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KINETIC STUDY OF THE ENZYMATIC HYDROLYSIS OF SUGARCANE BAGASSE

M. L. Carvalho[1], R. Sousa Jr.[1*], U. F. Rodríguez-Zúñiga[1], C. A. G. Suarez[1], D. S. Rodrigues[2], R. C. Giordano[1] and R. L. C. Giordano[1]

[1]Department of Chemical Engineering, Federal University of São Carlos,
Phone: + (55) (16) 3351 8713, Fax: + (55) (16) 3351-8266, Rod. Washington Luís,
Km 235, CP 676, CEP: 13565-905, São Carlos - SP, Brazil.
E-mail: ruy@ufscar.br
[2]EMBRAPA Agroenergy, Parque Estação Biológica, PqEB s/n,
CEP: 70770-901, Brasília - DF, Brasil.

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Abstract - This work presents a kinetic study of the enzymatic hydrolysis of three cellulosic substrates: filter paper (FP), used as a low recalcitrance substrate model; steam exploded sugarcane bagasse (SB); and weak acid pretreated SB (1:20 dry bagasse:H_2SO_4 solution 1% w/w), the last two delignified with 4% NaOH (w/w). The influence of substrate concentration was assessed in hydrolysis experiments in a shaker, using Accellerase® 1500, at pH 4.8, in 50 mM sodium citrate buffer. Cellulose loads ($weight_{substrate}/weight_{total}$) were changed between 0.5%-13% (for FP) and 0.99%-9.09% (for SB). For FP and low loads of steam exploded SB, it was possible to fit pseudo-homogeneous Michaelis-Menten models (with inhibition). For FP and higher loads of steam exploded SB, modified Michaelis-Menten models were fitted. Besides, it was observed that, after retuning of the model parameters, it is possible to apply a model fitted for one situation to a different case. Chrastil models were also fitted and they were the only feasible approach for the highly recalcitrant acid-treated SB.
Keywords: Cellulose enzymatic hydrolysis; Sugarcane bagasse; Kinetic study.

INTRODUCTION

In Brazil, the large production of ethanol from sugarcane juice turns bagasse into an attractive feedstock for second generation ethanol. Cellulose hydrolysis, acid or enzymatic, yields glucose, which is further fermented to provide ethanol, but enzymatic hydrolysis can be operated under milder conditions, avoiding formation of byproducts that may inhibit the fermentation, such as hydroxymethylfurfural (Granda *et al*., 2007). Modeling the enzymatic hydrolysis of lignocellulosic materials is probably one of the most challenging subjects in bioreactor engineering science. The difficulties of this problem may be grouped into three classes: the complexity of the substrate, of the action of the enzymes, and of the enzymes-substrate interaction.

The interactions involving the three biopolymers that comprise the cell tissue of biomass, i.e., lignin, hemicellulose and cellulose (Mansfield *et al*., 1999) are responsible for its recalcitrance against the action of degrading enzymes. Thus, pretreatment stages are required in industry to make the substrate more accessible to cellulolytic enzymes (Lynd *et al*., 2002). These processes add more degrees of freedom to the problem, since the pretreatment will impact the accessibility of the enzymes.

A second sort of difficulties for the modeler comes from the multiple actions of the different enzymes. The pool of commercial enzymes generally has endoglucanases, which hydrolyze the amorphous regions of cellulose and open the way for exogluca-

*To whom correspondence should be addressed

nases (cellobiohydrolases I and II), which attack the reducing and non-reducing ends of crystalline cellulose, respectively; cellobiases (beta-glucosidases) hydrolyze cellobiose (and probably cellotriose and other small celluolygomers) that result from the previous reactions, delivering glucose (Walker and Wilson, 1991; Woodward, 1991).

The third difficulty is the enzyme-substrate interaction, including spatial hindrances. Problems like steric obstruction (Rothschild, 1998), jamming of enzymes (Willams, 2005), and unproductive adsorption are in focus here. And this problem becomes more complex when one recalls that the substrate is changing dynamically as the reaction advances.

It becomes evident from the experimental information that the demands on a detailed phenomenological model for the estimation of its parameters would be overwhelming (Sousa Jr *et al*., 2011). Such an effort probably would not be justified to simulate a process that yields a low-cost commodity, such as a liquid biofuel.

Zhang and Lynd (2004) grouped kinetic models for the enzymatic hydrolysis of biomass based on the level of detail of their description of the substrate and/or of the activities of the different enzymes that are acting. According to these authors, models can be classified as nonmechanistic, semimechanistic, functionally based and structurally based.

Within this framework, this paper intends to assess how simple semimechanistic models (most probably, the class of model that will be used for the design and optimization of an industrial reactor nowadays) conform to experimental data of enzymatic hydrolysis of different cellulosic substrates. Three kinetic models were used in this assessment, as follows:

The most common semimechanistic approach is based on pseudo-homogeneous Michaelis-Menten models, i.e., the substrate, despite actually being a solid, is treated as a soluble reactant, characterized by its concentration. The enzyme is soluble as well, and the Brigs-Haldane steady-state assumption is adopted. Bezerra and Dias (2004) investigated the kinetics of one exoglucanase (Cel7A, from *Trichoderma reesei*) in the presence of cellobiose, with different enzyme/substrate (Avicel) ratios. It was found that the cellulose hydrolysis velocity, V, followed a model that takes into account competitive inhibition by cellobiose (as in Equation (1)).

$$V = \frac{V_{max} S}{K_m[1 + (P / K_{ic})] + S} \tag{1}$$

V_{max} is the maximal velocity = $k.E_0$, S is cellulose (transformed in potential product concentration), P is the product; K_m is the Michaelis-Menten constant and K_{ic} is a (competitive) product inhibition constant. The authors studied the inhibition of purified Cel7A by cellobiose when hydrolyzing crystalline cellulose; thus, their product was this disaccharide.

