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Study of kinetics of enzymatic hydrolysis of cellulose materials

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Abstract : In this paper, the kinetics of enzymatic hydrolysis of cellulose samples with different structural characteristics has been studied using the equation of Avrami-Kolmogarov-Erofeev (AKE): $\ln(1-\alpha) = -K t^n$, where α is conversion degree; K is effective rate constant; t is time, and n is effective order of the kinetic process. It was shown that AKE-equation adequately describes the experimental kinetic curves. In case of hydrolysis of highly crystalline microcrystalline cellulose, the coefficient n in the AKE-equation is 0.5, which is typical for diffusion mechanism of the process. With the decrease of crystallinity degree of cellulose, the coefficient n increases and reaches 1 for completely amorphous cellulose in a wet state that indicates on the reaction of first-order. The intermediate n-value from 0.5 to 1 shows that the enzymatic hydrolysis of the sample is limited by diffusion of the large enzyme molecules into the cellulose structure. Drying of cellulose samples causes a decrease of pore volume and amplifies the contribution of diffusion to integral hydrolysis process. Effective rate constant K of enzymatic hydrolysis also increases with decreasing of crystallinity of the cellulose sample. Furthermore, the K-value for the wet sample was higher than for the dry sample. The use of parameters of AKE-equation allows predicting the kinetics of cellulose conversion into glucose during enzymatic hydrolysis.

Keywords : Cellulose; Enzymatic hydrolysis; Kinetics; Mechanism.

INTRODUCTION

One of the important branches of biochemistry involves enzymatic hydrolysis of cellulose into glucose with subsequent fermentation to obtain various valuable bioproducts or biochemical. Extraction of fermentable sugar - glucose, from non-food cellulose materials has been regarded as a promising way to obtain glucose without competing with food and feed industry. Process of enzymatic hydrolysis of cellulose was described in numerous publications. In particular, an effect of various structural factors (porosity, crystallinity, degree of polymerization, presence of residual lignin and other admixtures, etc.) on hydrolysability of cellulose has been discussed[1-6]. Among various factors the crystallinity was considered to be an important structural parameter that hinders the enzymatic hydrolysis[4-6, 7, 8].

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Amorphization of cellulose by its dissolution followed by regeneration from the solutions leads to extremely rise in hydrolysis rate and conversion degree[8-10]. Dependence of enzymatic digestibility on the solid content of cellulose substrate and enzyme loading has been also studied[11, 12].

Though numerous investigations, some problems of the cellulose hydrolysis were not solved yet, and among them – kinetic mechanism of the enzymatic hydrolysis. As known, a rate of the enzymatic hydrolysis of cellulose samples decreases during time of the process like to kinetics of other reactions. However, a reaction order of the cellulose enzymatic hydrolysis is usually lower than 1. This is explained by action of additional factors such as increase in the content of less digestible crystalline part or/and accumulation of inhibiting products during the hydrolysis process, etc.[13-15]. Besides, a diffusion limitation of the hydrolysis reaction can occur.

To describe the complicated kinetic curves of the cellulose hydrolysis, various models have been proposed. Unfortunately, the most models have focused on one specific aspect of the hydrolysis process, but have excluded the others simultaneously occurring processes. Various models and equations were proposed to describe kinetics of enzymatic hydrolysis of cellulose substrates[13-15, 17-20]. These equations can be used for mathematic analysis of the experimental kinetic curves, but these are not valid for disclosing of the real kinetic mechanism of the enzymatic hydrolysis. As shown, the initial stage of cellulose hydrolysis can be described by the equation of pseudo-first order kinetics; however, this equation does not describe the whole kinetic curve[7]. The Michaelis-Menten kinetic model and its modifications developed for homogeneous enzymatic reactions in solutions are not valid for the heterogeneous hydrolysis of cellulose[16, 17].

Diffusion process plays an important role in heterogeneous systems, comprising a soluble enzyme and insoluble substrate. Since the molecules of cellulolytic enzymes are large having an increased MW ranging usually from 40000 to 80000, the hydrolysis reaction may be limited by diffusion of large molecules of cellulases into a cellulose substrate. Furthermore, the contribution of reverse diffusion of the reaction products should be also taken into account. Thus, the kinetics of the hydrolysis reaction can depend on the diffusion of enzyme molecules into the solid cellulose and on the reverse diffusion of formed sugars into the aqueous phase[14].

As is known, when a combination of chemical reaction and diffusion process takes place, the equation of Avrami-Kolmogorov-Erofeev (AKE) can be used to describe the integral kinetic process[15, 21-23]:

$$\ln(1-\alpha) = -K t^n \quad (1)$$

where α is conversion degree; K is effective rate constant; t is time, and n is effective order of the process that reflects the kinetic mechanism, and namely: if n = 1, then it is a reaction of the first-order; if n=0.5, then it is a diffusion process; and if n is in the range from 0.5 to 1, then it is a diffusion-limited reaction.

Main purpose of this paper was to verify the suitability of AKE-equation for the enzymatic hydrolysis of cellulose samples in order to disclose the real kinetic mechanism of hydrolysis process.

