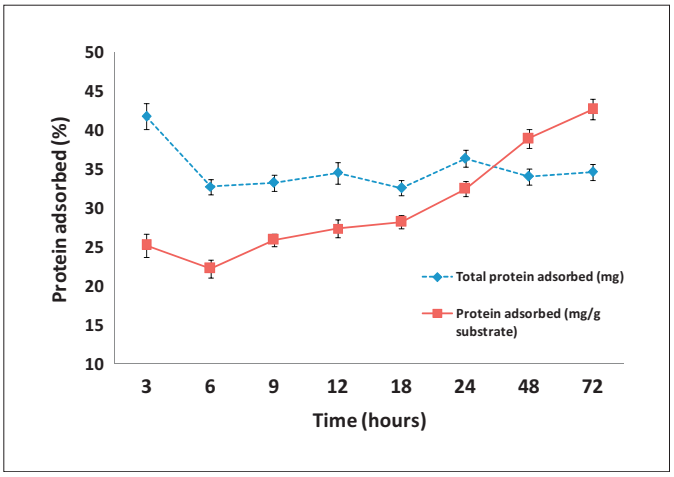

FIGURE 3

Protein adsorbed during EH kinetics of steam pretreated SCB. Total protein adsorbed (◇), total protein adsorbed per residual material, mg protein/g material (□) in each kinetics time.

concentrations of cellobiose (0.36 g/L) and xylose (1.2 g/L) were observed in the supernatants of the EH after 72 hours (data not shown).

The protein adsorption pattern was investigated to determine possible differences in substrate reactivity as the hydrolysis progresses. The specific amount bound to substrate was calculated as the amount of bound enzyme relative to remaining substrate (mg/g substrate). The enzymes showed rapid adsorption to the material at the beginning of the fast hydrolysis phase, with 32.81 mg of total protein adsorbed (35%) after 6 hours of EH (Fig. 3). After this, equilibrium between enzyme adsorption and desorption was reached and there was no significant change in the total amount of bound protein (34.71 mg, corresponding to 37% of the initial enzyme loading). These results are in line with other steam exploded herbaceous biomass [23]. However, when considering the specific protein adsorption in terms of residual substrate (mg protein/g substrate), the degree of protein adsorption increased from 28.3 to 42.8 mg/g substrate from the intermediate phase (18 hours) to the recalcitrance phase (72 hours) of EH.

Chemical composition of enzymatic hydrolysis solid residue

The SCB used in this study contained 44% glucan, 28.4% hemicellulose and 22.3% lignin (Table 1). The average chemical composition of SCB in the present study was similar to previous reports [24]. Steam explosion solubilized most of the hemicelluloses (acetyl, arabinose and xylose) from the SCG (Table 1). As a result, the WIS obtained from pretreatment of SCG was enriched in glucan

TABLE 1

Chemical composition of untreated, steam pretreated at 210°C for 5 min, and solid residue from EH using substrate loading 2% (w/v), enzyme dosage 15 FPU/g WIS, 50°C, pH 5.0, 100 rpm

