Enzyme kinetics of cellulose hydrolysis of *Miscanthus* and oat hulls

Ekaterina I. Makarova¹ · Vera V. Budaeva¹ · Aleksey A. Kukhlenko¹ · Sergey E. Orlov¹

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Abstract Experiments were done to model enzymatic hydrolysis of *Miscanthus* and oat hulls treated with dilute solutions of nitric acid and sodium hydroxide in direct and reverse sequences. The enzymatic hydrolysis kinetics of the substrates was studied at an initial solid loading from 30 to 120 g/L. The effects of feedstock type and its pretreatment method on the initial hydrolysis rate and reducing sugar yield were evaluated. The fitting results by the developed models showed good agreement with the experimental data. These models designed for developing the production technology of concentrated glucose solutions can also be applied for glucose fermentation into ethanol. The initial solid loading of 60–90 g/L provides the reducing sugar concentration of 40–80 g/L necessary for ethanol synthesis. The kinetic model can also be applied to investigate enzymatic hydrolysis of other substrates (feedstock type, pretreatment method) under the similar conditions used herein, with adjusted empirical coefficient values.

Keywords *Miscanthus* · Oat hulls · Cellulose · Enzymatic hydrolysis · Kinetics · Mathematical model

Abbreviations

C_{ES} Concentration of enzyme–substrate complex (g/L)
 C_m Equilibrium concentration of reducing sugars (g/L)

C_P Concentration of reducing sugars (g/L)
 C_S Concentration of substrate (g/L)
 k_1 Formation constant of enzyme–substrate complex (h^{-1})
 k_2 Breakdown constant of enzyme–substrate complex [g/(L h)]
 k_3 Formation constant of reducing sugars [g/(L h)]
 k_m Michaelis constant (g/L)
 k_s Disassociation constant of enzyme–substrate complex (g/L)
NAM Nitric-acid method
CM Combined method
RS Reducing sugars
MC *Miscanthus* cellulose
OHC Oat hull cellulose

Introduction

Sustainable production of cellulosic ethanol from ligno-cellulose materials is one of the greatest challenges in biorefinery (Sun et al. 2016). A key step in the conversion of lignocellulosic biomass into biofuels is the pretreatment of feedstocks to facilitate the hydrolysis of polysaccharides into monomeric sugars, which can then be converted into fuel (Himmel et al. 2007; Oh et al. 2015). The main goal of pretreatment is to increase the enzyme accessibility improving digestibility of cellulose. Each pretreatment has a specific effect on cellulose, hemicellulose and lignin fraction; different pretreatment methods and conditions should therefore be chosen according to the process configuration selected for the subsequent hydrolysis and fermentation steps (Alvira et al. 2010). The feedstock is

✉ Ekaterina I. Makarova
makarova@ipcet.ru

¹ Laboratory of Bioconversion, Laboratory of Chemical Engineering Processes and Apparatuses, Institute for Problems of Chemical and Energetic Technologies, Siberian Branch of the Russian Academy of Sciences (IPCET SB RAS), Biysk, Altai Krai, Russia 659322

processed with different reagents ranging from ionic liquids to supercritical dioxide and hydrothermally (without using reagents) (Shill et al. 2012; Park et al. 2001; Rocha et al. 2017). In particular, the combined pretreatment strategies have been reviewed for improving the enzymatic hydrolysis of lignocellulose and realizing the comprehensive utilization of lignocellulosic materials (Sun et al. 2016). As to *Miscanthus*, various pretreatments have been tried to produce substrates: sulfuric acid/ethanol/water; formic acid/acetic acid/water; formic acid/hydrogen peroxide/water; aqueous NaOH; ethylenediamine/DMSO; ethylenediamine/1-butyl-3-methylimidazolium dimethyl phosphate; 1-ethyl-3-methylimidazolium acetate with ammonia and/or oxygen; autohydrolysis in water with and without 2-naphthol; ozone/ethanol and electrolyzed water with or without alkaline peroxide; aqueous ammonia with or without hydrogen peroxide (Yu et al. 2014); various alkalis and acids (Si et al. 2015); and concentrated sodium benzoate solution (Pavlov et al. 2015; Denisova et al. 2016). Although the combined pretreatment methods uniting biological, chemical, physical and other techniques have been reported (Sun et al. 2016), there is no account on pretreatment of a feedstock, particularly *Miscanthus* and oat hulls, in two stages: with dilute solutions of such simple and cheap chemicals as nitric acid and sodium hydroxide in direct and reverse sequences. We chose the two-stage pretreatment because we strived to obtain chiefly a glucose hydrolyzate.

