COMPARATIVE KINETIC ANALYSIS OF ENZYME HYDROLYSIS OF STEAM-EXPLODED WHEAT STRAW

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The present work represents a comparative kinetic analysis of enzyme hydrolysis, using two kinetic variables to describe both reducing sugars and glucose. The preliminary treatment of wheat straw by steam explosion was followed by enzyme hydrolysis using the NS 50013 and β-glucosidase NS 50010 enzyme cellulase complex. The hydrolysis has been described by an exponential kinetic equation valid for processes developed on uniformly inhomogeneous surfaces. The wheat straw–enzyme system has been observed to behave as an energetically homogeneous one. The preexponential factor decreased with the increase of the hydrolysis degree, some kind of compensation effect between the change of activation energy and the preexponential factor being derived. The isokinetic temperature at which a complete compensation was observed was calculated, the rates of reducing sugars and glucose formation being equal.

Keywords: wheat straw, cellulose, cellulase, exponential equation, correlations, compensation effect

INTRODUCTION

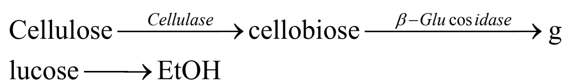
The agricultural lignocellulosic raw materials obtained from wheat straw are potential sources for the production of ethanol. The biomass is inexpensive, renewable, widely available and environmentally friendly. The bioconversion of wheat straw is favored by its relatively low lignin content and high carbohydrate content.[1,2] Due to the close association of cellulose and hemicellulose with lignin in the plant cell wall, pretreatment is necessary to make these carbohydrates available for enzymatic hydrolysis and fermentation. Thermochemical pretreatments, such as dilute acid hydrolysis and steam explosion, which solubilise the hemicellulose components and increase cellulose accessibility, are commonly used to prepare lignocelluloses for enzymatic saccharification.[3,4] Steam explosion (autohydrolysis) is one of the most cost-effective and widely used pretreatment methods for wheat straw.[5] According to this method, the size-reduced biomass is rapidly heated by high pressure steam for a short period of time, after which pressure is suddenly reduced, causing an explosive decomposition of the materials.[6]

Hydrolysis using appropriate enzymes represents the most effective method to liberate simple sugars from cellulosic materials. Cellulose hydrolysis is catalyzed by a class of enzymes, known as cellulases. Three major groups of enzymes, namely endo-gluconase, exo-gluconase and β-glucosidase, are involved in cellulose hydrolysis. Enzymatic hydrolysis can be influenced by substrate and end-product concentration, enzyme activity and reaction conditions. β-glucosidase plays a significant role in the hydrolysis process, since cellobiose is an end-product inhibitor of many cellulases.[7,8] The products of hydrolysis are usually reducing sugars, including glucose. The reducing sugars degraded by cellulases can be fermented by yeasts or bacteria to ethanol.[9]

Enzymatic hydrolysis is a hurdle because of the heterogeneous nature of the cellulose substrate and because degradation of the cellulose chain progresses in only one direction for each cellobiohydrolase, effectively reducing the reaction to a one-dimensional process. As known, cellulose consists of relatively easily accessible amorphous regions with few lateral interactions among the cellulose chains, as well as of crystalline domains much more difficult to hydrolyze.

In addition, the hydrolysis product of the cellulase reaction, cellobiose (glucosyl β-1-4

glucose) is well-known to severely inhibit cellulase. Therefore, β-glucosidase is added to cleave cellobiose to glucose:[10]



$$\text{Cellulose} \xrightarrow{Cellulase} \text{cellobiose} \xrightarrow{\beta-Glu\cos idase} \text{glucose} \longrightarrow \text{EtOH}$$

The optimization of lignocellulosic bioconversion by cellulase enzymes requires good knowledge of the reaction kinetics. The complexity of the enzymatic hydrolysis of lignocellulosic wastes consists from the fact that they are heterogeneous insoluble substrates, and thus, their enzymatic hydrolysis is always limited.[1] Usually, enzymatic kinetics is studied with the Henri-Michaelis-Menten equation,[11] even if it has been shown as not suitable for the analysis of enzymatic reactions of the heterogeneous system.[12-14]

The aim of this work is to investigate the kinetics of enzyme hydrolysis of steam-exploded wheat straw, with respect to the reducing sugars and glucose obtained. Based on the kinetic relationships established, a comparative analysis, for elucidating the mechanism of enzymatic hydrolysis, was performed.