Material	Glucan (%)	Xylose (%)[a]	Arabinose (%)[a]	Acetyl (%)[a]	Lignin (%)	Phenolic (mg/g)	S/G	Ash (%)	Glucan (g/100 g WIS)	Lignin/glucan ratio
Raw bagasse	44.0 ± 0.6	22.2 ± 0.3	1.5 ± 0.5	4.7 ± 0.2	22.3 ± 0.2	nd	nd	1.3 ± 0.3	nd	0.5
Steam pretreated bagasse	57.7 ± 2.2	6.0 ± 0.1	0.3 ± 0.0	1.2 ± 0.02	29.0 ± 0.5	12.99 ± 0.07	0.25	1.3 ± 0.09	57.73	0.5
3 hours EH residue	53.5 ± 1.2	3.3 ± 0.5	0.1 ± 0.02	1.3 ± 0.75	33.5 ± 0.8	9.91 ± 0.27	0.26	2.8 ± 0.39	46.56	0.6
6 hours EH residue	46.5 ± 0.7	3.9 ± 0.6	0.2 ± 0.04	0.8 ± 0.11	36.9 ± 0.3	12.21 ± 0.05	0.23	3.7 ± 0.66	36.68	0.8
9 hours EH residue	42.8 ± 0.5	2.5 ± 1.0	0.1 ± 0.06	0.7 ± 0.12	41.7 ± 1.0	13.59 ± 0.23	0.22	3.9 ± 0.0	29.92	1.0
12 hours EH residue	35.9 ± 1.8	2.9 ± 0.7	0.1 ± 0.04	0.7 ± 0.16	47.4 ± 0.6	14.55 ± 1.00	0.22	3.6 ± 0.01	22.08	1.3
18 hours EH residue	34.9 ± 1.3	3.2 ± 0.5	0.2 ± 0.03	0.9 ± 0.06	49.5 ± 4.0	15.04 ± 0.08	0.22	5.8 ± 0.31	20.54	1.4
24 hours EH residue	29.2 ± 0.4	2.7 ± 0.2	0.2 ± 0.01	0.7 ± 0.07	54.8 ± 1.2	17.08 ± 0.44	0.22	4.8 ± 0.42	15.53	1.9
48 hours EH residue	24.2 ± 0.2	3.0 ± 0.4	0.2 ± 0.03	0.8 ± 0.06	56.2 ± 2.1	15.88 ± 0.21	0.22	5.7 ± 0.32	12.54	2.3
72 hours EH residue	19.4 ± 0.6	1.8 ± 0.7	0.1 ± 0.08	0.6 ± 0.21	61.9 ± 1.6	15.96 ± 0.76	0.22	5.8 ± 0.16	9.14	3.2

[a] Hemicellulose represented by the monomers of xylan, arabinan and acetyl; EH residue, solid residue from enzymatic hydrolysis; S/G, syringyl/guaiacyl ratio (mol/mol). nd: not determined. All data represented was the average value of triplicate experiments; glucan (%): determined by chemical composition; glucan (g/100 g WIS): determined based on EH glucose yield.