Enzymatic hydrolysis is known to significantly contribute to the cost of cellulosic ethanol and, from the economic perspective, improvement in enzymatic hydrolysis is a prerequisite. The major obstacles to enzymatic hydrolysis are low reaction rate, high enzyme cost, low product concentration, and incomprehension of the cellulose kinetics on lignocellulosic substrates. One way to overcome this problem is to run enzymatic hydrolysis with a high consistence of the insoluble solid. However, the saccharification reaction at a high insoluble solid consistence will have to encounter the problems of increased viscosity, higher energy input for stirring, shear inactivation of cellulases, and poor heat transfer due to rheological properties of the dense fibrous suspension (Gupta et al. 2012; Ioelovich and Morag 2012).

Considerable interest in kinetics of biotechnological processes stems from the necessity to scientifically predict the complicated system in a production environment (Ye and Berson 2011; Fan et al. 2015; Peri et al. 2007; Prathyusha et al. 2016). The topicality of investigating the enzymatic hydrolysis kinetics of substrates is governed by the necessity to predict the reactivity of pretreated lignocellulosic feedstock (Gonzalez et al. 1989; Marcos et al. 2013). In this respect, there are several reports on enzymatic saccharification, which mainly deal with the

development of appropriate kinetic models to describe the phenomena (Shill et al. 2012; Kadam et al. 2004; Zhang et al. 2014). Several kinetic models of enzymatic hydrolysis of cellulose have been developed and reviewed (Park et al. 2001; Sousa et al. 2011). They include non-mechanistic, semi-mechanistic, functionally, and structurally based models, applied to different enzymes and lignocellulosic materials (Park et al. 2001). Non-mechanistic models, which are not based on a definable enzyme/substrate interaction model, can be very useful for data correlation, although they do not enhance the phenomenological understanding of the system. The most common semi-mechanistic approach is based on pseudo-homogeneous Michaelis–Menten model: a definable enzyme/substrate interaction model, but use only concentrations as the substrate state variable and/or with only one overall enzymatic activity (Sousa et al. 2011). Functionally based models go further, including additional substrate state variables, such as crystallinity, and considering the action of multiple enzymes. Nevertheless, functionally based models can lead to an overwhelming number of parameters, demanding a large amount of experimental data for a consistent model-fitting and validation procedure that may discourage their application (Sousa et al. 2011; Bansal et al. 2009).

A kinetics study at high substrate loadings is necessitated by scaling up enzymatic hydrolysis by volume and creating the industrial technology. There also exists an opinion that enzymatic hydrolysis of biomass at a high solid loading (>10% w/w dry mass) has become increasingly important as a key step in the production of second-generation bioethanol (Olsen et al. 2011; Alvira et al. 2013).

The present work aimed to experimentally explore the enzyme kinetics of hydrolysis of lignocellulosic substrates obtained by pretreatment of *Miscanthus* and oat hulls with dilute solutions of nitric acid and sodium hydroxide in direct and reverse orders at different solid loadings ranging from 30 to 120 g/L.

Materials and methods

Feedstock and enzyme

The utilization of non-woody raw materials, say energy crops or crop residues, as the source of cellulose arouses a growing interest among researchers. *Miscanthus* (*Miscanthus sinensis* Andersson) is an energy crop capable of annually producing 10–15 ton/ha/year of dry biomass in the same field for 15–20 years, which is equivalent to 4–6 ton/ha of pure cellulose (Jones and Walsh 2001; Shumny et al. 2010). Oat hulls (*Avena sativa*) are an

Table 1 Enzymatic activities of enzymes

Enzyme	Enzymatic activity
CelloLux-A (cellulose-standardized)	Cellulase: 2000 CMCase AU/g
	Xylanase: 8000 X AU/g
	β -Glucanase: 1500 β -gl AU/g
BrewZyme BGX (hemicellulose-standardized)	Cellulase: 2100 CMCase AU/g
	Xylanase: 4200 X AU/g
	β -Glucanase: 530 β -gl AU/g

CMCase AU/g carboxymethylcellulase activity units per gram, X AU/g xylanase activity units per gram, β -gl AU/g β -glucanase activity units per gram

abundant and available raw source in agricultural regions of the world. The high cellulose content (up to 35%), worldwide availability, and “zero” cost of oat hulls determine their potential use as a substrate for enzymatic hydrolysis (Chaud et al. 2012; Yadav et al. 2016).