A second class of semimechanistic rate equations pictures a system closer to the real one, considering that the substrate is in solid form and that the soluble enzyme has to adsorb to (and desorb from) it. Carrillo *et al*. (2005) studied the kinetics of the hydrolysis of pretreated (with sodium hydroxide) wheat straw using different concentrations of a commercial cellulase (Novozymes A/S). Initial velocities of a rate equation derived from a Michaelis-Menten mechanism were measured, but with solid substrate and soluble enzyme. The initial hydrolysis velocity can be expressed as a function of the initial enzyme concentration (as in Equation (2)).

$$V_0 = \frac{V_{e\,max} E_0}{K_m + E_0} \tag{2}$$

V_0 is the initial hydrolysis velocity, $V_{emax} = k.S_0$ is the maximal velocity for the initial concentration of adsorption sites on the substrate, S_0, and K_m is the corresponding half-saturation constant. In this work, Equation (2) will be used not only for initial rates but in batch experiments as well as assuming competitive inhibition (of beta-glucosidase) by glucose (Equation (2a)). In this case, the concentration of adsorption sites on the solid, S, changes with time ($S=S_0 - P$). In addition, the hypothesis assumed here is that the total enzyme concentration is equal to E_0 ($E_{ads} <<< E_0$).

$$V = \frac{k.E_0.S}{K_m.(1 + \frac{I}{Ki}) + E_0} \tag{2a}$$

Finally, Chrastil´s approach (1988 and 1988b, as in Equation (3)) was assessed in this work. All time constants for the rate of product formation are ranked, taking into account that, in a heterogeneous system, the time curves depend strongly on the heterogeneous rate-limiting phenomena that are present in the substrate-enzyme system, including enzyme diffusion and adsorption.

$$P = P_\infty [1 - \exp(-k' E_0 t)]^n \tag{3}$$

P and P_∞ are the products which diffuse at time t and at equilibrium, respectively, k' is a rate constant proportional to the diffusion coefficient, and n is a

parameter related to structural diffusion-adsorption resistance, dependent on the steric structure of the system. When enzyme diffusion-adsorption is not the rate-limiting step, n tends to 1. If the reaction rate is strongly limited by the diffusion-adsorption of the enzyme, n decreases (becoming less than 0.6, Carrillo, 2005).

OBJECTIVE

Considering this scenario, the objective of this work was to assess the kinetics of the enzymatic hydrolysis of cellulose for three different substrates: filter paper (FP), used as a model of a de-lignified substrate with low recalcitrance; steam-exploded sugarcane bagasse (SB); and acid-treated SB, the last two treated with 4% NaOH. The underlying idea was to verify whether the three semi-mechanistic models described beforehand could represent the hydrolysis for these different substrates.

MATERIALS AND METHODS

Enzymes and Substrates

The commercial complex of cellulases Accellerase® 1500, from *Trichoderma reesei*, donated by Genencor® (Palo Alto, CA), was used. According to the manufacturer, this complex contains multiple enzymatic activities, different exoglucanases and endoglucanases, beta-glucosidase and hemicellulase. This complex operates between 50- 65 °C and pH 4.0- 5.0. However, in long term assays, 50 °C is a better choice to minimize thermal inactivation. To determine the overall activity of the complex, the method of total reducing sugars (TRS) was applied, making use of 3,5-dinitrosalicylic acid (DNS) and Whatman filter paper No. 1 (Miller, 1959; Ghose, 1987; Adney and Baker, 1996).

Three cellulosic materials were used in the experiments of hydrolysis: qualitative filter paper (Satelit, Brazil) and sugarcane bagasse, *in natura* (*Saccharum officinarum*) and steam exploded, donated by the Center for Sugarcane Technology (CTC) – Piracicaba/SP.

Pretreatment Procedures

Hemicellulose was removed from *in natura* sugarcane bagasse using a ratio of 1:20 (dry bagasse:H_2SO_4 solution 1% w/w); the suspension was autoclaved for 30 min, at 1 atm and 121 °C, then washed with water until neutralization. Next, delignification was performed using a ratio of 1:20 (dry bagasse:NaOH solution 4% w/w), in autoclave for 30 min, at 1 atm and 121 °C. After the removal of lignin, the remaining solid was thoroughly washed with hot water, the last wash being with 50 mM citrate buffer, pH 4.8.

Steam exploded bagasse was washed using a ratio of 1:20 (dry bagasse:boiling H_2O). Delignification followed the procedure used for *in natura* bagasse.

Samples of *in natura* pretreated bagasse (with acid and NaOH) and steam-exploded pretreated bagasse (with NaOH) were characterized for chemical composition according to the analytical methodology for sugarcane bagasse developed by Rocha *et al*. (1997) and validated by Gouveia *et al*. (2009).

Filter Paper Studies

Initially, the influence of agitation on the hydrolysis and the influence of substrate concentration, using qualitative filter paper, were studied. To study the influence of agitation, hydrolysis experiments were performed in 250 mL Erlenmeyer flasks, with a total reaction volume of 20 mL; 5 $FPU.g^{-1}_{cellulose}$ of Accellerase® 1500 were added, corresponding to 0.5 $FPU.mL^{-1}_{solution}$, at pH 4.8, in 50 mM sodium citrate buffer. The experiments were run for about 30 minutes using a refrigerated incubator (Marconi MA-832) with a range of agitation from 0 to 300 rpm, at 50 °C, setting the substrate concentration at 9.1% (w/w), corresponding to a potential of 111.11 $g_{RS}.L^{-1}_{solution}$. To determine the effect of substrate concentration, hydrolysis experiments were performed in 250 mL Erlenmeyer flasks, in a total reaction volume of 20 mL, with 0.5 $FPU.mL^{-1}_{solution}$, at pH 4.8, in 50 mM sodium citrate buffer. The experiments were run using a refrigerated incubator (Marconi MA-832) with agitation set at 250 rpm, at 50 °C, varying the substrate concentration in a range from 0.5 to 13% (w/w), corresponding to a potential of 5.55 to 166.67 $g_{RS}.L^{-1}_{solution}$.

