Fed-batch Enzymatic Hydrolysis of Steam-exploded Sugarcane Bagasse

Marlon Gualberto Ganter de Moura,[a] Thiago Alessandre da Silva,[b] Arion Zandoná Filho,[b] Marcos Lúcio Corazza,[b] and Luiz Pereira Ramos [a,b,]*

Statistical design and mathematical modeling were used to investigate the fed-batch enzymatic hydrolysis of steam-exploded sugarcane bagasse (195 °C, 7.5 min). First, a Box-Behnken experimental design was used to evaluate the effect of enzyme loading (8, 24, and 40 FPU g^{-1} glucans of Cellic CTec3®), stirring speed (100, 150, and 200 rpm), and substrate total solids (5, 12.5, and 20 wt%) on the release of glucose equivalents (GlcEq, mostly glucose) after hydrolysis for 48 h in batch mode. A simplified kinetic model was used to fit the experimental data, in which specific activities in Cellic CTec3 were not differentiated, enzyme adsorption was ignored, and end-product inhibition was only attributed to glucose accumulation. The adjusted kinetic model was used to predict the effects of substrate and enzyme intermittent feedings in fed-batch hydrolysis experiments. Compared with batch experiments at 20 wt%, the proposed fed-batch procedure was able to increase GlcEq productivity by nearly 68% using the same enzyme loading, producing substrate hydrolysates containing 91.8 g L^{-1} GlcEq.

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Contact information: a: Graduate Program in Chemistry, Federal University of Paraná (UFPR), P.O. Box 19032, Curitiba, PR - 81531-970, Brazil; b: Graduate Program in Chemical Engineering, Federal University of Paraná (UFPR), P.O. Box 19011, Curitiba, PR - 81531-970, Brazil;
** Corresponding author: luiz.ramos@ufpr.br*

INTRODUCTION

The use of renewable sources for energy generation has gained increasing interest due to the possibility of minimizing the environmental impact of fossil fuels. Renewable liquid fuels such as ethanol are good alternatives for sustainable fuel for ground transportation (Bui *et al*. 2023). However, current ethanol production is based on edible sources such as sugarcane sucrose and corn starch. Production of these first-generation feedstocks in large scale compete with the availability of arable lands and may adversely impact the local biodiversity. To overcome these issues, a promising strategy is to produce ethanol from second-generation feedstocks such as lignocellulosic materials that are derived from non-food crops (*e.g.*, *Miscanthus sinensis*, switchgrass, willow, energy cane, and wood residues, among others) and agro-industrial residues such as wheat straw, corn cobs, rice straw, rice husks, plantain pseudostem, and sugarcane bagasse (Chovau *et al.* 2013; Hernández-Beltrán and Hernández-Escoto 2018; Correa *et al.* 2019; Hernández-Beltrán *et al.* 2021).

Lignocellulosic materials are comprised of highly ordered and tightly packed microfibrillar structures containing cellulose, hemicelluloses, and lignin. Lignin is an aromatic polyether that provides plant cell wall rigidity, improves its mechanical strength, and protects cellulose and hemicelluloses from chemical and biological attack. Cellulose is a natural polysaccharide with a repeating unit comprised of D-glucopyranosyl residues joined together by β(1→4) glycosidic linkages, forming linear chains that interact with one another by a tightly-packed intra- and intermolecular hydrogen bonding network (Ansell and Mwaikambo 2009). Hemicelluloses are heteropolysaccharides containing both hexoses such as mannose, galactose, and glucose, pentoses such as xylose and arabinose, and uronic acids such as D-glucuronic and 4-O-methyl-D-glucuronic acids in their composition. This latter structural macromolecular component contributes significantly to both wood and fiber quality (Gregory and Bolwell 1999; Bajpai 2018).

Several pretreatment techniques target lignin removal to improve the accessibility of cellulose and hemicelluloses to enzymatic hydrolysis (*e.g.*, organosolv, alkali washing, oxidative pretreatments, extraction with ionic liquids or deep eutectic solvents, ammonia fiber explosion), while others focus on hemicellulose removal while coalescing lignin into small fragments, leaving cellulose more exposed to the concerted action of endo and exoglucanases (*e.g.*, hydrothermolysis, steam explosion, dilute acid hydrolysis, acid-catalysed organosolv). Fermentable sugars are released in pretreatment liquors in both monomeric or oligomeric forms, while glucans (mostly cellulose) must be retained in the pretreatment solids in high yields for their subsequent hydrolysis and fermentation to liquid biofuels, platform chemicals and functional biomaterials (Arantes and Saddler 2011; Hernández-Beltrán and Hernández-Escoto 2018; Hernández-Bentrán *et al.* 2021).

Compared with acid hydrolysis, enzymes require milder temperatures and normal pressure conditions to break down plant cell wall polysaccharides into water-soluble sugars. This hydrolysis takes place in a noncorrosive environment, resulting in low operation and maintenance costs. However, the economic feasibility of this process is limited by the high cost of enzymes (Zhu and Zhuang 2012; Hernández-Beltrán and Hernández-Escoto 2018; Hernández-Beltrán *et al.* 2021). A feasible strategy to reduce costs in enzymatic hydrolysis is to perform it at high total insoluble solids (TS), which is reached when biomass concentration is higher than 15 wt% (dry basis) (Fockink *et al.* 2016; Chen and Liu 2017). Higher concentrations of fermentable sugars are achieved in biomass hydrolysates, allowing higher productivities and product recovery after fermentation. For cellulosic ethanol, ethanol concentrations above 4 wt% must be achieved to enable its recovery by distillation, lowering its corresponding energy requirements (Wingren *et al.* 2003; Chen and Liu 2017; de Godoy *et al.* 2019). In addition, water consumption is lowered, and wastewater treatment costs are reduced.

Despite its several advantages, the use of high TS implies in increased system viscosity, poor mass and heat transfers, higher enzyme loading requirements, and special equipment for hydrolysis (Wingren *et al.* 2003; Gao et al. 2014; Fockink *et al.* 2016; Mukasekuru *et al.* 2018). However, these drawbacks can be partly overcome by adopting a fed-batch feeding strategy, whereby hydrolysis would begin at 5-10 wt% TS and end at TS values as high as 30-40 wt%. Problems with high viscosity and heat and mass transfers are reduced compared to loading the substrate all at once in the reaction beginning. This latter practice increases the energy costs for homogenization of the reaction medium and delays the initial stages of substrate saccharification (Zhang and Lynd 2004; Liu *et al.* 2015; Mukasekuru *et al.* 2018; de Godoy *et al.* 2019).

