3.5 Kinetic behavior of cellulolytic CellicCTec2 enzyme cocktail on Avicell and organosolv pretreated hemp hurds

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Introduction

The upgrading of lignocellulosic material is usually designed via four major consecutive steps: pretreatment, cellulose hydrolysis, sugars fermentation, and product recovery. Enzymatic hydrolysis of cellulose has advantages over chemical methods for its high sugar yield, low energy consumption and mild operating conditions [1]. Although cellulase enzymes price has been recently decreased, and efficient and stable cellulase "cocktails" has been introduced, its consumption is still one of the major costs in biorefinery. Industrial cellulase cocktail typically contains three major types of activities: endoglucanases, cellobiohydrolases and β-glucosidase, which synergistically degrade cellulose into glucose; moreover others accessory enzymatic activities, such as esterases and oxidases are usually added to enhance lignocellulosic hydrolysis. Despite advanced formulation are commercial available, the rate of cellulose hydrolysis by enzymes decreases rapidly with conversion, leading to decreased yields, long processing times, and high enzyme usage [2]. Different explanations of this behavior have been formulated. Among them changes in substrate properties (i.e. crystallinity index, degree of polymerization and surface area), enzyme inactivation, product inhibition, and interference by irreversibly attached proteins on the substrate are the most critical [3].

A mathematical model is an important tool to understand the mechanism of a complex reaction in the developing of large-scale process models [4].

Extensive studies of lignocellulosic hydrolysis kinetics have been carried out. However understanding the dynamic nature of this reaction is still limited since most of the developed models are correlation-based, thus reliable only under the conditions they were developed for [5]. Fractal-like methods are particularly useful to study heterogeneous reactions that involve the diffusion of catalyst on non-ideal substrate surfaces [6]. In enzymatic hydrolysis of cellulosic substrates the fractal exponent (*h*) is usually correlated to the structural organization of the substrate [7].

In this work, a fractal-like kinetic model was employed to investigate how enzymatic saccharification is affected by different organosolv pretreatment conditions. Experimental data were analyzed and the pretreatment severity was tentatively related to the change observed in the fractal parameters.

Material and methods

Organosolv Pretreatment

Organosolv pretreatment of hemp hurds (OSHH) was carried out according to Gandolfi et al. [5]. The two different pretreatment severities here considered, were achieved by changing the catalyst loading; 1% w/w for CS 0.6 and 2.5% w/w for CS 1.3, whereas others pretreatment variables i.e. temperature, reaction time and solvent concentration were keep constant (165 °C, 20 min, 60% MeOH v/v, respectively). Removal of re-deposited lignin from pretreated OSHH was carried out using a Soxhlet apparatus (50 °C, 5 h ~2 cycle h^{-1}). Methanol was used as solvent. After extraction, biomass was extensively washed whit deionized water and air dry. Biomass compositional analysis was performed as previously published [8].

Enzymatic hydrolysis

Enzymatic hydrolysis was performed in duplicate using 30 mL glass vials with a total working volume of 10 mL. Substrate loading was 5% w/v and a 50mM citrate buffer (pH 4.8) was used. The reaction was carried out in a thermostated shaker for 144 hours at 50 °C

and 150 rpm. The cellulase enzyme (Cellic CTec2, Novozyme) loadings varied from 5 to 80 FPU g^{-1} of substrate. Samples of 100 μL were taken periodically to monitor the hydrolysis. Samples were heated at 99 °C for 10 min to denature the enzyme and then centrifuged for 10 min at 15000 g. The liquid was filtered through 0.45 μm nylon membranes and glucose concentration was analyzed by HPLC. The FPU of the enzyme stock solution was periodically assessed according to NREL standard protocol [9].