Enzymatic Hydrolysis of Sugarcane Bagasses

To study the influence of substrate concentration for the exploded sugarcane bagasse treated with 4% NaOH, hydrolysis experiments were performed in 250 ml Erlenmeyer flasks, with a liquid volume of 30 mL, by adding 0.85 $FPU.mL^{-1}_{solution}$, at pH 4.8, in 50 mM sodium citrate buffer, with the stirring speed set at 250 rpm (refrigerated incubator Marconi MA-832) and the percentage change from 0.99% to 9.09% ($w_{cellulose}$ / w_{total}), corresponding to potential glucose concentrations from 11.11 to 111.11 $g_{Glucose}.L^{-1}_{solution}$. Experiments were also conducted in 250 ml

Erlenmeyer flasks, with a liquid volume of 30 mL, in 50 mM sodium citrate buffer, pH 4.8, with the stirring speed set at 250 rpm (refrigerated incubator Marconi MA-832), varying the quantity of enzyme in a range from 6 to 90 $FPU.g^{-1}_{cellulose}$ of Accellerase® 1500 (corresponding to 3.9 to 60 $g_{enzyme}.L^{-1}_{solution}$) and keeping the substrate constant at 6.54% ($w_{cellulose}$ / w_{total}, corresponding to a potential glucose concentration of 77.77 $g_{Glucose}.L^{-1}_{solution}$).

For the bagasse treated with 1% H_2SO_4 and delignified with NaOH 4%, the last procedure for the exploded bagasse (variation in the amount of enzyme, keeping constant the substrate at 6.54% ($w_{cellulose}$ / w_{total})) was repeated. Also, an assay was performed by setting the substrate at 2.9% ($w_{cellulose}$ / w_{total}), corresponding to a potential glucose concentration of 33.33 $g_{Glucose}.L^{-1}_{solution}$, and adding 0.85 $FPU.mL^{-1}_{solution}$.

Reducing Sugars and Glucose Quantification

For the experiments using the filter paper as substrate, the method of total reducing sugars was used (DNS). For the determination of glucose concentration from samples taken during the hydrolysis assays, an enzymatic kit (GOD-PAP) containing a reagent ready for use and also a standard solution of glucose, 100 $mg.dL^{-1}$, were used.

Cellobiose Quantification

Cellobiose was determined by High Performance Liquid Chromatography (HPLC) using a Shimadzu SCL-10A chromatograph with a Shimadzu RID-10A refractive index detector. The separation column used was Aminex HPX-87H (300 x 7.8 mm, Bio-Rad) with 0.005 mol L^{-1} H_2SO_4 as mobile phase, at a flow rate of 0.6 mL min^{-1} and oven temperature of 45 °C. For the construction of calibration curves for the sugars, pattern samples were injected. Samples of the liquid fraction were filtered through a Sep-Pak C-18 (Waters) solid phase extraction cartridge before being injected to retain lignin and degradation products.

Mathematical Modeling

For kinetic modeling based on the experimental results, three different softwares were used: Origin Pro 7, in-house software written in Fortran and in-house software programmed in Matlab. Origin Pro 7 was used to fit experimental data to the models of Chrastil (1988 and 1988b), Lineweaver-Burk (LB) and Michaelis-Menten (MM), using the curve-fitting tool (Advanced fitting tool). To fit the inhibition parameter for Michaelis-Menten type models, the in-house Fortran software was used, employing the classical Marquardt algorithm. It must be mentioned that, because of the high activity of β-glucosidase in the enzymatic complex used in this work, only inhibition (competitive) by glucose was considered. The small accumulation of cellobiose in the medium, compared to glucose, was confirmed by High Performance Liquid Chromatography, as exemplified in Figure 1 (more detailed MM type models, one should however also consider the inclusion of inhibition by cellobiose).

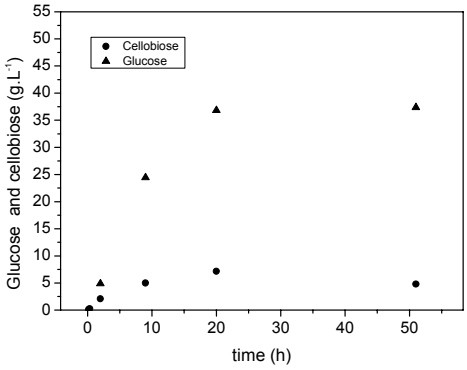


Figure 1: Time evolution of cellobiose (and glucose) concentration in a hydrolysis assay (for pretreated steam exploded bagasse) at 50 °C, pH 4.8, substrate load of 6.54% ($w_{cellulose}$ / w_{total}) in an Erlenmeyer flask, 1.4 $FPU.ml^{-1}_{solution}$ of Accellerase® 1500.

Additional fitting procedures (and statistical analyses) were also conducted for the parameters of the Michaelis-Menten model and for its inhibition constant by using an in-house global search algorithm, Simulated Annealing (Kirkpatrick *et al*., 1983), implemented in Matlab.

RESULTS AND DISCUSSION

To determine the chemical composition, the treated bagasses were hydrolyzed in concentrated acid. The values obtained in this characterization are presented in Table 1.

The results presented in Table 1 indicate that treatment of bagasse with steam explosion followed by delignification with 4% NaOH was able to remove all detectable hemicellulose. In addition, a low lignin content was observed. Pretreatment of bagasse with 1% H_2SO_4 followed by 4% NaOH delignification resulted in a material with residual hemicellulose and 17% of lignin. After treatment, the cellulose concentration increased in both cases.

Table 1: Characterization of the different pretreated sugarcane bagasses.

Samples	Cellulose %	Lignin %	Hemicellulose %	Ashes %
***In natura* Sugarcane bagasse**	38.80	21.70	29.40	5.10
Steam exploded sugarcane bagasse, treated with 4 % NaOH	88.20	5.37	-	0.70
Acid treated sugarcane bagasse, treated with 4 % NaOH	73.30	17.00	7.00	2.00

A careful study of the kinetics of enzymatic hydrolysis of cellulose must be preceded by the determination of the agitation speed appropriate to the system studied, i.e., the agitation speed at which the system is not limited by external diffusion. It must also be considered that excessive agitation can deactivate enzymes and reduce the conversion efficiency (Ingesson *et al*., 2001).

Figure 2 shows the initial hydrolysis reaction rates for 9.1% (w/w) of substrate at different agitations. The initial rates of hydrolysis ranged from 0.044-0.065 $g_{RS}.L^{-1}_{solution}.\ min^{-1}$.

