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Enzymatic Saccharification of Industrial and Agricultural Lignocellulosic Wastes

Main Features of the Process

A. V. GUSAKOV,* A. P. SINITSYN, J. A. MANENKOVA, AND O. V. PROTAS

Department of Chemistry, M. V. Lomonosov Moscow State University, Moscow 117234, Russia

ABSTRACT

Cellolignin, an industrial residue obtained during the production of furfural from wood and corn cobs, when pretreated by diluted H_2SO_4 at elevated temperature, may be considered as a potential substrate for enzymatic saccharification. Cellulose fractions of these substrates can almost completely be converted to glucose by cellulases from *T. viride* and *A. foetidus*. The concentration of glucose in hydrolyzates achieves 4–5.5%. A mathematical model for enzymatic hydrolysis of lignocellulose was developed. Using mathematical modeling of the process, an attempt was undertaken to estimate the relative influence of various factors on the efficiency of lignocellulose hydrolysis.

Index Entries: Lignocellulose; industrial and agricultural wastes; cellulases; enzymatic hydrolysis; mathematical modeling.

INTRODUCTION

Industrial and agricultural lignocellulosic wastes are potential sources for the production of ethanol, amino acids, and other useful products by enzymatic and microbial methods *(1,2)*.

*Author to whom all correspondence and reprint requests should be addressed.

The enzymatic cellulose hydrolysis is known to be affected by many factors that are closely interrelated. Mathematical modeling of the process may help to evaluate the individual roles of these factors. Previously, the kinetic model for hydrolysis of delignified cellulose had been developed (*3*) and used for a quantitative evaluation of some factors in batch and continuous reactors (*4*). However, the possible effects of lignin, which usually makes a significant fraction in natural cellulose-containing materials, were not taken into account in the model. Lignin may adsorb and inactivate the cellulolytic enzymes, leading to a decrease in the efficiency of the process (*5–7*).

The objective of this study was to assess the potential of two lignocellulosic residues in the enzymatic saccharification. We also tried to estimate the relative influence of major factors on the efficiency of enzymatic hydrolysis of lignocellulose, using a mathematical model of the process, which, together with various factors, takes into account the effects of lignin.

MATERIALS AND METHODS

Enzymes

The enzyme preparations from *T. viride* and *A. foetidus*, the industrial products of Privolzhsk Biochemical Plant (USSR), were used for hydrolysis. The *T. viride* preparation had filter paper activity, 80 U/g (pH 4.8, 50°C) (*8*), and β-glucosidase (cellobiase) activity assayed at pH 4.5 and 40°C by standard method (*9*), using 2 m*M* cellobiose, 5 U/g. The corresponding activities of *A. foetidus* preparation were 7 and 75 U/g. In all cases, 1 U of activity corresponds to the quantity of the enzyme that produces 1 μmol of product or hydrolyze 1 μmol of substrate/min.

Substrates

Lignocellulosic industrial residue (so-called cellolignin) obtained during the production of furfural from mixed wood at Manturovo Biochemical Plant (USSR), and corn cobs, pretreated by 1% H_2SO_4 at 130–150°C for 60 min, were used as substrates in the enzymatic hydrolysis. Cellulose hydrolyzable fractions in cellolignin and pretreated corn cobs, determined by the extensive enzymatic hydrolysis of the substrates at their concentrations of 5 and 10 g/L, were found to be 45 and 37%, respectively. The residual hemicellulose content in the substrates was 2 and 3%, respectively. Cellolignin was used for hydrolysis without wash; pretreated corn cobs were washed with water before the hydrolysis.

Hydrolysis Conditions

Lignocellulose was hydrolyzed at 50 °C in thermostated glass cells (50 mL vol) agitated on a shaker (250 shakings/min). The reaction was carried out in 0.1*M* acetate buffer, pH 4.5, using *T. viride* or composite *T. viride-A. foetidus* preparation of appropriate concentration.