Mathematical modeling of hydrolysis kinetics

To fitting the time course data of enzymatic hydrolysis Eq. 1 was used. This model is based on the first-order kinetics to obtain the enzymatic rate [4,10].

$$\frac{dC}{dt} = -k \cdot C$$

$$X = 100 \cdot (1 - EXP(-kt)) \quad (1)$$

C is the cellulose concentration, *X* is the degree of glucan conversion, *k* is the rate constant and *t* is time. The model described by Eq. 2 instead, was used for the fractal-like fittings (pseudo first order reaction kinetic). In which *X* is the degree of glucan conversion, *C* is the cellulose concentration, k_t is the rate coefficient, *k* is the rate constant and *h* is the fractal exponent [4].

$$\frac{dC}{dt} = -k_t \cdot C$$

$$k_t = k \cdot t^{-h}$$

$$X = 100 \cdot \left(1 - EXP\left(-k\left(1 + \frac{t^{(1-h)}-1}{1-h}\right)\right)\right) \quad (2)$$

The parameters *h* and *k* of the fractal-like model were determined by non-linear regression (*nls*) using R 3.0.2. A Gauss-Newton algorithm was used for error minimization.

Results and discussion

Enzymatic hydrolysis of Avicell PH-101, a pure form of cellulose, with different cellulase loadings was carried out, results are shown in Fig. 1. As observed the glucan conversion increased nonlinearly increasing the enzyme concentration. A plateau was reached between 20 to 80 FPU g^{-1} of enzyme.

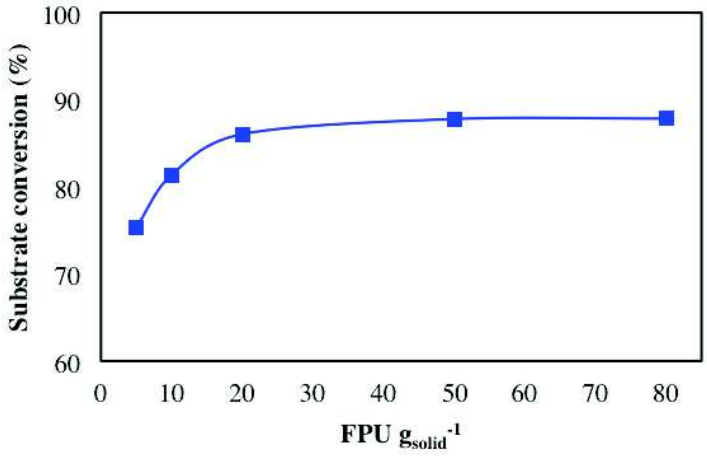

Figure 1. Effect of enzyme blend Cellic CTec2 loading on Avicell hydrolysis. Reaction time was 72 hours and substrate loading was 5% w/v.

When the enzymatic hydrolysis was subjected to time course analysis the application of a first order enzymatic rate failed to fit the experimental data, especially at high degree of conversion (Fig. 2a). This is in agreement on the results obtained by other authors by using different cellulases and different cellulose sources.

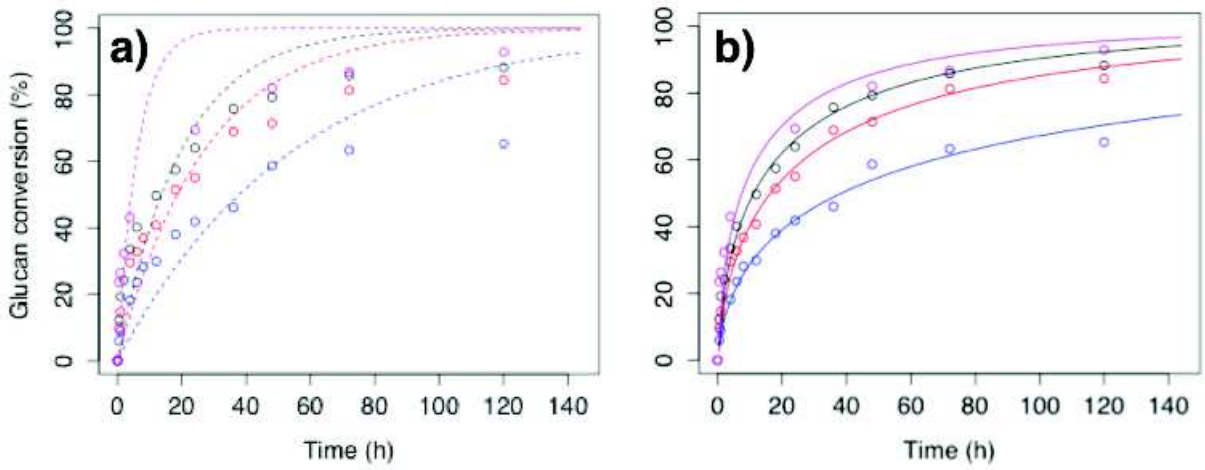

Figure 2. Time course hydrolysis profiles for different loadings of enzyme blend Cellic CTec2. Points represent the experimental data, lines represents fittings. Blue 5 FPU, red 10 FPU, black 20 FPU and violet 80 FPU. Fittings are based on Eq. 1 (a). Fittings are based on Eq. 2 (b).

