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Kinetic modeling of rapid enzymatic hydrolysis of crystalline cellulose after pretreatment by NMMO

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Abstract Pretreatment of cellulose with an industrial cellulosic solvent, *N*-methylmorpholine-*N*-oxide, showed promising results in increasing the rate of subsequent enzymatic hydrolysis. Cotton linter was used as high crystalline cellulose. After the pretreatment, the cellulose was almost completely hydrolyzed in less than 12 h, using low enzyme loading (15 FPU/g cellulose). The pretreatment significantly decreased the total crystallinity of cellulose from 7.1 to 3.3, and drastically increased the enzyme adsorption capacity of cellulose by approximately 42 times. A semi-mechanistic model was used to describe the relationship between the cellulose concentration and the enzyme loading. In this model, two reactions for heterogeneous reaction of cellulose to glucose and cellobiose, and a homogenous reaction for cellobiose conversion to glucose was incorporated. The Langmuir model was applied to model the adsorption of cellulase onto the treated cellulose. The competitive inhibition was also considered for the effects of sugar inhibition on the rate of enzymatic hydrolysis. The kinetic parameters of the model were estimated by experimental results and evaluated.

Keywords Enzymatic hydrolysis · Kinetic modeling · *N*-Methylmorpholine-*N*-oxide · Pretreatment · Substrate reactivity

List of symbols

E_b	Adsorbed cellulase (mg cellulase/l)
E_f	Free cellulase (mg cellulase/l)
K_{ads}	Dissociation constant (l/g cellulose)
S	Cellulose concentration (mg/ml)
C	Cellulose concentration at a given time (mg/ml)
C_0	Cellulose concentration at time zero (mg/ml)
E_{1b}	Bound concentration of cellulase on cellulose (mg protein/ml)
E_{if}	Concentration of free enzymes in solution (mg protein/ml) ($i = 1$ for cellulase; $i = 2$ for β-glucosidase)
E_{1T}	Total endogluconase/cellobiohydrolase concentration (mg protein/ml)
E_{2T}	Total β-glucosidase concentration (mg protein/ml)
G	Glucose concentration (mg/ml)
G_2	Cellobiose concentration (mg/ml)
k_{ir}	Reaction rate constants (ml/mg h), in which $i = 1$ for cellulose to cellobiose; $i = 2$ for cellulose to glucose; $i = 3$ for cellobiose to glucose
K_{iIG}	Inhibition constants of glucose on enzymes (mg/ml), in which $i = 1$, 2, and 3 (i is the same as that in k_{ir})
K_{iIG2}	Inhibition constants of cellobiose on enzymes (mg/ml), in which $i = 1$ and 2 (i is the same as that in k_{ir})
K_{3M}	Substrate (cellobiose) saturation constants (mg/ml)
r_i	Reaction rate (mg/ml h), where $i = 1$, 2, and 3 (i is the same as that in k_{ir})

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Introduction

Cellulose, the most abundant renewable resource, has a high potential for mass production of renewable biofuels and chemicals. However, the cellulose consists of linear chains of linked β-D-glucose units. It forms a highly ordered structure mainly due to the strong hydrogen bonding and as a result, is very resistant to enzymatic and microbial attacks. Therefore, the hydrolysis of cellulose is a main and challenging part of the process for economically feasible conversion of cellulose to fermentable sugar [14]. Cellulose hydrolysis process is usually performed by enzymes or chemicals. The enzymatic hydrolysis is preferred due to higher sugar yields, lower byproduct formation, and milder operation conditions [22, 23].

Since the sugar yield in hydrolysis of native celluloses is very low, pretreatment of cellulose prior to enzymatic hydrolysis is necessary in order to modify the structure of cellulose and render its enzymatic conversion. A number of pretreatments have been suggested and developed, including biological, physical, chemical, and physicochemical methods [7, 24, 28]. Among these processes, pretreatment with cellulose solvents are shown to be the most effective methods [12, 21].

N-methyl-morpholine-*N*-oxide (NMMO), a commercial cellulose solvent used in the Lyocell process for cellulose dissolution, has recently been investigated for the pretreatment of cellulose. NMMO-pretreatment improves the yield of enzymatic hydrolysis up to several orders of magnitude [12, 21]. Furthermore, in comparison to other pretreatment methods, it can be performed at milder operation conditions with lower energy consumption. In this process, it is possible to dissolve cellulose without any chemical derivatization [19], and recycle NMMO by more than 99% [10]. These advantages led to the development of a process for the separation of the cellulosic part of waste textiles for biofuels productions [9]. However, pretreatment with NMMO is still passing its primary development stages and so far, only a few researchers have investigated the different aspects of this process.

Developing a suitable kinetic model based on observable and macroscopic properties of the overall system can be used as a tool for design, optimization, and economic evaluation of the enzymatic hydrolysis [11]. To our knowledge, no kinetic study has been conducted regarding the enzymatic hydrolysis of NMMO-pretreated cellulose.

This study was aimed to study the kinetics of enzymatic hydrolysis of high crystalline cellulose, which was pretreated by NMMO. A kinetic model that incorporated the enzyme adsorption and end-product inhibition was used and experimentally evaluated.