Mathematical modeling and process simulation are important tools to understand enzymatic hydrolysis at high TS and optimize sugar yields. Kadam *et al.* (2004) developed a remarkable kinetic model to simulate the enzymatic hydrolysis of dilute sulfuric acid pretreated corn straw in batch mode using commercial cellulases (CPN commercial cellulase, Iogen Corp., Ottawa, Ontario, Canada). The aim was to formulate and validate an enzymatic hydrolysis kinetic model capable of predicting performance over a range of operating conditions encompassing various background sugar concentrations, reaction temperatures, and mixing regimes using a kinetic model with eighteen adjustable parameters, three overall hydrolysis reactions, and one adsorption reaction based on a Langmuir isotherm. The model assumes no difference in accessibility between amorphous and crystalline cellulose during enzymatic hydrolysis, avoiding the need for measuring two different enzyme adsorption equilibria simultaneously.

Later, Hodge *et al.* (2009) used a modified version of the Kadam's model to develop a feeding strategy for the fed-batch hydrolysis of corn stover after pretreatment with dilute sulfuric acid. Spezyme CP from Genencor International (Palo Alto, CA, USA) was used for this study, and the enzyme loading was 40 mg protein g^{-1} glucans, which corresponded to 10.7 FPU g^{-1} glucans. The model used a mechanistic approach of the hydrolysis kinetics by applying an open-loop fed-batch feeding into a stirred tank reactor (STR) to increase the cumulative level of insoluble solids in the reaction chamber. This approach mimics the hydrolysis performance at high TS while maintaining the operating parameters of reactions at low TS. Morales-Rodriguez *et al.* (2010) used a modeling approach to recalibrate the experimental data of Hodge *et al.* (2009) and applied it to reduce the amount of enzyme required for optimal fed-batch hydrolysis of cellulosic materials.

de Godoy *et al*. (2019) developed a simplified kinetic model for the enzymatic hydrolysis of hydrothermally pretreated sugarcane bagasse (190 °C for 10 min). Hydrolysis was carried out at four different substrate loadings (5, 10, 15 and 20 wt.% TS) using Cellic CTec2 (Novozymes, Bagsværd, Denmark) at 15 FPU g^{-1} TS. The model proposed by de Godoy *et al*. (2019) makes no distinction among different specific activities in cellulase preparations (endoglucanases, exoglucanases, and β-glucosidases), disregards enzyme adsorption on the substrate surface, and hypothesizes that inhibition is only due to glucose accumulation. The latter assumption was based on an earlier observation that cellobiose accumulation is not a limiting step for kinetic modeling because more advanced cellulase preparations contain high β-glucosidase activities that keep cellobiose concentration under non-inhibitory levels (Tervasmäki *et al.* 2017). The simplified kinetic model achieved a high goodness-of-fit for the enzymatic hydrolysis in batch mode, but the proposed kinetic parameters were not predictive for fed-batch hydrolysis experiments. This was solved by training the model with a new set of experimental data based on intermittent substrate feeding, in which enzymes were charged only at the reaction beginning over an initial substrate TS of 10 wt.%. Hence, predictability of the model for fed-batch hydrolysis was only achieved by introducing a new set of kinetic parameters.

In this work, the simplified model developed by de Godoy *et al.* (2019) was applied to predict the enzymatic hydrolysis of steam-exploded sugarcane bagasse at various TS and enzyme loadings. The kinetic model was trained initially with a robust experimental data set from batch experiments, defined in a typical Box-Behnken experimental design, and the resulting parameters were applied to predict the effects of intermittent substrate and enzyme feedings.

EXPERIMENTAL

Materials

Sugarcane bagasse was obtained from a local industrial site (Piracicaba, SP, Brazil). The commercial preparation (Cellic CTec3) used for hydrolysis, which is composed mainly by cellulases, hemicellulases, and other auxiliary enzymes, were kindly donated by Novozymes Latin America (Araucária, PR, Brazil). Reagents, standards, and organic solvents were purchased in analytical and chromatographic and/or spectrometric grade for biomass pretreatment and analytical procedures, respectively. All chemicals were used as received without any further treatment.

Methodology

Pretreatment of sugarcane bagasse

Sugarcane bagasse with a final moisture content of ~50 wt% (wet basis) was pretreated by steam explosion in a 10-L stainless-steel reactor. The reference reaction conditions were obtained from previous optimization studies in which pretreatment was carried out by auto-hydrolysis (no added catalyst) at 195 °C for 7.5 min (Pitarello *et al.* 2016; Fockink *et al.* 2018). Steam explosion was performed in duplicate by loading approximately 500 g bagasse (dry basis) in the preheated reactor chamber. After completing the residence time at the desired temperature, the pretreated material was released from the pressurized reaction vessel into a stainless-steel cyclone and collected from a reservoir placed underneath. The resulting slurry was centrifuged to separate the water-insoluble fibers from the water-soluble fraction or pretreatment liquor (C5 stream). The obtained fibers were suspended in water (5 wt% TS), washed under mechanical agitation for 1 h at room temperature and centrifuged once again, and both C5 stream were combined. The mass recovery yield of both water-soluble and water-insoluble fractions was determined after oven-drying a representative aliquot at 105 °C until constant mass. Water-insoluble steam-exploded fibers were vacuum-sealed in plastic bags and kept under refrigeration until use for both chemical characterization and enzymatic hydrolysis.