Among the various models developed to describe enzymatic hydrolysis of cellulose, the fractal-like method has been successfully applied [4-7]. The fitting of experimental data with Eq. 2 is shown in Fig. 2b, and a good correlation was observed between experimental data and simulation. In the fractal-like kinetic model used the rate constant k (Eq. 1) is replaced by a transient rate coefficient $k_t = kt^{-h}$ that decays over the time with a fractal exponent h [6]. In Fig. 3 the relationship between the fractal kinetic parameters and enzyme loading is depicted. As shown, the rate constant k rises as a function of enzyme concentration till a platform value of ~0.15. This can be ascribed to an increasing adsorption of enzymes to cellulose, whereas at high enzyme loadings saturation phenomena may occurs, causing the observed plateau. Differently, the fractal exponent h is usually related to substrate features e.g. cellulose crystallinity and lignin content, but also to crowding effect [11]. However the increase of the fractal exponent is associated to a rate slowdown [7]. For Avicell hydrolysis experiments h

values ranging from 0.49 to 0.51 were obtained, these values well agree with values found in the literature [4]. Furthermore, at mathematical level, the fractal exponent value of 0.5 is referred for reactions that take place in different phases [12].

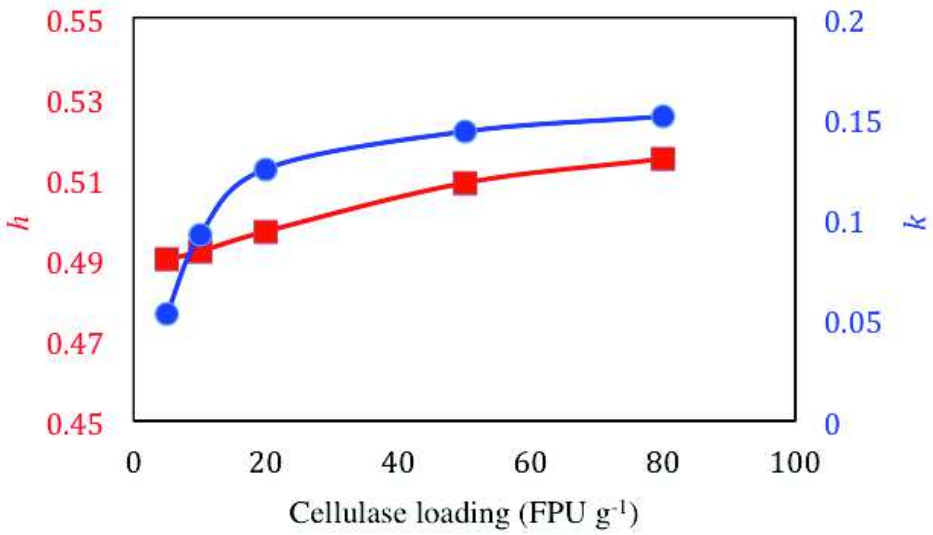

Figure 3. Relationship between fractal kinetic parameters and enzyme loading.

Time course enzymatic hydrolysis analysis of OSHH has been used to evaluate the effect of organosolv pretreatment on biomass saccharification. Sample containing a different ratio of cellulose to lignin were used (Tab. 1) and hydrolysis data were fitted using the fractal-like model (Eq. 2) to obtain the parameters *k* and *h*. (Fig. 4).

Sample	CS[a]	Glucan (wt.%)	Xylan (wt.%)	Lignin (wt.%)
HH	n.a	43.27 ± 1.4	22.48 ± 0.9	26.37 ± 0.8
OSHH1[b]	1.3	78.05 ± 1.5	8.24 ± 1.0	6.96 ± 0.7
OSHH2	1.3	70.44 ± 1.6	8.32 ± 0.9	14.36 ± 1.0
OSHH3[b]	0.6	55.72 ± 1.5	17.85 ± 1.4	18.74 ± 0.9
OSHH4	0.6	53.23 ± 1.1	16.85 ± 1.0	21.67 ± 0.9

Table 1. Compositional analysis of untreated hemp hurds (HH) and differently pretreated hemp hurds (OSHH). [a] Combined Severity Factor. [b] Shoxhlet extracted samples.

