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# Determination of Enzyme (Cellulase from *Trichoderma reesei*) Kinetic Parameters in the Enzymatic Hydrolysis of $H_2SO_4$-Catalyzed Hydrothermally Pretreated Sugarcane Bagasse at High-Solids Loading

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Enzymatic kinetic studies of celulignin material obtained from acid-catalyzed hydrothermally pretreated sugarcane bagasse at high-solids loading (S / 25%), acid concentration (*A*) equal to 1.0; 2.0 and 3.0% w/v and pretreatment time ($t^P$) equal to 30; 90 and 150 min were carried out using the synergistic action of cellulase from *T. reesei* and cellobiase from *Aspergillus niger*.
The kinetic experiments were carried out at 50 °C, 150 rpm and 3 h with substrate (water insoluble solids after pretreatment - WIS) loading range between 30 $g_{substrate}/L_{sol}$ and 100 $g_{substrate}/L_{sol}$, and mixed with cellulase (at 5.0; 15.0; 30.0 and 60.0 FPU cellulase/$g_{substrate}$) and β-glucosidase (at 33.0 IU β-glucosidase/$g_{substrate}$) enzymes. A Michaelis-Menten dependence on the cellulase from *T. reesei* concentration and substrate concentration plots were obtained based on experimental data. The rate constant, $K_m$ and maximum reaction rate attainable, $V_{max}$ were determined to characterize the cellulase-catalyzed reaction.
Results showed that the cellulase from *T. reesei* behavior was highly specific for each WIS fraction obtained under a combination of [*A*-*S*-$t^P$] where $V_{max}$ varies greatly as enzyme (cellulase) concentration rises. For example, $V_{max}$ values when considered a substrate obtained at [1.0% w/v - 25% - 30 min] and [3.0% w/v - 25% - 30 min] using 5.0 FPU cellulase/$g_{substrate(WIS)}$ and [1.0% w/v - 25% - 90 min] and [3.0% w/v - 25% - 90 min] using 60.0 FPU cellulase/$g_{substrate(WIS)}$ were 5.96±0.27 $g_{GLC}/L_{SOL}h$; 2.90±0.05 $g_{GLC}/L_{SOL}h$; 5.16±0.18 $g_{GLC}/L_{SOL}h$; 7.48±0.81 $g_{GLC}/Lh$, respectively. Dependence on the $K_m$ of enzyme-catalyzed reaction exhibited a considerably variation with the substrate nature and the enzymatic kinetic conditions establishing that $K_m$ for the cellulase from *T. reesei* –substrate complex was between 54.81 $g_{GLC}/L_{SOL}$ and 209.99 $g_{GLC}/L_{SOL}$.

## 1. Introduction

The enzymatic hydrolysis is a heterogeneous reaction catalyzed by cellulases, distinguished by an insoluble substrate (cellulose) and a soluble catalyst (enzymes). The complete hydrolysis of the cellulose requires the combined action of multiple enzymes with different substrate specificities (Kumar et al., 2008), and offers the potential for a long term reduction in costs, since it is possible to attain high yields under less critical conditions of pressure, temperature and chemical attack (Canilha et al., 2011).

Kinetic modeling of enzymatic saccharification results in a complex procedure comprising the kinetics of enzyme-catalyzed reactions: Michaelis-Menten kinetics, enzyme Inhibition and the enzyme-substrate interactions among others. Bansal et al. (2009) provides an overview of the models published in the open literature about enzymatic mechanism on cellulose hydrolysis. In their review are presented eighteen works about empirical models where the main predicted variables were: the glucose concentration, glucan to glucose

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conversion and hydrolysis yield. On the other hand, they presented Adsorption and Michaelis – Menten based models clustered in five groups: Michaelis – Menten, Product inhibition, Quasi Steady State assumption, Adsorption based approach, BG –β-glucosidase).

For the adsorption based approach to approximate the kinetic equations governing the enzymatic hydrolysis and analyzing the enzyme-substrate reactions, Philippidis and Hatzis (1997) and Philippidis et al. (1992), defined a mathematical model based on quality and concentration of the cellulosic-substrate; quality and concentration of the cellulase and β-glucosidase enzyme system and the mode of interaction between substrate and enzyme (Figure 1).