EXPERIMENTAL

Kinetic investigations were performed on wheat straw with the following chemical composition: 44% cellulose,[15] 33.5% pentosans (TAPPI T19m-54 Standard), 24.3% lignin (TAPPI-T222 om-88 Standard), easily-hydrolysable polysaccharides and water-soluble substances – 20.7%[16] and 4.6% ash.

Steam explosion, used as a pretreatment of wheat straw, was performed in a 2 L stainless steel laboratory installation, under the following conditions: a hydromodule ratio of 1:10; initial temperature of 100 °C; maximum temperature of 190 °C; pressure of 12.8 bar; heating time of 60 min, followed by additional 10 min at the maximum temperature.

The residue was washed with distilled water and the obtained hydrolysate was filtered. The solid residue produced during the steam explosion pretreatment was subjected to enzymatic hydrolysis as a second treatment stage.

The cellulase complexes NS 50013 with 700 EGU/g activity and β-glucosidase NS 50010 with 250 CBU/g activity, produced by Novozymes AS, were used for enzymatic hydrolysis. The enzyme charge of NS 50013 was of 5%, while that of NS 50010 was of 0.5%.

Cellulasic hydrolysis was carried out in polyethylene bags in a water bath previously heated to the desired temperature.

The kinetics of cellulasic hydrolysis was examined at temperatures of 30, 40 and 50 °C, consistency of 10%, $pH_{initial}$ of 5.0-6.0 and pH_{final} of 4.2-4.6 and reaction time ranging from 1 to 24 h.

The reducing sugars in the hydrolysates obtained were determined according to the DNS method.[17]

The glucose, xylose and cellobiose contents were analyzed with a Dionex HPLC system, according to the NREL standard biomass analytical procedure.[18] The amount of glucose formation was calculated.

RESULTS AND DISCUSSION

The preliminary treatment of wheat straw by steam explosion is followed by enzymatic hydrolysis, using the enzyme cellulase complex NS 50013 and β-glucosidase NS 50010. The enzyme complex used breaks the β-1,4-glucoside bonds in the cellulose molecule, leading to the formation of cellobiose, an intermediate included in the total amount of reducing sugars. Cellobiose hydrolysis with the participation of the second enzyme (β-glucosidase NS 50010) results in glucose formation (Fig. 1).

The type and amount of reduced substances are determined by HPLC. Steam explosion for 10 min at 190 °C is used as a preliminary treatment. Enzymatic hydrolysis with the cellulase complex NS 50013 and β-glucosidase is carried out to give a hydrolysate containing glucose, xylose and cellobiose. The chromatogram presented in Figure 1 shows that the presence of glucose is the highest.

The increase, with time, of the obtained amount of reducing sugars, R_s (%), and glucose, G (%), is followed at different temperatures, for studying the kinetics of raw material enzymatic hydrolysis. Figure 2 plots the comparative kinetic curves obtained (Fig. 2).

Figure 2 shows that the amount of reducing sugars is higher than that of glucose, irrespectively of the time and temperature applied. For example, $R_S = 23.1\%$, while $G = 18.46\%$ in the 24th hour of the experiment, carried out at 50 °C (an $R_S/G = 1.25$ ratio thus resulting). Figure 2 also shows that the kinetic curves obtained for both products have a similar shape which, in turn, assumes a correlation whose elucidation is important for understanding the mechanism of the hydrolysis process.