Chemical characterization of water-insoluble fractions

Ash, moisture, and total extractives Total extractives, moisture and ash contents were determined according to the following National Renewable Energy Laboratory (NREL) procedures: NREL/TP-510-42622 (Sluiter *et al.* 2008a), NREL/TP-510-42621 (Sluiter *et al.* 2008b), and NREL/TP-510-42619 (Sluiter *et al.* 2008d), respectively. Extractive-free sugarcane bagasse and water-insoluble fractions derived from pretreatment were analyzed for total carbohydrates, acid-insoluble lignin, and acid-soluble lignin following the NREL/TP-510-42618 analytical procedure (Sluiter *et al.* 2008c). Carbohydrates, organic acids, and dehydration by-products were determined by high performance liquid chromatography (Shimadzu LC-20AD HPLC workstation) using an Aminex HPX-87H column (Bio-Rad) that was preceded by a Cation-H^+ guard column. The column was stabilized at 65 °C and eluted with 5 mmol L^{-1} H_2SO_4 at a flow rate of 0.6 mL min^{-1}. Sample injection (15 μL) was carried out using an autosampler (Shimadzu SIL-10AF). Cellobiose, glucose, xylose, arabinose, and formic, acetic and levulinic acids were detected by differential refractometry (Shimadzu RID-10 A), while furfural and 5-hydroxymethylfurfural (5-HMF) were monitored by UV spectrophotometry at 273 nm (Shimadzu SPD-M10AVP). Quantitative analysis was always carried out by external calibration using multicomponent calibration curves based on primary standards.

Enzyme characterization

The total cellulase activity of Cellic CTec3 (Novozymes) was determined according to Ghose (1987). Protein content in this enzyme preparation was based on the BCA (bicinchoninic acid) protein assay (He 2011).

Enzymatic hydrolysis in batch mode

Enzymatic hydrolysis of steam-exploded sugarcane bagasse was initially investigated through a Box-Behnken design (Pasma *et al.* 2013), which involved three factors in three levels for a total of 15 experiments including three replicates at the center point. Enzyme loadings (Cellic CTec3, Novozymes) of 8, 24, and 40 FPU g^{-1} glucans and substrate TS of 5, 12.5 and 20 wt.% were adopted as independent variables as shown in Table 1. Statistical analyses were carried out using the R software package 4.2.2 with the RStudio 576 build graphical interface (R Foundation, Vienna, Austria) to generate models that were able to describe changes in both glucose and xylose release by enzymatic hydrolysis (in g L^{-1}).

Table 1. Variables of the Box-Behnken Experimental Design

Experiment	Substrate Total Solids (wt%)		Enzyme (FPU g^{-1} glucans)		Agitation (rpm)	
	Level	Value	Level	Value	Level	Value
1	-1	5	-1	8	0	150
2	-1	5	1	40	0	150
3	0	12.5	-1	8	0	150
4	1	20	1	40	0	150
5	1	20	-1	8	-1	100
6	0	12.5	1	40	-1	100
7	0	12.5	-1	8	1	200
8	0	12.5	1	40	1	200
9	-1	5	-0	24	-1	100
10	1	20	0	24	-1	100
11	-1	5	0	24	1	200
12	1	20	0	24	1	200
13	0	12.5	0	24	0	150
14	0	12.5	0	24	0	150
15	0	12.5	0	24	0	150

All hydrolysis experiments were carried out at 50 °C and 150 rpm in 250 mL Erlenmeyer flasks using 50 mmol L^{-1} sodium acetate buffer (pH 5.2). The reactions took place in an orbital shaker incubator (Ecotron, Infors HT, Bottmingen, Switzerland). Aliquots were collected after 0, 3, 6, 9, 12, 24, 48, 72, and 96 h of hydrolysis, centrifuged at 7000 rpm for 5 min, filtered through a 0.45 mm syringe filter, and analyzed in the same HPLC system mentioned above. Glucose release (in g L^{-1}) after enzymatic hydrolysis was always expressed as glucose equivalents (GlcEq, Equation 1), in which cellobiose is also accounted as a reaction product.

$$GlcEq = Glc + 1.056 \cdot [Cellobiose] \quad (1)$$

Kinetic modeling of enzymatic hydrolysis in batch mode

The kinetic model developed by de Godoy *et al.* (2019) was used to fit the batch enzymatic hydrolysis kinetic data of steam-exploded sugarcane bagasse. Model implementation was carried out using the Matlab® R2015a software environment, where the *ode23s* subroutine was used to solve the ordinary differential equations (kinetic model), and the kinetic parameters were optimized using the fm*insearch* subroutine by minimizing the objective function of least squares presented in Eq. 2,

$$f = \sum_{i=1}^{NOBS}\left(G_{i,calc} - G_{i,exp}\right)^2 \quad (2)$$

where $G_{i,calc}$ and $G_{i,exp}$ are the glucose concentration at each reaction time and NOBS is the number of observations. Four kinetic datasets were considered for parameter estimation: 8 FPU and 5 wt% TS; 40 FPU and 5 wt% TS; 40 FPU and 20 wt% TS; 24 FPU and 12.5 wt% TS. All other conditions were applied for model validation.

This model was initially applied to fit the hydrolysis data using the parameters proposed by de Godoy *et al.* (2019), in which cellobiose accumulation is not inhibitory, all enzyme activities are concentrated in a single unit (FPU g^{-1}), and the effects of enzyme adsorption are ignored. Based on these assumptions, the inhibition constant was calculated according to Eq. 3,

$$K_1^* = K_{1G}\left[1 - a\frac{(G^n)}{C_T}\right] \quad (3)$$

where K_1^* is the modified product inhibition constant, K_{1G} is the inhibition constant for glucose (g L^{-1}), G is the glucose concentration (in g L^{-1}), a is a parameter with dimension (g $L^{-1})^{(1-n)}$, n is a dimensionless parameter, and C_T is the total glucans (mostly cellulose) added to the reaction medium.