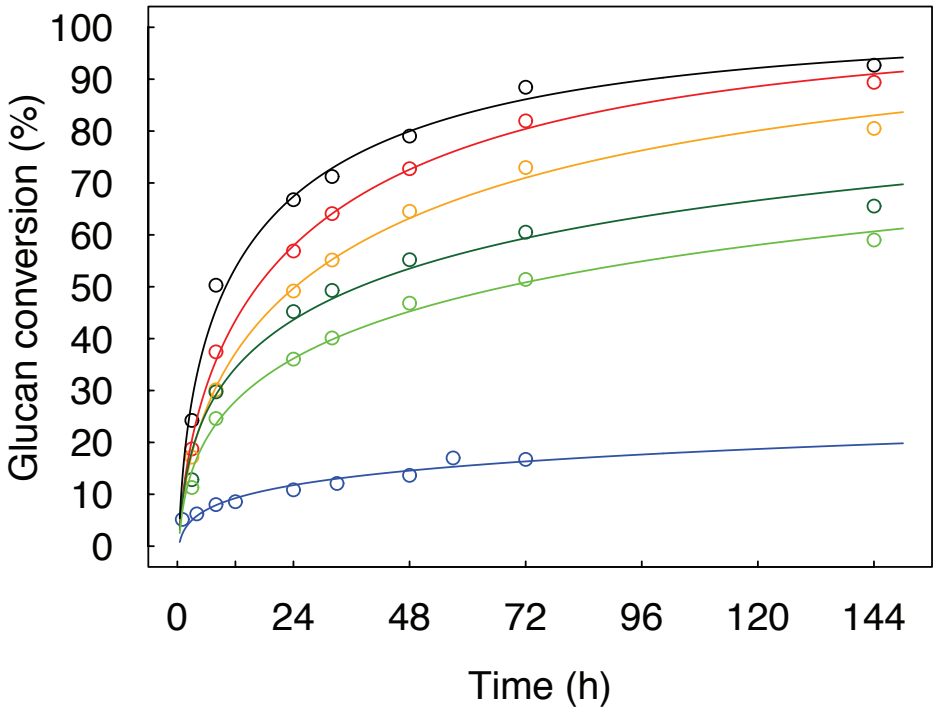

Figure 4. Time course of enzymatic hydrolysis. Points represent the experimental data, lines represents fittings and are based on Eq. 2. Black Avicell, Red OSHH1, orange OSHH2, dark green OSHH3, green OSHH4 and blue untreated HH. Enzyme loading was 20 FPU g^{-1}.

Lignin re-deposition during biomass pretreatment is usually observed [13]. This phenomenon was also considered and extraction of re-deposited lignin

was carried out for samples OSHH1 and OSHH3. The dependence of *k* and *h* as a function of lignin content in OSHH is shown in Fig. 5.