For enzymatic hydrolysis, an enzyme cocktail of CelloLux-A (in the form of powder, Sibbiopharm Ltd, Russia) and BrewZyme BGX (in the form of solution, Tarchomin Pharmaceutical Works Polfa S.A., Poland), standardized against cellulase, xylanase and β -glucanase activities in compliance with the certificates of analysis, was used. The enzyme cocktail was injected as follows: CelloLux-A 40 FPU/g solid and BrewZyme BGX 15 FPU/g solid. FPU was determined by the international procedure (Ghose 1987). The enzyme activities are given in Table 1.

Pretreatment of feedstock

The cellulose specimens were obtained by chemical treatment of *Miscanthus* and oat hulls by the nitric-acid method (NAM) and combined method (CM) at our pilot production site. In the nitric-acid process, the feedstocks were subjected to successive treatment with dilute solutions of nitric acid (4% (w/w) in a solid-to-liquid ratio of 1:20 (w/v) under atmospheric pressure at 94–96 °C for 4–8 h) and sodium hydroxide (2% (w/w) in a solid-to-liquid ratio of 1:20 w/v) under atmospheric pressure at 94–96 °C for 2–4 h (Gismatulina et al. 2015). The combined method used the same reagents but in reverse order: 4% (w/w) sodium hydroxide solution in a solid-to-liquid ratio of 1:20 (w/v) under atmospheric pressure at 94–96 °C for 4–10 h and then 4% (w/w) nitric acid solution in a solid-to-liquid ratio of 1:20 (w/v) under atmospheric pressure at 94–96 °C for 2–8 h (Budaeva et al. 2016). Both methods employ the simple and available reagents, nitric acid and sodium hydroxide, more specifically dilute solutions thereof, as well as standard equipment for chemical treatment.

Enzymatic hydrolysis

In the enzymatic hydrolysis experiments, a weighed portion of the substrate (cellulose), 0.15 dm³ acetate buffer (pH = 4.7), and the enzymes calculated as 0.004 kg CelloLux-A/kg substrate and 0.004 dm³ BrewZyme BGX/kg substrate were used. The hydrolysis was run at 45 ± 2 °C under continuous stirring on a PE-6410 M horizontally oriented shaking platform (ECROS, Russia) at 150 rpm. To monitor the concentration of reducing sugars in the hydrolyzate, samples of 0.002 dm³ in volume were collected in every 8 h. After the enzymatic hydrolysis was completed (72 h), the reaction mass was filtered to give a ready-to-use hydrolyzate and a solid residue of the unreacted substrate. Each experiment was run in triplicate under the same conditions.

Analytical methods

The α -cellulose content of the substrates was determined by treating the celluloses with a 17.5% NaOH solution for 45 min and washing with a 9.5% NaOH solution (Obolenskaya et al. 1991). Klason lignin (acid-insoluble) was quantified pursuant to TAPPI T222 om-83 (1999). Pentosans were transformed in boiling 13% HCl solution to furfural, which was collected in the distillate and determined on a xylose-calibrated UNICO UV-2804 spectrophotometer (United Products & Instruments, USA) at a wavelength of 630 nm using the orcinol-ferric chloride reagent (Obolenskaya et al. 1991). The ash content was quantified by incineration of the celluloses at 600 °C for 3 h (Obolenskaya et al. 1991).

The index of crystallinity was estimated by the Ruland method following X-ray diffraction analysis (a DRON-3 M diffractometer, Bourestnik, Inc., Russia, using FeK α radiation with a pyrolytic graphite monochromator) (Thygesen et al. 2005).

The cellulose degree of polymerization was determined from the viscosity of solutions in cadoxene on a VPZh-3 viscometer (ECROS, Russia) with a capillary of 0.92 mm in diameter.