The mass balance was determined by Eqs. 4 and 5,

$$\frac{dC}{dt} = -r \quad (4)$$

$$\frac{dG}{dt} = 1.111r \quad (5)$$

while the reaction rate (r) was given by Eq. 6,

$$r = \frac{k \cdot E \cdot C \cdot R}{K_M\left[1 + \left(\frac{G}{K_1}\right)\right] + C} \quad (6)$$

where k is the kinetic constant, E is the enzyme concentration (in g L^{-1}), C is the glucan concentration (in g L^{-1}), R is the substrate reactivity parameter, and K_M is a parameter for cellulose saturation. In this model, the term R was included to consider that the reaction rate decreases with an increase in substrate conversion (Kadam *et al.* 2004). R was given by Eq. 7,

$$R = \frac{C}{C_0} \quad (7)$$

where C_0 is the initial glucan concentration (in g L^{-1}). Finally, the enzyme concentration (in g L^{-1}), identified as E in Eq. 8, was given by,

$$E = (TS_0 \cdot CT_0) \times \frac{(FPUc \cdot TP_0 \cdot 1000)}{FPU_0} \quad (8)$$

where TS_0 is the substrate total solids (g L^{-1}), CT_0 is the glucan content (in mass fraction), $FPUc$ is the initial cellulase activity charged to the reaction medium (in FPU g^{-1} glucans), TP_0 is the protein content of Cellic CTec3 (mg mL^{-1}), and FPU_0 is total cellulase activity of this same enzyme preparation (in FPU mL^{-1}),

The model verification was made by calculating the root-mean-square deviation (RMSD) as described in Eq. 9.

$$RMSD = \sqrt{\frac{\sum_{i=1}^{NOBS} \left(G_{i,\exp} - G_{i,calc} \right)^2}{NOBS}} \quad (9)$$

Fed-batch enzymatic hydrolysis

After adjustment to the enzymatic hydrolysis of steam-exploded sugarcane bagasse in batch mode, the simplified model developed by de Godoy *et al.* (2019) was applied to simulate fed-batch hydrolysis experiments based on intermittent solids and enzyme feedings. Runs A to E in Table 2 were performed to evaluate the predictability of the model using three different initial TS (12.5, 10 and 20 wt%, dry basis) and two different initial enzyme loadings (12 and 24 FPU g^{-1} glucans). Substrate feeding started after 6 h of hydrolysis. Four loads of bagasse and enzymes were added within time intervals of 3 h. Every load added to the reaction vessel corresponded to the solids and enzyme equivalents for a single batch process at 5 wt% TS. In two experiments, enzymes were added only at the reaction beginning. Different enzyme loadings were tested (6, 9, 12, and 24 FPU g^{-1} glucans) to investigate their effect on hydrolysis yields.

Table 2. Experimental Conditions Used in Fed-Batch Enzymatic Hydrolysis of Steam-Exploded Sugarcane Bagasse

Run	Initial TS[1] (wt%)	Initial EL[2] (FPU g^{-1})	Additional TS		Additional EL[2]	
			t (h)	TS (wt%)	*t* (h)	EL (FPU g^{-1})
A	20	24	None		9	2.4
					12	2.4
					24	2.4
B	12.5	24	None		9	2.4
					12	2.4
					24	2.4
C	10	24	6	5	None	
			9	5		
			12	5		
			24	5		
D	10	12	6	5	9	12
			9	5		
			12	5		
			24	5		
E	10	24	6	5	6	-
			9	5	9	2.4
			12	5	12	2.4
			24	5	24	2.4

[1] TS, substrate total solids;
[2] EL, enzyme loading with activities always expressed in relation to the total substrate glucans

Fed-batch experiments were carried out in an Infors-HT Labfors-5 stirred tank bioreactor (STBR) with a 2.3 L working volume. The vessel was 462 mm in height and had an internal diameter of 464 mm. The STBR had a Hamilton pH sensor for automatic measurement and control. Agitation was provided by a combination between a modified Rushton turbine and a helical propeller. Once the temperature inside the STBR vessel reached 52 °C, the enzymatic cocktail was added, and the reaction system was thoroughly mixed to provide system homogeneity. Aliquots were collected at different reaction times, centrifuged at 7000 rpm for 5 min, filtered through a 0.45 μm syringe filter, and analyzed by HPLC using the same system mentioned above.

RESULTS AND DISCUSSION

Chemical Characterization of Sugarcane Bagasse

The chemical characterization of untreated and pretreated sugarcane bagasse revealed changes in fiber composition in response to high pressure steaming (Table 3).

Table 3. Chemical Composition of Sugarcane Bagasse Before and After Steam Explosion at 195 °C for 7.5 min and Water Washing to Remove the Pretreatment Liquor (C5 stream)

Component (%)	Untreated	Pretreated
Glucans (mostly cellulose)	35.8 ± 0.8	54.5 ± 0.4
Xylans	21.3 ± 0.7	2.7 ± 0.2
Arabinosyl residues[1]	1.5 ± 0.2	bdl[7]
Acetyl groups[1]	4.3 ± 0.1	bdl
Dehydrated hexoses[2]	1.1 ± 0.1	0.6 ± 0.1
Dehydrated pentoses[3]	0.5 ± 0.1	0.4 ± 0.1
Acid-soluble lignin	2.8 ± 0.1	2.4 ± 0.3
Acid-insoluble lignin	22 ± 2	31.2 ± 0.8
Extractives in water	3.7 ± 0.6	nd[8]
Extractives in ethanol	1.9 ± 0.3	nd
Ashes	3.7 ± 0.5	4.8 ± 0.2
Total	98 ± 2	96.6 ± 0.7
Total glucan content[4]	36.9 ± 0.8	55.1 ± 0.4
Total hemicellulose content[5]	27.6 ± 0.7	3.1 ± 0.2
Total lignin content[6]	25 ± 2	33.6 ± 0.4

[1] Present as heteroxylan components (hemicelluloses);
[2] Dehydration by-product from hexoses measured as hydroxymethylfurfural (HMF);
[3] Dehydration by-product from pentoses measured as furfural;
[4] Sum of anhydroglucose and unidentified hexoses that were detected as HMF;
[5] Sum of xylans, arabinosyl residues, acetyl groups and unidentified pentoses detected as furfural;
[6] Sum of acid-soluble and acid-insoluble lignin;
[7] bdl: below the detection limit of the method;
[8] nd: not determined.

Glucans and acid-insoluble lignin were higher in pretreated bagasse mainly due to the removal of hemicelluloses that were susceptible to acid hydrolysis, while acid-soluble lignin remained almost the same. Xylans decreased from 21.3 wt% in untreated bagasse to 2.7 wt% in water-washed, steam-exploded fibers. Also, acetyl groups and arabinose were

not detectable in pretreated materials. Release of acetyl groups from hemicelluloses as acetic acid in the pretreatment liquor was due to the lability their ester bonds to acid hydrolysis (Silveira *et al.* 2015; Pitarello *et al.* 2016). Likewise, the glycosidic bonding of arabinose to the xylan backbone is weak, and free arabinose was partially dehydrated to furfural in the pretreatment liquor due to its low conformational stability (Shrestha *et al.* 2019).

