Appl Biochem Biotechnol (2010) 162:1471–1482
DOI 10.1007/s12010-010-8926-z

Kinetic Study on the Pretreatment and Enzymatic Saccharification of Rice Hull for the Production of Fermentable Sugars

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Received: 4 November 2009 / Accepted: 1 February 2010 /

Abstract The production of fermentable sugars from rice hull was studied by dilute acid pretreatment and enzymatic saccharification. Rice hull (15%, *w*/*v*) was pretreated by 1% (*v*/*v*) sulfuric acid at high temperature (120~160°C) for 15, 30, 45, and 60 min, respectively. The maximum sugar concentration from rice hull in the prehydrolysate was obtained at 140°C for 30 min, but the enzymatic saccharification yield from the corresponding pretreated rice hull is not high. To another aspect, the maximum enzymatic saccharification yield was achieved at 160°C for 60 min, while the recovery of fermentable sugars was the poorest. To take account of fermentable sugars from pretreatment and enzymatic saccharification, the maximum yield of sugars was obtained only when rice hull was treated at 140°C for 30 min. Under this condition, 72.5% (*w*/*w*) of all sugars generated from the raw material can be recovered. The kinetic study on the enzymatic saccharification of dilute acid pretreated rice hull was also performed in this work by a modified Michaelis–Menten model and a diffusion-limited model. After calculation by a linear and a non-linear regression analysis, both models showed good relation with the experimental results.

Keywords Rice hull · Pretreatment · Hydrolysis · Saccharification · Cellulase · Kinetics

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Introduction

Rice hull, one of the agricultural residues, can be used as a renewable feedstock for the production of bio-ethanol, which will greatly cater for the insufficiency of fossil energy source in the future because of the cheapness and wide availability of this lignocellulosic biomass [1–3]. Rice hull is abundant in rice-producing countries such as China, Korea, and USA, where its disposal by combustion to get energy entails an environmental problem. The bioconversion of this agricultural by-product to ethanol and other chemicals will, therefore, possess economic, environmental, and strategic advantages [4, 5]. Rice hull contains high quantities of lignin and ash, which constrain its use for the bioconversion. It also comprises more than 50% (according to dry weight) of polysaccharides (hemicellulose and cellulose), which can be converted into monomeric sugars, even though its conversion still has some difficulty to be performed due to the crystalline structure of cellulose and the presence of lignin barrier [6]. To take advantage of rice hull as a renewable source for the production of fuel ethanol, it is necessary to transform carbohydrate to fermentable sugars as much as possible. Pretreatment should firstly be carried out to remove lignin, reduce cellulose crystallinity, and increase the porosity of the material. As a result, the pretreated residues will have higher accessibility to cellulase, which is in good favor of the following saccharification [7]. Up to now, many pretreatment methods, such as comminution, steam explosion, ammonia fiber explosion, acid hydrolysis, and alkaline digestion have been extensively investigated [8, 9]. Among them, dilute acid has usually been used to recover sugars from hemicellulose [10], and the most commonly used acid is sulfuric acid [9, 11].

The process for the enzymatic saccharification of pretreated lignocellulosic materials is very complicated, and the reaction rate is always low or limited for the substrate is insoluble [12]. The classical Michaelis–Menten equation [13] had usually been used to study the kinetics of enzymes by the initial velocity, but it is not suitable for the enzymatic hydrolysis of heterogeneous substrate such as rice hull, especially when the enzymatic reaction is diffusion-limited [14]. Therefore, an alternative approach (Eq. 1) based on the Michaelis–Menten equation was proposed by Baley [15], which can be used for the description of rice hull saccharification. According to Eq. 1, the initial hydrolysis rate (v_0) is related with the initial enzyme concentration (E_0):

$$v_0 = \frac{V_{\mathrm{emax}} E_0}{K_{\mathrm{e}} + E_0} \tag{1}$$

where V_{emax} is the maximum rate of reaction at saturation with enzyme, and K_{e} is the half-saturation constant.