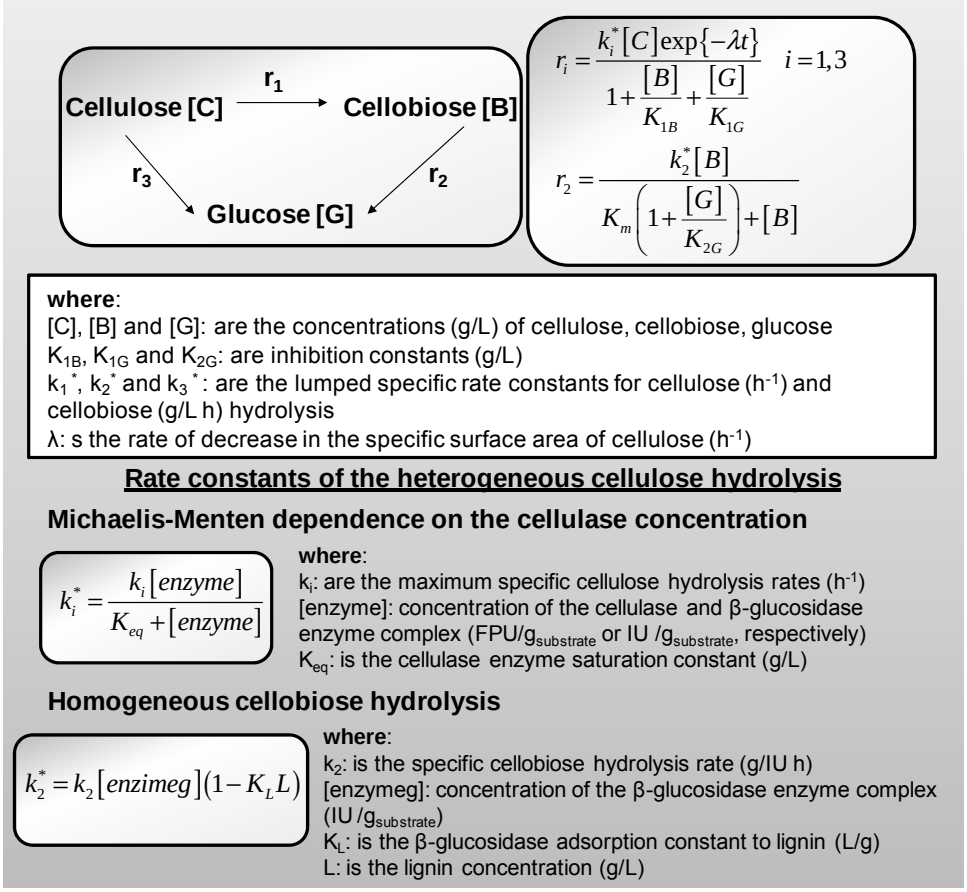


*Figure 1: Philippidis based kinetic model: adsorption of cellulose and β-glucosidase onto lignin*

Moreover, aiming to determine lumped specific rate constants for cellulose using the Philippidis and Hatzis (1997) and Philippidis et al. (1992) model associated to a Michaelis-Menten dependence on the cellulose, Kumar et al. (2008) described a methodology to ensure the validity of the steady state assumption for a standard enzyme-substrate reaction. In this sense, based on Philippidis and Hatzis (1997) and Philippidis et al. (1992) model to describe the enzymatic hydrolysis from lignocellulosic biomass, the present manuscript is an attempt to analyze the kinetic activator constant ($K_m$) and maximum reaction rate attainable or theoretical maximal velocity, $V_{max}$ based on enzymatic kinetic investigation of celulignin material from $H_2SO_4$-catalyzed hydrothermally pretreated sugarcane bagasse at high-solids loading (S / 25%), acid concentration ($A$) equal to 1.0; 2.0 and 3.0% w/v and pretreatment time ($t^P$) equal to 30; 90 and 150 min. For such an enzymatic complex of cellulase from *T. reesei* and cellobiase from *Aspergillus niger* was used.