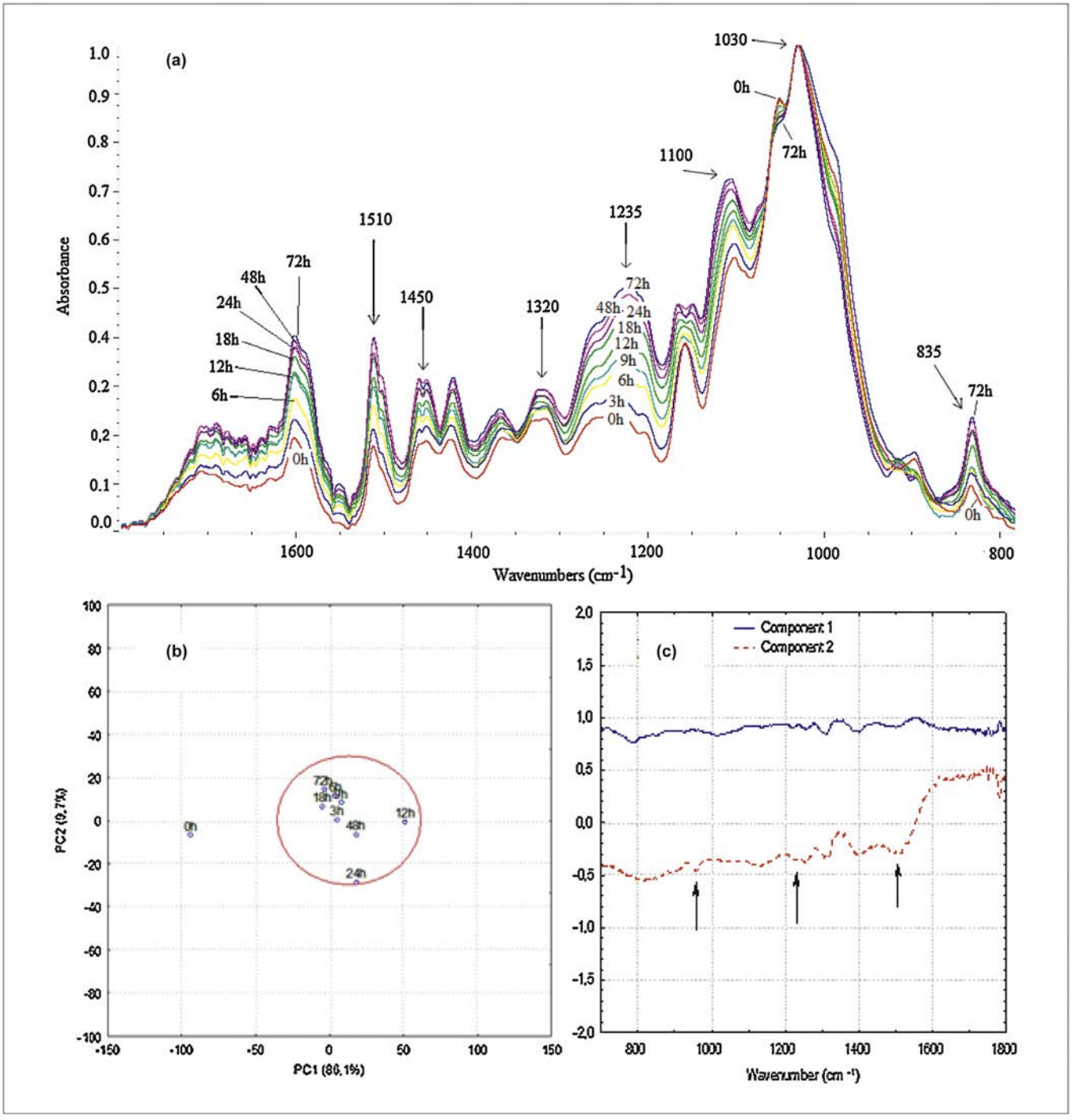

FIGURE 4

FT-IR spectra (a), FT-IR score plots (b) indicating the pretreated material (0 hour) and the solid residue from EH (3–72 hours) and FT-IR data matrix load plots (c) of the residue from EH of SCB.

(57.7%) and lignin (29%), with low residual hemicelluloses content (6% xylose, 0.3% arabinose and 1.2% acetyl). The composition of the remaining solids was also monitored during the progress of EH. The chemical composition of the substrate is being modified during EH. The glucan content was reduced with the subsequent enrichment of other components such as lignin and insoluble ash during EH. The final residue from EH had 3 times less glucan (17.6%), double lignin (61.9%) and more structural ash (5.8%) compared with the starting WIS (Table 1). However, the residual amount of hemicellulose in pretreated SCG was removed by the action of the hydrolytic enzymes.

The phenolic content of the material gradually increased from 12.99 to a maximal value of 17.08 mg/g,after 24 hours, while the syringyl/guiacyl (S/G) ratio decreased from 0.25 to minimum of 0.22 after 9 hours (Table 1). Since cellulases should not have action on the lignin structure, the removal of polysaccharides could partially affect lignin fragment solubilization, by acting on lignin–carbohydrate complexes (LCCs).