The concentration of reducing sugars calculated as glucose in the hydrolyzate was measured spectrophotometrically (UNICO UV-2804 spectrophotometer) using the 3,5-dinitrosalicylic acid (DNS) reagent (Miller 1959). This method relies on the reduction of DNS by the reducing sugar to 3-amino-5-nitrosalicylic acid which is then determined spectrophotometrically at a wavelength of 530 nm. The absolute (systematic) error in measuring the concentration of reducing sugars was 0.02 g/L in the range of 0.2–2.0 g/L.

The yield of reducing sugars was calculated with allowance for a coefficient of 0.90 attributed to the water

molecule addition to anhydroglucose residues of the corresponding monomer units as a result of enzymatic hydrolysis.

Kinetics modeling

Mathematical models describing the enzymatic hydrolysis kinetics of the substrates were built from the experimental data using the modified Michaelis–Menten equation suggested by Briggs and Haldane (1925). The enzymatic hydrolysis reaction under question was carried out under unsteady-state conditions of the system, which is due to inhibition of the hydrolysis by the excess substrate. Under such conditions, it is inadmissible to ignore the change in concentration of the enzyme–substrate complex.

The initial rate of the formation of reducing sugars was calculated by the method of double inverse coordinates using the Michaelis–Menten relationship:

$$v_0 = \frac{V_m S_m}{K_m + S_m},$$

where v_0 —initial rate; V_m —maximum rate.

The reaction rate constants were estimated through approximation of the experimental data by the least squares method (Hastie et al. 2009). The effect of cellobiose on enzymatic hydrolysis was disregarded in the modeling. Verification was performed by comparing the calculated data with the experimental. The adequacy of the mathematical model was confirmed by statistical manipulation of the theoretical and experimental data.

Results and discussion

Physicochemical characterization of substrates

The *Miscanthus* and oat hull celluloses (MC and OHC) were composed mainly of hydrolyzable ingredients (Table 2): 90–92% cellulose and 2–7% pentosans. The minor ingredients were non-hydrolyzable impurities: 0.5–3.6% residual lignin and 0.1–4.2% ash content. The

cellulose specimens obtained by the combined method were observed to have a higher content of hemicelluloses because of the decreased time of the nitric-acid treatment step. The cellulose samples obtained from the same feed-stock but by the different methods had similar degrees of polymerization, which allows us to assume that the pre-treatment method has no impact on this parameter.

Enzyme kinetics of cellulose hydrolysis

The concentration and yield of reducing sugars in 72 h of enzymatic hydrolysis as a function of the initial loading of the four substrates (*Miscanthus* and oat hull celluloses) are summarized in Table 3. For all of the initial substrate loadings, the yields of reducing sugars ranged from 44 to 92%. For all of the cellulose samples, increasing the initial solid loading from 30 to 120 g/L was found to decrease the yield of reducing sugars by a factor of 1.3–1.5, which was due to the substrate inhibition. It is obvious that the concentration of reducing sugars (40–80 g/L) required for the ethanol synthesis is achieved in the initial solid loading range of 60–90 g/L.

According to the classical Michaelis–Menten theory of enzymatic catalysis, the end product (reducing sugars) is generated from the substrate (cellulose) through the formation of the enzyme–substrate complex, the formation reaction of the enzyme–substrate complex being characterized by formation rate constant k_1 , breakdown rate constant k_2 , and formation rate constant k_3 of end product P . Such a mechanism is described by the following chemical reaction equation:

where S —substrate, E —enzyme; ES —enzyme–substrate complex; P —product (Briggs and Haldane 1925).

In such a case, having denoted the concentrations of the starting and intermediate substances and of the reaction product (reducing sugars) as C_S , C_{ES} , and C_P , respectively, an equation system can be written to describe the enzymatic hydrolysis kinetics. When mathematically written,