## 2. Material and methods

Sugarcane bagasse (SCB) was donated by a Brazilian sugar-alcohol-co-generation mill (Usina São João, Araras, São Paulo - Brazil) and its composition is presented in Figure 2.

$H_2SO_4$-catalyzed hydrothermal pre-treatment and enzymatic digestibility experiments were carried out as exhibited in Figure 2. Water insoluble solids (WIS) and liquid streams were analyzed based on standard procedures (Canilha et al., 2011, Gouveia et al., 2009, Sluiter et al., 2008).

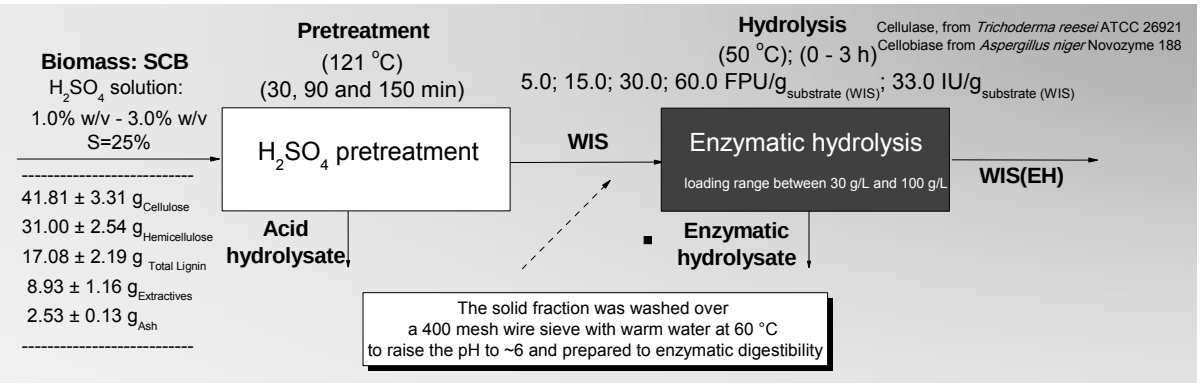


*Figure 2: Enzymatic kinetic studies of celulignin material obtained from acid-catalyzed hydrothermally pretreated sugarcane bagasse*

### 2.1 Method of modelling: Michaelis-Menten kinetics

The Michaelis-Menten (MM) equation is the model of enzyme kinetics for a one-substrate (S) enzyme-catalyzed (E) reaction to product (P) which quantitatively relates the initial rate, the maximum rate, and the initial substrate concentration to the Michaelis constant $K_m$ (Figure 3). Furthermore, enzyme-catalyzed reaction is characterized by the formation of a complex between the enzyme and its substrate (ES) (Berg et al., 2002).