The dimensionless term α_R, used as a kinetic variable with respect to reducing sugars, defines the relative change in their amounts, according to Eq. (1):

$$\alpha_R = R_S / R_{S,\max} \quad (1)$$

where R_S is the current amount of reducing sugars and $R_{S,\max}$ is the maximum amount of reducing

sugars obtained after 48 h of treatment, found equal to 35%.

The kinetic variable α_G, which can be used in the case of glucose, expresses the relative change in the amount of glucose, according to Eq. (2):

$$\alpha_G = G / G_{max} \quad (2)$$

where G is the current amount of glucose obtained and G_{max} is the maximum amount obtained after 48 h of treatment, found equal to 26%.

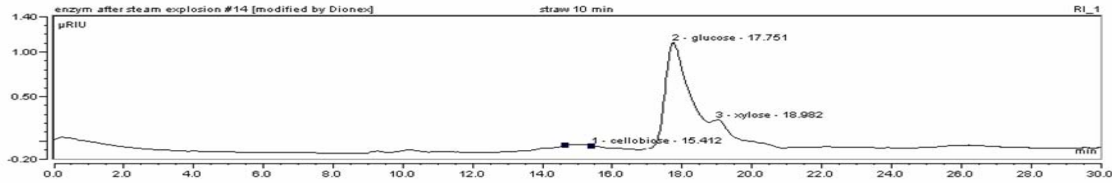


Figure 1: Chromatogram of wheat straw enzyme hydrolysate. Steam explosion for 10 min has been applied as a preliminary treatment

The kinetic variables α_R and α_G express the degree of wheat straw enzymatic hydrolysis. Figure 3 plots the kinetic curves of both variables, which increase with increasing time and temperature (Fig. 3).

In both cases, the hydrolysis process can be described by an exponential kinetic equation valid for processes taking place on uniformly inhomogeneous surfaces and successfully applied in studies of enzymatic hydrolysis.[19-21] According to the model of uniformly inhomogeneous surfaces, the active centers on the surface are distributed linearly, referring to their energy and entropy. The exponential kinetic equation is applied in the form:

$$v = v_0 e^{-a\alpha} \quad (3)$$

where $v = d\alpha / dt$ and v_0 are the current and the initial rate of enzymatic hydrolysis, respectively. The kinetic coefficient a accounts for the energy and entropy inhomogeneity of the system, being actually a temperature-dependent term. The same coefficient is related to the number of active centers, to their accessibility and spatial orientation.

All kinetic curves are linearized in coordinates α *vs*. ln$t$, according to the approximate integral form of Eq. (3), *i.e.*:

$$\alpha = \frac{1}{a} \ln v_0 a + \frac{1}{a} \ln t \quad (4)$$

The linear dependencies obtained are presented in Figure 4. The slope of the lines obtained permits the evaluation of the inhomogeneity coefficients. It is found out that a_G = 6.7, while a_R = 7.7.

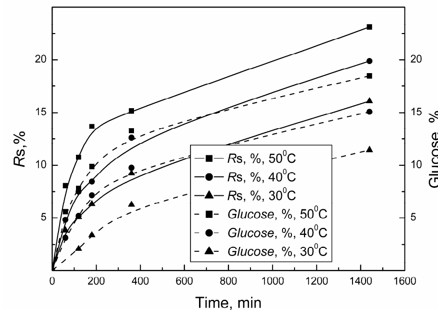


Figure 2: Kinetic curves of the amount of reducing sugars R_S (%) and glucose obtained G (%) at different temperature values

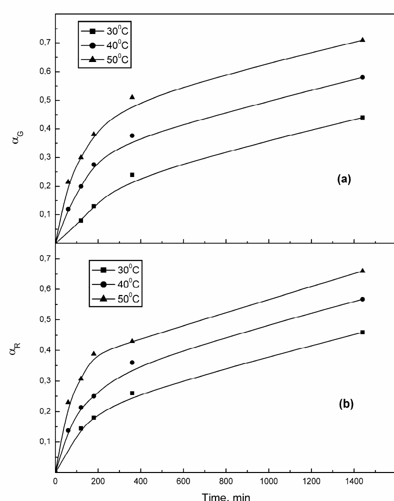


Figure 3: Kinetic curves of α_G (a) and α_R (b)