Pitarello *et al.* (2016) pretreated sugarcane bagasse at the same experimental conditions (195 °C for 7.5 min) and found similar results for glucan (47.4%) and lignin (33.5%) contents after pretreatment, in addition to low hemicellulose and acetyl contents below the detection limit of the method. Also, Fockink *et al*. (2017) pretreated sugarcane bagasse at 195 °C for 7.5 min and obtained steam-exploded materials containing 55.4% glucans, 3.8% hemicelluloses, and 33.8% lignin, with acetyl groups being undetectable in their acid hydrolysates.

Rocha *et al*. (2015) pretreated sugarcane bagasse by steam explosion at 190 °C for 15 min and obtained substrates with 58.0, 4.5, and 33.0 wt% glucan, hemicellulose, and total lignin contents, respectively. Although the conditions are slightly different, these results show that there is an order of correspondence among macromolecular components that should be found in pretreated materials after steam explosion. The most abundant material should be glucan, followed by lignin and hemicelluloses (Fockink *et al.* 2018).

Enzyme Activity and Protein Content

Total cellulase activity measured as filter paper units (FPU) by the IUPAC method (Ghose 1987) was 225 FPU mL^{-1} for Cellic CTec3. Considering the density of the enzyme preparation (1.19 g mL^{-1}), this value approached 188 FPU g^{-1}, which was close to the value reported by Sun *et al.* (2015) for Cellic CTec3 (165 FPU g^{-1}) using the same experimental procedure. The protein content based on the bicinchoninic acid (BCA) method was 252.3 mg mL^{-1}. Mok *et al.* (2015) found a protein content of 227 mg mL^{-1} using the $NaBH_4$ coupled ninhydrin-based assay. The modified ninhydrin assay was claimed to be better for protein quantification compared to traditional colorimetric protein assays such as BCA.

Enzymatic Hydrolysis of Steam-exploded SEB in Batch Mode

Enzymatic hydrolysis was performed according to the conditions described in Table 1. Table 4 presents both predicted and observed values for both glucose and xylose release after 48 h of hydrolysis. This reaction time was chosen to facilitate the observation of differences in hydrolysis performance among the test conditions involved in this study.

Glucose concentration was the response variable used for statistical analysis, since it was by far the most predominant fermentable sugar in enzymatic hydrolysates. Also, compared to glucose, cellobiose was always found in small concentrations (below 2 to 3%) throughout the reaction course. Table 5 presents the analysis of variance (ANOVA) of glucose release (g L^{-1}) after 48 h of hydrolysis. Xylose release is also reported in Table 4, but its ANOVA was not performed because xylans were a minor component in steam-exploded sugarcane bagasse.

Based on the F-test, the low tabulated F-value in relation to the calculated F-value for regression indicated a model with good predictability of the experimental data within a 95% confidence level. In addition, the model presented a low lack of fit and the high R^2 value (0.97) indicated that most of the system variance was explained by the model.

Table 4. Predicted and Observed Values for Glucose and Xylose Release (g L^{-1}) after 48 h of Hydrolysis

Run	Glucose (g L^{-1})		Xylose (g L^{-1})	
	Observed	Predicted	Observed	Predicted
1	2.9	3.3	0.2	0.4
2	32.1	26.8	0.8	0.8
3	51.1	64.4	2.7	2.8
4	76.4	88.9	2.9	2.8
5	41.4	45.3	2.3	2.3
6	41.9	45.3	1.3	1.6
7	36.3	47.3	2.3	2.3
8	68.9	76.0	2.8	2.8
9	28.6	32.0	1.0	0.9
10	72.1	82.4	3.2	3.3
11	29.0	30.3	1.2	1.4
12	84.3	92.4	3.2	3.4
13 (CP)	54.5	62.7	2.3	2.4
14 (CP)	56.3	62.3	2.3	2.3
15 (CP)	57.7	65.5	2.4	2.3

CP, Center Point of the experimental design.

Table 5. Analysis of Variance (ANOVA) for Glucose Release (g L^{-1}) after 48 h of Hydrolysis

Source	SS	DF	MS	F	F_{tab}
Regression	6499.9	9	722.2	46.1	4.8
Residues	78.4	5	15.7		
Lack of fit	73.0	3	24.3	9.2	19.2
Pure error	5.3	2	2.7		
R^2 = 0.97; %Var = 0.99					

SS, sum of squares; DL, degrees of freedom; MS, mean of squares; F, calculated Fischer distribution; F_{tab}, tabulated Fischer distribution.

Equation 10 represents the mathematical model that provided the best fit for glucose release (g L^{-1}) after 48 h of hydrolysis, where *a*, *b*, and *c* are the contributions of substrate TS, enzyme loading, and stirring speed, respectively.

$$R_{Glc} = 56.14 + 10.94a + 23.90b + 4.32c - 1.03ab + 8.02ac + 2.29bc - 10.92a^2 - 4.56b^2 + 1.89c^2 \quad (10)$$

Data indicated that the highest glucose release was obtained at the highest substrate TS and stirring speed, using intermediate to high enzyme loadings (experiments 4 and 12). For instance, experiment 12 (20 wt% TS, 200 rpm and 24 FPU g^{-1} glucans) provided the highest glucose concentration of 90 g L^{-1} after 48 h of hydrolysis. According to Galbe and Zacchi (2007), this concentration is enough to make ethanol distillation economically feasible after fermentation.

Modeling of batch enzymatic hydrolysis

Figure 1 shows the experimental data obtained in this study for steam-exploded sugarcane bagasse at 5, 12.5 and 20 wt% TS using 8 (A), 24 (B), and 40 (C) FPU g^{-1} glucans. Then, the experimental data are compared to the values calculated using the kinetic model proposed by de Godoy *et al.* (2019), with parameters adjusted for the hydrolysis of hydrothermally pretreated sugarcane bagasse (dashed lines), and the kinetic model derived from a new set of parameters that were based on four kinetic data obtained in this work (solid lines). The other conditions listed in Table 1 were used for the model fitting validation and the resulting kinetic curves are expressed in relation to the release of glucose equivalents in g L^{-1}, despite that low cellobiose concentrations (below 2-3% of glucose release) were found in all reaction aliquots.