Materials and methods

Materials

Defatted and bleached cotton linter (Apoliva Co., Sweden) was used as the high-crystalline cellulose in this study. The water content of cotton was determined as 3.3% by drying at 105°C until constant weight was reached. Two commercial enzymes were used for hydrolysis, including cellulase (Celluclast 1.5L, Novozyme, Denmark) and β-glucosidase (Novozyme 188, Novozyme, Denmark). The cellulase activity, measured according to Adney and Baker [1], was 72 FPU/ml. β-glucosidase activity was 257 CBU/ml according to the method presented by Ximenes et al. [26]. The enzyme protein content was determined by the Bradford method [4], and both of the enzyme solutions had the same protein content of 42 mg/ml.

NMMO pretreatment

A commercial NMMO (50% w/w, BASF, Ludwigshafen, Germany) was concentrated using a rotary vacuum evaporator (Heidolph, Germany) equipped with a vacuum pump (Wertheim, Germany) up to 85% w/w NMMO concentration. Cotton linter (3 g) was added to 97 g of 85% NMMO solution in an oil bath at 120°C and mixed with a glass rod for 2 h to dissolve the cellulose. It was then regenerated with the gradual addition of solution to 50 ml of hot water, while the suspension was mixed continuously. Afterwards, the pretreated cellulose was filtrated and washed with hot water until clear filtrate was observed. Finally, it was stored in a cold room at 4°C until use. The water content of the pretreated cellulose was determined to be 91% by drying at 105°C.

In a similar pretreatment, separation of cellulose from waste textiles was performed [9]. The waste textiles were a polyester/cotton (50/50%) and a polyester/viscose blend (40/60%). The textiles were cut into small pieces (approximately 3×3 cm^2), and its cellulose was dissolved in NMMO under the identical conditions as for the cotton linter. The polyester parts of the textiles were then separated from the solution using a metal sieve (1-mm pore size), and the dissolved cellulose was regenerated similar to cotton linter with hot water.

Enzymatic hydrolysis

The pretreated cellulose (1.2 g) was suspended in 40 ml citrate buffer (0.05 M, pH 4.8) in 125-ml screw-cap Erlenmeyer Flasks. Sodium azide (0.05%) was supplemented to inhibit microbial growth during the enzymatic hydrolysis [33]. The enzyme loadings were 15 FPU (Filter Paper Unit) cellulase and 30 CBU (cellobiase unit)

β-glucosidase per g cellulose. The hydrolysis was carried out at 50°C in a reciprocating shaker water bath with agitation speed of 120 rpm. All experiments were performed in duplicate. The sugar profiles were followed by sampling at predefined intervals, separation of the supernatants using centrifugation for 5 min at 5,000 rpm, and keeping the samples at −20°C for subsequent sugar analysis. The total sugar yields of the enzymatic hydrolyses were calculated as:

$$\text{Yield}(\%) = \frac{[\text{Glucose}(\text{g/l}) + \text{Cellobiose}(\text{g/l}) \times 1.053] \times 0.9}{\text{Cellulose}(\text{g/l})} \quad (1)$$

Enzyme adsorption

The adsorption isotherm of cellulase on the pretreated and untreated cotton was conducted by adding different amounts of cellulase to the substrate (%1 w/v). In order to avoid the hydrolysis of cellulose, the citrate buffer (0.05 M, pH 4.8) and the enzymes were pre-cooled to 4°C for 1 h before addition to the solids. The adsorptions were then performed for 2 h in a shaking water bath at 4°C and 100 rpm. Substrate blanks without cellulase, and cellulase blanks without substrate were also run in parallel. The mixtures were centrifuged at 12,500×g for 7 min, and their free proteins were measured [4]. The bound protein was calculated by subtraction of the free protein from the initial total protein. Each experiment was performed in triplicate. It is noteworthy that β-glucosidase was not adsorbed on the NMMO-treated cotton when it was tested separately.

The Langmuir isotherm was used to describe the adsorption of cellulase on cellulose [29]:

$$E_b = \frac{E_{max} K_{ads} E_f S}{1 + K_{ads} E_f} \quad (2)$$

Substrate reactivity

In order to measure the substrate reactivity (SR) during the enzymatic hydrolysis of cellulose, enzymatic hydrolyses were performed with the same method explained in the "Enzymatic hydrolysis" section. The reaction was interrupted after 0.25, 0.5, 1.0, 1.5, 3.0, and 4.0 h hydrolysis, and fresh enzymes were added to the residual substrate, and then the experiments were restarted. For interruption of the hydrolysis reaction, the hydrolysis flask was immediately placed in boiling water for 7 min to deactivate the enzymes. Boiling the substrate has been shown to have no side effects on the hydrolysis or enzyme adsorption [6]. Thereafter, the hydrolyzate was separated from the residual solid by centrifuging at 12,500×g for 7 min. The residual substrate was washed twice with distilled water, and once with citrate buffer (0.05 M, pH 4.8) to replace the distilled water in the substrate. Then, the restarted experiment was conducted by addition of fresh buffer, cellulase, and β-glucosidase to the residual substrate. The enzyme loadings were chosen such that the ratio of enzymes to cellulose was constant, identical to the uninterrupted hydrolysis experiments. The restarted hydrolysis experiments were conducted for an additional 1.0 h. During the restarted hydrolysis, samples were withdrawn after 1, 5, 10, 30, and 60 min of hydrolysis for measurement of glucose and cellobiose concentrations. The SR was calculated using the following equation:

$$\text{SR} = \frac{R}{R_0} \quad (3)$$

where R_0 and R represent the initial hydrolysis rate of uninterrupted and restarted hydrolysis rates, respectively. The following equation was used to correlate the SR to cellulose conversion potential:

$$\text{SR} = \alpha \frac{C}{C_0} \quad (4)$$

where C_0 is the initial cellulose concentration, C is cellulose concentration at a given time (mg/ml), and α is a dimensionless constant [11].

Analytical methods

The samples from enzymatic hydrolysis experiments were analyzed using HPLC (Waters, Milford, MA), equipped with RI detector (Waters 2414). Glucose and cellobiose were analyzed on an Aminex HPX-87H column (Bio-Rad, Hercules, CA) at 60°C with 0.6 ml/min eluent of 5 mM sulfuric acid. The change in the structure of the pretreated and untreated cotton linter was analyzed using FTIR spectrometer (Impact 410, Nicolet Instrument Corp., Madison, WI). The analyses were carried out in the wavelength range of 400–4,000 cm^{-1}, with 4 cm^{-1} resolution, and 64 scans per sample. OMNIC 8.1 software (Thermo-Nicolet Corp.) was used to determine peak positions and intensities. The Bradford assay [4], with Bovine serum albumin (BSA) as a protein standard, was used for determination of protein concentration.

Kinetic modeling

Different kinetic models have been developed and investigated for enzymatic hydrolysis of various cellulosic materials over the past several decades [5, 15, 18, 25, 30], mainly on acid-treated cellulosic materials [11, 13, 20, 32]. One of the most comprehensive models for the enzymatic hydrolysis of lignocelluloses was developed by Kadam et al. [11], and validated for acid-pretreated corn stover. The model showed promising results for kinetic modeling of creeping wild ryegrass [32]. This model incorporated

several parameters including, SR, structure of enzyme preparation, end-product inhibition (cellobiose and glucose), and productive and unproductive adsorption of enzymes on cellulose and lignin. In the current study, this model was modified and used for kinetic study of hydrolysis of NMMO-pretreated cellulose.

Kinetic model description

The simplified kinetic model, its reactions, and mass balances are presented in Table 1 and Fig. 1. The model considers two pathways for conversion of cellulose to glucose: (a) direct conversion of cellulose to glucose (r_2), and (b) conversion of cellulose to cellobiose (r_1) first and then to glucose (r_3). Each enzymatic reaction is potentially inhibited by its sugar products. The competitive inhibition was chosen to show the sugar inhibition phenomena in the current model, since it is mechanistically more realistic than other inhibition modes [11]. The multiple enzyme system was divided into two different parts, based on the performance of the enzymes: (a) cellulase that convert cellulose to cellobiose and glucose, and (b) β-glucosidase that hydrolyzes cellobiose to glucose. This model has been proposed and validated for different lignocellulosic biomass [11, 32].

Estimation of kinetic parameters

MATLAB® programming (MATLAB version 2009b, the Math Works, Natick, MA) was used to solve the kinetic model. The MATLAB optimization function of "lsqnonlin", which is able to solve the nonlinear least-squares and data-fitting problems, was applied to simultaneously estimate various kinetic parameters. The Langmuir parameters were determined independently and served as input.

Table 1 Reactions and mass balances for hydrolysis of cellulose

Reactions	Description
$r_1 = \frac{k_{1r} \times E_{1b} \times C}{1+\frac{G_2}{K_{1IG2}}+\frac{G}{K_{1IG}}}$	Cellulose-to-cellobiose reaction with competitive glucose and cellobiose inhibition
$r_2 = \frac{k_{2r} \times E_{1b} \times C}{1+\frac{G_2}{K_{2IG2}}+\frac{G}{K_{2IG}}}$	Cellulose-to-glucose reaction with competitive glucose and cellobiose inhibition
$r_3 = \frac{k_{3r} \times E_{2f} \times G_2}{K_{3M}\left[1+\frac{G}{K_{3IG}}\right]+G_2}$	Cellobiose-to-glucose reaction with competitive glucose inhibition
Mass balance	
$\frac{dC}{dt} = -r_1 - r_2$	Mass balance for cellulose
$\frac{dG_2}{dt} = 1.056r_1 - r_3$	Mass balance for cellobiose
$\frac{dG}{dt} = 1.1116r_2 + 1.053r_3$	Mass balance for glucose
$E_{1T} = E_{1f} + E_{1b}$	Mass balance for cellulase
$E_{2T} = E_{2f}$	Mass balance for β-glucosidase

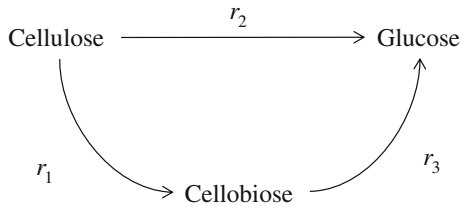

Fig. 1 Reaction scheme for modeling of cellulose hydrolysis (adopted from [12])

Model evaluation

After estimation of the model parameters, the kinetic model was evaluated for the data that were not used for parameters estimation. For evaluation of the model, experiments were performed at different conditions, beyond the conditions used to calculate the model parameters, with different enzyme and substrate loadings, and also waste textiles as substrate. For this purpose, the enzyme loadings of 5–100 FPU/g were used, while the β-glucosidase is always used as double activity similar to the cellulase.