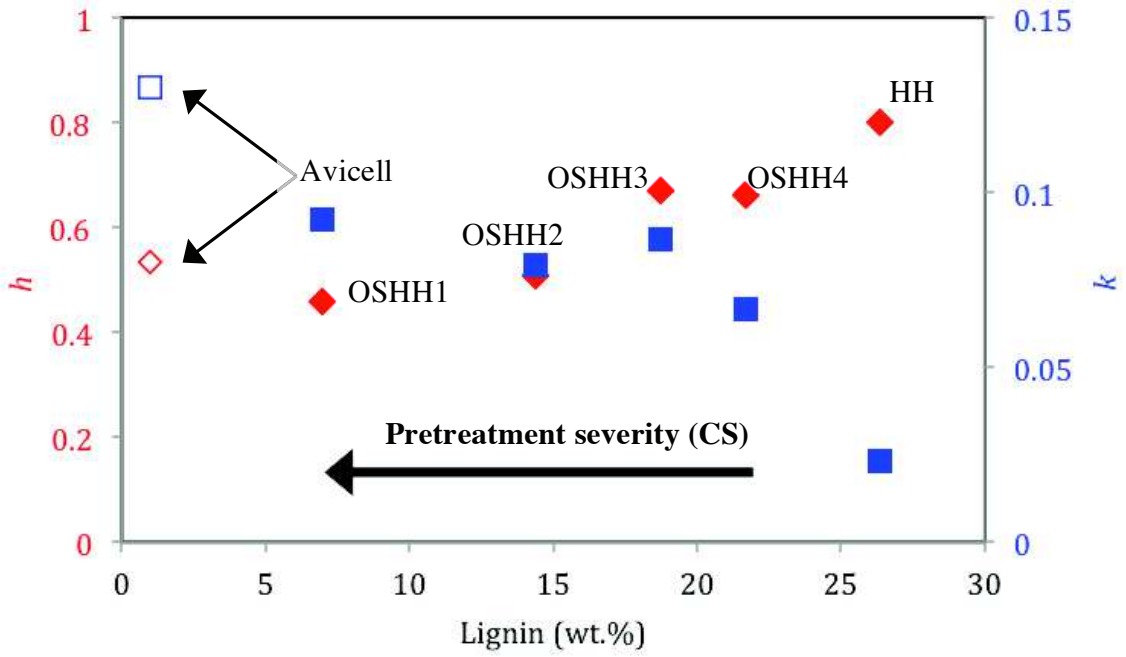

Figure 5. Relationship of fractal exponent (*h*) and rate constant (*k*) to the lignin content of OSHH and HH samples. Values obtained from Avicell hydrolysis (open symbols) were used as reference.

The calculated fractal exponent for Avicell is relatively low 0.53 while that of untreated hemp hurds is high 0.80; this shows the dependence of the *h* parameter to the nature of substrate. Comparing the *h* values obtained from sample OSHH2 and OSHH4 (different pretreatment CS) an increase from 0.50 to 0.66 was obtained. This markedly affected the degree of cellulose conversion, which decreased from 80.5 to 60.0%. Differently, comparing samples OSHH1 and OSHH2 (same pretreatment CS), which have different lignin content (Tab. 1) the obtained *h* values were similar 0.45 and 0.50 respectively, and the final degrees of cellulose conversion were close (Fig. 4). Likewise, samples pretreated at CS of 0.6 (OSHH3 and OSHH4) shows

the same behavior. Therefore, lignin re-deposition seems not affect the fractal parameter as residual lignin content do. Nevertheless, the Soxhlet extracted samples gave higher hydrolysis degree (Fig. 4).

To better understand the singular and mutual effect of fractal parameters on enzymatic hydrolysis more data are needed. As the enzymatic hydrolysis process is strongly related both to enzymes behaviors and substrate structural features, which are heavily modified during pretreatment; the study of enzymatic adsorption profiles, cellulose crystallinity index and substrate surface accessibility of pretreated samples, will provide a better overview of the process. Moreover, in this way the relationship of *h* and *k* parameters, to the enzymes and substrate behaviors, could be evaluated.

Conclusion

Fractal-like kinetic model has been successfully applied to study the time course of enzymatic saccharification of Avicell and organosolv pretreated hemp hurds. The good fitness of the experimental data indicates that the model can be used to describe the complex profile of enzymatic hydrolysis with two variables *k* and *h*.

References

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4 Discussion

Renewable production of chemicals can provide a great variety of benefits ranging from reducing environmental impact to development of a green economy, which bypass the use of fossil source as a feedstock. Research and innovations are needed along the entire development pipeline, beginning with the biomass sources that will serve as inputs, till the recognition of platform chemicals that could satisfy a sizable share of the market. Waste constitutes an enormous potential resource, among them lignocellulosic biomass residues are estimated to exceed 2×10^{11} ton per year worldwide. Such feedstocks could provide the basis of bio-renewable chemical production by means of biorefinery facilities. Fermentation of sugars, to usefully platform chemicals is a relatively robust and efficient industrial process. However, bulk production of low-price sugars from sustainable source (i.e. lignocellulosic residue) is a main objective and bottleneck of second-generation biorefineries. Advances over the past several years have resulted in a deeper understanding of the impact of lignin and polysaccharide structure on recalcitrance and cellulose digestibility of lignocellulosic biomass. Nonetheless, many challenges remain in developing a strong industrial process to deconstruct lignocellulosic biomass to liberate these sugars.