Analysis of the Hydrolysis Products

The hydrolysis products were analyzed by high-pressure liquid chromatography (HPLC) with a Knauer HPLC system (FRG) using a Silasorb-NH_2 column (4.6 mm × 25 cm) and acetonitrile-water (70:30) as the mobile phase. The hydrolyzate was filtered before the analysis.

Hydrolysis Modeling and Determination of Kinetic Parameters

A system of differential equations used to predict the kinetics of lignocellulose hydrolysis was solved numerically by a fourth-order Runge-Kutta method (*10*). Kinetic parameters were determined by least-squares fitting of theoretical kinetic curves of cellobiose and glucose formation to experimental data. A simplex method combined with random searching was used in the minimization algorithm (*10*). All computations were carried out on a Tandon PCA 286 computer using Fortran programs.

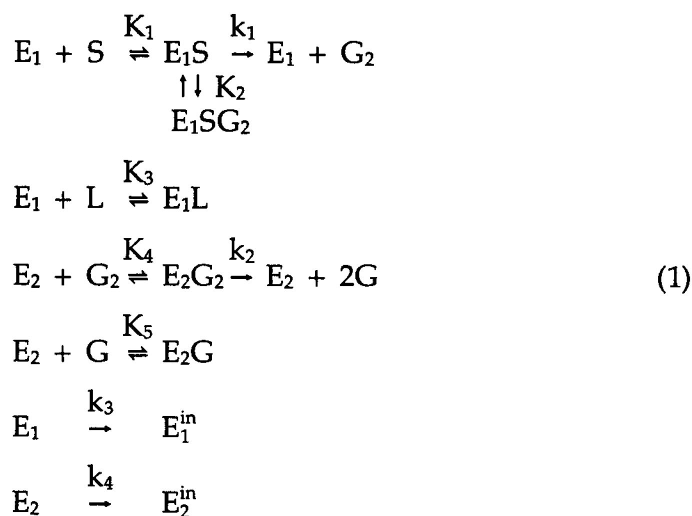
THEORY

The main principles of the mathematical model for lignocellulose hydrolysis by enzymes from *T. viride* (most of them were confirmed experimentally) are as follows:

1. The products of the hydrolysis are glucose and cellobiose.
2. Cellobiose is produced by cellulases (cellobiohydrolases and endoglucanases) adsorbed on cellulose fraction of lignocellulose. Cellobiose is hydrolyzed in the solution to glucose by β-glucosidase. Such reaction scheme was confirmed by independent experiments on cellolignin hydrolysis in the presence of δ-gluconolactone (selective inhibitor of β-glucosidase).
3. Cellobiose-producing cellulases may be considered as one enzyme (E_1). Though this assumption is not true in reality, such a simplification is used in most models for enzymatic cellulose hydrolysis (*see*, e.g., ref. *11*), and work fairly well.

4. Cellulase (E_1) may be also bound to lignin fraction of lignocellulose. This fact was reported by various researchers (*5–7*), and was confirmed in our experiments where cellulase adsorption on lignacious residue, obtained after the extensive hydrolysis of lignocellulose, was studied.
5. Adsorbed cellulase is inhibited by cellobiose. Enzyme sites for adsorption and catalysis (inhibition) are different and independent (*12,13*). β-Glucosidase is competitively inhibited by glucose.
6. The reactivity of cellulose fraction is not altered during the hydrolysis. This was shown by independent experiments carried out as described in ref. (*3*).
7. In the course of hydrolysis, inactivation of cellulase and β-glucosidase occurs. The inactivation is caused by several factors, discussed below. The exponential equation is supposed to be valid to describe the inactivation.