Results

High crystalline cellulose, i.e., cotton linter, was pretreated with NMMO in order to increase the yield and reduce the time of enzymatic hydrolysis of cellulose. Besides, the hydrolysis was modeled in order to investigate the effect of NMMO-pretreatment on the kinetic of hydrolytic reactions. The macroscopic measured data was used for the estimation of kinetic parameters. The model was evaluated for the NMMO-pretreated cellulose at different conditions of hydrolysis and also for pretreated denim as well as separated cotton and viscose from waste textiles.

Effect of NMMO pretreatment on the structure and enzymatic hydrolysis of cellulose

Fourier transform infrared spectroscopy (FTIR) was used to investigate the changes in the structure of cellulose as a result of the pretreatment. Figure 2 shows the FTIR spectra of pretreated and untreated cotton, which are quite different. There is a sharp peak at 894 cm^{-1} for pretreated cotton, while this peak is small for the untreated one. The absorption band at 894 cm^{-1} is assigned as C–O–C stretching and it is a characteristic of β-linked glucose polymers. This band is weak and wide for cellulose I, while it is strong and sharp in cellulose II [8, 17]. The higher absorbance strength at 894 cm^{-1} (Fig. 2) indicates transformation of cellulose I to cellulose II.

The change in crystallinity of pretreated and untreated cotton was investigated. An empirical crystallinity index

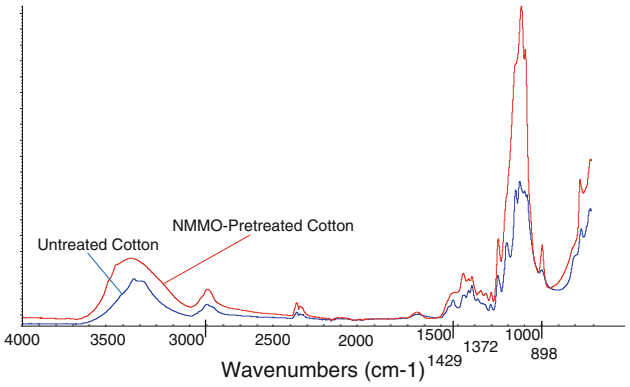

Fig. 2 FTIR spectra of NMMO-pretreated and untreated cotton

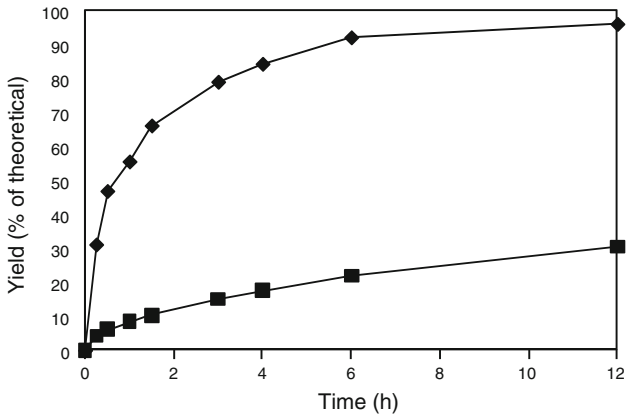

Fig. 3 Yield of enzymatic hydrolysis (cf. Eq. 1) of NMMO-pretreated *filled diamond* and untreated *filled square* cotton with 3% (w/v) cellulose concentration and 15 FPU cellulase and 30 CBU β-glucosidase loading per g cellulose

was defined as a ratio of absorption at 1,429 and 894 cm^{-1}, which was known as lateral order index (LOI) [8, 17]. Furthermore, total crystallinity index (TCI) was also defined as absorption ratio at 1,372–2,900 cm^{-1} [16]. The TCI and LOI decreased from 7.1 and 2.7 for untreated cotton to 3.3 and 1.1, respectively, for the pretreated cotton. Both of these reductions propose a significant decrease in the crystallinity of the cellulose during the NMMO pretreatment.

A 3% cotton linter was pretreated with NMMO at 120°C. It was completely dissolved in less than 3 h. Then, the dissolved cellulose was regenerated with hot water and subjected to enzymatic hydrolysis at 15 FPU/g cellulase for 12 h. The profiles of hydrolyses yields of pretreated and untreated cotton are presented in Fig. 3. The results show significant improvement in the rate of enzymatic hydrolysis of pretreated cellulose compared to that of the untreated one. As a result of the pretreatment, the sugar yield was increased from 31 to 96%. Furthermore, the major fraction of cellulose, which constitutes 92% of its weight, was converted during 6 h of hydrolysis. The experimental conditions and yield of hydrolysis in present study are compared with similar studies [12, 31] in Table 2. Since a major part of the cellulose was converted in less than 6 h, the duration of 6 h was chosen for the rest of the hydrolyses experiments. Moreover, glucose was the major part of the hydrolyzed cellulose, while the amount of cellobiose was negligible (data not shown).