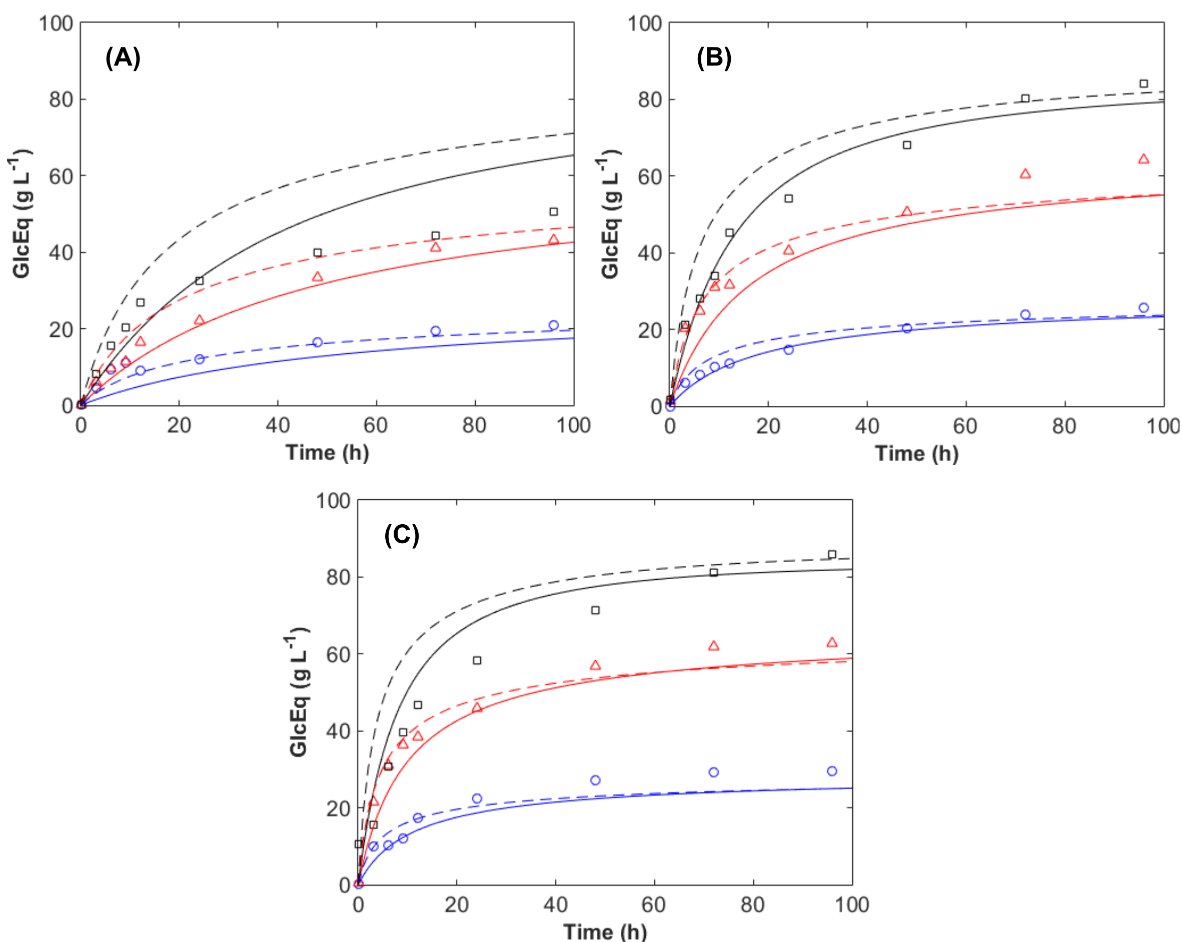

Fig. 1. Enzymatic hydrolysis of steam-exploded sugarcane bagasse in batch mode using 8 (A), 24 (B) and 40 (C) FPU g^{-1} glucans. Symbols are experimental values for 5% TS (blue circles), 12.5% TS (red triangles) and 20% TS (black squares). Dashed lines were calculated based on the Godoy *et al.* [8] kinetic model parameters, and solid lines were calculated with the kinetic parameters adjusted in this work (Table 6).

The kinetic parameters proposed by de Godoy *et al.* (2019) were not able to adequately fit the enzymatic hydrolysis of steam-exploded sugarcane bagasse in batch mode. However, this earlier study was based on a different enzyme (Cellic CTec 2) acting on a pretreated substrate with different chemical composition. Cellic CTec2 has a total

cellulase activity of 112.6 FPU mL^{-1} and a protein content and 74.6 mg mL^{-1}, and these are lower than those observed for Cellic CTec3 (Sun *et al.* 2015). Also, Cellic CTec3 may be more resilient to end-product inhibition than Cellic CTec2. Also, compared to the hydrothermal pretreatment, steam explosion does not deliver the same thermochemical effect on the structure and chemical composition of sugarcane bagasse.

Table 6 shows the new set of modeling parameters that were determined for steam-exploded sugarcane bagasse based on the simplified kinetic model described above. Also, the kinetic parameters that were adjusted for the hydrolysis of hydrothermally pretreated sugarcane bagasse are given for comparison (de Godoy *et al.*, 2019). Although both kinetic studies were based on the same feedstock, differences were observed in the kinetic parameters because hydrolyses were carried out under different conditions using different substrates and enzyme preparations.

Table 6. Original Modeling Parameters Proposed by de Godoy *et al.* (2019) and those Adjusted in this Study for Steam-exploded Sugarcane Bagasse

Parameter	Estimated Values	
	This study	de Godoy *et al.* (2019)
k (h^{-1})	1.7974	4.367
K_M (g L^{-1})	1.6663 x 10^{-2}	0.018
$K_{I,G}$ (g L^{-1})	1.3575 x 10^{-2}	8.300 x 10^{-3}
a	1.8825 x 10^{-3}	0.100
n	2.4717	1.557
$RMSD$ (g L^{-1})	5.8	7.9

k, Reaction constant (h^{-1}); K_M, glucan saturation constant; $K_{I,G}$, glucose inhibition constant; a, parameter with dimensions (g L^{-1})$^{(1-n)}$; n, dimensionless parameter.