As a heterogeneous enzymatic reaction system, diffusion limitation between enzyme and substrate should be taken into account. Chrastil had carried out many researches on this aspect, proposing a diffusion-limited kinetic model [16, 17] as described in Eq. 2:

$$P = P_\infty \left(1 - e^{-kE_0 t}\right)^n \tag{2}$$

where P and P_∞ are the products which are generated from the reaction at time t and at equilibrium, respectively, k is a rate constant proportional to the diffusion coefficient, E_0 is the initial enzyme concentration, and n is a structural diffusion resistance constant dependent on sterical structure of the system. When $n<1$, the system is limited by diffusion resistance, and the smaller the n is, the higher the limitation will occur. However, when $n>1$, it is a consecutive reaction, and diffusion resistance should never be considered [17].

In recent years, many efforts have been made to use rice hull as the feedstock for the production of fermentable sugars, followed by the fermentation of sugars to produce ethanol by recombinant *Escherichia coli* or *Saccharomyces cerevisiae* [3, 18, 19]. Also some progress had been made on the utilization of rice hull, including pretreatment and saccharification [1, 20], whereas few attention was paid to the kinetics on the enzymatic hydrolysis of rice hull. In this work, the pretreatment of rice hull by dilute acid was investigated, with special focus on the recovery of fermentable sugars decomposed from hemicellulose under different reaction conditions. The kinetics of enzymatic saccharification of pretreated rice hull was also studied, and the experimental data were analyzed by an alternative approach to Michaelis–Menten equation, together with a kinetic model for diffusion-limited enzymatic reactions.

Materials and Methods

Materials

Rice hull was obtained from a local farm, which belongs to National Horticulture Research Institute of Korea. It was milled in a hammer mill after being air-dried in an oven at 70 °C for 12 h, and a fraction (<425 μm) was separated using a 40-mesh sieve and collected for further use.

Celluclast 1.5 L was purchased from Novozymes and used in the enzymatic hydrolysis of rice hulls. The protein content, carboxylmethylcellulase (CMCase) activity, and filter paper activity of the crude enzyme were measured to be 60 g/L, 853 U/mL, and 18.8 filter paper unit (FPU) per milliliter, respectively. Cellulase C-1184, glucose, xylose, mannose, galactose, arabinose, carboxylmethylcellulose (CMC), sodium azide, and 3,5-dinitrosalicylic acid (DNS) were purchased from Sigma-Aldrich Co., St Louis, MO, USA. Other chemicals used were all of standard analytical grades.

Pretreatment

Powdered rice hull was loaded in 1% H_2SO_4 at a solid/liquid ratio of 15% (*w*/*v*) and pretreated in a specially made autoclave (0~1.0 MPa, Aid Engineering Company, Korea) at 120~160 °C for 15 min to 1 h. The slurry was separated by filtration for the recovery of solid and liquid fractions, and the filtrate was collected to determine sugars. After washing twice with distilled water and drying, the cake was utilized as the substrate for kinetic study on the enzymatic saccharification.

Enzymatic Hydrolysis

The pretreated rice hull was hydrolyzed by cellulase (Celluclast 1.5 L/cellulase C-1184= 5:1, *w*/*w*) in a rotating shaker for 48 h with constant temperature of 50 °C and agitation rate of 150 rpm. Rice hull was suspended in 100 mL sodium citrate buffer (0.05 mol/L, pH5.0) containing 5 mg sodium azide. Unless stated otherwise, enzyme to substrate ratio was maintained at 20 FPU/g of rice hull, while the solid to liquid ratio of 2% (*w*/*v*) in the reaction mixture was always used. In order to get full knowledge on the kinetics on the enzymatic saccharification of rice hull, the reaction was performed just for 7 h, and the initial cellulase loading rate was, therefore, altered in the range from 5 to 100 FPU/g. Samples were withdrawn at the desired time and quickly mixed with DNS solution after properly diluted.