Table 2 Comparison of experimental conditions and yield of hydrolysis of the present study with two similar studies which used other pretreatments

Authors	Substrate	Yield (%)	Hydrolysis conditions	
			Time of hydrolysis (h)	Pretreatment method
Current work	Cotton	92	6	NMMO
Kuo and Lee [12]	Cotton	85	48	NaOH/urea
Zhao et al. [31]	Avicel	72	48	Ionic liquids

Results of modeling

Enzyme adsorption

The Langmuir equilibrium isotherm was used to correlate the bound (adsorbed) enzyme concentration with the free enzyme concentration. The experimental data were obtained from the results of the hydrolysis of pretreated and untreated cotton with 3% cellulose, 15 FPU/g cellulase, and 30 CBU/g β-glucosidase. The Langmuir adsorption constants were obtained using nonlinear regression and are presented in Table 3. The results indicated more than doubling of E_{max}, when the cotton was pretreated. It means more adsorption of enzymes on the cellulose, while the other Langmuir constant, K_{ads}, was not significantly changed by the pretreatment.

Substrate reactivity

Substrate reactivity parameter has been incorporated in the kinetic model of enzymatic hydrolysis of cellulosic materials to represent the effect of different structural features of the substrate. The SR of NMMO-pretreated cellulose was measured in order to investigate the relation between the reactivity of cellulose and the extent of cellulose conversion during enzymatic hydrolysis. Thus, at different extent of cellulose conversion, the reaction was interrupted and a fresh enzyme was added to the spent substrate and the reaction was restarted. The SR was calculated according to Kadam et al. [11], and the initial rate of restarted reaction was divided by the initial rate of uninterrupted

Table 3 Experimental conditions for model development, Langmuir adsorption parameters, estimated kinetic parameters, and relative inhibition in the hydrolytic reaction rates

Substrate	In this study		Zheng et al. [32]	Kadam et al. [11]
	Untreated cotton	NMMO-pretreated cotton	Acid-pretreated corn stover	Acid-pretreated CWR[a]
Total hydrolysis time (h)	96	6	168	166
Cellulase (FPU/g substrate)	15	15	15	15
β-Glucosidase (CBU/g substrate)	30	30	15	0
E_{max} (mg protein/g substrate)	100.50	212.00	42.55	60
K_{ads} (ml/mg protein)	5.00	5.70	0.60	400
k_{1r} (ml/mg h)	5.64	32.10	16.50	22.30 (g/mg h)
K_{1IG2} (mg/ml)	1.58	7.52	0.04	0.02 (g/kg)
K_{1IG} (mg/ml)	0.01	0.34	0.10	0.10 (g/kg)
k_{2r} (ml/mg h)	10.98	13.56	7.10	7.20 (g/mg h)
K_{2IG2} (mg/ml)	211.74	38.41	132.50	132.00 (g/kg)
K_{2IG} (mg/ml)	0.28	1.58	0.01	0.04 (g/kg)
k_{3r} (h^{-1})	102.86	263.89	267.60	285.50 (h^{-1})
K_{3M} (mg/ml)	184.82	11.63	25.50	24.30 (g/kg)
K_{3IG} (mg/ml)	1.92	3.19	2.10	3.90 (g/kg)
K_{1IG2}/K_{1IG}	117.66	22.17	0.40	0.20
K_{2IG2}/K_{2IG}	755.15	24.29	13,250.00	3,300.00
K_{3M}/K_{3IG}	96.02	3.64	12.14	6.23

[a] Creeping wild ryegrass

reaction (cf. Eq. 3). The results are presented in Table 4. After 15 min of starting the hydrolysis and conversion of 30% of the substrate, SR remained almost constant, i.e., 0.6 ± 0.1, until conversion of 84% of cellulose in 4 h hydrolysis was obtained. This indicates that the SR was not a function of substrate conversion for NMMO-pretreated cellulose, and it might be eliminated from the kinetic model proposed by Kadam et al. [11].

Kinetic parameters

The kinetic parameters were calculated by MATLAB® best-fit regression using "lsqnonlin" function. The data of typical hydrolysis conditions and two values for SR were used as the program inputs. The calculated parameters considering SR with $\alpha = 1$ are presented in Fig. 4. In this case, the calculated coefficients of determination (R^2) for glucose, cellobiose, and cellulose were 0.98, 0.91, and 0.98, respectively. The modeling by considering SR equals to one was also performed (Fig. 4). In this case, the obtained R^2 for predicted concentrations were more than 0.99 for glucose, cellobiose, and cellulose. Therefore, it can be clearly concluded that the SR did not play a significant role during the enzymatic hydrolysis of the NMMO-pretreated cotton.