TABLE 2

Dye adsorption, biomass crystallinity index (CrI) and degree of polymerization (DP) of solid residue from EH using substrate 2% (w/v), enzyme loading 15 FPU/g WIS, 50°C, pH 5.0, 100 rpm

EH time (h)	Adsorbed dye (mg/g fiber)			Ratio DO/DB	CrI (%)	DP	Un-hydrolyzed glucan
	DO	DB	Total				
0	32.57	73	105.57	0.45	54.97	515.17	57.7
3	71.43	79.70	151.13	0.90	53.32	494.22	53.5
6	80.0	85.47	165.47	0.94	51.67	501.36	46.5
9	70.0	90.0	160.0	0.78	48.10	463.88	42.8
12	100.0	115.0	215.0	0.87	47.74	439.01	35.9
18	105.26	139.86	245.12	0.75	45.24	421.24	34.9
24	160	129.43	289.43	1.24	46.63	–	29.2
48	153.85	125	278.85	1.23	35.78	–	24.2
72	91.0	91.32	182.32	1.0	30.17	–	19.4

DO, direct orange dye; DB, direct blue dye; CrI, crystallinity index; DP, degree of polymerization; –, not determined.

FT-IR analysis on the solid residue from enzymatic hydrolysis

The FT-IR analysis was used to investigate the structural changes in WIS during EH. The FT-IR analysis of the WIS during EH indicated that the absorption bands at 1600, 1510, and 1450 cm^{-1} increased drastically as EH proceeded (Fig. 4a). These bands correlate to skeletal vibrations in the aromatic C=C stretches, which were attributed to the lignin structure [25]. Similarly, the increase in the band at 1235 cm^{-1} indicated an increase in the lignin content as this band represents guaiacyl ring, the C–O guaiacyl aromatic methoxyl linkage as well as C–O lignin stretching [25]. Furthermore, the band at 835 cm^{-1} represents the C–H out of plane vibrations in the C2 and C6 positions of the syringyl units of lignin [25]. Additionally, the band at 900 cm^{-1} decreased, representing the C–O–C stretching of β-1,4-glucosidic bonds between glucose monomers in glucan, indicating the glucan content decreased in the material as EH proceeded.

Principle component analysis (PCA) of the FT-IR data identified the most significant differences between the absorption spectra of the solid residue from EH. The PCA score plot showed that samples obtained from the solid residue of EH (3–72 hours) were distinctly different from the unhydrolyzed material (0 hour) (Fig. 4b). The loadings plot for PCA showed discrepancies in the regions of 900, 1235 and 1510 cm^{-1} were responsible for most of the spectral differences observed among the samples (Fig. 4c). Those bands are attributed to glucosidic linkage (C–O–C), methoxyl linkage in the guaiacyl ring (C–O), and aromatic ring (C=C) found only in the lignin structure, respectively, indicating that both changes in glucan and lignin were associated with the slowdown (decreased rate and ultimate yield) of EH.

Substrate crystallinity and glucan degree of polymerization

A decrease in the crystallinity index (CrI) of the pretreated WIS material was observed as EH proceeded. The initial CrI of the WIS was 55% that decreased gradually to 30.17% after 72 hours of EH (Table 2). The CrI of the whole solid residue is diminished during glucan conversion to glucose by EH, since this process actually removes glucan, enriching the material with the amorphous fraction (Table 1). In fact, the CrI decreased with glucan content with a linear correlation (data not shown). No correlation was observed between CrI and enzymatic hydrolysis glucose yield, however the decreasing rate was directly correlated to CrI increases (Supplemental Fig. 1).

The degree of polymerization (DP) of the glucan isolated from the WIS used in the present study decreased from 515 to 421.25 after 18 hours of EH (Table 2). The chemical isolation of the glucan was not practical in residues beyond 18 hours of EH. The increasing lignin to glucan ratio during EH (Table 1) hampered the chemical isolation of glucan after the initial fast hydrolysis phase (18 hours), thus not allowing measure of DP beyond 18 hours of EH. Even though the reduction in the DP indicated greater amounts of reducing ends available for exo-glucanase to hydrolyze [9], the glucan to glucose conversion rate decreased. No correlation was observed with DP and enzymatic hydrolysis glucose yield; however the decreasing rate was direct correlated to DP increases (Supplemental Fig. 1).