Analytical Methods

Chemical compositions of rice hull were determined by Feeds & Foods Nutrition Research Center of Pukyong National University of Korea, according to the procedures recommended by NREL [21] of USA. CMCase activity was assayed in a reaction mixture (0.5 mL) containing 1% (*w*/*v*) CMC, 0.05 mol/L sodium citrate buffer (pH5.0), and appropriately diluted enzyme solutions. After incubation at 50°C for 20 min, the reducing sugar liberated in the reaction mixture was measured by DNS method [22]. Filter paper activity was assayed by incubating a reaction mixture containing a strip of Whatman No.1 filter paper (1×6 cm) immersed in 1 mL of 0.05 mol/L sodium citrate buffer (pH5.0) and 0.5 mL of appropriately diluted enzyme solution at 50°C for 30 min. One unit of CMCase and filter paper activity (FPU) were both defined as the amount of enzyme that generates 1 μmol of reducing sugar as glucose in the reaction mixture per minute under the assay conditions. All acid hydrolysis and enzymatic saccharification experiments were carried out in duplicate, and average results are given.

High-Pressure Ion Chromatography

Individual sugar (arabinose, galactose, glucose, and xylose) concentrations were analyzed by a Bio-LC system, which was equipped with AS autosampler, pulsed amperometry gold electrode detector, and Rheodyne injector. The column used in this study was a CarboPack PA1 analytical column (4×250 mm) with a CarboPack PA1 guard column (4×50 mm). The mobile phase was 12 mmol/L NaOH at a flow rate of 1.2 mL/min. The injection loop size was 5 μL, and the temperatures of column heater and cell heater were 25 and 35°C, respectively. The measurement of furfural and hydroxymethylfurfural was also carried out by HPIC, expect that the mobile phase of 3 mmol/L NaOH was used in this study.

Estimation of Kinetic Parameters

Kinetic studies were conducted with the experimental data from enzymatic hydrolysis, and the kinetic parameters (V_{emax} and K_{e}) involved in the modified Michaelis–Menten equation were calculated using a linear regression analysis method. As the initial velocity v_0 is undefinable when the time $t \rightarrow 0$, the concentration of reducing sugar after 30 min saccharification was taken as an estimate. The parameters (k and n) in the diffusion-limited model under different initial enzyme concentrations were estimated by a non-linear regression analysis of the time-course plots for enzymatic saccharification. The software used to determine parameters is Statistica 6.0 (StatSoft, OK, USA) together with the quasi-Newton method.

Results and Discussion

Pretreatment of Rice Hull by Dilute Acid

Pretreatment of rice hull by 1% (*v*/*v*) sulfuric acid to recover fermentable sugars from rice hull was observed to be optimum in the former study, which will be published elsewhere. Here, only temperature and reaction time for rice hull hydrolysis were investigated in order to find how the monosaccharide (such as xylose, glucose, arabinose, galactose, and other sugars) in the prehydrolysate changed under specified conditions, and the concentration of sugars that resulted from pretreatment were illustrated in Table 1. It is clear that xylose is the most abundant sugar in filtrate due to the high content in the extractive fraction of rice hull that will release to the liquid after pretreatment, and the highest xylose concentration can be obtained under appropriate condition (140 °C and 30 min). If temperature increased, xylose concentration decreased dramatically. This should be attributed to sugar-degradation reactions, and the by-products of furfural and hydromethylfurfural formed using pentose like xylose as substrate [20, 23] together with a darker color of the hydrolysate. The by-products will have inhibitory effect on the following fermentation process by microorganism [24], and hence this must be taken into account in the pretreatment. Glucose is another abundant sugar if higher temperature such as more than 140 °C is introduced, and the concentration of glucose increased concomitantly with temperature and hydrolysis time. The highest glucose concentration was found when using higher temperature and longer time (e.g., 160 °C and 60 min). Under this severe condition, part of cellulose in rice hull was decomposed to glucose [3]. As a result, the highest total sugar concentration of 40.6 g/L was achieved at 140 °C for 30 min (Table 1), which is about 63% of the yield of sugar from crude fiber (consisted of cellulose and hemicellulose). Under this milder condition, pentose can be recovered as much as possible.

