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An Extension of the Harano–Ooshima Rate Expression for Enzymatic Hydrolysis of Cellulose to Account for Changes in the Amount of Adsorbed Cellulase

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ABSTRACT

Three empirical rate expressions, Kinetics I, II, and III, for the enzymatic hydrolysis of cellulose were evaluated in an effort to develop a easy-to-use rate expression. They are based on the following equation: $-dV/dX = kV$, where V and X are the hydrolysis rate and the fractional conversion. In Kinetic I, k is constant. In Kinetic II, a linear relatinship between k and t is assumed. In Kinetic III, an exponential relationship is assumed. The three expressions were applied to enzymatic hydrolysis carried out under seven different conditions in which the kinds of substrates, enzymes, and initial concentrations were varied. All of the examined rate expressions were applied to the hydrolysis with success, but the better accuracies were obtained by Kinetic III, Kinetic II, and Kinetic I in this order. The variations of k with time found in this study, especially the exponential relationship, were consistent with the effect of the measured changes in the concentration of adsorbed enzyme as predicted by theory developed previously by Ooshima et al. (*1*).

Index Entries: Enzymatic hydrolysis of cellulose; kinetics; adsorption of cellulase.

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INTRODUCTION

Many kinetic models for the enzymatic hydrolysis of cellulosic materials have been developed, based on the substrate characteristics, product inhibition, the deactivation of enzyme, the adsorption of enzyme on cellulose, the multiplicity of enzyme components, and so on (2). However, it is difficult to explain the enzymatic hydrolysis of many kinds of cellulosic materials sufficiently in terms of any one of those models alone or even their combination. The true mechanism of the hydrolysis probably includes all of the factors discussed in the models and other more complex factors, such as changes in the surface area accessible to enzyme during the hydrolysis (3). Even if it could be explained perfectly, the rate expression would be complicated by too many variables. Far from these approaches, another possible approach is to derive an empirical rate expression. Walseth's equation that the fractional conversion is in proportion to the nth power of the hydrolysis time is a typical empirical rate expression developed for the enzymatic hydrolysis of cellulose (4).

One of the present authors and the coworkers have proposed the following empirical rate expression, Eq. (1).

$$-dV/dX = kV \tag{1}$$

where V is the hydrolysis rate, X is the fractional conversion, and k is the overall rate constant (dimensionless) (5). The integral formula of Eq. (1) has been also derived:

$$P = (S_0/k)\, ln\, [1 + (V_0/S_0)kt] \tag{2}$$

where P, S, and t are the product concentration, the substrate concentration, and the reaction time, respectively. The subscription "0" means the initial value of the corresponding parameter. Equations (1) and (2) have been applied to the enzymatic hydrolysis of many cellulosic materials with some success (6). However, it has been found through further investigations that some cases of the hydrolysis cannot be expressed by Eq. (2) with much accuracy.

We report herein a modified rate expression that is widely held. The rate expression was developed on basis of Eq. (1) and by allowing k to depend on time, linearly and exponentially.

MATERIALS AND METHODS

Materials

Mixed hard wood, Wilner 060 (90% birch and 10% maple) was obtained from Wilner Wood products (Norway, ME). Cellulose powder Avicel was purchased from Asahi Chemical Co., Japan. Cellulase from *Trichoderma*

Table 1
Reaction Conditions in the Enzymatic Hydrolyses of Cellulosics

Run	Reaction condition				
	Cellulosic	Enzyme	S_0,[a] mg/mL	E, mg/mL	pH
1	Avicel	Meicelase	22.3	2.00	7.0
2	PTW180	F-β	13.9	0.50	4.8
3	PTW200	F-β	15.5	0.50	4.8
4	PTW220	F-β	14.0	0.50	4.8
5	PTW180	GC123	26.0	2.53	4.8
6	PTW200	GC123	34.2	2.52	4.8
7	PTW220	GC123	34.2	2.52	4.8

[a]Initial concentration of cellulose based on cellobiose.

reesei, GC123, and cellulase from *Trichoderma viride*, Meicelase, were obtained from Genencor in the United States and Meiji Seika Kaisha Ltd. in Japan, respectively. Cellulase removed β-glucosidase from Meicelase by DEAE-Sepharose chromatograpy also was used. The cellulase was called F-β and did not have β-glucosidase activity as long as the activity was measured by *p*-nitrophenylglycoside method (*7*). All other chemicals were used of reagent grade.