Surface morphological change during enzymatic hydrolysis

Interior and exterior specific surface area was estimated to confirm structural changes in the material during EH. This method uses two different dyes: the direct blue (DB) dye populates the smaller inner pores of the material associated with the interior fiber surface area; the direct orange (DO) populates the external substrate surface area, due to its larger molecular size and greater affinity for glucan than DB [26]. Thus, the total amount of absorbed dye can be used as indicative of glucan accessibility of the material. Pores with diameter smaller than cellulases (minimal diameter of 5.1 nm) will limit conversion while those with larger diameter benefit glucan conversion [19].

The amount of dye adsorbed on the substrate showed a higher increase for DO in the first 3 hours of reaction (Table 2), the period with higher EH rate (Fig. 1). During this period of EH, a lower increase was observed for DB dye adsorption, and the material could be characterized as rich in internal surface area due to predominant DB adsorption. The total dye adsorption gradually increased from an initial 105.57 mg dye/g fiber up to a maximum value of 289 mg dye/g fiber at 24 hours of EH. The greater internal surface area coincided with the initial fast phase (up to 18 hours) observed during EH (Fig. 1). After 18 hours, in the intermediate

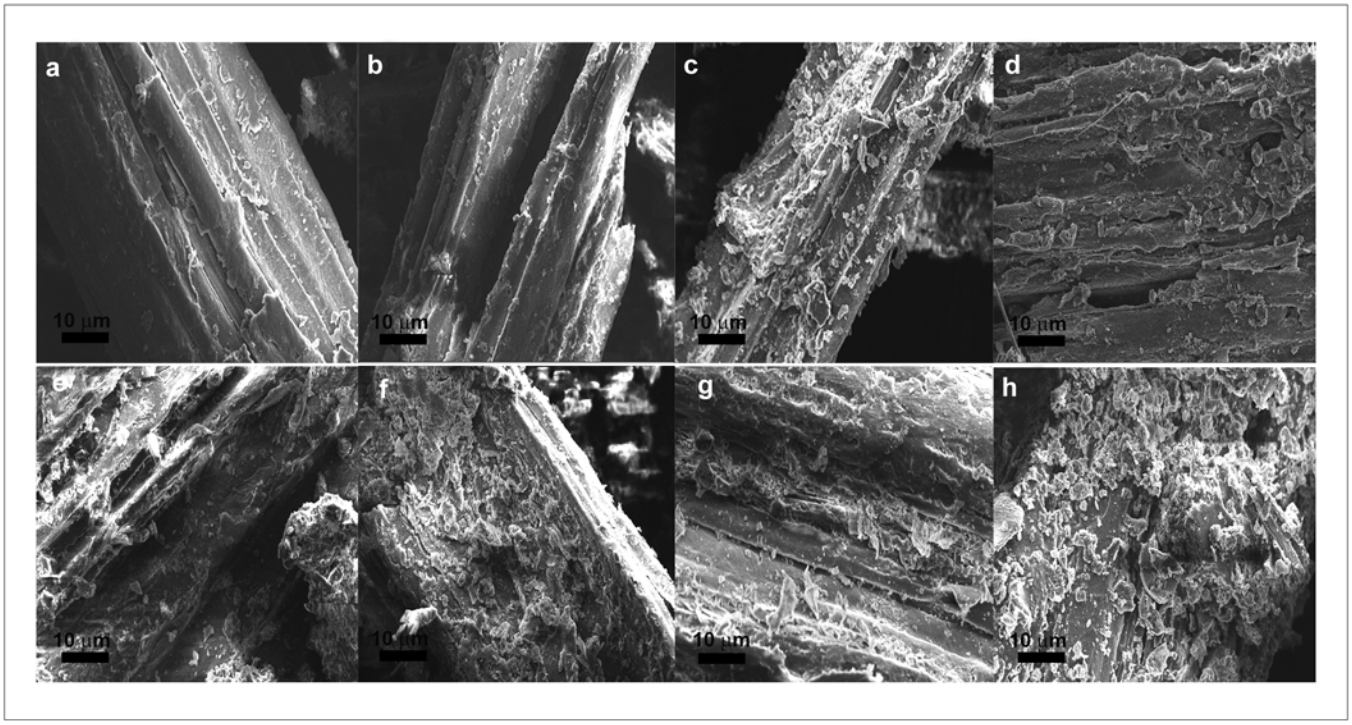

FIGURE 5

Scanning electron microscopy images of the residual solids from EH kinetic. (a) 0 hour, (b) 3 hours, (c) 6 hours, (d) 9 hours, (e) 12 hours, (f) 18 hours, (g) 48 hours and (h) 72 hours represent the solid residue from EH at the indicated time at 2000× magnification.

phase, the material was characterized with lower internal area than external area, indicating changes in the material morphology.

The solid residue from EH showed gradual increase in material damage/porosity as the reaction progressed, evidencing morphological differences (Fig. 5a–h). The visual damage observed in samples from different EH reaction times is in agreement with the glucose yield and the total dye adsorption trend (Figs 1,5 and Table 2). The dye adsorption indicated the accessible surface area of the residues was changing impacting the material digestibility as EH progressed. The enzyme action could increase the pore damage observed by SEM; with predominant exterior surface area compared to interior surface area, determined by dye adsorption. Since the material changed in terms of porosity, damage and interior and exterior surface area, it is expected that the glucan availability and accessibility also changed.

Discussion