Table 2 Physicochemical properties of cellulose specimens

Attributes	<i>Miscanthus</i>		Oat hulls	
	NAM	CM	NAM	CM
α -Cellulose (%)	90.3 $\pm$ 0.4	90.5 $\pm$ 0.4	91.0 $\pm$ 0.4	92.3 $\pm$ 0.4
Residual lignin (%)	3.6 $\pm$ 0.1	1.4 $\pm$ 0.1	0.8 $\pm$ 0.1	0.5 $\pm$ 0.1
Ash (%)	4.2 $\pm$ 0.0	0.7 $\pm$ 0.0	5.8 $\pm$ 0.0	0.1 $\pm$ 0.0
Pentosans (%)	1.7 $\pm$ 0.1	6.4 $\pm$ 0.1	2.0 $\pm$ 0.1	6.9 $\pm$ 0.1
Degree of polymerization	660 $\pm$ 10	760 $\pm$ 10	1140 $\pm$ 10	1140 $\pm$ 10
Index of crystallinity (%)	70 $\pm$ 5	65 $\pm$ 5	67 $\pm$ 5	62 $\pm$ 5

Table 3 Concentration and yield of reducing sugars (RS) in 72 h of enzymatic hydrolysis versus initial solid loading from 30 to 120 g/L

Hydrolysis time (h)	RS concentration (g/L)							
	<i>Miscanthus</i> cellulose (NAM)				<i>Miscanthus</i> cellulose (CM)			
	30	60	90	120	30	60	90	120
8	7.8 ± 0.2	14.8 ± 0.2	19.6 ± 0.2	25.1 ± 0.5	13.8 ± 0.2	22.7 ± 0.5	28.4 ± 0.5	26.0 ± 0.5
16	13.3 ± 0.2	18.9 ± 0.2	23.5 ± 0.5	27.3 ± 0.5	22.4 ± 0.5	32.7 ± 0.5	42.5 ± 0.5	47.1 ± 0.5
32	18.0 ± 0.2	26.7 ± 0.5	36.3 ± 0.5	41.1 ± 0.5	27.3 ± 0.5	45.5 ± 0.5	58.3 ± 1.0	68.2 ± 1.0
40	19.6 ± 0.2	33.0 ± 0.5	41.8 ± 0.5	47.1 ± 0.5	28.3 ± 0.5	50.2 ± 0.5	63.1 ± 1.0	74.0 ± 1.0
48	21.0 ± 0.5	35.3 ± 0.5	46.0 ± 0.5	51.2 ± 1.0	29.0 ± 0.5	53.2 ± 1.0	67.2 ± 1.0	78.0 ± 1.0
64	22.2 ± 0.5	38.0 ± 0.5	50.0 ± 0.5	58.7 ± 1.0	30.0 ± 0.5	55.0 ± 1.0	71.8 ± 1.0	84.3 ± 1.0
72	22.2 ± 0.5	38.0 ± 0.5	50.0 ± 0.5	58.7 ± 1.0	30.0 ± 0.5	55.0 ± 1.0	72.0 ± 1.0	84.3 ± 1.0
RS yield (%)	66.7 ± 1.4	57.0 ± 0.8	50.0 ± 0.5	44.0 ± 0.8	90.0 ± 1.5	82.5 ± 1.5	72.0 ± 1.0	63.2 ± 0.8

Hydrolysis time (h)	Oat hull cellulose (NAM)				Oat hull cellulose (CM)			
	30	60	90	120	30	60	90	120
	30	60	90	120	30	60	90	120
8	11.5 ± 0.2	20.2 ± 0.2	22.2 ± 0.5	40.0 ± 0.5	22.8 ± 0.5	26.9 ± 0.5	38.4 ± 0.5	39.3 ± 0.5
16	17.0 ± 0.2	28.5 ± 0.5	38.4 ± 0.5	53.1 ± 1.0	27.8 ± 0.5	35.2 ± 0.5	52.5 ± 1.0	60.4 ± 1.0
32	23.2 ± 0.5	38.7 ± 0.5	59.1 ± 1.0	65.1 ± 1.0	30.0 ± 0.5	45.3 ± 0.5	68.3 ± 1.0	81.3 ± 1.0
40	25.3 ± 0.5	41.7 ± 0.5	66.5 ± 1.0	72.0 ± 1.0	30.4 ± 0.5	48.3 ± 0.5	73.1 ± 1.0	87.3 ± 1.0
48	26.5 ± 0.5	46.0 ± 0.5	70.9 ± 1.0	79.5 ± 1.0	30.5 ± 0.5	52.7 ± 1.0	77.2 ± 1.0	91.3 ± 1.0
64	27.5 ± 0.5	53.7 ± 1.0	77.6 ± 1.0	87.5 ± 1.0	30.8 ± 0.5	60.0 ± 1.0	81.3 ± 1.0	96.9 ± 1.0
72	27.5 ± 0.5	53.8 ± 1.0	77.9 ± 1.0	87.5 ± 1.0	30.8 ± 0.5	60.0 ± 1.0	81.3 ± 1.0	96.9 ± 1.0
RS yield (%)	82.5 ± 1.5	80.7 ± 1.5	77.9 ± 1.0	65.6 ± 0.8	92.4 ± 1.5	90.0 ± 1.5	81.3 ± 1.0	72.7 ± 0.7