Substrate Pretreatment

Wilner hardwood was pretreated by acid hydrolysis in a continuous plug flow reactor as described by McParland et al. (*8*). Residence time for a 5% w/w wood slurry containing 1% w/w H_2SO_4 was 8.5 s for a reactor temperature of 180, 200, and 220°C. The pretreated slurry was stored in acidified form at 0–5°C. The substrates pretreated at 180, 200, and 220°C were named PTW180, PTW200, and PTW220, respectively.

Enzymatic Hydrolysis

Pretreated wood was prepared for hydrolysis by washing and neutralizing the acidified slurry with distilled water until the pH of the supernatant was between 5 and 7. Hydrolysis was carried out in 158-mL serum bottles (GC123) or 50-mL test tubes (F-β). Avicel was buffered to pH 7.0 with 0.1*M* phosphate, although it is not the optimum pH of cellulase used. Hydrolysis was started by adding cellulase solution. The serum bottle and the test tube were sealed and incubated in shaking bath at 40°C. Other reaction conditions are summarized in Table 1.

Determination of Concentration of Sugar and Protein During Hydrolysis

Total reducing sugar concentration was determined during hydrolysis by the DNS method (*7*). Total sugar as cellobiose in mg/mL was determined from calibration curves for cellobiose and glucose. Glucose was determined separately by an enzymatic method using the glucose oxidase/peroxidase reagent. The conversion was calculated using the potential cellobiose.

The concentration of protein in the liquid phase was determined by Bradford colorimetric assay from Bio-Rad Co., using bovine serum albumin as a standard. The concentration of enzyme adsorbed on cellulose was calculated from the concentration of protein in the liquid phase.

THEORY

Equation (1) represents the decline of the reaction rate during hydrolysis. The derivation of Eq. (2) from Eq. (1) is based on the assumption that k is constant. On the other hand, the same equation as Eq. (1) has been derived separately in a quantitative study of the change of the enzymatic activity of cellulase adsorbed on cellulose during hydrolysis (*1*). In this study, the parameter k was found to be a function of the concentration of adsorbed cellulase, E_{ads}, and its change during hydrolysis, dE_{ads}/dX, as shown in Eq. (3).

$$k = -n\,[ln\,(E_{ads}/E_t) + d\,lnE_{ads}/d\,lnX] \tag{3}$$

where E_t is the total enzyme concentration, $n = n_0 \exp(qS_0)$, and n_0 and q are constant. The parameter k is constant only when the concentration of adsorbed enzyme does not change during reaction, namely $d\,lnE_{ads}/d\,lnX = 0$. Therefore, Eq. (2) with k constant can be expected to represent the data accurately only when the adsorbed enzyme concentration is constant during the reaction.

When the adsorbed enzyme concentration changes during the reaction, k should be evaluated as a variable. In order to do so, the changes in E_{ads} during hydrolysis must be known. The adsorption of cellulase on cellulose has been investigated mainly in terms of isotherms. In order to evaluate E_{ads} as a function of X (or t), not only the adsorption isotherm, but also the changes in the surface area accessible to enzyme during the hydrolysis must be evaluated. However, the change in the accessible surface area is not as simple (*3,9*). Therefore, it is not easy to derive a general expression for the adsorbed enzyme concentration.

In the present work, a different approach was attempted to evaluate the change of k. It is to formulate the change of k empirically as a function of hydrolysis time t. Since E_{ads} is a function of t intrinsically, the k–E_{ads} relationship results in a k–t relationship.

When k is a function of t (and hence a function of X) in Eq. (1), we obtain:

$$(1 / V) = (1 / V_0) + (1 / S_0) \int_0^t k dt \quad (4)$$

We assume three kinetics for the relationship of k vs t, Kinetics I, II, and III, to examine better the estimation for the change of k during the reaction.