Despite a high glucan conversion yield was achieved (86%) for steam-pretreated SCB (Fig. 1), the analysis of the residual solid indicated that there was still 19.4% of unhydrolyzed glucan (9.14 g/100 g WIS) after 72 hours. As EH proceeded the glucan was continuously hydrolyzed and removed from the solid residue of steam pretreated SCB. Since glucan and hemicellulose were removed, the lignin content in the residual solid increased as the EH proceeded. It was thus hypothesized that: (i) residual lignin affects the substrate recalcitrance, as the increasing lignin: lignin/carbohydrate ratio is a physical barrier to EH; (ii) residual lignin could adsorb enzymes increasing the unproductive linkages, decreasing the enzyme available to the catalysis. A reduction in the EH yield increment and rate could be a consequence of both cases (i) and (ii) considered as direct effect of lignin. The experiments were done to minimize enzyme factors, but thermal inactivation was observed. In fact, the EH of pure cellulose, avicel, showed the same three phases that represents the process kinetics in the steam exploded SCB: fast, intermediate and recalcitrant phases of EH. However, the EH of steam pretreated SCB showed shorter fast and intermediate phases when compared to avicel kinetics, reaching the recalcitrance phase earlier. In the case of avicel, the total theoretical yield could not be reached either [27,28]. This suggests that the slowdown is not only due to intrinsic properties of the substrate as enrichment of lignin, but also to intrinsic property of the glucan and enzyme-related factors. However, there are a combination of enzyme and substrate-related factors influencing the EH rate and yield. On the other hand, works evaluating enzyme-related factors suggest the relevant role of substrate related-factors such as heterogeneity [29,30]. The present work results showed that this heterogeneity is mainly because of lignin and ash enrichment.

Results suggested the enzyme cocktail lost activity as a function of time due to loss of total available enzyme as adsorbed [28] and as thermal instability as shown in Fig. 2. These results are comparable to previous studies at similar conditions where Spezyme CP have been shown to retain 80–85% of cellulase and CMCase activity for 24 hours [23] and 48 hours [31]. Likewise, the severe reduction of endo-xylanase activity is consistent with other studies that have proven its thermal instability even after a 6 hours incubation period [23]. The enzyme mixture only kept 33% of the starting endo-xylanase activity and 60% of FPase after 72 hours. This

reduction in the enzyme activities and therefore loss of synergism (exo-glucanase:endo-glucanase, cellulase:endo-xylanase) probably contributed to glucan conversion to glucose rate decrease. However, it is not the only factor contributing to the reaction slowdown, since high cellulase activity (70%) remained available for the enzymatic reaction after 24 hours incubation. According to Levine et al. [29], an enzyme half-life much shorter and strong end-product inhibition are required to explain the reduction in glucan hydrolysis.