the equations describing enzymatic hydrolysis of all the cellulose specimens will be identical and be differing only in values of coefficients k_1 , k_2 , k_3 , and C_m (C_m —equilibrium concentration of reducing sugars).

At the initial instant $t = 0$: $C_S = C_m$ and $C_{ES} = C_P = 0$.

The kinetic equations describing the substrate transformation into reducing sugars will be written as follows:

$$\begin{aligned}\frac{dC_S}{dt} &= -k_1 C_S + k_2 C_{ES} \\ \frac{dC_{ES}}{dt} &= k_1 C_S - (k_2 + k_3) C_{ES} \\ \frac{dC_P}{dt} &= k_3 C_{ES}\end{aligned}\quad (2)$$

The equation system (2) is solved via the expressions (Briggs and Haldane 1925):

$$C_S = C_m \left\{ \frac{\lambda_2 - k_1^{-1} k_3}{\lambda_2 (\lambda_2 - \lambda_3)} \exp(-\lambda_2 k_1 t) + \frac{k_1^{-1} k_3 - \lambda_3}{\lambda_3 (\lambda_2 - \lambda_3)} \exp(-\lambda_3 k_1 t) \right\} \quad (3)$$

$$C_{ES} = \frac{C_m}{\lambda_2 - \lambda_3} [\exp(-\lambda_2 k_1 t) - \exp(-\lambda_3 k_1 t)], \quad (4)$$

$$\begin{aligned}\text{where } \lambda_2 &= \frac{1}{2}(\alpha + \beta); \quad \alpha = 1 + \frac{k_2}{k_1} + \frac{k_3}{k_1}; \quad \lambda_3 = \frac{1}{2}(\alpha - \beta); \\ \beta &= \left(\alpha^2 - \frac{4k_3}{k_1} \right)^{0.5}.\end{aligned}$$

To build the mathematical model, it is necessary that C_m , k_1 , k_2 , and k_3 values be determined. The reaction rate coefficients, k_1 , k_2 , and k_3 , are dependent on the substrate nature and ambient conditions (temperature, pH, substrate stirring conditions during enzymatic hydrolysis, etc.), which were unchanged. Since the enzyme in this work was in excess (it was decided to use the enzymes in excess because they were procured 1.5 years prior to the experiments), those coefficients do not depend on the enzyme-to-substrate ratio. Coefficient C_m , besides the aforesaid influencing factors, is also dependent on the initial substrate loading; therefore, these circumstances were taken into account in finding the coefficients of the equation system for *Miscanthus* and oat hull celluloses. The experimental data were processed as follows: Eqs. (3) and (4) were combined into Eq. (5) that describes the change in the concentration of reducing sugars:

$$C_P = C_m - (C_S + C_{ES}) \quad (5)$$

Equation (5) was used as an approximating function that defines the models' coefficients for the substrates. The approximation of the experimental data listed in Table 3 was performed via Eq. (5) by the least squares method (Hastie et al. 2009).