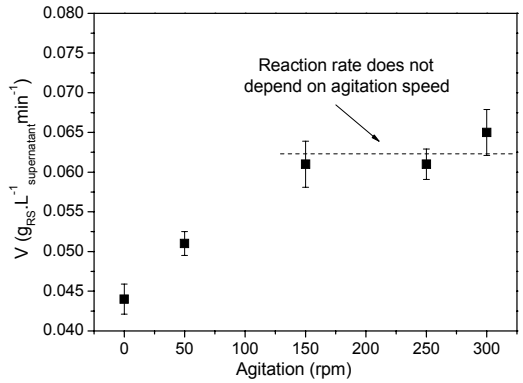


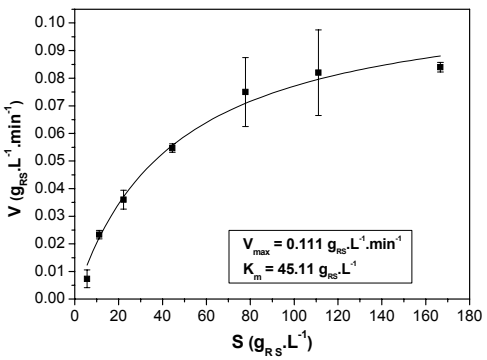
Figure 2: Initial rates for different agitation speeds for 9.1% of substrate (w_{paper} / w_{total}) at 50 °C, pH 4.8, enzyme Accellerase® 1500, 5 FPU / $g_{cellulose}$ (0.5 $FPU.mL^{-1}_{solution}$).

Through the tests using filter paper it was found that there is a significant variation in the rate of hydrolysis with increasing agitation up to 150 rpm, when it becomes approximately invariant (up to 300 rpm). Therefore 250 rpm was chosen as the stirring speed.

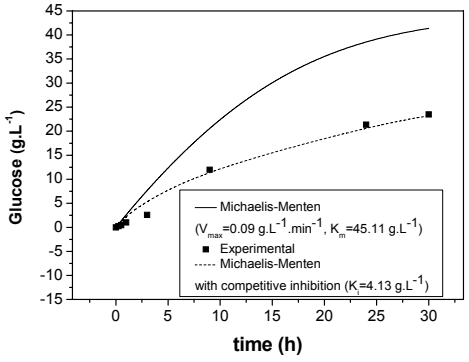
The parameter values obtained from a Lineweaver-Burk (LB) plot (V_{max} = 0.105 $g_{RS}.L^{-1}_{solution}.min^{-1}$ and K_m = 39.78 $g_{RS}.L^{-1}_{solution}$), not shown here, were used as initial estimates when the Michaelis-Menten model (Figure 3(a)) was fitted to the initial rates of hydrolysis. The parameter values obtained for the MM model with FP were: V_{max} = 0.111 $g_{RS}.L^{-1}_{solution}.min^{-1}$ and K_m = 45.11 $g_{RS}.L^{-1}_{solution}$. By using data for potential glucose instead of reducing sugars, the parameter values were: V_{max} = 0.09 $g_{Glucose}.L^{-1}_{solution}.min^{-1}$ and K_m = 45.11 $g_{Glucose}.L^{-1}_{solution}$ (in what follows, concentrations and velocities will be expressed in terms of glucose).

It can be observed that the MM model (pseudo-homogeneous) fits well the experimental data when no product is present. The effect of inhibition by glucose was assessed from long-term hydrolysis assays. The pool of enzymes (endoglucanases, exoglucanases and cellobiases) is doped with betaglucosidase, and measured concentrations of cellobiose were small throughout the reaction course. The formalism of Equation (1), with competitive inhibition by the product, was applied here using glucose as the inhibitory product. Figure 3(b) clearly shows the importance of inhibition. The inhibition parameter was evaluated as K_i = 4.13 ± 0.59 $g.L^{-1}$ (for an initial substrate load of 3.85%, w_{paper} / w_{total}).

Figure 4 summarizes the results obtained for all the cases presented in this paper.



(a)



(b)

Figure 3: Michaelis Menten (pseudo-homogeneous) model for the enzymatic hydrolysis of filter paper (FP) at 50 °C, pH 4.8, enzyme Accellerase® 1500 ($0.5FPU.mL^{-1}_{solution}$): (a) initial rates with substrate loads from 0.5 to 13% (w_{paper} / w_{total}); (b) long-term hydrolysis assay (with accumulation of the inhibitory product), models with and without the inhibition effect.