In terms of these principles, enzymatic hydrolysis of lignocellulose can be represented by the following kinetic scheme:



$$E_1 + S \overset{K_1}{\rightleftharpoons} E_1S \overset{k_1}{\rightarrow} E_1 + G_2$$
$$E_1S \overset{K_2}{\rightleftharpoons} E_1SG_2$$
$$E_1 + L \overset{K_3}{\rightleftharpoons} E_1L$$
$$E_2 + G_2 \overset{K_4}{\rightleftharpoons} E_2G_2 \overset{k_2}{\rightarrow} E_2 + 2G \qquad (1)$$
$$E_2 + G \overset{K_5}{\rightleftharpoons} E_2G$$
$$E_1 \overset{k_3}{\rightarrow} E_1^{in}$$
$$E_2 \overset{k_4}{\rightarrow} E_2^{in}$$

where E_1 denotes cellulase, E_2 denotes β-glucosidase, E_1^{in} and E_2^{in} are inactivated enzymes, S denotes cellulose fraction of lignocellulose, L is lignin fraction, G_2 is cellobiose, and G is glucose.

Time courses of the concentrations of the substrate and products are described by the following system of differential equations:

$$\begin{aligned} d[S]/dt &= -v_1 \\ d[G_2]/dt &= v_1/R_1 - v_2 \\ d[G]/dt &= 1.05 \cdot v_2 \end{aligned} \qquad (2)$$

Table 1
Kinetic Parameters for Hydrolysis of Cellolignin by Cellulases from *T. viride* at 50°C and pH 4.5

Parameter	Value	Units
Specific activity of E_1	70	$U\ g^{-1}$
Specific activity of E_2	12.4	$U\ g^{-1}$
K_1	12	$U\ L^{-1}$
K_2	3.58	$g\ L^{-1}$
K_3	15	$U\ L^{-1}$
K_4	0.79	$g\ L^{-1}$
K_5	0.43	$g\ L^{-1}$
k_1	0.0118	min^{-1}
k_2	0.000342	$g\ U^{-1}\ min^{-1}$
k_3	0.00171	min^{-1}
k_4	0.00034	min^{-1}
R_1	27.9	$U\ g^{-1}$
R_2	15.1	$U\ g^{-1}$

where $v_2 = k_2[E_2]_0[G_2] \cdot e^{-k_4 t} / (K_4(1+[G]/K_5)+[G_2])$ is the rate of cellobiose hydrolysis to glucose under the action of β-glucosidase, and $v_1 = k_1[E_1S]$ is the rate of cellobiose formation from cellulose.

In the system of differential equations (2), the concentrations of E_1 and E_2 are expressed in U/L. In order to operate in equivalent units, the concentrations of cellulose, lignin, and complexes, E_1S, E_1L, and E_1SG_2, are also expressed in U/L. For cellulose and lignin

$$[S]_0 = R_1 \times [\text{weight concentration of cellulose fraction}]$$

$$[L]_0 = R_2 \times [\text{weight concentration of lignin fraction}]$$

The coefficients R_1 and R_2, used to pass from weight concentrations of cellulose and lignin (g/L) to units (U/L), represent, in fact, maximum adsorption of cellulase on cellulose and lignin fractions (U/g). Concentrations of glucose and cellobiose are expressed in g/L. The units for kinetic parameters are presented in Table 1.

The concentration of $[E_1S]$ in the equation for v_1 can be defined in the following way:

On the one hand, the total adsorbed cellulase exists in two forms (material balance by enzyme catalytic or inhibition site):

$$[E_1S]_0 = [E_1S] + [E_1SG_2] \tag{3}$$

Using the equation for the equilibrium constant

$$K_2 = [E_1S][G_2] / [E_1SG_2] \tag{4}$$

Equation (3) may be transformed to:

$$[E_1S] = [E_1S]_0 / (1 + [G_2]/K_2) \quad (5)$$

On the other hand, the concentration of total adsorbed cellulase can be expressed through the equilibrium constant K_1, where enzyme adsorption site is involved:

$$[E_1S]_0 = [E_1]\,[S] / K_1 \quad (6)$$

Taking into account equations (5) and (6), together with inactivation factor, the expression for the rate v_1 becomes:

$$v_1 = k_1[E_1]\,[S]\cdot e^{-k_3t} / K_1 (1 + [G_2]/K_2) \quad (7)$$

In this equation, the values of concentrations of free enzyme and cellulose, $[E_1]$ and $[S]$, should be defined in order to perform numerical integration. This can be done in the following way. The material balance equations for E_1, S, and L, taking into account the cellulase adsorption on cellulose and lignin fractions of lignocellulose, where the adsorption site of the enzyme is involved, are:

$$\begin{aligned} [E_1]_0 &= [E_1] + [E_1S]_0 + [E_1L] \\ [S]_0 &= [S] + [E_1S]_0 \\ [L]_0 &= [L] + [E_1L] \end{aligned} \quad (8)$$