The calculation of the mathematical models' coefficients allowed us to have determined disassociation

Table 4 Calculated coefficients and constants of mathematical models for enzymatic hydrolysis of substrates

Coefficient	<i>Miscanthus</i> cellulose (NAM)				<i>Miscanthus</i> cellulose (CM)			
	30	60	90	120	30	60	90	120
C_m , g/L	24.90	40.76	53.78	62.28	32.43	56.79	73.30	84.81
k_1 , h ⁻¹	0.269				0.323			
k_2 , g/(L h)	1.378				1.359			
k_3 , g/(L h)	0.273				0.330			
k_s , g/L	5.125				4.208			
k_m , g/L	6.141				5.228			
	Oat hull cellulose (NAM)				Oat hull cellulose (CM)			
	30	60	90	120	30	60	90	120
C_m , g/L	29.73	53.31	79.33	90.72	33.78	56.69	81.12	95.80
k_1 , h ⁻¹	0.299				0.363			
k_2 , g/(L h)	1.369				1.348			
k_3 , g/(L h)	0.305				0.370			
k_s , g/L	4.577				3.715			
k_m , g/L	5.598				4.733			

constant k_s of the enzyme–substrate complex (suggested by Michaelis and Menten) and Michaelis constant k_m as suggested by Briggs and Haldane (1925):

$$k_s = k_2/k_1;$$

$$k_m = k_s + k_3/k_1$$

It follows from the results summarized in Table 4 that when the substrate loading is increased, the C_m value characterizing the final concentration of reducing sugars is higher for the substrates obtained by the combined method than for those obtained by the nitric-acid method. That is, the former substrates are transformed into reducing sugars in a higher yield.

Figure 1 illustrates a graphical interpretation of the results from the numerical modeling in the form of particular solutions to Eq. (5) with superimposed experimental points.

It follows from the results in Fig. 1 that the built mathematical models fit well with the experimental data. The reproducibility of all the experimental results has been corroborated through the Cochran test. The adequacy of the models constructed from Eq. (5), with the model coefficients in Table 3 taken into account, has been validated by the Fisher criterion (Hastie et al. 2009). The calculated Fisher criterion values for the models describing the enzymatic hydrolysis of the *Miscanthus* cellulose (MC) and oat hull cellulose (OHC) were as follows:

$$F_{MC(NAM)} = 1.24 < F_{table} = 1.76; F_{MC(CM)} = 1.67 < F_{table} = 1.78$$

$$F_{OHC(NAM)} = 1.38 < F_{table} = 1.76; F_{OHC(CM)} = 1.60 < F_{table} = 1.76, \text{ at the significance level } \alpha = 0.05.$$

In doing so, the reproducibility (rep.) variance for the models was as follows:

$$\sigma_{MC(NAM)rep.}^2 = 5.15; \sigma_{MC(CM)rep.}^2 = 4.73;$$

$$\sigma_{OHC(NAM)rep.}^2 = 7.74; \sigma_{OHC(CM)rep.}^2 = 6.64, \text{ with the number of degrees of freedom of 36.}$$

The adequacy (adeq.) variance for the models was as follows:

$$\sigma_{MC(NAM)adeq.}^2 = 6.38; \sigma_{MC(CM)adeq.}^2 = 2.84;$$

$$\sigma_{OHC(NAM)adeq.}^2 = 10.68; \sigma_{OHC(CM)adeq.}^2 = 10.64, \text{ with the number of degrees of freedom: } f_{MC(NAM,CM)adeq.} = f_{OHC(NAM,CM)adeq.} = 36 \text{ (Ioelovich 2015).}$$

The values of the confidence intervals of the reducing sugar concentrations at the significance level $\alpha = 0.05$ were as follows: $\Delta_{MC(NAM)} = \pm 3.83 \text{ kg m}^{-3}$; $\Delta_{MC(CM)} = \pm 3.68 \text{ kg m}^{-3}$; $\Delta_{(NAM)} = \pm 4.70 \text{ kg m}^{-3}$; $\Delta_{OHC(CM)} = \pm 4.35 \text{ kg m}^{-3}$.

The determination coefficient for the built mathematical models is within $R^2 = 0.85\text{--}0.93$, which attests to a good convergence between the calculated and experimental data.

Thus, it can be deduced from the statistical manipulation of the experimental data and from their comparison with the calculated values of the functions of reducing sugar concentrations that the mathematical model constructed is verified.

Since the mathematical model coefficients were derived by approximation of the experimental data, the model

Fig. 1 Concentration (C) of reducing sugars versus enzymatic hydrolysis time for different solid loadings: *Miscanthus* cellulose (**a** NAM, **b** CM) and oat hull cellulose (**c** NAM, **d** CM). Experiment: white circle 30 g/L, filled circle 60 g/L, white square 90 g/L, filled square 120 g/L; calculation: straight line 30 g/L, 60 g/L, - - - - 90 g/L, 120 g/L

constraints in both cases were the vicinities of the initial substrate loading from 30 to 120 g/L and of the enzymatic hydrolysis time from 0 to 72 h.