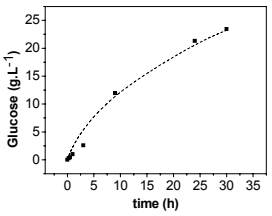
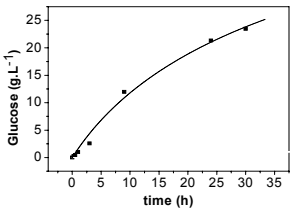
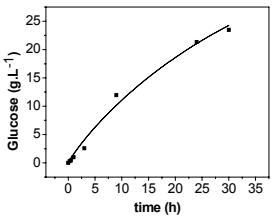
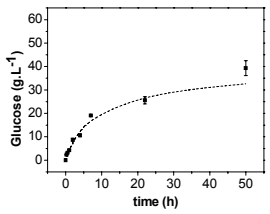
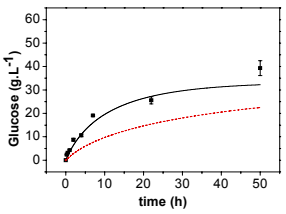
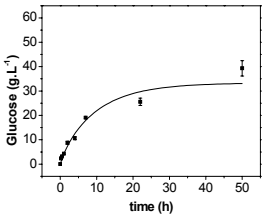
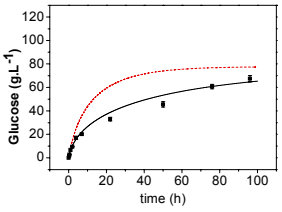
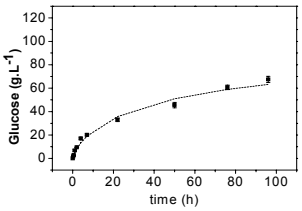
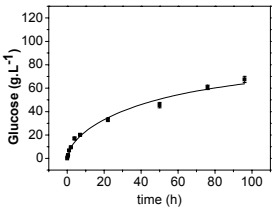
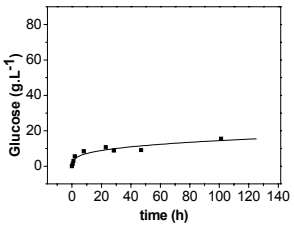
Filter paper	 (a) MM with inhibition, with V_{max}=0.09 g.L^{-1}.min^{-1}, K_m=45.11 g.L^{-1}, K_i=4.13 g.L^{-1}	 (b) Modified MM with inhibition, with k=0.0035 min^{-1}, K_m=21.01 g.L^{-1}, K_i=23 g.L^{-1}	 (c) Chrastil, with k'=9x10^{-5} (Lg^{-1}.min^{-1}) and n=0.89
Steam exploded bagasse (low load, 2.90%)	 (d) MM with inhibition, with V_{max}=0.105 g.L^{-1}.min^{-1}, K_m=8.78 g.L^{-1}, K_i=3.34 g.L^{-1}	 (e) Modified MM with inhibition (thin line: non-retuned, with parameters as in quadrant h; thick line: retuned, with k=0.0054 min^{-1}, K_m=13.37 g.L^{-1}, K_i=20 g.L^{-1})	 (f) Chrastil, with k'=2x10^{-4} (Lg^{-1}.min^{-1}) and n=0.85
Steam exploded bagasse (high load, 6.54%)	 (g) MM with inhibition (thin line: non-retuned, with parameters as in quadrant d; thick line: retuned, with the same k and K_m as in d, but with K_i=0.7 g.L^{-1})	 (h) Modified MM with inhibition, with k=0.0033 min^{-1}, K_m=22.06 g.L^{-1}, K_i=7.61 g.L^{-1}	 (i) Chrastil, with k '=2x10^{-5} (Lg^{-1}.min^{-1}) and n=0.57
Acid treated bagasse	MM with inhibition (KI tends to zero; model not adequate)	Modified MM with inhibition (KI tends to zero; model not adequate)	 (j) Chrastil, with k'=4.96x10^{-8} (Lg^{-1}.min^{-1}) and n=0.30

Figure 4: Complete picture of the results obtained in this kinetic study.

Although the pseudo-homogeneous MM model fitted very well the experimental data of FP hydrolysis (Figure 4(a)), the modified heterogeneous (solid substrate and soluble enzyme) Michaelis-Menten model (with inhibition) was also tested (manually fitted). Figure 4(b) shows that this modified MM model also adheres very well to the experimental data.

Additional tests with filter paper were carried out, which allowed quantifying the diffusion-adsorption effects on the rates of hydrolysis, by analyzing the parameters of the Chrastil model (1988 and 1988b). Figure 4c shows the result of fitting the Chrastil model to the filter paper long term assay. The values of n and k' were 0.89 (close to 1) and $9x10^{-5}$ ($Lg^{-1}.min^{-1}$), which indicate low diffusion-adsorption resistance for the enzymes through the substrate matrix.

As it is possible to observe, the three semimechanistic models fitted well to the experimental data for FP hydrolysis. At this point, a question arose: can one expect the same behavior when working with the real industrial substrate (sugarcane pretreated bagasse)?

Preliminary tests with exploded sugarcane bagasse treated with 4% NaOH were carried out for substrate loads of 0.99%, 2.90%, 3.85%, 6.54% and 9.09% ($w_{cellulose}$ / w_{total}), corresponding to the range of concentrations of potential glucose from 11.11 $g_{Glucose}.L^{-1}_{solution}$ to 111.11 $g_{Glucose}.L^{-1}_{solution}$. The importance of diffusion-adsorption effects was assessed after fitting the model of Chrastil (1988 and 1988b). For loads below 3.85%, intra-matrix enzyme diffusion-adsorption was not the rate limiting step: the values of Chrastil's parameter n monotonically increased, approaching the threshold of n=1. Figure 4f and 4i exemplify this trend for cellulose loads of 2.90% and 6.54%, respectively. The values were n=0.85, with $k'=2x10^{-4}$ ($Lg^{-1}.min^{-1}$), for a cellulose load of 2.9%, and n=0.57 (below 0.6) and $k'=2x10^{-5}$ ($Lg^{-1}.min^{-1}$), for a cellulose load of 6.54%.

For a number of lignocellulosic substrates, working at higher solids concentrations can lead to significant decreases in the yield of hydrolysis. Of course, the resistance for mass transfer through the external film and deviations from the well-mixed reactor pattern may play a role here. But even when stirring overcomes these effects, the trend of a decrease in the apparent reaction rate for higher loads of solids still remains. Internal diffusion of the enzyme that is already inside the solid matrix does not depend on the bulk concentration of solids in the reactor; some other phenomenon must explain this unexpected behavior – which was confirmed, as previously discussed, by our experimental data.

One of the possible causes for this 'solid effect' could be the inhibition of the adsorption of the enzyme on the substrate by the product of the reaction. According to Carrillo *et al*. (2005) and Chrastil (1988), the enzymes must diffuse through the structure of the substrate and, on their way to further reaction centers (i.e., sites on the cellulose chain that are susceptible to enzymatic attack), some enzyme molecules may adsorb on this chain, either in a productive or in an inactive conformation. The product that is formed in the activated centers will diffuse to the bulk flow. If not, these molecules may act as inhibitors of the transport of the enzyme to further reaction centers. Indeed, Lee and Fan (1983) reported that these products might cause the deactivation of the adsorbed enzyme (acting as "uncompetitive" inhibitors of the exoglucanase activity in the case of cellulose hydrolysis). Consequently, internal diffusion-adsorption of the enzyme, which does not depend on the bulk concentration of substrate (i.e., weight of cellulose per weight of reaction medium), would depend on the bulk concentration of product: if this concentration in the reactor increases, the retrieval of product from the neighborhood of the enzyme becomes slower, thus decreasing the apparent reaction rates.