Expressing $[E_1S]_0$ and $[E_1L]$ through the equilibrium constants, and solving the system of algebraic equations, we obtain:

$$[E_1]_0 = [E_1] + [E_1]\,[S]_0 / (K_1 + [E_1]) + [E_1]\,[L]_0 / (K_3 + [E_1]) \quad (9)$$

Transforming this expression gives cubic equation:

$$A[E_1]^3 + B[E_1]^2 + C[E_1] + D = 0 \quad (10)$$

where $A = 1$
$B = K_1 + K_3 + [S]_0 + [L]_0 - [E_1]_0$
$C = K_1K_3 + K_3[S]_0 + K_1[L]_0 - K_1[E_1]_0 - K_3[E_1]_0$
$D = -K_1K_3[E_1]_0$

Having solved the cubic Eq. (10) by well known analytical methods (*14*), one can obtain the value $[E_1]$ and then the value [S], that are used in the expression for v_1.

RESULTS AND DISCUSSION

Determination of Kinetic Parameters for β-glucosidase from *T. viride*

Kinetic parameters for β-glucosidase from *T. viride* were determined from three independent experiments on cellobiose hydrolysis by *T. viride* preparation, where the concentration of cellobiose varied in the range of

5–20 g/L, and the enzyme concentration was 10 g/L. Using the progress kinetic curves of cellobiose consumption and glucose formation, the values of K_4, K_5, and $V_{max} = k_2[E_2]_0$ were found by least-squares minimization. The value of V_{max} was found to be 0.0424 g/L/min. This corresponds to specific β-glucosidase activity of 12.4 U/g of enzyme preparation. The values of kinetic parameters are presented in Table 1. It should be noted that, since in lignocellulose hydrolysis modeling the concentration of β-glucosidase is expressed in U/L, and concentrations of glucose and cellobiose are expressed in g/L, k_2 is, in fact, a coefficient used to transform U/L into g/L.

Modeling the Kinetics of Cellolignin Hydrolysis by *T. viride* Cellulases

The other *T. viride* kinetic parameters were found by least-squares minimization using the data of 15 experiments, where the concentrations of cellolignin and enzyme preparation varied in the range of 10–150 g/L and 5–40 g/L, respectively. In some of these experiments, cellobiose (10 g/L) or glucose (20 g/L) was added to the reaction mixture at the beginning of the hydrolysis. The values of kinetic parameters are presented in Table 1.

Figures 1 and 2 show the theoretical kinetics of cellolignin hydrolysis by *T. viride* cellulases and the corresponding experimental data points for some experiments where enzyme and substrate concentrations were varied. As can be seen from Fig. 1, the theoretical kinetic curves of cellobiose and glucose formation coincide satisfactorily with experimental points. The best fitting may be observed for initial rates of products' formation and rather satisfactory, for final concentration of sugars after 48 h of hydrolysis (Fig. 2). However, for some of the experiments (Fig. 2), the model gave some overestimation in the middle region of kinetic curves (10–30 h). This can be explained by the fact that enzyme inactivation is taken into account in the model in a very simple (exponential) form. More detailed consideration of factors responsible for the inactivation may help to develop a more adequate mathematical model.