All the kinetic parameters calculated based on a constant SR and also the experimental conditions, in which the data were obtained, are presented in Table 3. Significant changes in the kinetic parameters were observed between pretreated and untreated cellulose. All the reaction rate constants, i.e., k_{1r}, k_{2r}, and k_{3r}, were increased as a result of pretreatment with NMMO. In Table 3, the kinetic parameters of enzymatic hydrolysis of acid-treated lignocellulosic materials, which have been obtained at almost the same conditions as in previous studies by Zheng et al. [32], and Kadam et al. [11], are compared with the data obtained in the present study. The reaction rate constant of conversion of cellulose to glucose (k_{2r}) was more than twice that of hydrolysis of cellulose present in the corn stover or creeping wild ryegrass. However, the reaction rate constant of conversion of cellobiose to glucose (k_{3r}) was almost in the same range as in the hydrolysis of pretreated cotton and pretreated lignocelluloses (Table 3). On the other hand, the relative inhibition effects were also different for the cotton and the lignocelluloses. K_{1IG2}/K_{1IG}, which indicates the inhibition effect of glucose to that of cellobiose in r_1, was decreased from 118 to 22, after

Table 4 Substrate reactivity (cf. Eq. 3) of NMMO-pretreated cotton during enzymatic hydrolysis

Time (h)	C/C_0	Relative glucose production rate in secondary hydrolysis (SR)
0.00	1.00	1.00
0.25	0.69	0.43
0.50	0.53	0.62
1.00	0.45	0.59
1.50	0.34	0.61
4.00	0.16	0.57

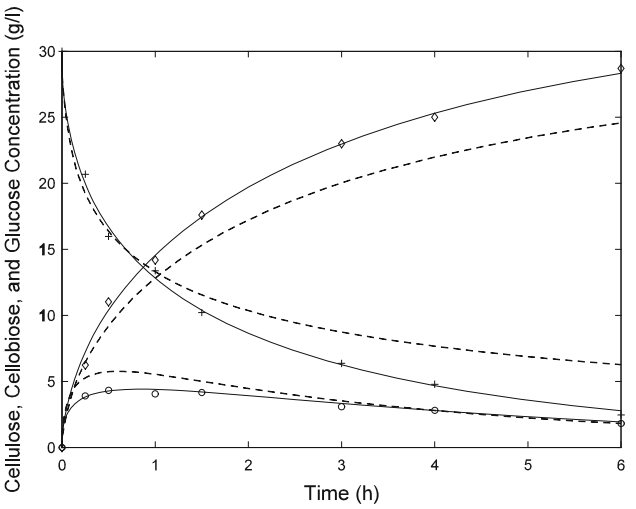

Fig. 4 Enzymatic hydrolysis of NMMO-pretreated cotton under typical conditions. The *symbols* represent the experimental data of: *open diamond* glucose, *open circle* cellobiose, and *plus sign* cellulose concentration. The *solid lines* are predicted by the model without the effect of SR and the *dashed lines* are predicated using $\alpha = 1$ in SR formula

NMMO-pretreatment of cotton. This indicates higher inhibition effects of cellobiose on conversion of cellulose to cellobiose after pretreatment. Similar results were also obtained for the inhibition of cellobiose on the other reactions. Higher rate of conversion of cellulose to cellobiose was also observed. The value of k_{1r} was obtained as 32 (ml/mg h) for NMMO-pretreated cotton compared to 6 (ml/mg h) for cotton, which shows a faster cellobiose production from pretreated cotton. At this condition, the inhibition of cellobiose for r_1 is not surprising.

Effect of substrate and enzyme loading on enzymatic hydrolysis of NMMO-pretreated cotton

The performance of the modified model in the present study was evaluated under a wider range of experimental conditions. Experiments at different substrate loadings and enzyme loadings were conducted, and the predicted glucose concentrations by the model were compared with the experimental results. Furthermore, three types of cellulose from waste textiles including denim, cotton/polyester, and viscose/polyester waste textiles were subjected to NMMO pretreatment. The results of the hydrolysis of these celluloses were then used to examine the model.

The results of glucose concentration in enzymatic hydrolysis of NMMO-pretreated cotton linter at various substrate loadings of 1, 3, 5, and 7% (w/v) are shown in Fig. 5. When substrate loading was increased from 1 to 7%, no significant change in the initial rate of enzymatic hydrolysis was obtained. However, an increase in substrate loadings led to a decline in the overall rate of enzymatic hydrolysis. The yield for 1, 3, and 5% substrate loading after 6 h hydrolysis was in the ranges of 95–85%, whereas this yield dampened to 78% for 7% cellulose loading (Fig. 5). The predicted data by the model are also presented in Fig. 5. The coefficient of determination (R^2) was also calculated for glucose and cellulose concentration at different substrate loadings and the results are shown in Table 5. The data showed that the predictability of the model for lower substrate loadings, in the range of 1–5% (w/v) was better than that for higher substrate loadings, i.e., 7% (w/v). The glucose concentration was slightly underestimated at lower substrate loadings, whereas this was overestimated at higher substrate loadings. One possible reason for this observation could be the differences in the efficiency of mixing at different substrate concentrations.