1. Kinetic I: k is constant during the hydrolysis, namely $k = k_0$. The relationship between P and t is expressed by,

$$P = (S_0 / k_0) \ln [1 + (V_0 k_0 t / S_0)] \quad (5)$$

2. Kinetic II: a linear relationship was assumed, namely:

$$k = k_0 + at \quad (6)$$

where k_0 is the initial value of k, and parameter a is constant. Integration of Eq. (4) combined with Eq. (6) gives:

$$P = (S_0 / k_{m1}) \ln [1 + (V_0 k_{m1} t / S_0) \cdot (k_0 + k_{m1}) / (k_0 + k_{m1} + at)] \quad (7)$$

where k_{m1} is a modified parameter k, and given by:

$$k_{m1} = (k_0^2 - (2aS_0 / V_0)]^{1/2} \quad (8)$$

When $a = 0$ (zero), $k_{m1} = k_0$, and Eq. (7) is the same as Eq. (5).

3. Kinetic III: We assume another relationship between k vs t, that is, an exponential relationship:

$$k = k_0 \exp (bt) \quad (9)$$

where b is constant. Integration of Eq. (4) combined with Eq. (9) gives:

$$P = (S_0 / k_{m2}) \ln [1 + (V_0 k_{m2} t / S_0) \cdot \{1 - \exp (-bt)\} / bt] \quad (10)$$

where k_{m2} is another modified parameter k, and given by:

$$k_{m2} = k_0 - (bS_0 / V_0) \quad (11)$$

When $b = 0$ (zero), $k_{m2} = k_0$ and Eq. (10) is the same as Eq. (5).

Equations (7) and (10) contain three unknown parameters, respectively, namely V_0, k_0, and a in Eq. (7) and b in Eq. (10). However, the essentially unknown parameters are two, k_0 and a or b. These parameters are determined by numerical fitting. The initial reaction rate V_0 can be determined separately from the *P-t* curve experimentally obtained.

RESULTS

Four kinds of cellulosic materials were hydrolyzed under the seven different conditions presented in Table 1, as described in the experimental section. Numerical fitting of Eqs. (5), (7), and (10) to the experimentally obtained relationship of X vs t was carried out. Among the rate parameters to be determined, which are V_0, k_0, and a in Eq. (7) and b in Eq. (10), V_0

Table 2
Comparison of Accuracies of the Three Kinetics Derived on a Basis of the Change in k Value as a Function of Reaction Time. (Run Numbers Correspond to the Numbers Shown in Table 1)

		Estimated value of rate parameter					Standard error, %[b]		
		Kinetic					Kinetic		
	V_0[a]	I	II		III				
Run	mg/(mL·h)	k_0, –	k_0, –	a l/h	k_0, –	b l/h	I	II	III
1	2.55	21.5	24.4	−0.47	25.7	−0.034	1.00	0.54	0.42
2	0.62	9.58	7.97	0.17	8.05	0.016	1.64	1.28	1.26
3	0.65	6.01	3.82	0.22	4.02	0.031	3.00	1.72	1.64
4	0.61	5.38	3.89	0.14	4.03	0.023	2.62	1.63	1.54
5	12.5	5.86	6.44	−0.13	6.33	−0.020	4.57	3.96	3.88
6	21.8	5.23	3.57	0.50	3.92	0.063	6.72	2.16	2.38
7	40.0	5.65	4.46	0.41	4.65	0.059	7.27	3.50	3.36

[a]Determined from the initial slope of the experimentally obtained P-t curve.
[b]Based on the initial concentration of cellulose, i.e., based on the reaction conversion in percent.