The substrate also changes during the enzymatic hydrolysis becoming more recalcitrant. The final residue from EH had three times less glucan (19.4%), double lignin (61.9%) and four times more structural ash (5.8%) compared with the starting WIS (Table 1). These structural changes were further corroborated by FT-IR analysis of the EH residues. The bands indicated an increase in the lignin content, for example guaiacyl aromatic methoxyl linkage and syringyl units of lignin [25]. Guaiacyl enrichment means recalcitrance increased due to more stable bonds between lignin components [2]. The difficulty of glucan isolation to determine the degree of polymerization is also an evidence of the increasing recalcitrance as EH proceeded. The exponential decay of the glucan to glucose conversion rate (Fig. 1) is simultaneously related to the increasing lignin (Supplemental Fig. 1), lignin/glucan ratio and increasing phenolic/glucan ratio (Fig. 2) of the material as EH progresses.

The changes in substrate composition resulted in changes of the enzyme adsorption pattern. The enzymes were still bound to the substrate during the slowdown as indicated by similar values of total adsorbed protein (about 36% of initial enzyme) during the EH (Fig. 3). But the amount of protein bound per remaining substrate increased from 32.81 to 43 mg/g substrate. Since limited glucan conversion was observed after 24 hours (Fig. 1), it could probably be due to unspecific enzyme binding to lignin [10] and/or trapping of the exo-glucanases (CBH) in a more recalcitrant carbohydrate fraction. The CBH can work better on crystalline substrate increasing the rate due the binding module affinity [31], what is important as EH progress, but coincide the moment of enzymes has activity reduced by thermo deactivation. The endo-glucanase can be affected by substrate adsorption, what is suggested to collaborate to slowdown due enzyme trapping [32,33]. Some functional groups such as phenolic hydroxyl groups can strongly bind to enzymes [34]; carboxylic acid can contribute to decrease of the hydrophobicity, however could provoke ionic interaction with the protein [35]. In fact, the FTIR band at the region of 1380 and 1630 cm^{-1}, attributed respectively to phenolic OH and C=O, showed increased intensity as EH proceeded (Fig. 4a), increasing the possible interaction sites. Likewise, lignin was enriched in guaiacyl linkages that showed more protein adsorption than syringyl rich lignin [35]. Therefore, there was evidence of increase unproductive binding of the enzymes to the lignin during the course of EH, once the lignin-to-glucan ratio was significantly increased. The protein linkage with lignin occurs based on hydrophobic interactions, and the adsorption patterns depends on both lignin and enzyme characteristics [36]. Enzymes that present a carbohydrate binding domain (CBD) are strongly inhibited by lignin. For example, the degree of inhibition of lignin is higher in CBH, followed by xylanases and EG, while β-glucosidases is the least inhibited enzyme. The unproductive binding coupled with thermal deactivation, mainly of the exo-glucanase activity, may play a significant role in slowdown of glucan hydrolysis.

The amorphous fraction of glucan is hydrolyzed first and faster than the crystalline component as previously reported [37]. The total CrI change of the solid residual material during EH could not be correlated to the decreasing glucan to glucose conversion rate (Table 2) even though a linear correlation between the CrI of pure cellulose and its effect on the rate of glucan conversion to glucose has previously been established [38]. However, for lignocellulosic material that is composed of crystalline (glucan) and amorphous (hemicellulose and lignin) components, a direct correlation is not observed [31]. Indeed, it has been reported that the CrI is proportional to the α-cellulose content in the materials [39], explaining the CrI decrease as EH progress (Table 2).