The application of the pseudo-homogeneous MM model to the enzymatic hydrolysis of exploded sugarcane bagasse (treated with 4% NaOH) at 50 °C, pH 4.8, with substrate loads from 0.99 to 3.85% ($w_{cellulose}$ / w_{total}), gave the parameter values of: V_{max} = 0.105 $g_{Glucose}.L^{-1}_{solution}.min^{-1}$ and K_m = 8.78 $g_{Glucose}.L^{-1}_{solution}$. From the temporal evolution of the glucose concentration obtained in long-term hydrolysis assays (with the maximum substrate concentration within the range from 0.99% to 3.85%), it was possible to analyze the effects of inhibition by glucose. At this point, it must once again be mentioned that, because of the high activity of β-glucosidase in the enzymatic complex used in this work, only inhibition (competitive) by glucose was considered. The presence of the inhibition term (with $K_i = 3.34 \pm 1.30$ $g.L^{-1}$) in the representation of (the steam exploded) bagasse hydrolysis by the MM model was also very important, as in the case of FP hydrolysis. Figure 4(d) shows the pseudo-homogeneous MM model with competitive inhibition fitted to experimental data for the enzymatic hydrolysis assay (with maximum substrate concentration within the range from 0.99 to 3.85%, $w_{cellulose}$ / w_{total}).

Additional tests were also conducted to analyze the Michaelis-Menten parameters and the inhibition constant using a global search algorithm, Simulated Annealing (Kirkpatrick *et al*., 1983), implemented in-house using Matlab®. The results of the simultaneous estimation of K_m, V_{max} and K_i, confirmed the results initially obtained, i.e.:

K_m = 8.78 $g_{Glucose}.L^{-1}_{solution}$, V_{max} = 0.105 $g_{Glucose}.L^{-1}_{solution}.min^{-1}$ and K_i = 3.34 $g.L^{-1}_{solution}$ (Origin + Fortran, Marquardt algorithms);

K_m = 7.52 $g_{Glucose}.L^{-1}_{solution}$, V_{max} = 0.090 $g_{Glucose}.L^{-1}_{solution}.min^{-1}$ and K_i = 3.38 $g.L^{-1}_{solution}$ (Matlab, Simulated Annealing).

The in-house Matlab tool can also provide non-linear confidence intervals (Schwaab *et al*., 2008) for the kinetic parameters (represented in two dimensions, corresponding to combinations of pairs of parameters in the vicinity of the point of minimal sum of squares of errors). Figure 5 shows one of the results obtained.

Figure 5 shows that the confidence regions can be broad, which is coherent with the fact that Michaelis-Menten parameters are highly correlated (correlation coefficients with an absolute value close to 1). The nonlinear character of the problem becomes clear in this Figure, since linear models would provide elliptical confidence regions.

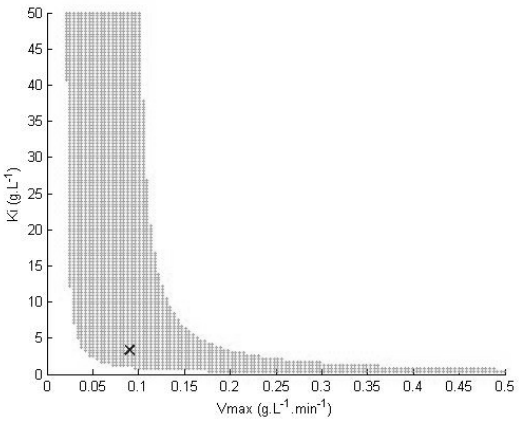


Figure 5: Confidence region (K_i / V_{max}), according to the Beale criterion (not restricted to an elliptical shape).

For substrate (cellulose in treated exploded sugarcane bagasse) concentrations above 3.85% (w/w), the application of a modified heterogeneous (solid substrate and soluble enzyme) Michaelis-Menten model (with inhibition) was initially considered. This implies that the concentrations of enzyme and substrate are exchanged in the Michaelis-Menten equation, and the enzyme is released into the medium at the end of the reaction. Thus, the initial rate of hydrolysis can be expressed as a function of initial enzyme concentration, as shown in Equation (2).

For enzymatic hydrolysis of exploded sugarcane bagasse (treated with 4% NaOH) at 50 ° C, pH 4.8, with a substrate load of 6.54% ($w_{cellulose}$ / w_{total}), the parameter values obtained for the modified MM model were: V_{emax} = 0.256 $g_{Glucose}.L^{-1}_{solution}.min^{-1}$, with a corresponding k=0.0033 min^{-1}, and K_m = 22.06 $g_{enzyme}.L^{-1}_{solution}$.

From experimental data for the temporal evolution of glucose concentration in long-term hydrolysis assays (with an initial substrate concentration of 6.54%), it was possible to analyze effects of inhibition by glucose.

Figure 4h shows the fitting of the modified MM model with competitive inhibition by the product (Equation (2a)) to an enzymatic hydrolysis assay with an initial substrate concentration of 6.54% ($w_{cellulose}$ / w_{total}). Again, the inhibition term (with $K_i = 7.61 \pm 0.87$ $g.L^{-1}$) is of paramount importance.

For the bagasse treated with 1% H_2SO_4 and delignified with 4% NaOH, an assay was first carried out by setting the substrate concentration at 2.9% ($w_{cellulose}$ / w_{total}). Fitting the experimental data to the Chrastil model, it was observed that the medium presented high intra-matrix effects. In sequence, higher substrate loads were focused. Recalcitrance in these cases was so effective that only a Chrastil model could be used to fit the experimental data (Figure 4(j)). The use of the MM model with product inhibition was not appropriate (K_i tended to zero).

It is important to note that some improvement in terms of yield can be achieved for *in natura* bagasse treated with 1% H_2SO_4 followed by delignification with 4% NaOH if some kind of milling of the biomass (not considered here) is associated with this pretreatment.