As far as the material solubilization is concerned, total gravimetric recovery (solids remaining after pretreatment divided by original oven-dried weight) decreased when using

Table 1 Compositions of the prehydrolysate resulting from pretreatment of rice hull (15%, *w*/*v*) by 1% sulfuric acid under different temperature and time.

Temperature (°C)	Time (min)	Sugar concentration (g/L)					Total sugar (g/L)	Furfurals (g/L)
		Glucose	Xylose	Arabinose	Galactose	Other sugars		
120	15	0.5	23.3	2.0	1.1	0.3	27.2	0
	30	0.7	26.7	2.4	1.3	0.2	31.3	0
	45	0.9	28.1	2.3	1.4	0.6	33.2	0
	60	1.1	28.8	2.3	1.4	1.4	35.0	0
130	15	1.1	29.5	2.2	1.4	1.5	35.6	0
	30	1.4	30.8	2.3	1.4	2.2	38.2	0.1
	45	1.7	31.9	2.5	1.5	2.0	39.7	0.1
	60	1.9	31.1	2.5	1.4	1.6	38.5	0.3
140	15	1.8	30.7	2.4	1.5	2.6	39.0	0.2
	30	2.4	32.3	2.4	1.4	2.1	40.6	0.3
	45	2.8	27.2	2.3	1.1	3.8	37.2	0.8
	60	3.5	27.0	2.4	1.2	2.7	36.8	0.8
150	15	3.3	27.3	2.3	1.1	3.0	37.0	0.8
	30	5.5	24.5	2.3	1.2	2.2	35.7	1.0
	45	6.4	15.9	1.7	0.9	4.9	29.9	2.7
	60	9.4	15.3	1.9	1.0	0.6	28.2	2.8
160	15	8.6	15.6	2.3	1.2	5.0	32.7	2.8
	30	13.7	9.9	1.4	0.9	5.0	31.0	4.0
	45	18.1	5.3	1.1	0.8	5.4	30.6	4.2
	60	20.7	2.6	0.7	0.6	5.8	30.4	4.5

higher temperature and longer time (Fig. 1a). For example, at 160°C, the material solubilization was 43.7% when hydrolyzed for 60 min, however just 33.1% for 15 min. This result is similar to that under different temperature, for instance, if the reaction time was fixed at 60 min, the material solubilization was only 22% at 120°C. The decrease observed in the total gravimetric recovery is in agreement with the increase in the content in hemicellulose-derived sugars (xylose, arabinose, and galactose) assayed in the prehydrolyzate (Table 1) as long as the temperature and time increased. According to Fig. 1b, the highest yield of 270 mg/g of sugar from rice hull was obtained at 140°C and 30 min, where more fermentable sugars can be achieved.

The chemical compositions of untreated and dilute acid pretreated rice hull were shown in Table 2. It is evident that the content of crude fiber increased by more than 40% after pretreatment. Because most of hemicellulose was transformed to mono-sugars by dilute