Whit this aim, the objective of this PhD project has been the study of an integrated process for the fermentative production of platform chemicals (i.e. butanol and lactic acid) from a lignocellulose material, hemp hurds (HH). Beginning with a detailed feedstock compositional analysis, the valuable structural components of lignocellulose (i.e. cellulose, hemicellulose and lignin) has been separated and recovered by organosolv pretreatment (OS) optimization and by enzymatic hydrolysis step.

Like the petroleum refinery industry, a biorefinery's ultimate goal is to make value from all the functionality of the feedstock. Accurate compositional analysis enabled the identification and quantification of polymers and molecules in the feedstock. HH showed high carbohydrates content (~75%) and a very low amount of ash (1.2%); the former value is of importance as it decreases the value of the feedstock. Furthermore, the HH saccharidic fraction has been fully characterized and glucose (57%) and xylose (31%) has been identified as major monosaccharides. Beside major components content, that is of main importance as they are proportional to the yields of the platform to be produced, minor components, can become significant at industrial-scale level. Although some of these compounds such as proteins and sterols could represent a side income for biorefinery, their presence is considered to have a negative effect during the pretreatment: accordingly a removal step is required, this increase the process costs. The total amount of hydrophobic and hydrophilic extractives found was very low (4%); furthermore, no valuable compounds were identified. Results from this PhD study disclosed the potential of HH as a substrate for biorefinery.

Pretreatment processes, suitable for an integrated biorefinery should afford the selective separation of each component of the lignocellulosic biomass and allow the easy isolation and high-yield recovery of fractionated compounds that should therefore be ready to be converted into valuable products with additional minimal purification steps. During this PhD study, the effect of OS process variables (i.e. temperature, catalyst, reaction time and solvent concentration) on HH has been evaluated under different pretreatment severities (CS), ranging from -0.5 to 1.6. The degree of biomass solubilization, the amount of xylose recovered in the process liquid, the

production of inhibitory compounds, the lignin removal and the enzymatic hydrolysis of pretreated biomass showed a good correlation with the CS, thus allowing to obtain a desirable separation of the components. Additionally, the quality of the produced streams has been shown to be controllable and adjustable by tuning process variables. In order to evaluate the effect of OS on biomass saccharification the time course of enzymatic hydrolysis pretreated HH has been carried out. The fractal-like model has been successfully applied to the experimental data; the kinetic analysis highlighted that the increased process severity resulted in a higher rate constant. These results suggested that OS pretreatment improved substrate accessibility towards cellulolytic enzymes compared to untreated samples. Moreover, the sample obtained at CS of 1.3 showed fractal parameters (*k* and *h*) close to those obtained using pure cellulose as substrate. The results of this PhD project highlighted the potential of OS pretreatment as leading technology for the effective lignocellulosic biomass fractionation.

OS pretreatment at CS of 1.3 allowed obtaining the best results in terms of glucan recovery (98%), enzymatic hydrolysis of pretreated biomass (70%) and hemicellulosic sugars recovery (61%). Moreover, the generated low concentration of microorganism inhibitory compounds, such as furfural, HMF and levulinic acid in the hemicellulosic stream permitted its direct fermentation without any detoxification step.

During this PhD study, lactic acid (LA) was used as an example for bulk chemical production from HH-derived sugars streams. Non sterile fermentation of the C6 and C5 sugars by *B. coagulans* strain XZL4 has been resulted in high LA yields (0.90 and 0.84 g g^{-1}), high titers (141 and 109 g L^{-1}), and high enantiomeric excess (> 99%). Overall, 42 g of L-LA were

obtained from 100 g of raw hemp hurds. These results can be considered promising for HH valorization toward the production of polymer-grade LA.

In conclusion, the results of this PhD project demonstrated that the proposed process represents a suitable biorefinery approach for platform chemicals production because of significant yields in the recovery of main components from lignocellulosic biomass. Further investigations using different mixtures of lignocellulosic residues will help to exploit the potential of the process, whereas the upgrading of the lignin fraction, into aromatics building blocks, will help to increase the economy of the process making it industrially attractive.