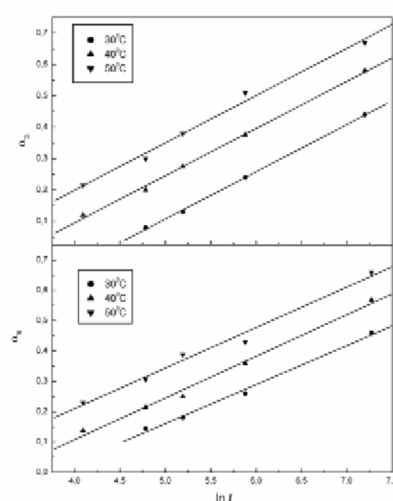


Figure 4: Linear dependencies α_G *vs*. ln*t* (a) and α_R *vs*. ln*t* (b)

As seen in Figure 4, the linear dependencies presented run in parallel, *i.e.* the kinetic coefficients a_G and a_R do not depend on temperature and, hence, account only for the entropy inhomogeneity of the system[20-23] (Fig. 4).

The juxtaposition of the integral forms of Eq. (4) for both kinetic variables provides the correlation between the degrees of hydrolysis α_R and α_G, defined as:

$$\alpha_R = \frac{1}{a_R}\ln\frac{v_{0R}a_R}{v_{0G}a_G} + \frac{a_G}{a_R}\alpha_G \tag{5}$$

being valid at any time of the process and at any temperature considered. The linear correlation between α_R and α_G is illustrated in Figure 5.

The slope of the line permits to determine the ratio of the coefficients of inhomogeneity $\frac{a_G}{a_R}$, which does not depend on the temperature, as both a_G and a_R are temperature-independent. The line goes through the origin of the coordinate system, which leads to the following most probable ratio:

$$\alpha_R / \alpha_G = a_G / a_R = 0.87 \tag{6}$$

The relation just derived can be presented as the ratio of the current values of R_S and G evaluated according to Eqs. (1) and (2). This results in:

$$\frac{\alpha_R}{\alpha_G} = \frac{(R_S/35)}{(G/26)} = 0.87 \tag{7}$$

There follows then that:

$$R_S = 1.2G \tag{8}$$

The relation just established agrees with the experimental data R_S and G obtained at any time of the process, and at any temperature value studied (Fig. 2). The theoretical model derived can be strictly applied at 50 °C to the kinetic variables α_R and α_G, while some deviations are observed at lower temperatures.

Eq. (4) is used to estimate the initial rates of the hydrolysis process v_0. The values obtained, summarized in Table 1, show that they increase with increasing temperature.

The values of the activation energy E_0 and of the preexponential factor A_0 are evaluated using the Arrhenius equation applied to the initial rate of the process studied, *i.e.*:

$$v_0 = A_0 e^{-\frac{E_0}{RT}} \tag{9}$$

The corresponding linearized dependencies are presented in Figure 6. The value of the activation energy calculated on the basis of temperature dependence of the initial rate, determined with respect to reducing sugars, *i.e.* $E_{0,R}$ is found equal to 46.5 kJ/mol, while that corresponding to glucose, *i.e.* $E_{0,G}$ is found equal to 66.5 kJ/mol. The values just reported refer to the whole temperature interval studied. The preexponential factors found are as follows: $\ln A_{0,R}$ = 12.4 and $\ln A_{0,G}$ = 19.9 for reducing sugars and glucose, respectively.

As already pointed out, glucose is formed during the hydrolysis of the intermediate cellobiose catalysed by β-glucosidase. The

experimental results obtained show that this reaction is energetically hindered at the beginning of the process. The lower initial rates of hydrolysis (Table 1) support this conclusion. It is also important to note that the hydrolysis rates, for α_R and α_G, are equal at 50 °C, which indicates that the initial characteristics at this temperature do not depend on the kinetic variable used.