was determined separately from the numerical estimation by measuring the initial slope of the experimentally obtained P-t curves and tabulated in Table 2. The other parameters were estimated by Maraquart method with a commercial computer software MultiFit 2.0. All data were treated evenly in the weight, i.e., the variance of the dependent variable was treated as nonexistent. The estimated values of rate constants were tabulated in Table 2. Standard errors in percent were evaluated by:

$$\text{Standard error} = 100\{\sum_{i=1}^{N}(X_{\text{exp,i}} - X_{\text{calc,i}})^2 / N\}^{1/2} \quad (12)$$

where X_{exp} and X_{calc} are fractional conversions ($=P/S_0$) obtained experimentally and calculated by putting the values of rate constants shown in Table 2 to Eq. (5), (7), or (10), respectively. N is the number of data in each set of experiments. The standard error represents how large the difference is between the experimental and the calculated values of reaction conversion in percent. The standard error shows that Kinetic II has better accuracy than Kinetic I, and Kinetic III is slightly better than Kinetic II.

Two results are presented in Figs. 1A and B, which correspond to Runs 1 and 6, respectively. As evaluated by the standard error in Table 2, the X-t curves calculated by Eq. (7) (i.e., by Kinetic II) and Eq. (10) (Kinetic III) showed better fits with the experimental data. Similar results were obtained for the hydrolysis of Runs 2, 3, 4, 5, and 7.

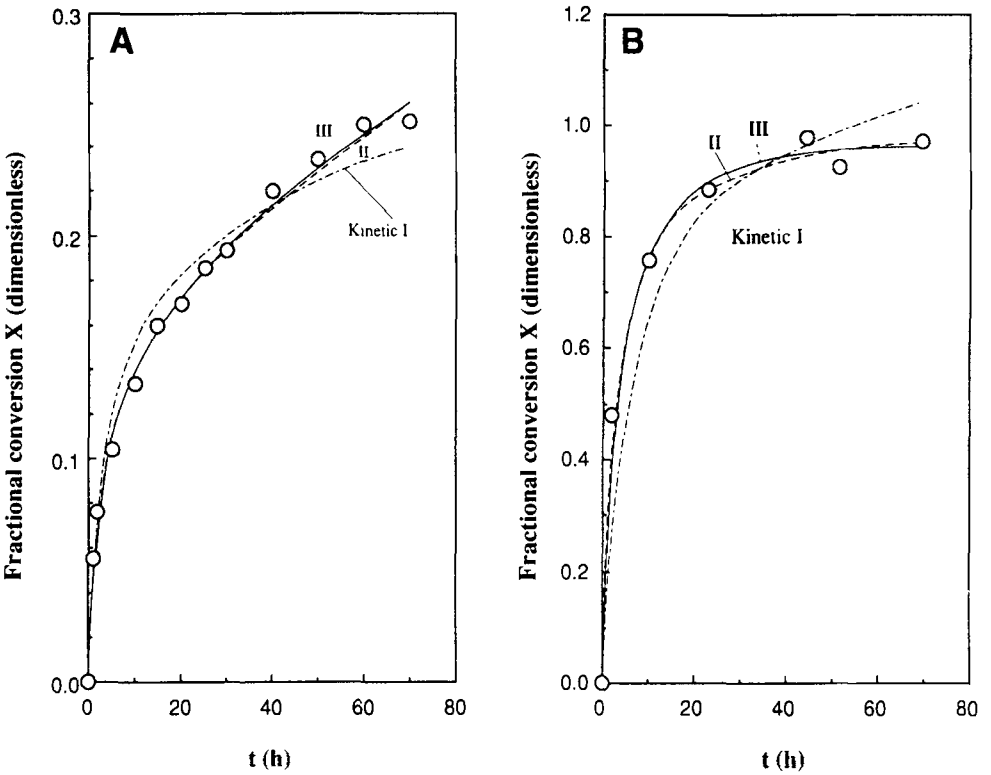

Fig. 1. Fitting the calculated *X-t* curves with the experimental data obtained in Run 1 (**A**) and Run 6 (**B**). Calculation: solid curve by Eq. (5) (Kinetic III), dotted curve by Eq. (7) (Kinetic II), and broken curve by Eq. (10) (Kinetic I).