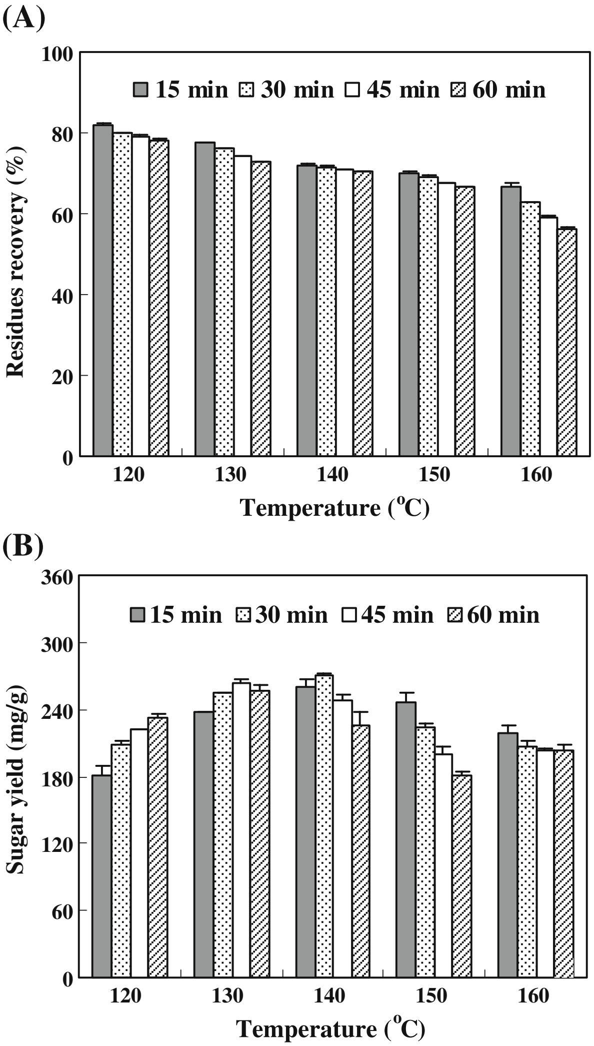

Fig. 1 **a**–**b** Total gravimetric recovery and sugar yield from rice hull (15%, *w*/*v*) after dilute acid (1% H_2SO_4) hydrolysis at different temperatures

Table 2 Chemical compositions (%, *w*/*w*) of untreated and dilute acid pretreated rice hulls.

Composition	Rice hull	
	Untreated (raw material)	Pretreated by 1% H_2SO_4
Moisture	18.23±0.11	5.57±0.26
Crude lipid	0.51±0.12	0.15±0.00
Crude protein	1.92±0.04	0.83±0.05
Crude ash	12.95±0.39	10.81±0.32
Crude fiber	42.97±0.28	60.75±0.09
Cellulose	28.59±0.12	57.46±0.09
Hemicellulose	14.38±0.20	3.29±0.05
Salt	0.08±0.00	–
Lignin	23.71±0.24	21.89±0.12

acid, mostly, the residual fiber in pretreated rice hull is cellulose. Besides, another interesting result is that there is still a high content of lignin remaining just by acid pretreatment, which will surely prevent cellulase from combining with cellulose in the following enzymatic saccharification process [20]. Monosaccharides from hemicellulose and cellulose in rice hull after acid pretreatment are clearly shown by HPIC data (Fig. 2), wherein the peaks matched completely with the standards injected.