It is also worth noting at this point that all values of the parameter n in the Chrastil models of this work were consistent with those presented in Carrillo *et al*. (2005), i.e., n close to 1 for systems without important intra-matrix effects and n less than or equal to 0.6 for systems where diffusion-adsorption of the enzymes is the rate controlling step (n around 0.3 for highly recalcitrant systems). Finally, for the latter case (acid treated SB), neural networks (Annema, 1995; Hagan *et al*., 1996; Zhang *et al*., 2009) are suggested to model the hydrolysis reaction rates as a function of substrate concentration. This is

because, in fact, one could attribute the behavior observed for the highly recalcitrant acid treated SB (and delignified with NaOH 4%) not only to product inhibition of the productive adsorption of the enzyme, but also to other factors such as non-productive adsorption of the enzyme on lignin (since the latter pretreatment showed no efficiency in the removal of lignin compared to the exploded bagasse treated with 4% NaOH). Some authors have utilized neural networks in studies of enzymatic hydrolysis of sugarcane bagasse, like Rivera *et al*. (2010).

Fitted parameters (not including manually fitted parameters) are presented in Table 2 along with standard deviations, residuals, χ^2 tests and regression coefficients.

Finally, an interchange between modeling strategies was tried, that is, the application of a model characterized for one situation to a different situation. More specifically:

1. The pseudo-homogeneous Michaelis-Menten model (with inhibition), defined for lower loads of steam exploded bagasse (Figure 4d), was applied for higher substrate (steam exploded bagasse) loads;
2. The Modified Michaelis-Menten model (with inhibition), defined for higher loads of steam exploded bagasse (Figure 4(h)), was applied for lower substrate (steam exploded bagasse) loads.

In general, the direct application of a model in a different situation was not possible (the fit to the experimental data was not satisfactory). However, with (manual) retuning of the parameters, one can satisfactorily apply a model characterized for one situation to a different situation (Figure 4(e) and 4(g)). With this interchange among the modeling strategies, we intend to show that different semi-mechanistic models may be successfully applied to real-world reactor design, catalyst optimization and cost evaluation.

As indicated in Figure 4, (4(d)-4(i)), the three semimechanistic models also fitted well the experimental data of steam exploded bagasse hydrolysis, but only after retuning of MM models when the cellulose load was changed from low load to high load (pairs 4(d)-4(g) and 4(e)-4(h)). Chrastil models also present different parameters for low and high loads of substrate (4(f) and 4(i)). Regarding the bagasse treated with 1% H_2SO_4, only the Chrastil model was adequate to model cellulose hydrolysis (4(j)).

Table 2: Kinetic parameters along with standard deviations, residuals, χ^2 tests and regression coefficients.

	Michaelis-Menten models with inhibition, pseudo-homogeneous or modified					**Chrastil model**		
	Initial velocity assays			**Long term assays**		**Long term assays**		
Substrate	V_{max} * ($g.L^{-1}.min^{-1}$)	k ** (min^{-1})	K_m ($g.L^{-1}$)	K_i ($g.L^{-1}$)	Total residual, $\sum(P_{exp}-P_{calc})^2$ $(g.L^{-1})^2$	k' ($L.g^{-1}.min^{-1}$)	n	χ^2 and R^2
Filter paper	0.090 ± 0.006	-	45.11 ± 7.85	4.13 ± 0.59	12.42	$9.0x10^{-5} \pm 1.0x10^{-5}$	0.89 ± 0.10	1.10285 0.99042
Low load exploded bagasse (2.90%, $w_{cellulose}/w_{total}$)	0.105 ± 0.015	-	8.78 ± 4.89	3.34 ± 1.30	62.24	$2.0x10^{-4} \pm 0.3x10^{-4}$	0.85 ± 0.10	7.99470 0.95314
High load exploded bagasse (6.54% $w_{cellulose}/w_{total}$)	-	0.0033 ± 0.0007	22.06 ± 10.28	7.61 ± 0.87	80.33	$2.0x10^{-5} \pm 0.2x10^{-5}$	0.57 ± 0.05	6.66681 0.98980
Acid treated bagasse	-	-	-	-	-	$4.96x10^{-8} \pm 5.94x10^{-8}$	0.30 ± 0.05	2.40691 0.91389

* For pseudo-homogeneous Michaelis-Menten
** For modified (heterogeneous) Michaelis-Menten

CONCLUSIONS

For qualitative filter paper the role of mass transport resistance in the external film was not significant when the agitation speed was in the range of 150-300 rpm. It was possible to fit a pseudo-homogeneous Michaelis-Menten model and a modified MM model (both with inhibition). The Chrastil model was also fitted.

For a real industrial substrate (exploded bagasse treated with 4% NaOH, 88% of cellulose content), it was possible to fit a pseudo-homogeneous MM model, with initial estimates obtained from a Lineweaver-Burk diagram for a range of substrate loads from 0.99% to 3.85% ($w_{cellulose}$ / w_{total}). It was also possible to set a competitive inhibition constant to complete the MM model. For exploded bagasse treated with 4% NaOH and considering a higher substrate load (6.54%), hydrolysis assays were used in the fitting of a modified MM model, with inhibition. Again, Chrastil models were also fitted. Finally, it was shown that, after retuning of the model parameters, one can satisfactorily apply a model characterized for one situation to a different situation.

A "solid effect" was observed when the load of substrate in the reactor was increased. The parameter n of Chrastil's model was affected by this load, indicating that the intra-matrix delaying effects (diffusion-adsorption of the enzymes through the solid matrix) depended on the bulk concentration of substrate. One hypothesis to explain this behavior is the inhibitory effect that the product may exert on the active adsorption of the enzyme on the solid substrate.

For the bagasse pretreated with 1% H_2SO_4 and with 4% NaOH, which proved to be very recalcitrant, it was only possible to fit a Chrastil model.

We believe that, at present, engineering practice will probably rely more on different empirical and semi-empirical approaches (nonmechanistic or semimechanistic), including simple rate equations like those presented here, with two or three parameters. These simple equations are based on assumptions that are far from the real situation, but they nonetheless fit well to the data and can be utilized for practical optimization studies.