Modeling the Kinetics of Lignocellulose Hydrolysis by Composite Cellulase Preparation

The hydrolyzates of lignocellulose obtained under the action of *T. viride* cellulases contained large amounts of cellobiose (*see*, e.g., Fig. 1), since the *T. viride* preparation was deficient by β-glucosidase. The addition of β-glucosidase from *Aspergillus* strains may be useful for obtaining the hydrolyzates with high glucose content. We used the enzyme preparation from *A. foetidus*. The kinetics of cellobiose hydrolysis by β-glucosidase from *A. foetidus* have been studied previously (*15*). In this article, simplified kinetic model for cellobiose hydrolysis by *A. foetidus* β-glucosidase was used. In this model, substrate and product inhibition (in

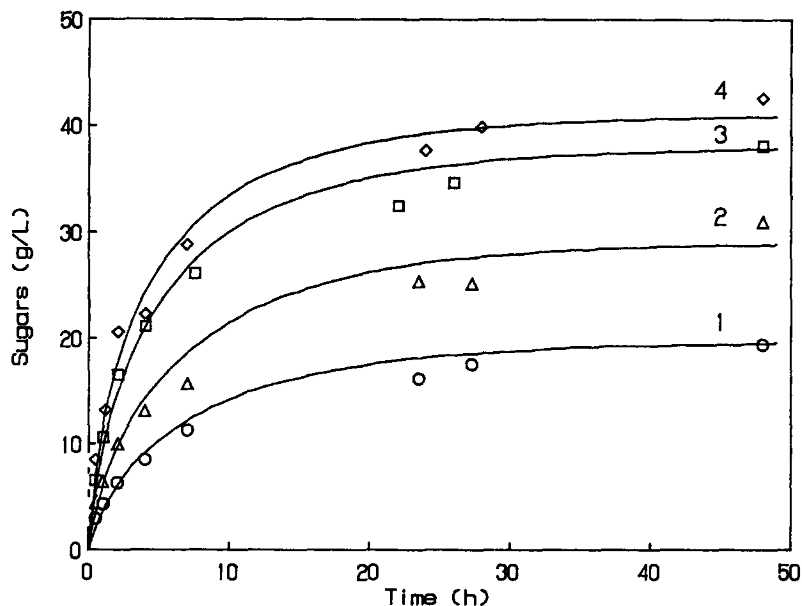


Fig. 1. Theoretical kinetic profiles of cellobiose (1), glucose (2), and total sugar (3) formation during enzymatic hydrolysis of cellolignin (150 g/L) by cellulases from *T. viride* (20 g/L) and experimental data. ○, cellobiose; △, glucose; □, total sugars (glucose + cellobiose).

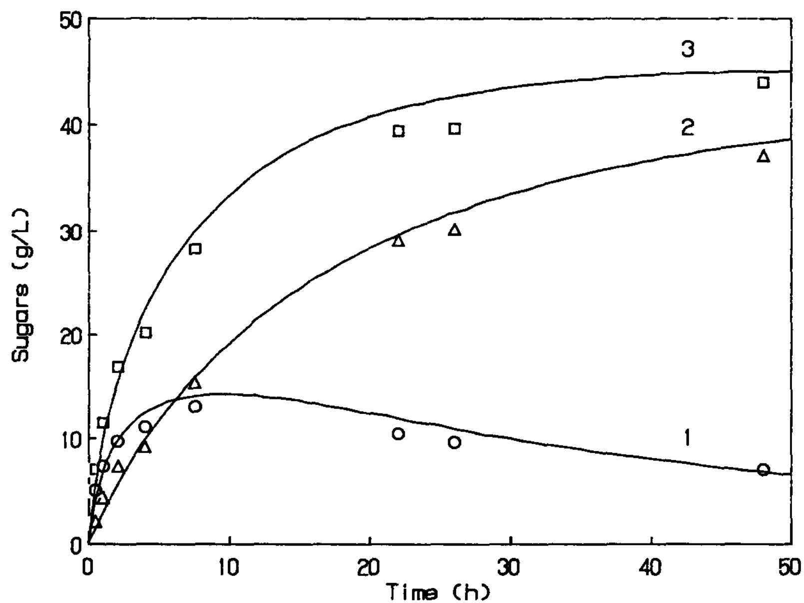


Fig. 2. Theoretical kinetic profiles of formation of sugars (cellobiose + glucose) during enzymatic hydrolysis of cellolignin (100 g/L) by cellulases from *T. viride* and experimental data. Concentration of the enzyme preparation: 1, 5 g/L; 2, 10 g/L; 3, 20 g/L; 4, 35 g/L.