DISCUSSION

As described above, Eqs. (1) and (2) have been applied to the enzymatic hydrolysis of many cellulosic materials with some success (*6*). However, there were cases in which higher accuracy was desired to represent the experimental data more exactly. In the previous paper, the error was found to be caused by the fact that the value of k in Eq. (1) is not always constant owing to changes in the concentration of adsorbed enzyme. In the present study, the time dependency of k was simply expressed by Eqs. (6) and (9). As the result, it was shown that a better estimation for the P (or X)-t curve can be attained rather than when k is hypothesized to be constant. Furthermore, it was shown that Eq. (9) is slightly better than Eq. (6) as evaluated by the standard error in Table 2.

As Table 2 shows, both minus and plus values in both constant a in Kinetic II and constant b in Kinetic III were obtained. The minus value indicates that k decreases as the hydrolysis proceeds, and it is expected

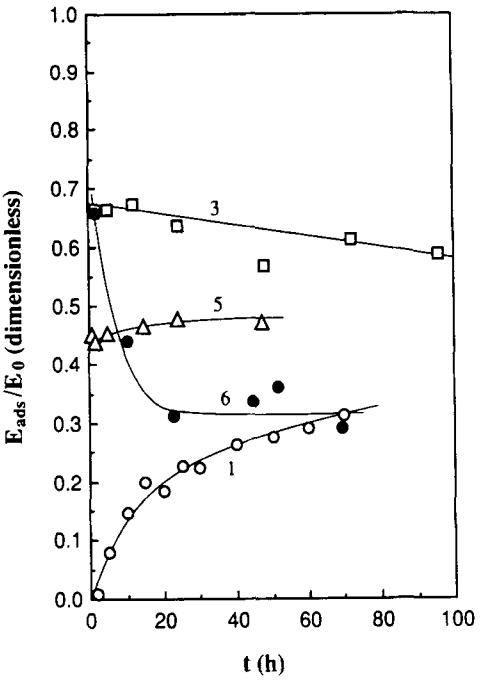

Fig. 2. Adsorption of enzyme on the substrate during hydrolysis in Runs 1, 3, 5, and 6. Numbers in the figure are Run numbers.

from Eq. (3) that the decrease in k value must be caused by an increase in the adsorption of enzyme. Several adsorption data are shown in Fig. 2. Runs 1 and 5 are the cases in which the concentration of adsorbed enzyme increases as the hydrolysis proceeds, and as expected above, the signs of a and b are minus. Furthermore, it is also expected from Eq. (3) that a quick change in the concentration of adsorbed enzyme gives a large absolute value of a and b. In the adsorption data shown in Fig. 2, Runs 1 and 6 are the cases in which the concentration of adsorbed enzyme changes quickly. Run 7 was the similar case, although the adsorption data are not shown in Fig. 2. In these cases, as shown in Table 2 and as expected above, large absolute values in a and b have been obtained. These results suggest that the k–t relationships given by Eqs. (6) and (9) are representing the k–E_{ads} relationship expressed by Eq. (3) in some degree.

In order to study this further, k/n was calculated by substituting the adsorption data of Run 1 (the hydrolysis of Avicel) in Eq. (3). The results are plotted in Fig. 3. The solid curve was obtained by an exponential curve fitting with least-square approximation as expressed by:

$$k/n = 1.11 \exp(-0.035t) \tag{13}$$

The exponent of -0.035 is close to the b value -0.034 determined for the corresponding reaction and shown in Table 2. The curve obtained by using -0.034 as the exponent, which is shown in Table 2, is almost the