The current rates of the process, estimated with Eq. (3), are listed in Table 1. As seen in Table 1, the current hydrolysis rate decreases during the process, irrespectively of the kinetic variable used. The temperature dependence of the current rate is observed with the Arrhenius equation, the results obtained being presented in Figure 6. The values of the activation energy found for the whole temperature range coincide with those estimated from the temperature dependence of the initial rate. This refers both to the reducing sugars and glucose obtained, *i.e.* $E_R = E_{0,R} = 46.5$ kJ/mol and $E_G = E_{0,G} = 66.5$ kJ/mol. The results just reported indicate that the wheat straw–enzyme system behaves as an energetically homogeneous one, irrespectively of the increase of the hydrolysis degree. The process of glucose formation is more energetically hampered over the whole temperature range studied, compared to the formation of reducing sugars, which is most probably due to the specific enzyme action and less to the activity of *β*-glucosidase.

Unlike the activation energy, the preexponential factor *A* decreases with the increase of the hydrolysis degree, for both reducing sugars and glucose. This dependence can be described by:

$$\ln A = \ln A_0 - a\alpha \tag{10}$$

The data obtained according to Eq. (10) are presented in Table 2.

Table 1
Dependence of enzymatic hydrolysis rate v, min^{-1} on $\alpha_R = \alpha_G = \alpha$ at different temperature values

$\alpha_R = \alpha_G = \alpha$	v_Rx10^3, min^{-1}			v_Gx10^3, min^{-1}		
	T = 30 °C	T = 40 °C	T = 50 °C	T = 30 °C	T = 40 °C	T = 50 °C
0.0	3.16	5.70	10.10	1.99	4.90	10.20
0.2	0.68	1.10	2.17	0.53	1.37	2.68
0.3	0.32	0.51	1.00	0.27	0.70	1.37
0.4	0.14	0.23	0.46	0.13	0.36	0.70

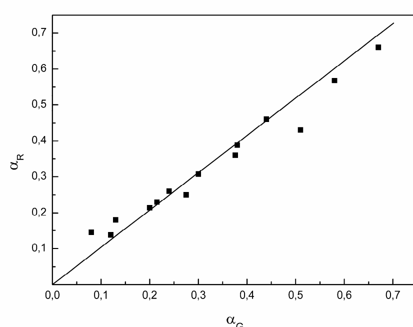


Figure 5: Correlation between the degrees of hydrolysis α_R and α_G

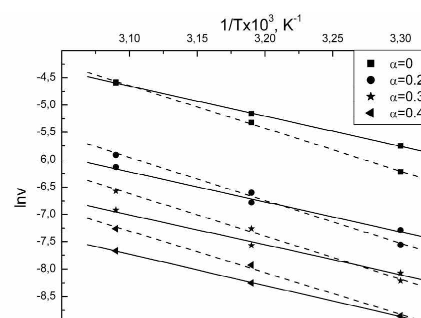


Figure 6: Temperature dependence of current rate at $\alpha_R = const$ (—) and $\alpha_G = const$ (---)

Table 2
Dependence of lnA_R and lnA_G logarithms of preexponential factor on hydrolysis degree α

α	lnA_R	lnA_G
0	12.4	19.9
0.2	10.86	18.56
0.3	10.09	17.89
0.4	9.32	17.22

The preexponential factor is related to the number of active centers of the examined system, to their accessibility and spatial orientation. Generally, this accounts for the entropy inhomogeneity of the wheat straw–enzyme system.[20-23]

The process rates are affected by both activation energy and preexponential factor.

When these counteracting factors are changed simultaneously and unidirectionally in the course of the process, the compensation effect, well-known in heterogeneous kinetics, is observed, being described by the expression:[24]

$$\Delta \ln A = m\Delta E \quad (11)$$

This effect is expressed as the proportionality between the changes produced in the logarithm of the preexponential factor and in the activation energy. The coefficient of proportionality m is related to the so-called isokinetic temperature, T^*, at which the rate remains constant, due to the complete compensation of the counteracting action of E and ln$A$:

$$m = 1/RT^* \quad (12)$$

During the enzymatic hydrolysis, investigated related to the kinetic variables α_R (Eq. (1)) and α_G (Eq. (2)), the change in the activation energy $\Delta E = E_G - E_R$ remains constant.