Table 2
Kinetic Parameters for Hydrolysis of Cellobiose by β-glucosidase from *A. foetidus* at 50°C and pH 4.5

Parameter	Value	Units
Specific activity of E_3	190	$U\ g^{-1}$
K_6	0.68	$g\ L^{-1}$
K_7	0.36	$g\ L^{-1}$
K_8	7.2	$g\ L^{-1}$

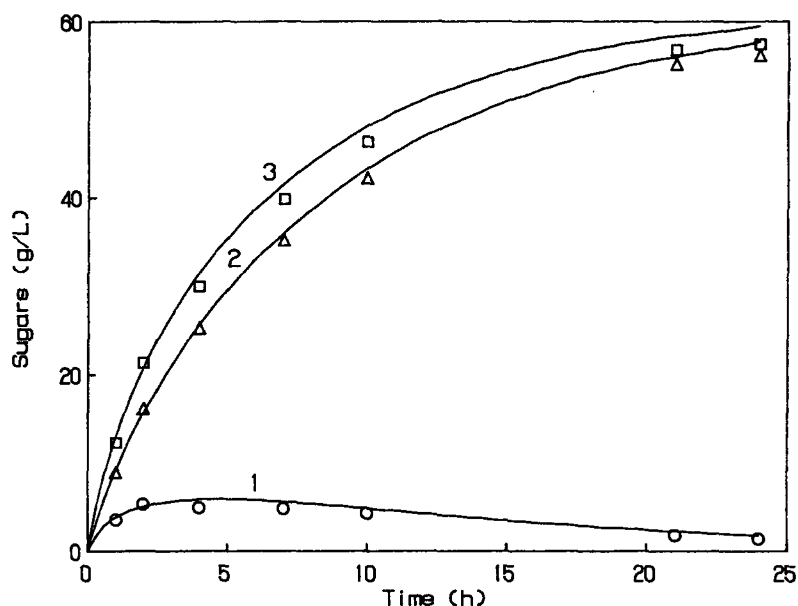


Fig. 3. Theoretical kinetic profiles of cellobiose (1), glucose (2), and total sugar (3) formation during enzymatic hydrolysis of cellolignin (150 g/L) by composite enzyme preparation *T. viride* (20 g/L), *A. foetidus* (10 g/L), and experimental data. ○, cellobiose; △, glucose; □, total sugars (glucose+cellobiose).

simplified form) is taken into account, the transglycosylation effects are neglected, and the equation for cellobiose hydrolysis, which is used in differential equations for cellobiose consumption and glucose accumulation in system (2), together with v_2, is the following:

$$v_3 = k_2[E_3]_0[G_2]\cdot e^{-k_4 t} / (K_6 (1 + [G]/K_7) + [G_2]^2/K_8 + [G_2])$$

where $[E_3]_0$ denotes activity of β-glucosidase from *A. foetidus* (U/L), K_6 is Michaelis constant, and K_7 and K_8 denote product and substrate inhibition constants. It was supposed that the inactivation constant (k_4) for E_3 is the same as for E_2. The values of other kinetic parameters are presented in Table 2.