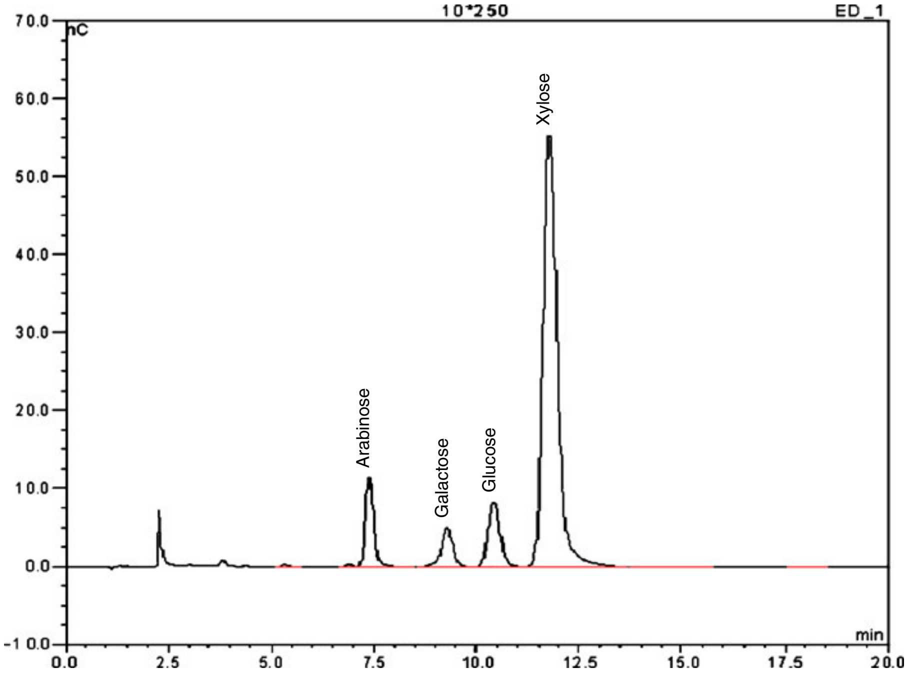

Fig. 2 HPIC analysis of the sugars in prehydrolysate produced by 1% H_2SO_4 hydrolysis of rice hull (typical sample from reaction conditions of 140°C and 30 min), showing arabinose, galactose, glucose, and xylose

Enzymatic Saccharification of Pretreated Rice Hull by Cellulase

The enzymatic hydrolysis yield obtained when the pretreated rice hull was submitted to the action of commercial cellulase (Celluclast 1.5 L supplemented with Cellulase C-1184) was summarized in Table 3. It is evident that increasing either the temperature or hydrolysis time in pretreatment has a positive effect on the following saccharification of pretreated rice hull biomass cellulose fraction.

The highest enzymatic hydrolysis yield that was found at the harshest pretreatment conditions (160°C, 60 min) reached 102.1 mg/g dry matter after 48 h of saccharification. It is clear that even at severe pretreatment condition, there is still a fraction of cellulose recalcitrant to the enzyme attack, because many fibers (most is cellulose) have not hydrolyzed in the saccharification process, as there is about 60% of fiber in the pretreated rice hull. When milder pretreatment conditions were used, the resulting solids were hydrolyzed with just a little poorer performance in terms of enzymatic hydrolysis yields. For example, the enzymatic hydrolysis yield at 160°C and 60 min is 102.1 mg/g, which is about 2.7-fold of that at 120°C and 15 min. The improvement in enzymatic hydrolysis yields concomitantly with pretreatment temperature, and hydrolysis time may be attributed to the solubilization of the hemicellulose fraction of the residue [25], because enzyme accessibility to cellulose is favored.

Sugars were generated from two steps studied in this work (pretreatment and enzymatic hydrolysis), and yields referred to the raw material that has been determined for each step,

Table 3 Yield of sugar from rice hull after pretreatment by 1% (*v*/*v*) H_2SO_4 followed with enzymatic saccharification.

Temperature (°C)	Time (min)	Enzymatic hydrolysis yield (mg/g dry matter)	Yield of sugar (mg/g dry matter)	Total yield of sugar (%)
120	15	37.9	219.6	51.1
	30	38.0	246.7	57.4
	45	38.4	259.8	60.5
	60	39.8	273.0	63.5
130	15	37.7	275.2	64.0
	30	38.2	291.6	67.9
	45	38.4	302.8	70.5
	60	41.2	298.0	69.4
140	15	41.2	302.2	70.3
	30	41.9	311.6	72.5
	45	43.9	291.9	67.9
	60	43.9	269.4	62.7
150	15	48.9	295.6	68.8
	30	48.7	273.2	63.6
	45	54.9	254.1	59.1
	60	58.3	239.8	55.8
160	15	63.8	281.9	65.6
	30	94.6	301.2	70.1
	45	97.9	301.6	70.2
	60	102.1	304.8	70.9
Untreated	–	37.9	19.0	4.4