In the intermediate phase of EH, the material was characterized with less internal than external specific surface area, indicating that the smaller pores were overcome by bigger pores by enzyme action. Fungal cellulolytic enzymes could not act on the small pores, but its action on the fiber could have fragmented the material creating external surface area. The maximal adsorption of total dye on substrate (24 hours) (Table 2), represented the maximum enzyme accessibility to the glucan, where after there was a decrease in the adsorption of dyes as a result of the significant decrease in glucan content (Table 1). Therefore, it seems logical that the pores and damage provoked by the enzymes action could increase the internal and external surface area. It can be considered that during EH cellulose chains can be solubilized exposing new sites to enzyme action [40]. Moreover, it has been reported high damage and fractionated degree on residues from enzymatic hydrolysis observed by scanning electron microscopy [40]. The substrate accessibility is not responsible only to yield and rate on EH. This substrate related-factor also could interferer on enzymes action, since the synergism between endo-glucanase and exo-glucanase depends on the substrate availability [29]. Mechanistic modeling of enzymatic hydrolysis suggested that the rate and final yield can increase with increase of accessible surface area [41].

The enzyme–substrate interactions and the mechanism involved in the enzymatic hydrolysis of biomass influenced by enzyme and substrate factors have been the focus of several works. However, these factors together can influence enzyme–substrate interactions that are not complete understood [42]. Moreover, the present work results showed how the recalcitrance of material changes during the EH: lignin and ash enrichment, and cellulose accessibility modification. Considering extensive hydrolysis of cellulose desired for process feasibility some points could be considered: (i) development of more thermostable enzymes. These enzymes can guarantee enough action during enzymatic hydrolysis over long reaction time; (ii) development of exo-glucanases with less reactive bonding module avoiding substrate unspecific adsorption; and (iii) lignin modification to reduce unspecific bond. A hydrophilic rich structure could facilitate the lignin solubilization during pretreatment and enzyme adsorption. These factors also should be evaluated at high solid loading enzymatic hydrolysis, where the enzyme related factors will be more relevant as influent on the EH slowdown.

Conclusion

During the EH of steam pretreated SCG some of the enzymes in the cocktail loose activity, and the substrate enriched in lignin content, significantly increasing the lignin/glucan ratio. The increased lignin/glucan ratio, increased lignin content and changes in the specific surface area (accessibility) of the WIS as EH progressed, were the major substrate changes correlated to the slowdown of glucan to glucose conversion rate during EH of steam pretreated SCG. Overall, in the initial phase of EH the material is rich in internal surface area, until the intermediate phase, changing to predominantly external surface area thereafter. As a result, the chemical nature of the remaining unhydrolyzed material became increasingly recalcitrant due to the greater presence of lignin content. These hindrances ultimately decreased the accessibility (internal and external surface area), explained the characteristic slowdown phases of glucan to glucose conversion rate and incomplete glucan to glucose conversion yield as the reaction progressed.

The material recalcitrance should be overcome to improve the success of 2G bioethanol process. The use of more thermostable enzymes may improve the degradation of more recalcitrant cellulose, and lignin modification to more hydrophilic structure could improve its pretreatment removal and avoid enzyme unspecific adsorption. Otherwise, it could be accepted to stop the reaction at the intermediate phase, using a cost effective enzyme and pretreatment condition and using the EH residue for energy cogeneration.

Acknowledgments

The authors would like to acknowledge the financial support from South Africa Sugarcane Research Institute (SASRI), the National Research Foundation (NRF) and the Technology and Human Resources for Industry Program of the Department of Trade and Industry of South Africa (DTI). Furthermore, the authors gratefully thank CIEMAT– Renewable Energies Department – Biomass unit, especially Ignacio Ballesteros, for pretreating the biomass.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.nbt.2016.01.004.

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