Figure 3 shows the time course of cellolignin hydrolysis by composite *T. viride-A. foetidus* preparation. As can be seen from the figure, the main

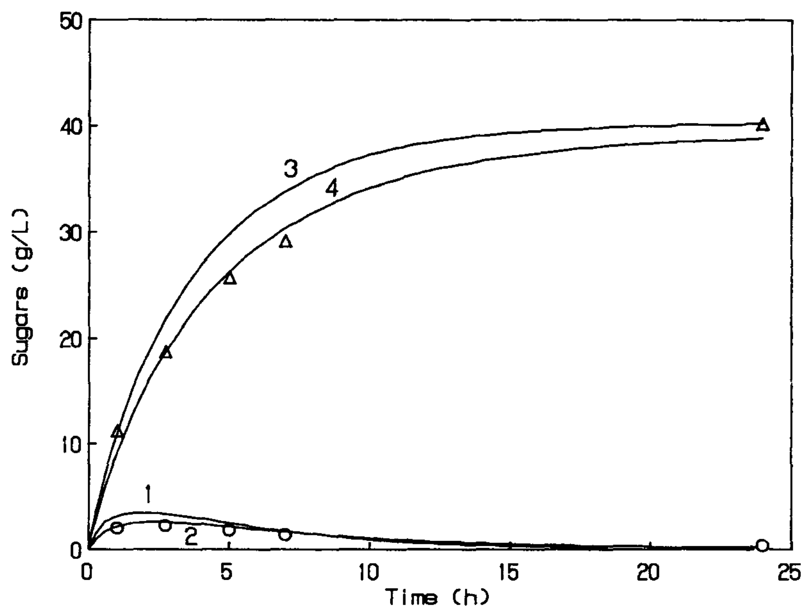


Fig. 4. Theoretical kinetic profiles of cellobiose (1,2), and glucose (3,4) formation during enzymatic hydrolysis of pretreated corn cobs (110 g/L) by composite enzyme preparation *T. viride* (30 g/L), *A. foetidus* (15 g/L), and experimental data. ○, cellobiose; △, glucose. 1 and 3, $k_1 = 0.0118\ min^{-1}$; 2 and 4, $k_1 = 0.008\ min^{-1}$.

product of hydrolysis is glucose, and theoretical kinetic curves coincide fairly well with experimental data points.

Using the kinetic parameters for cellolignin hydrolysis, an attempt was made to describe the time course of hydrolysis of pretreated corn cobs under the action of composite enzyme preparation. With the same kinetic parameters, the model gave some overestimation (Fig. 4, curves 1,3). However, after changing only one parameter, k_1 (0.008 instead of 0.0118 min^{-1}), rather satisfactory fitting was achieved (Fig. 4, curves 2,4). Satisfactory fitting was also observed with other concentrations of corn cobs and enzyme preparation (data are not presented here). This fact gives a hope that the model may be used with very slight modifications to predict the kinetics of hydrolysis of other lignocellulosic substrates.

Figures 3 and 4 show that 4–5.5% glucose solution can be obtained after 1 d of hydrolysis of cellolignin and pretreated corn cobs with composite enzyme preparation having filter paper activity of 1.6–2.4 U/mL (activity of *T. viride* cellulases. *A. foetidus* cellulose solubilizing activity may be neglected since it was very low). The degree of cellulose conversion was 82 and 89%. So, these lignocellulosic residues may be considered as very prospective for saccharification.