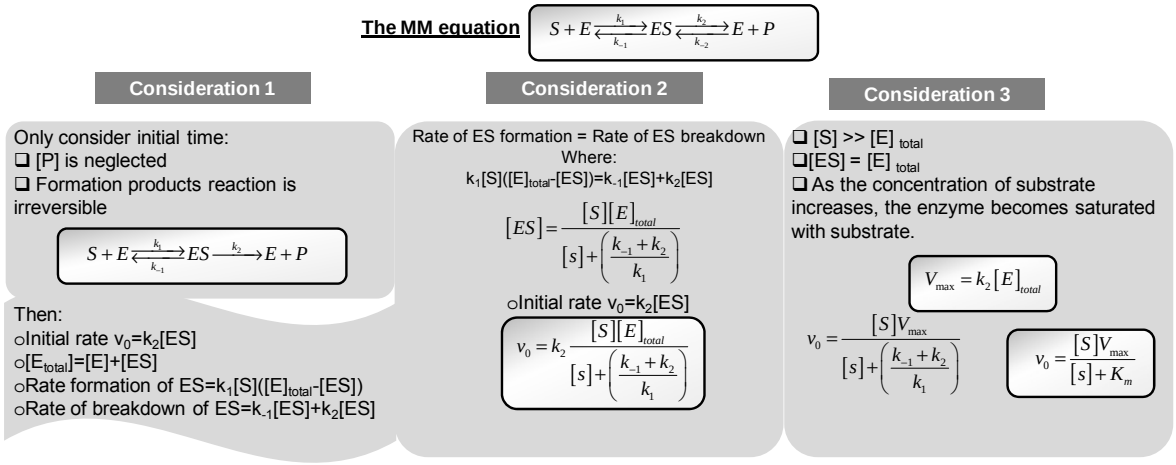


*Figure 3: Considerations to use the Michaelis-Menten equation to estimate Philippidis and Hatzis (1997) and Philippidis et al. (1992) model parameters*

Based on Michaelis-Menten equation, it is possible to estimate $k_1$*, $k_2$* and $k_3$* (Figure 1) and described the kinetic behavior of enzyme by Eq(1) considering a variation in solid substrate at different soluble enzyme concentration (Carvalho et al., 2013, Magalhães et al., 2010).

$$v_0 = \frac{V_{max}[S]}{K_m + [S]} \quad (1)$$

where: $V_{max}$ is the maximal velocity for the initial concentration of adsorption sites on the substrate ($g_{GLC}/L_{SOL}h$); $K_m$ is the corresponding half-saturation constant ($g_{GLC}/L_{SOL}$), and [S] is the substrate concentration ($g_{substrate}/L$).

## 3. Results and discussion

### 3.1 Estimation of kinetic parameters for cellulase

The initial rate of the glucose released from cellulose presented in the $H_2SO_4$-catalyzed hydrothermally pretreated sugarcane bagasse (under a combination of [$A$-$S$-$t^p$] equal to [1.0% w/v - 25% - 90 min]; [2.0% w/v - 25% - 90 min] and [3.0% w/v - 25% - 90 min]) by the synergistic action of cellulase from *T. reesei* and cellobiase from *Aspergillus niger* as a function of substrate concentration is shown in Figure 4.

Experimental data showed the effect of the $H_2SO_4$-catalyzed hydrothermal pretreatment of sugarcane bagasse, the substrate concentration and the enzyme concentration on the initial enzymatic hydrolysis during 3 h (Figure 4). Due to polymeric nature, it is observed that it is not a linear behavior and it rises as the substrate concentration and enzyme concentration increase up to substrate become limiting (about 80 $g_{substrate}/L_{SOL}$).

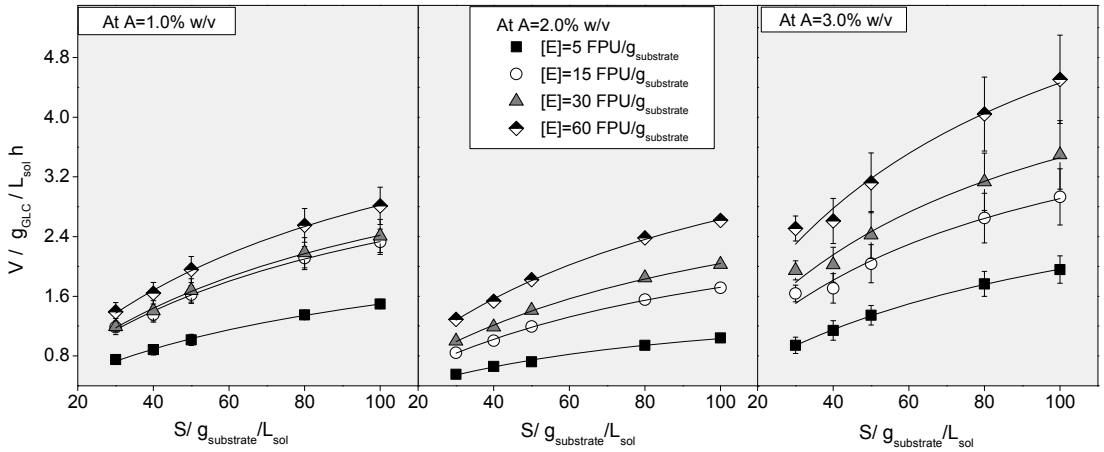


*Figure 4: Initial rate of the glucose released from cellulose presented in the $H_2SO_4$-catalyzed hydrothermally pretreated sugarcane bagasse*