In evaluating the efficiency of enzymatic hydrolysis of the substrates, the behaviors of the initial hydrolysis rate and of the reducing sugar yield should be considered jointly. It follows from the results listed in Table 3 that the relation between coefficients k_2 and k_1 (k_s value) for the NAM cellulose specimens is higher than for the CM celluloses. The k_s value is inversely proportional to the formation rate of the enzyme–substrate complex: the greater the k_s , the slower the formation of the enzyme–substrate complex. It can be inferred from the findings that the enzyme–substrate complex will be formed faster from the CM celluloses, which will lead to an increase in the formation rate of reducing sugars.

The formation rate of reducing sugars depends on the k_3 coefficient value and C_{ES} value, in which case the k_3 coefficient is higher for the enzyme–substrate complexes generated from the CM celluloses than for those generated from the NAM celluloses. Thus, the formation rate of

reducing sugars in the processes under study will be higher for the CM celluloses than for the NAM ones.

Figure 2 displays a graphical representation of the initial reaction rates of the processes under question.

It follows from the data in Fig. 2 that the initial hydrolysis rate is slower for the NAM celluloses than for the CM ones, regardless of the feedstock type and initial substrate loading ranging from 30 to 120 g/L. Here also, a tentative estimate is demonstrated for the initial rate of enzymatic hydrolysis, derived from the kinetic M–M equation.

By comparing in a similar fashion the celluloses obtained from the different feedstocks, it was found that the cellulose specimens produced from oat hulls exhibit a faster hydrolysis rate. The introduced mathematical model for the unsteady-state enzymatic hydrolysis at high loadings of the substrates obtained from the two different biomasses by the two chemical pretreatment methods incorporates new knowledge of the process under study. Coefficients k_1 , k_2 , k_3 , k_s , and k_m were found to be independent on the initial solid loading for the four substrates, in contrast to coefficient C_m . Among the cellulose

Fig. 2 Initial hydrolysis rate versus solid loading for **a** *Miscanthus* cellulose and **b** oat hull cellulose: solid line—NAM and dashed line—CM

specimens examined, oat hulls and combined method exhibit advantages as the substrate and pretreatment, respectively.

The present study has demonstrated advantages of oat hulls as substrate and of the combined method as pretreatment. The proposed mathematical model describes well the kinetics of the enzymatic hydrolysis process by the multi-enzyme complex (CelloLux-A + BrewZyme BGX) when the initial solid loading varies from 30 to 120 g/L at pH = 4.7, temperature of 45 ± 2 °C, under constant agitation.

Overall, the model introduced can be useful in investigating enzymatic hydrolysis of any substrates (feedstock type, pretreatment methods) under the similar conditions used herein, with refined values of the empirical coefficients.

The findings are novel in that a mathematical model has been developed for unsteady-state enzymatic hydrolysis at high loadings of the substrates obtained from the two feedstocks by the two different chemical pretreatments. The use of oat hulls as substrate and of the combined method as pretreatment has scientifically been justified as being the most promising.

The results presented herein are significant because they substantiate the choice of feedstock type, its pretreatment methods, and initial solid loading for subsequent, efficient transformation into bio-based products; more particularly, they are significant in that the mathematical model can be applied to other plant biomasses.

The estimated relationships can successfully be used in optimizing enzymatic hydrolysis and scaling up the process by volume.

Conclusion

The experiments conducted in this study to model enzymatic hydrolysis of cellulose specimens obtained from two different feedstocks by the nitric-acid method (NAM) and

combined method (CM) suggest that the oat hull substrates exhibit a higher hydrolysis rate. The initial rate of enzymatic hydrolysis was found to be greater for the CM cellulose samples than for the NAM ones, regardless of the feedstock type and initial solid loading ranging from 30 to 120 g/L. The reducing sugar concentration (40–80 g/L) needed for ethanol synthesis is achieved in the initial solid loading range of 60–90 g/L.

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Compliance with ethical standards

Conflict of interest The authors declare that there is no conflicts of interest regarding the publication of this article.

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