and the results are summarized in Table 3. It is found that although severe pretreatment conditions are favored than the enzymatic hydrolysis yield, it is not a good manner for the total yield of fermentable sugars to the raw material, because too many pentoses were degraded in the pretreatment step [20]. Therefore, if the total sugar yield is taken into account, the pretreatment condition of 140 °C and 30 min seemed to be the most optimum one. Under this condition, the yield of sugar reached 311.6 mg/g dry material, which is 72.5% of the ideal yield determined from the data of fiber composition of rice hull. The enhancement in overall sugar yield is mainly attributed to a significant increase of hemicellulose-derived sugars in prehydrolysis when using dilute sulfuric acid. Furthermore, compared with untreated rice hull, the pretreated material was surely more favored to cellulase attack, and much higher enzymatic hydrolysis yield can be achieved after prehydrolysis.

Kinetics on Enzymatic Saccharification of Pretreated Rice Hull

Enzymatic hydrolysis of untreated and pretreated rice hull by cellulase was carried out under diverse initial enzyme concentrations, and the time courses of the reaction were illustrated in Fig. 3. It was shown that the yield of reducing sugars was considerably improved if the rice hull was pretreated. Compared with the saccharification of pretreated and untreated rice hull at the same cellulase concentration of 1.2 g/L for 7 h, the sugar concentration was increased about 4-fold after pretreatment. A higher yield of reducing sugar was also observed if increasing the enzyme concentration. However, no significant increase was achieved when cellulase concentration was higher than 1.2 g/L, probably due to the saturation of substrate surface [26]. The maximum hydrolysis rate for each enzyme concentration was found at the beginning of the reaction, followed with a gradual decrease as the saccharification was carried on. This is mainly because of the combination of free-state cellulase on substrate and the inhibition of by-product accumulation [27].

The effect of initial cellulase concentration on the initial hydrolysis rate is shown in Fig. 4. The kinetic parameters of the modified Michaelis–Menten model (V_{emax}=0.075 g/L min, k_{e}= 0.36 g/L) were successfully estimated by linear regression analysis of the experimental data. Using these kinetic parameters, the relationship between the initial cellulase concentration and the initial hydrolysis rate can be drawn as a continuous curve in Fig. 4. It was shown that the modified Michaelis–Menten model, here, can also give a satisfactory predictive result on the

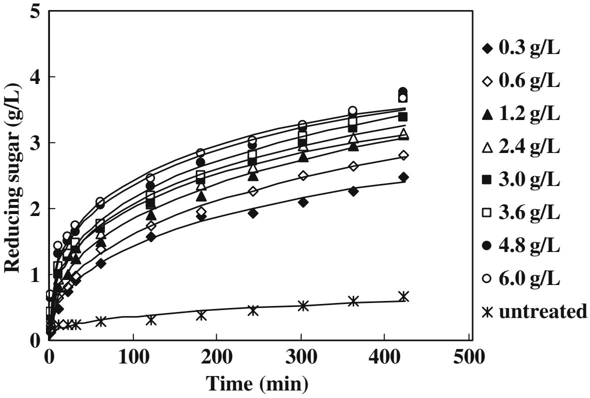

Fig. 3 Enzymatic hydrolysis of rice hulls under different initial cellulase concentrations. *Markers* represent experimental data, while *continuous curves* are the non-linear regression results obtained from the Chrastil equation. The initial cellulase concentration for untreated rice hull is 1.2 g/L

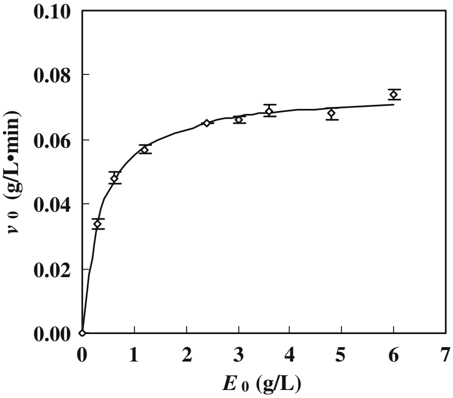

Fig. 4 The initial enzymatic hydrolysis rate (v_0) of pretreated rice hull under diverse initial cellulase concentrations (E_0). The experimental data were illustrated as *marker*, and the *curve* was calculated from Michaelis–Menten equation

enzymatic saccharification of pretreated rice hull, even though the diffusion of enzyme system and structural properties of substrate were ignored.