Initial rates were based on the quantification of glucose after 3 h of hydrolysis guarantying a linear region of the time curve. Table 1 lists the calculated $V_{max}$ and $K_m$ from the enzymatic hydrolysis data by nonlinear regression following the Eq(1). For high values of enzyme concentration (60.0 FPU/$g_{substrate}$), the ratio substrate and solution (SOL) became limited and the initial rate asymptotically approaches to a maximum as being: 2.81±0.25 $g_{GLC}/L_{SOL}$ h, 2.62± - $g_{GLC}/L_{SOL}$ h and 4.51±0.59 $g_{GLC}/L_{SOL}$ h, respectively. Similar trends were observed for the others substrates obtained from different pretreatment conditions.

Comparing the hydrolysis of substrate pretreated at acid concentration equal to 1.0, 2.0 and 3.0% w/v at the same cellulase enzyme concentration, it is observed that acid concentration in the pretreatment increases the total sugar (glucose) yield on the saccharification process about two times when acid concentration rises from 1.0 to 3.0% w/v. Moreover, results evidenced that increasing enzyme (from 5.0 to 60.0 FPU/$g_{substrate}$), it is produced higher amount of soluble reducing sugars, although for enzyme concentration between 15.0 and 30.0 FPU/$g_{substrate}$, for $H_2SO_4$-catalyzed hydrothermally pretreated sugarcane bagasse at 1.0% w/v, small differences were observed because of the substrate saturation.

Kinetics parameters were determined using Eq(1). A maximum rate, $V_{max}$ values, when considered a substrate obtained at [1.0% w/v - 25% - 30 min] and [3.0% w/v - 25% - 30 min] using 5.0 FPU cellulase/$_{gsubstrate(WIS)}$ and [1.0% w/v - 25% - 90 min] and [3.0% w/v - 25% - 90 min] using 60.0 FPU cellulase/$_{gsubstrate(WIS)}$ were 5.96±0.27 $g_{GLC}/L_{SOL}$h; 2.90±0.05 $g_{GLC}/L_{SOL}$h; 5.16±0.18 $g_{GLC}/L_{SOL}$h; 7.48±0.81 $g_{GLC}/L_{SOL}$h, respectively. Initial rate of enzymatic hydrolysis were calculated from the MM model and compared with the experimental results (Figure 4) and the MM model fits well with the experimental results reporting a adjustable-$R^2$ higher than 0.93.

*Table 1: Estimated the maximal velocity for the initial concentration of adsorption sites on the substrate ($V_{max}$) and the corresponding half-saturation constant ($K_m$) for $H_2SO_4$-catalyzed hydrothermally pretreated sugarcane bagasse obtained at different operational conditions (S=25%)*