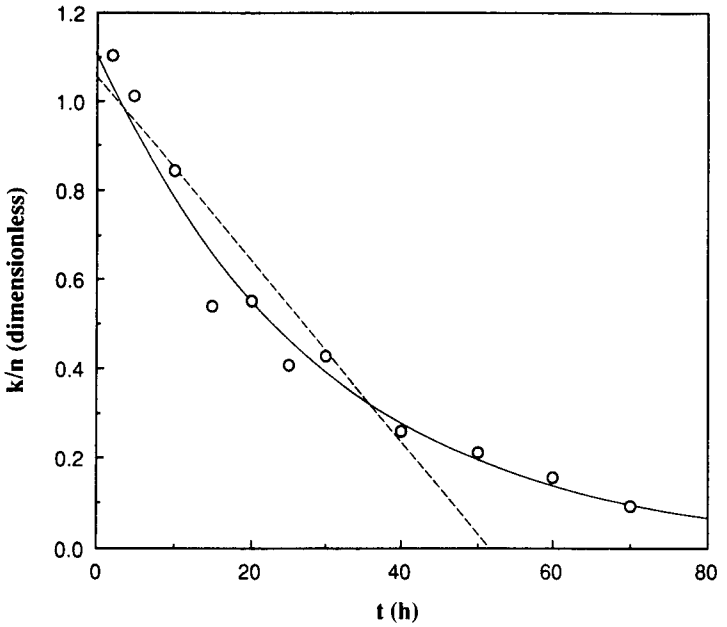

Fig. 3. Relationship between k/n and t calculated by substituting the enzyme adsorption data in Run 1, shown in Fig. 3, for Eq. (3). The solid exponential curve was obtained by the least-square approximation. The relationship could be expressed as $k/n = 1.11 \exp(-0.035\ t)$, corresponding to Eq. (9). The n-value estimated to be 23.1 from 25.7 of k_0 and 1.11 of the pre-exponential factor. The dotted line was obtained by Eq. (6). The relationship could be expressed as $k/n = 1.06 - 0.02t$ by using the same n-value 23.1.

same as the solid curve shown in Fig. 3, although the former curve is not presented here. Incidentally, the value of n was determined to be 23.1 from the values of k_0 and the value of the pre-exponential factor of Eq. (13) 1.11. The dotted straight line presents the k–t relationship determined by substituting -0.02 of a/n and 1.06 of k_0/n for Eq. (6). The estimation of the k–t relationship by Eq. (6) gives a slightly worse accuracy than that by Eq. (9). The standard errors shown in Table 2 represent this situation, although the two fits shown in Fig. 1A are almost the same visually. At least in Run 1, Eqs. (6) and (9) were proven to be good approximations of Eq. (3).

Kinetics II and III were successfully applied to the enzymatic hydrolysis under a variety of conditions. Those variations also include the difference in the enzyme adsorption. PTWs 180, 200, and 220 contain the lignacious residue on which enzyme adsorbs (*10*), but Avicel does not contain it. The enzymes, namely cellulase GC123 and Meicelase used for Runs 1, 5, 6, and 7 contain a protein, β-glucosidase, which does not adsorb on cellulose (*10,11*), but F-β used for Runs 2, 3, and 4 does not contain such a protein. Equation (3) suggests that the change of k depends on the change

in the concentration of adsorbed enzyme during the hydrolysis, rather than the concentration itself. Probably this is the reason Kinetics II and III could be successfully applied to a variety of hydrolyses. The proof will be carried out in another paper.

The favorable feature of Kinetics II and III is not only that they are applied to the hydrolysis under a variety of conditions, but also that they are applied to the hydrolysis attaining high conversions of almost 100% as can be seen in Fig. 1B. Finally it should be emphasized that the simplest equation, that is Eq. (5) (the same as Eq. [1]), is also useful because of the ease in determination of k-value as previously proven (*5,6*).

CONCLUSIONS

Three empirical rate expressions for the enzymatic hydrolysis of cellulose, Kinetics I, II, and III, were developed. They differ in the manner that k depends on hydrolysis time t, Kinetic I assumes that k is constant. Kinetic II assumes a linear relationship between k and t, and Kinetic III, an exponential relationship. The three kinetics were examined for the enzymatic hydrolysis carried out under seven different sets of conditions in which the substrate, enzyme, and those initial concentrations were varied. Better accuracies were obtained by Kinetics II and III as can be seen in Table 2 and Figs. 1A and 1B. Kinetic III gives better accuracy than Kinetic II, although the difference is not as large. The time dependencies of k were found to be consistent with the effect on k of the measured changes in the concentration of adsorbed enzyme as predicted by theory developed previously (*1*).

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