If diffusion limitation was taken into account, Chrastil equation should be used to show the extent of diffusion resistance. Based on the experimental data from Fig. 3, the parameters in Chrastil model were determined, as is shown in Table 4. A good agreement with the experimental results was also obtained (R^2>0.95), compared with the continuous curves (Fig. 3) drawn by non-linear regression analysis method. It is also possible to evaluate the diffusion characteristic of the enzyme system by analyzing the kinetic parameters of n and k.

The diffusion resistance of the enzymatic saccharification system can be illuminated by the changes in the diffusion coefficient n. Firstly, n increased from 0.292 to 0.394 for the hydrolysis of untreated and pretreated rice hull at the same initial cellulase concentration of 1.2 g/L, which was due to the structural modification by acid pretreatment. On the other hand, in the cases of different initial enzyme concentrations for the pretreated rice hull, higher n was obtained than untreated rice hull, which means less diffusion resistance in acid pretreated materials than untreated one.

Furthermore, the acid pretreatment of rice hull produces an improvement on the enzymatic hydrolysis property to the system, as is shown in the increase of rate constant k at 1.2 g/L of cellulase. Also, k for untreated rice hull is lower than the pretreated samples,

Table 4 Kinetic parameters determined from non-linear regression analysis of experimental data using Chrastil equation.

Rice hull	E_0/(g/L)	k/(L/g min)	n	R^2
Pretreated	0.3	5.91×10^{-3}	0.440	0.997
	0.6	1.44×10^{-3}	0.417	0.997
	1.2	7.69×10^{-4}	0.394	0.995
	2.4	5.83×10^{-4}	0.353	0.992
	3.0	4.03×10^{-4}	0.365	0.990
	3.6	3.87×10^{-4}	0.366	0.985
	4.8	3.72×10^{-4}	0.334	0.972
	6.0	3.39×10^{-4}	0.300	0.968
untreated	1.2	3.18×10^{-4}	0.292	0.959

showing that the pretreated substrate was less resistant to the hydrolysis and more accessible to cellulase.

According to the results of Table 4, another interesting thing is that *k* and *n* for pretreated rice hull decreased when the initial enzyme concentration increased. The possible reason for the decrease of *n* lies in the restriction of the diffusion process between molecules of enzyme and substrate, which was caused by the enzyme initially adsorbed on the surface of fibers [28]. Moreover, the decrease of *k* at high enzyme concentrations is just because of the feed-back inhibition effect of product on enzyme [29], which reduces the catalytic ability of cellulase.

Conclusions

The generation of fermentable sugars from rice hull by dilute acid pretreatment and enzymatic saccharification together with the kinetics of saccharification process was studied in this work. Mild pretreatment conditions led to a higher fermentable sugar content in the prehydrolysate, while severe pretreatment conditions resulted in a higher enzymatic saccharification yield, although sugar recovery in prehydrolysate decreased. For the sake of total fermentable sugars, the rice hull (15%, *w*/*v*) was prehydrolyzed by dilute acid (1% sulfuric acid, *v*/*v*) at 140°C for 30 min. Under this condition, 72.5% (*w*/*w*) of all sugars presented in rice hull biomass is available after pretreatment and enzymatic saccharification. A modified Michaelis–Menten model and a diffusion-limited model were utilized to study the kinetics of enzymatic saccharification of retreated rice hull. Both models showed good relation with experimental results, and kinetic parameters had also been determined with high accuracy.

Acknowledgment This work is financially supported by BK21 Bio-Silver Project of Dong-A University, Korea.

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