The greatest adjustment was made in the dimensionless parameter n. According to Equation 4, this parameter influences the inhibition constant exponentially during mathematical modeling. Thus, it seems that the importance of end-product inhibition, mostly by glucose since cellobiose was always a minor component in substrate hydrolysates, was underestimated by de Godoy *et al*. (2019). Hence, the consideration of a higher value for the parameter n was crucial to adjust the model, decreasing the deviation between both experimental and simulated data. Also, the goodness of fit, evaluated by the root mean square deviation (RMSD), was smaller for the new parameters compared to those presented by de Godoy *et al*. (2019).

Fed-batch Enzymatic Hydrolysis of Steam-exploded SCB

After building, adjusting, and validating the model for the batch enzymatic hydrolysis of steam-exploded sugarcane bagasse, this tool was applied to predict the behavior of fed-batch hydrolysis using the same substrate and enzyme preparation. Experiments were carried out with intermittent substrate and enzyme feedings as shown in Table 2, while the resulting release of glucose equivalents after 96 h of hydrolysis and the corresponding fed-batch kinetic curves are presented in Figs. 2 and 3, respectively. Glucose and cellobiose concentrations were monitored by HPLC, and the results incorporate the dilution caused by adding moist substrate (35 wt% TS) and enzymes at every feeding stage.

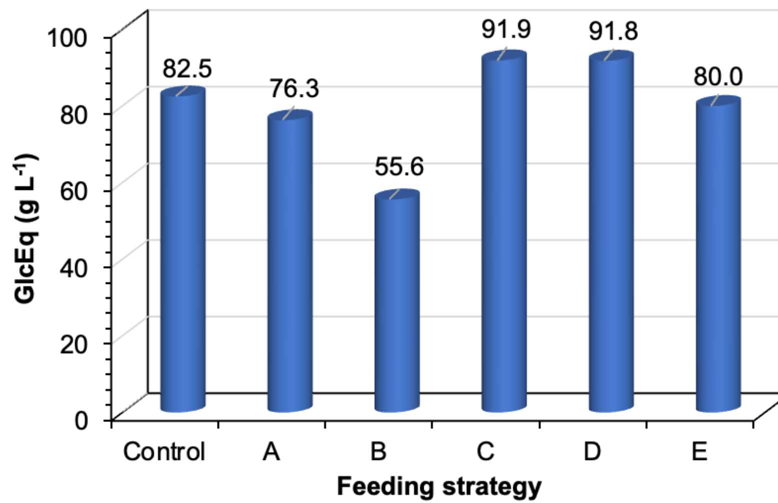

Fig. 2. Final concentration of glucose equivalents (GlcEq, mostly glucose) after enzymatic hydrolysis of steam-exploded sugarcane bagasse 96 h using the feeding strategies described in Table 2

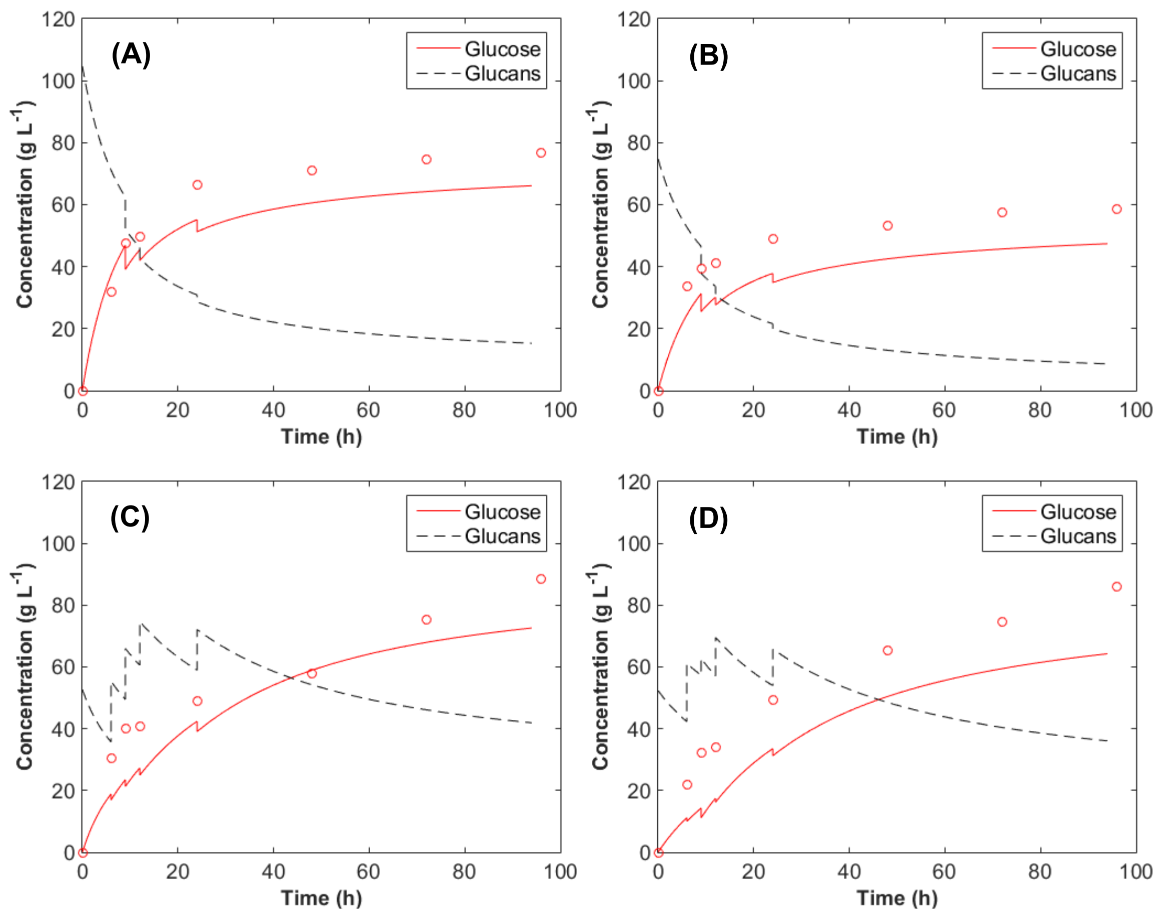

Fig. 3 (A-D). Predictability of the kinetic model for fed-batch enzymatic hydrolysis of steam-exploded sugarcane bagasse at different reaction conditions described in Table 2 (A to E). Markers indicate the experimental data and lines represent the calculated values.