Enzyme loading is known to be a crucial factor that significantly affects the yield and rate of enzymatic hydrolysis. Figure 6 demonstrates the effects of different enzyme loadings from 5 to 100 FPU/g cellulose on the hydrolysis of NMMO-pretreated cellulose over a period of 6 h. The cellulase-to-β-glucosidase activity ratio was kept at 0.5 for all enzyme loadings. A rapid hydrolysis was perceived within the first 15 min, followed by slower rates. For high enzyme loadings, including 30 and 60 FPU/g cellulose, after 4 h, the hydrolysis was almost completed, whereas 100 FPU/g loading resulted in 100% conversion in less than 3 h. The final conversion increases from 66.5% to almost 100% with an increase in enzyme loadings from 5 to 60 FPU. However, the difference in the final conversion between 15 and 30 FPU/g loading was only 7%. The results of the evaluation of the modified model at various enzyme concentrations are shown in Fig. 6 and the calculated R^2 are presented in Table 5. The predicted glucose

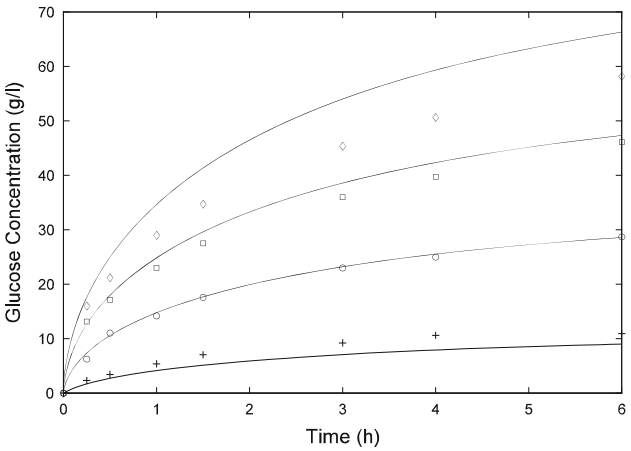

Fig. 5 Effect of substrate loading on enzymatic hydrolysis of NMMO-pretreated cellulose. The *symbols* represent: *plus sign* 1%, *open circle* 3%, *open square* 5%, and *open diamond* 7% cellulose concentration. The *solid line* is the predicted glucose concentration by the kinetic model

Table 5 Coefficient of determination (R^2) for prediction of glucose and cellulose concentration at different enzyme and substrate loadings

Conditions of enzymatic hydrolysis	3% Solid and different enzyme loadings				15 FPU cellulase/g cellulose and different substrate loadings		
	5 FPU	30 FPU	60 FPU	100 FPU	1%	5%	7%
Glucose	0.9974	0.9972	0.9994	0.9993	0.9456	0.9962	0.9652
Cellulose	0.9873	0.9858	0.9646	0.9723	0.9754	0.9736	0.9491

concentrations indicated that the model performed very well in a wide range of enzyme concentrations.

In a previous study, NMMO pretreatment was used to separate the cellulose part of waste textiles to produce ethanol or biogas [9]. In this study, the performance of the modified model was examined for prediction of the hydrolysis of separated cellulose from waste textiles. The profiles of glucose concentrations in hydrolysis under typical conditions are shown in Fig. 7. The performance of the model for pretreated denim, separated cotton and separated viscose was very well acceptable. Furthermore, the presence of dyes and reagents in the textiles did not have a significant effect on the enzymatic hydrolysis and the kinetic modeling parameters of cellulosic waste textiles after pretreatment with NMMO.

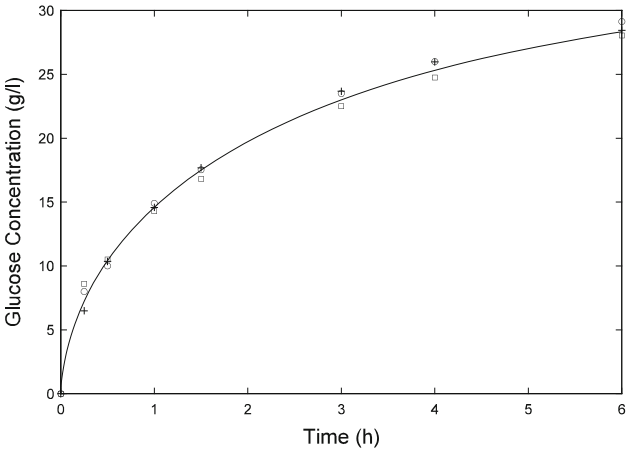

Fig. 7 Enzymatic hydrolysis of NMMO-pretreated waste textiles. The *symbols* represent: *plus sign* denim, *open circle* separated cotton, and *open square* separated viscose. The *solid line* is the predicted glucose concentration by the kinetic model

Discussion

The current study reveals that the enzymatic hydrolysis of high crystalline cellulose can be considerably improved by NMMO-pretreatment. The pretreatment resulted in 92% hydrolysis of cotton in 6 h with relatively low enzyme concentration (15 FPU/g), while merely 62% of the untreated one was hydrolyzed after 96 h. According to the structural analysis by FTIR, the lower crystallinity of the treated cotton could be one of the main reasons for the higher rate of hydrolysis.