| A / % w/v | | 1.0 | | 2.0 | | 3.0 | |
|---|---|---|---|---|---|---|---|
| $t^p$ / min | [E] / FPU/$g_{substrate}$ | $V_{max}$ / $g_{GLC}/L_{SOL}h$ | $K_m$ / $g_{GLC}/L_{SOL}$ | $V_{max}$ / $g_{GLC}/L_{SOL}h$ | $K_m$ / $g_{GLC}/L_{SOL}$ | $V_{max}$ / $g_{GLC}/L_{SOL}h$ | $K_m$ / $g_{GLC}/L_{SOL}$ |
| 30 | 5 | 5.96± 0.27 | 109.55± 8.12 | 4.09± 0.08 | 90.39± 3.20 | 2.90± 0.05 | 86.12± 2.48 |
| 60 | 5 | 2.80± 0.07 | 79.18± 3.41 | 3.96± 0.31 | 54.81± 9.15 | 1.66± 0.08 | 20.84± 3.69 |
| 90 | 5 | 2.71± 0.10 | 81.62± 5.38 | 1.69± 0.08 | 63.72± 5.88 | 3.69± 0.07 | 87.92± 2.80 |
| 150 | 5 | 3.61± 0.13 | 67.69± 4.78 | 2.18± 0.15 | 56.38± 8.07 | 2.45± 0.09 | 60.45± 4.50 |
| 30 | 15 | 7.16± 0.15 | 81.65± 3.02 | 5.76± 0.13 | 81.56± 3.45 | 3.95± 0.23 | 71.48± 8.10 |
| 60 | 15 | 4.17± 0.05 | 76.44± 1.71 | 8.40± 0.29 | 85.07± 5.20 | 7.32± 1.50 | 128.84± 40.80 |
| 90 | 15 | 4.18± 0.21 | 79.37± 7.22 | 3.15± 0.09 | 83.19± 4.45 | 4.86± 0.51 | 67.09± 13.82 |
| 150 | 15 | 5.94± 0.20 | 73.88± 4.57 | 8.83± 2.14 | 192.63± 64.36 | 4.71± 0.13 | 83.76± 4.18 |
| 30 | 30 | 8.49± 0.19 | 82.95± 3.42 | 6.90± 0.20 | 83.61± 4.31 | 4.75± 0.24 | 73.67± 7.09 |
| 60 | 30 | 5.15± 0.12 | 83.28± 3.48 | 9.92± 0.35 | 84.57± 5.34 | 8.58± 1.68 | 126.83± 38.51 |
| 90 | 30 | 4.41± 0.16 | 82.56± 5.29 | 3.73± 0.12 | 83.01± 4.69 | 5.79± 0.63 | 67.42± 14.26 |
| 150 | 30 | 7.11± 0.22 | 82.99± 4.66 | 11.11± 5.72 | 203.45± 142.40 | 5.57± 0.17 | 83.53± 4.46 |
| 30 | 60 | 9.58± 0.32 | 82.80± 4.96 | 8.83± 0.25 | 82.14± 4.12 | 6.06± 0.36 | 72.00± 8.05 |
| 60 | 60 | 5.77± 0.19 | 82.84± 4.89 | 12.82± 0.45 | 84.79± 5.31 | 11.13± 2.23 | 128.02± 39.62 |
| 90 | 60 | 5.16± 0.18 | 82.74± 5.16 | 4.81± 0.15 | 82.98± 4.71 | 7.48± 0.81 | 67.62± 14.23 |
| 150 | 60 | 8.19± 0.28 | 82.71± 5.16 | 14.61± 7.28 | 209.99± 141.32 | 7.19± 0.21 | 83.52± 4.47 |

Carvalho et al. (2013) studied the influence of substrate concentration for the exploded sugarcane bagasse treated with 4% NaOH during the enzymatic hydrolysis. Analyses during enzymatic hydrolysis were carried out for substrate loads ranges between 11.11 $g_{substrate}/L_{SOL}$ and 111.11 $g_{substrate}/L_{SOL}$. Results reported a $V_{max}$ = 6.3 $g_{GLC}/L_{SOL}h$ and $K_m$ = 8.78 $g_{GLC}/L_{SOL}$. Thus, the dependence on the $K_m$ of enzyme-catalyzed reaction exhibited a considerably variation with the substrate nature and the enzymatic kinetic conditions establishing that $K_m$ for the cellulase from *T. reesei* – substrate complex was between 54.81 $g_{GLC}/L_{SOL}$ and 209.99 $g_{GLC}/L_{SOL}$. Higher $K_m$ values indicate that the cellulase from *T. reesei* binds the substrate weakly and due to wide variation in $K_m$, it could be an indication of the presence of an inhibitor and/or loss of enzyme activity through adsorption to the lignin present in the substrate.

## 4. Conclusions

The influence of enzyme concentration (between 5.0 and 60.0 $FPU/g_{substrate}$) and substrate concentration (between 30 and 100 $g_{substrate}/L_{SOL}$) on the production of glucose was studied by the Michaelis–Menten model showing good correlation with experimental data. The parameters $V_{max}$ and $K_m$ have been determined with high accuracy.

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