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NOMENCLATURE

E	enzyme concentration	$g.L^{-1}$
E_{ads}	adsorbed enzyme	$g.L^{-1}$
E_0	initial enzyme concentration	$g.L^{-1}$
K_i	(competitive) glucose inhibition constant	$g.L^{-1}$
K_{ic}	(competitive) cellobiose inhibition constant	$g.L^{-1}$
k	rate constant of cellulose hydrolysis	min^{-1}
k'	rate constant proportional to the diffusion coefficient	$L.g^{-1}.min^{-1}$
K_m	Michaelis Menten constant in the case of pseudo homogeneous MM model and is a half saturation constant for the modified MM model	$g.L^{-1}$
n	structural diffusion adsorption resistance constant dependent on the steric structure of the system	$g.L^{-1}$
P	product	
P_∞	products which diffuse at equilibrium	$g.L^{-1}$
S	cellulose (transformed in potential product concentration)	$g.L^{-1}$
S_0	initial potential product concentration	$g.L^{-1}$
V_{max} and V_{emax}	maximal velocities for pseudo homogeneous and modified MM models, respectively	$g.L^{-1}.min^{-1}$
V_0	initial hydrolysis velocity	$g.L^{-1}.min^{-1}$

REFERENCES

Adney, B. and Baker, J., Measurement of cellulase activities: chemical analysis and testing task. Laboratory analytical procedure (1996).

Annema, A., Feed-forward Neural Networks: Vector Decomposition Analysis, Modeling and Analog Implementation. Kluwer Academic Publishers, Boston (1995).

Bezerra, R. M. F. and Dias, A. A., Discrimination among eight modified Michaelis–Menten kinetics models of cellulose hydrolysis with a large range of substrate/enzyme ratios. Appl. Biochem. Biotech., 112, 173 (2004).

Carrillo, F., Lis, M. J., Colom, X., Lopez-Mesas, M., Valldeperas, J., Effect of alkali pretreatment on

cellulase hydrolysis of wheat straw: Kinetic study. Process Biochem., 40, 3360 (2005).

Chrastil, J. Enzymic product formation curves with the normal or diffusion limited reaction mechanism and in the presence of substrate receptors. Int. J. Biochem., 20, No. 7, 683 (1988).

Chrastil, J., Determination of the first order consecutive reaction rate constants from final products. Comput. Chem., 12, No. 4, 289 (1988b).

Ghose, T. K., Measurement of cellulase activity. Pure & Appl. Chem., 59, 257 (1987).

Gouveia, E. R., Nascimento, R. T., Souto-Maior, A. M., Rocha, G. J. M., Validação de metodologia para a caracterização química de bagaço de cana-de-açúcar. Química Nova, 32, 1500 (2009). (In Portuguese).

Granda, C. B., O'Dwyer, J. P., Zhu, L., Holtzapple, M. T., Enzymatic hydrolysis of lime-pretreated corn stover and investigation of the HCH-1 Model: Inhibition pattern, degree of inhibition, validity of simplified HCH-1 model. Bioresource Technology, 98, 2969 (2007).

Hagan, M., Demuth, H., Beale, M., Neural Network Design. PWS Publishing, Boston (1996).

Ingesson, H., Zacchi, G., Yang, B., Esteghlalian, A. R., Saddler, J. N., The effect of shaking regime on the rate and extend of enzymatic hydrolysis of cellulose. J. Biotech., 88, 177 (2001).

Kirkpatrick, S., Gelatt, C. D., Vecchi, M. P., Optimization by simulated annealing. Science, 220, No. 4598, 671 (1983).

Lee, Y. H. and Fan, L. T., Kinetic studies of enzymatic hydrolysis of insoluble cellulose: (II) analysis of extended hydrolysis times. Biotechnol. Bioeng., 25, 939 (1983).

Lynd, L. R., Weimer, P. J., van Zyl, W. H., Pretorius, I. S., Microbial cellulose utilization: Fundamentals and biotechnology. Microbiol. Mol. Biol. Rev., 66, 506 (2002).

Mansfield, S. D., Mooney, C., Saddler, J. N., Substrates and enzyme characteristics that limit cellulose hydrolysis. Biotechnol. Prog., 15, 804 (1999).

Miller, G. L., Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal. Chem., 31, 426 (1959).

Rivera, E. C., Rabelo, S. C., Garcia, D. D., Maciel, R., da Costa, A. C., Enzymatic hydrolysis of sugarcane bagasse for bioethanol production: Determining optimal enzyme loading using neural networks. J. Chem. Technol. Biotechnol., 85, 983 (2010).

Rocha, G. J. M., Silva, F. T., Araújo, G. T., Curvelo, A. A. S., A fast and accurate method for determination of cellulose and polyoses by HPLC. In: Proceedings of the V Brazilian Symposium on the Chemistry of Lignin and Other Wood Components, 5, 113 (1997).

Rothschild, W. G., Fractals in Chemistry. Wiley Interscience, New York (1998).

Schwaab, M., Biscaia, E. C., Monteiro, J. L., Pinto, J. C., Nonlinear parameter estimation through particle swarm optimization. Chem. Eng. Sci., 63, 1542 (2008).

Sousa, Jr. R., Carvalho, M. L., Giordano, R. L. C., Giordano, R. C., Recent trends in the modeling of cellulose hydrolysis. Braz. J. Chem. Eng., 28, No. 4, 545 (2011).

Walker, L. P., Wilson, D. B., Enzymatic hydrolysis of cellulose: An overview. Biores. Technol., 1991, 3 (1991).

Willams, J. C., In: Gartner, N. H., Messer, C. J., Rathi, A., (Eds.), Monograph on Traffic Flow Theory. Transportation Research Board Special Report, National Research Council, Washington, DC, 6-1 (2005). http://www.tfhrc.gov/its/tft/chap9.pdf.

Woodward, J., Synergism in cellulase systems. Biores. Technol., 36, 67 (1991).

Zhang, Y., Xu, J. L., Yuan, Z. H., Modeling and prediction in the enzymatic hydrolysis of cellulose using artificial neural network. Fifth Intern. Conf. Natural. Comput., Tianjin, China (2009).

Zhang, Y. H. P. and Lynd, L. R., Toward an aggregated understanding of enzymatic hydrolysis of cellulose: Noncomplexed cellulase systems. Biotechnol. Bioeng., 88, No. 7, 797 (2004).