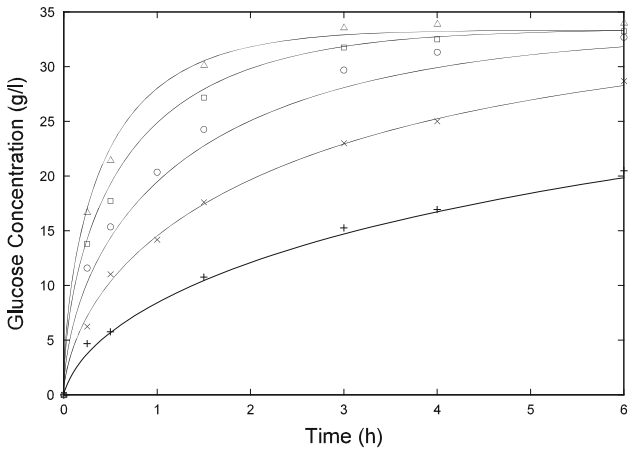

Fig. 6 Effect of cellulase loading on enzymatic hydrolysis of NMMO-pretreated cellulose. The symbols represent: *plus sign* 5, *multiplication sign* 15, *open circle* 30, *open square* 60, and *open triangle* 100 FPU cellulase per g cellulose. The *solid line* is the predicted glucose concentration by the kinetic model

Simulation and optimization of biofuel production from cellulose is a powerful tool for optimization, debottlenecking, providing the necessary data for the equipment design, and economic analyses. However, a reliable kinetic modeling for enzymatic hydrolysis is necessary to have a reliable simulation. On the other hand, enzymatic hydrolysis is a rather complicated process and several factors are involved in the kinetic of the reactions. The most important factors are adsorption of cellulase, inhibitions of the enzymes by glucose and cellobiose, enzyme loading, solid loading, SR, and productive and unproductive adsorption of enzymes on cellulose [2]. Kadam et al. [11] developed a complicated model based on a semi-mechanistic kinetic model that involved all of the above-mentioned factors and the model was validated for hydrolysis of lignocelluloses [32]. This model was used in the current study for predicting the hydrolysis of untreated cotton as well as NMMO-pretreated cotton and denim, and NMMO-separated cotton and viscose from waste textiles. The model with its original parameters was not successful to predict the applied substrate. However, it was successful when some modifications were applied to the model.

Adsorption of cellulase on the substrate is a prerequisite for the enzymatic hydrolysis. The maximum amount of

proteins that can be bound to cellulose during enzymatic hydrolysis is an important factor on the hydrolysis rate and yield of the reactions [15, 29]. Langmuir isotherm is the most common applied model for cellulase adsorption on cellulose and lignocelluloses [3]. This model was used in the current study to examine the cellulase binding on the pretreated and untreated cotton, and it could explain well the experimental data. The Langmuir constants obtained for the cotton were in line with several other studies on pure cellulose [29]. The results showed that the pretreatment increased the adsorption of cellulase on the cellulose. The maximum possible amount of protein adsorption, E_{max}, increased from 5 (mg/g substrate) for untreated to 212 (mg/g substrate) for the pretreated cellulose. Hence, the pretreatment resulted in an increase of 42 times in the maximum level of the adsorbed protein. This could be one of the reasons for a high enzymatic hydrolysis rate of NMMO-pretreated cotton.

Substrate reactivity is a parameter that has been defined to be a representative of structural features of cellulose, e.g., crystallinity, degree of polymerization, and substrate accessibility. In previous studies, fresh enzymes were introduced to partially converted cellulose and restarted the hydrolysis to investigate the reactivity of spent cellulose. The changes in hydrolysis rates were included in several models, instead of considering the individual structural features of cellulose, in order to explain the reduced digestibility of cellulose during hydrolysis [2, 11, 32]. Although the addition of SR as a function of conversion improved the models, the physical understanding of the constants in these equations is not possible [30]. The hydrolysis model that was developed by Kadam et al. [11] and used in this study was shown to be sensitive to SR in case of lignocellulosic materials. However, the kinetic data of cotton, either pretreated or untreated, in the current study were not explained by the model when the SR was measured and considered in the model. The model could explain the experimental data when SR was considered as a unity. Consequently, the decline in the hydrolysis rate of cellulose could not be attributed to changes in SR. The same conclusion has been suggested by Yang et al. [27]. The reason could be the complexity of the system. Thus, considering only one parameter for all changes in different properties of cellulose such as crystallinity, degree of polymerization, and enzymes accessibility may not be enough for modeling the hydrolysis of cotton. Furthermore, the SR measured by the method suggested by Kadam et al. [11] did not show any changes in the SR of NMMO-pretreated cellulose during enzymatic hydrolysis. It can be considered as the direct effect of regeneration-pretreatment. Once cellulose is dissolved and then regenerated, the resulted structure is more homogenous and the assumption of a biphasic substrate in its hydrolysis cannot be validated in case of the regenerated cellulose.

Conclusions

Pretreatment of cotton with NMMO significantly improved the yield and rate of enzymatic hydrolysis. The high-rated hydrolysis of cotton linter can be modeled by some modifications in the model presented by Kadam et al. [11]. Unlike the lignocelluloses, the model was fitted well when the SR was not included in the model. The model predicted different enzyme loadings very well, but the glucose concentration was underestimated at low substrate loadings and overestimated at high substrate loadings.

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