However, the logarithms of the preexponential factor are changed, because it determines the degree of hydrolysis, according to Eq. (10). At the same value of the hydrolysis degree, $\alpha_G = \alpha_R = \alpha$, for $\Delta \ln A = \ln A_G - \ln A_R$, the following expression is derived:

$$\Delta \ln A = \Delta \ln A_0 - \Delta a\, \alpha \quad (13)$$

where $\Delta \ln A_0 = \ln A_{0,G} - \ln A_{0,R}$ and $\Delta a = a_G - a_R$ represent the changes in the coefficients of inhomogeneity. Substituting Eq. (13) in Eq. (11), a form of compensation effect may be derived, in which the coefficient of proportionality m includes the degree of hydrolysis and the coefficients of inhomogeneity:

$$m = \left(\Delta \ln A_0 - \Delta a\, \alpha\right) / \Delta E \quad (14)$$

The solution of equations (12) and (14) leads to:

$$T^* = \frac{\Delta E}{\left(\Delta \ln A_0 - \Delta a\, \alpha\right) R} \quad (15)$$

For the system here under study, the isokinetic temperature T^* could be calculated with the following empiric equation:

$$T^* = \frac{20x10^3}{\left(7.5 + \alpha\right) R} \quad (16)$$

where ΔE = 20x10^3 J/mol, Δln$A$ = 7.5 and $\Delta a = -1$.

The isokinetic temperatures T^*, calculated with Eq. (16) at different values of the hydrolysis degree, are presented in Table 3. An important observation is that the theoretically derived values of T^* practically coincide with the experimentally obtained values presented in Figure 6.

At the beginning of the process, when $\alpha_R = \alpha_G = 0$, T^* = 47.7 °C. At temperatures below 47.7 °C, the initial rate evaluated for reducing sugars is higher than that found for glucose ($v_{0,R} > v_{0,G}$). The effect of the activation energy is determining over this temperature range.

The value of T^* decreases during the process, *i.e.* with the increase of the hydrolysis degree. For example, T^* is equal to 31.5 °C and $v_G = v_R$, when $\alpha_R = \alpha_G = 0.4$. At temperatures higher than 31.5 °C, the effect of the preexponential factor becomes predominant, leading, in turn, to a higher rate of glucose formation, *i.e.* $v_G > v_R$.

Table 3
Isokinetic temperatures T^* at different hydrolysis degree α and comparison of glucose v_G and reducing sugars v_R formation rates

α	T^*, K	Comparison of rates v_G, min^{-1} and v_R, min^{-1}	Factors determining v_G, min^{-1}
0	320.7	$v_{0,G} < v_{0,R}$ at $T < T^*$	Activation energy $E_G > E_R$
0.2	312.4	$v_G > v_R$ at $T > T^*$	Preexponential factor $\ln A_G > \ln A_R$
		$v_G < v_R$ at $T < T^*$	Activation energy $E_G > E_R$
0.4	304.5	$v_G > v_R$ at $T > T^*$	Preexponential factor $\ln A_G > \ln A_R$

CONCLUSIONS

The kinetics of enzyme hydrolysis of wheat straw after steam explosion was studied by two kinetic variables, for both reducing sugars and glucose. The process is described by an exponential kinetic equation valid for processes taking place on uniformly inhomogeneous surfaces. A linear correlation between two kinetic

variables was observed. The wheat straw–enzyme system behaves as an energetically homogeneous one. The process of glucose formation is more energetically hampered over the whole temperature range under study. The preexponential factor decreases with the increase of the hydrolysis degree, some form of compensation effect between the change of activation energy and the preexponential factor being derived. At temperatures higher than the isokinetic temperature, the preexponential factor becomes predominant, leading to a higher rate of glucose formation.

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