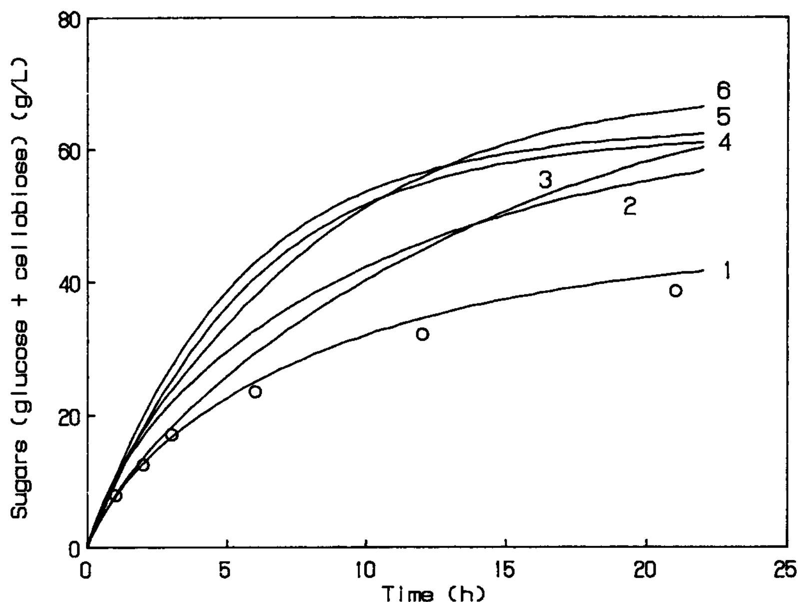


Fig. 5. Effects of various factors on kinetics of enzymatic hydrolysis of cellolignin by composite *T. viride* (10 g/L)-*A. foetidus* (5 g/L) preparation. Theoretical curves: 1, all factors are considered; 2, lignin is assumed to be absent in the reaction system; 3, the inactivation of E_1, E_2, and E_3 is not taken into consideration; 4, the inhibition of E_2 and E_3 by glucose is not taken into account; 5, the inhibition of E_1 by cellobiose is not taken into account; 6, lignin and the inactivation of E_1, E_2, and E_3 are assumed to be absent. The concentration of cellolignin is 150 g/L, cellulose concentration is 67.5 g/L. ○, experimental points.

Effect of Various Factors on Lignocellulose Hydrolysis

The mathematical model of the hydrolysis process allows us to estimate the effects of various factors on the efficiency of lignocellulose hydrolysis by simulating various situations in the reaction system and comparing the theoretical curves with experimental data. Theoretical kinetic curves 2–6 in Fig. 5, calculated with the assumptions that some of the factors, taken into account in the model, are absent in the reaction system, when compared with experimental data points, show the relative roles of these factors in hydrolysis.

Figure 5 shows that the most negative factors in hydrolysis of cellolignin by composite enzyme preparation are inhibition of the enzymes by cellobiose and glucose (compare curves 5 and 4 with curve 1). Significant negative effect is produced by enzyme inactivation (curve 3) and, to a lesser extent, by nonspecific binding of cellulase to lignin (curve 2).

Special attention should be paid to the inactivation of enzymes in the course of hydrolysis. There may be several reasons for the inactivation including (1) so-called pseudoinactivation of cellulase as a result of tight adsorption and loss of mobility of the enzyme (*3,16*), (2) lignin-induced inactivation (*5*), (3) inactivation by shear conditions (stirring or shaking) (*17*), and (4) thermoinactivation. The relative influence of various kinds of inactivation depends on properties of the enzyme and substrate, and conditions of the hydrolysis process. Our independent experiments, where the methods of inactivation study were similar to those described in refs. (*3,5,16*), have shown that during the hydrolysis of cellolignin, the first two types of inactivation (1 and 2) play a major role in comparison to the other kinds of inactivation. These two types of inactivation may be classified as substrate-induced inactivation. However, further work is needed to discriminate quantitatively the effects of cellulose and lignin, and to find out what portion of cellulase is inactivated irreversibly. Nevertheless, the loss of cellulase activity caused by the contact of the enzyme with lignin has been definitely observed. So, the negative effects of lignin, including both nonspecific cellulase binding and lignin-induced inactivation of the enzymes, may be close to the combined effect shown by the theoretical kinetic curve 6 in Fig. 5.

CONCLUSIONS

1. Cellolignin, an industrial residue obtained during the production of furfural from wood, and corn cobs, when pretreated by diluted H_2SO_4 at elevated temperature, may be considered as potential substrates for enzymatic saccharification. After 1 day of hydrolysis with composite *T. viride-A. foetidus* preparation, the hydrolysates with glucose concentration of 4–5.5% may be obtained, the degree of cellulose conversion being not less than 80%.
2. Kinetic analysis of cellolignin hydrolysis, using a mathematical model of the process, has shown that, together with product inhibition, nonspecific adsorption of cellulases on lignin fraction and their substrate-induced inactivation seem to affect negatively the hydrolysis efficiency.

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