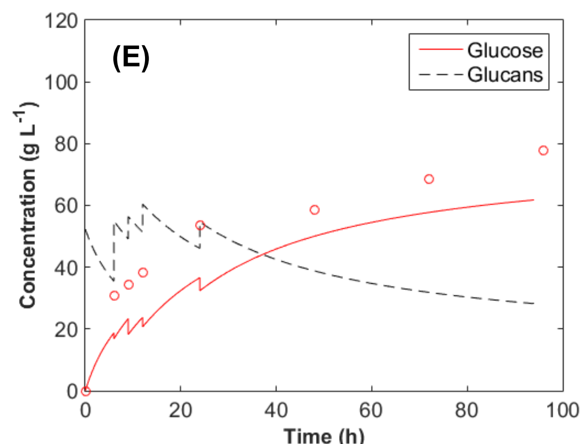

Fig. 3 (E). Predictability of the kinetic model for fed-batch enzymatic hydrolysis of steam-exploded sugarcane bagasse at different reaction conditions described in Table 2 (A to E). Markers indicate the experimental data and lines represent the calculated values.

In reactions A and B, substrate TS was only added at the beginning of the reaction, but enzyme addition was done stepwise: 24 FPU g^{-1} glucans at time zero (same as in the batch experiment at 20 wt% TS) plus supplementations of 10% in 9, 12, and 24 h. However, initial substrate TS was 20 wt% in reaction A, and 12.5 wt% in reaction B. Despite the high initial substrate TS of reaction A, glucose release was only 76.31 g L^{-1} at the end of hydrolysis. Therefore, compared to reaction B, a 60% higher substrate availability in reaction A resulted in an increase of only 37% in glucose release. This loss in reaction efficiency can be attributed to the solids effect, which is characterized by poor mass and/or energy transfer at high TS as already reported elsewhere (Liu *et al.* 2015; Chen and Liu 2017). Also, the goodness-of-fit of the mathematical model was best for reaction A, showing the adequacy of the proposed parameters to predict hydrolysis performance at high TS. It is worth noticing that small disturbances in the dotted line describing glucan availability in the reaction environment were due to the relatively small dilution factor of adding new batches of the enzyme dilution stepwise. Compared to the batch experiment at 20 wt% TS in Fig. 1, a slight increase in glucose yield was observed before 24 h of hydrolysis but, after that, enzyme performance remained very similar to the control experiment. Therefore, hydrolysis was not boosted by adding 10% more enzymes in three consecutive steps probably due to impact of end-product inhibition at high TS conditions, along with other inhibitory effects that are typical of highly lignified substrates such as irreversible and/or unproductive adsorption onto lignin fragments that build up in the hydrolysis environment.

Reactions C and D were performed using the same fed-batch approach to reach 30 wt% TS at the end, but the enzyme loading (24 FPU g^{-1} glucans) in C was entirely added at the reaction beginning, while in D enzymes were loaded in two stages (50% at time zero and 50% at 9 h). Enzyme addition in two stages was carried out to evaluate the possible loss of catalytic activity due to unproductive adsorption, thermal inactivation, and sheering along the reaction course. Release of glucose equivalents reached 30 g L^{-1} in 6 h when enzymes were only added in the reaction beginning, while for the other experiment, only 20 g L^{-1} was observed at the same reaction time. Despite this difference, glucan (mostly cellulose) consumption after 96 h of hydrolysis was similar in both experiments. Also, the model was more predictive for C compared to D and adding enzymes in two stages

impaired no difference to glucose concentration after 96 h of hydrolysis. Considering that the batch reaction at 20 wt% TS using the same enzyme loading released 82.5 g L^{-1} GlcEq for a total reaction volume of 900 mL, the proposed fed-batch procedure was able to increase glucose productivity by nearly 68% (82.5 g L^{-1} in 1000 mL for the batch compared to 91.8 g L^{-1} in 1272 mL for the fed-batch experiment). In this case, as mentioned above, the 27.2% increase in reaction volume was proportional to the moisture content of the pretreated substrate added to reach 35 wt.% TS in fed-batch mode.

Reaction E was performed by adding both substrate TS and enzymes at different reaction times, but the enzyme loading at the beginning was already 24 FPU g^{-1} glucans to complete 31.2 FPU g^{-1} glucans at 24 h of hydrolysis. During the first 24 h, this fed-batch strategy was useful to increase hydrolysis yields by roughly 10% compared to reaction C, in which 24 FPU g^{-1} glucans were added only at the beginning. This was so because the enzyme loading was increased by 10% in three consecutive steps, but after 24 h, the corresponding effect on hydrolysis performance was marginal, probably due to end-product inhibition. Also, the model predictivity in this case was close to that of reaction D.

CONCLUSIONS

1. A simplified mathematical model was adapted to describe the enzymatic hydrolysis of steam-exploded sugarcane bagasse in batch mode at various substrate total insoluble solids (TS) and enzyme loadings.
2. The model was not only predictive for batch experiments, but also for hydrolysis using intermittent substrate and enzyme feedings. This is the first time that events of a typical fed-batch hydrolysis experiments were described using the process parameters of a model developed for batch hydrolysis.
3. The model was not able to fit the experimental data, and this was attributed to inhibitory effects that are typical of enzymatic hydrolysis of highly lignified pretreated materials at high substrate TS.
4. The model was able predict changes in glucose release due to changes in substrate concentration and enzyme loading, but the latter seemed to have a marginal effect on reaction performance.
5. Despite lignin build-up in the reaction environment, fed-batch hydrolysis was able to boost glucose release from steam-exploded cane bagasse by 68%, reaching substrate total solids up to 30 wt% without impairing difficulties in substrate stirring during the entire reaction course.

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