EXPERIMENTAL

Materials

Various cellulosic materials were used for enzymatic hydrolysis. Bleached sulfite spruce pulp (SFI) was obtained from Weyerhaeuser Co, WA, USA. Undried bleached Kraft spruce pulp was delivered from Södra plant, Sweden. Linter of the middle-length cotton "Acala" cultivated in Israel was refined by a soda cooking. Filter paper No 1 of Whatman and microcrystalline cellulose (MCC) Avicel PH-301 also were used. Cotton linter was hydrolyzed with boiling 2.5N HCl for 30 min with subsequent washing up to neutral pH value. Regenerated cellulose (RC) was prepared by regeneration of the MCC solution in ortho-phosphoric acid[10]. Low-crystalline cellulose (LC) was obtained by treatment of SFI pulp with liquid ammonia for 30 min with following drying at 60 °C up to constant weight. Mercerized cellulose materials were carried by treatment with 18 wt.% NaOH at room temperature for 1 h with subsequent neutralization and washing up to neutral pH value. To remove excess

water, the not dried cellulose samples were preliminary squeezed up to solid content about 20-25 wt. %. Drying of the wet samples was carried out at 105 °C up to constant weight.

Enzymatic hydrolysis

Cellulose samples were hydrolyzed with a commercial cellulolytic enzyme preparation Cellic Ctec-2 (Novozymes A/S, Bagsvaerd, Denmark). Hydrolysis of the samples was carried out in 50-mL polypropylene tubes each containing the sample with concentration of 50 g/L in 50 mM acetate buffer (pH=4.8). The samples were thoroughly mixed with the buffer and then Ctec-2 was added to loading of 10 mg enzyme per 1g of dry cellulose. The closed tubes were placed in a shaker incubator at 50°C and agitated at 150 rpm during various times. Finally, the tubes were centrifuged in order to separate the glucose solution.

The concentration of the glucose (C_g) obtained as a result of enzymatic hydrolysis of the cellulose samples was determined by HPLC-apparatus of Agilent Technologies 1200 Infinity Series using the Amines HPX-87H column. Main conditions of the HPLC-analysis were: temperature 45°C; mobile phase 0.005 M sulfuric acid; flow rate 0.6 ml/min. The hydrolyzate was preliminary filtered through 0.45 μm Nylon filter and degassed. Conversion degree α of cellulose samples at enzymatic hydrolysis was calculated by the equation:

$$\alpha = C_g/C_m \quad (2)$$

where C_m = 308.64 mM or 55.55 g/L is maximum concentration of glucose after complete conversion of cellulose to glucose.

X-Ray diffraction

X-ray investigations of dried and swollen samples were carried out with a Rigaku-Ultima Plus diffractometer (CuK_α – radiation, λ=0.15418 nm). To hold the swollen structural state, the undried cellulose samples were washed with absolute ethanol, then with acetone and pentane, and finally dried at 60 °C up to constant weight. X-ray diffractograms were recorded in the $\varphi=2\Theta$ angle range from 5 to 80°. After recording of the diffractograms, the background was separated, and selected X-ray patterns were corrected and normalized. Then diffraction intensities from crystalline and non-crystalline regions were separated by a computerized method. The crystallinity degree (X) and the content of amorphous (non-crystalline) domains (Y) of cellulose sample were determined by the X-ray method[25, 26].

$$X = \int J_c \, d\varphi / \int J_o \, d\varphi \quad (3)$$

$$Y = 1 - X \quad (4)$$

where J_c and J_o are the corrected and normalized diffraction intensities for crystalline regions and sample respectively; φ=2Θ diffraction angle.

Three diffractograms were recorded for the each cellulose type to calculate average X and Y values and standard deviations that were in the range ±0.02.

Chemical and physicochemical tests

The content of alpha-cellulose and average degree of polymerization (DP) of the cellulose samples were studied by standard TAPPI methods T-203 and T-230. Water retention value (WRV) of the samples characterizing total volume of pores (V_p) in the water medium was tested by the method of Jayme et al.[24] using a centrifugal force 3000 G for 15 min (see SCAN-C 62:00 procedure). SD at determination of alpha-cellulose content was at most ± 1%, of DP ± 10, and of WRV ± 0.1 cm^3 /g.

RESULTS AND DISCUSSION

Characteristics of cellulose materials

Some characteristics of the dried cellulose samples are shown in TABLE 1. The samples contained relatively high level of alpha-cellulose indicating that they were sufficient pure. Samples of commercial MCC Avicel and hydrolyzed cotton linter were highly crystalline and characterized by a low content of non-crystalline domains. Treatment of cellulose samples with 18 wt.% sodium hydroxide caused irreversible disruption of the crystalline structure and increased the content of non-crystalline domains. Sample of regenerated cellulose (RC) had the most amorphized structure among the all investigated cellulose samples (TABLE 1, Figure 1).

Investigations of water retention value (WRV) of various cellulose samples were carried out to estimate total volume of pores (V_p). Undried cellu-

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TABLE 1 : Characteristics of dried cellulose samples

Samples	Alfa-Cellulose, %	DP	X	Y
Sulfite pulp (SFI)	95	1100	0.63	0.37
Kraft pulp (KP)	92	960	0.65	0.35
Mercerized Kraft pulp (KPM)	99	910	0.53	0.47
Filter paper (FP)	99	1200	0.71	0.29
Refined cotton linter (CL)	98	1600	0.69	0.31
Acid-hydrolyzed cotton linter (CLH)	86	180	0.77	0.23
Mercerized cotton linter (CLM)	99	1520	0.55	0.45
Avicel MCC (AV)	87	170	0.75	0.25
Mercerized Avicel (AVM)	98	160	0.57	0.43
Low-crystalline SFI pulp (LC)	93	1000	0.38	0.62
Regenerated cellulose (RC)	-	150	0.25	0.75

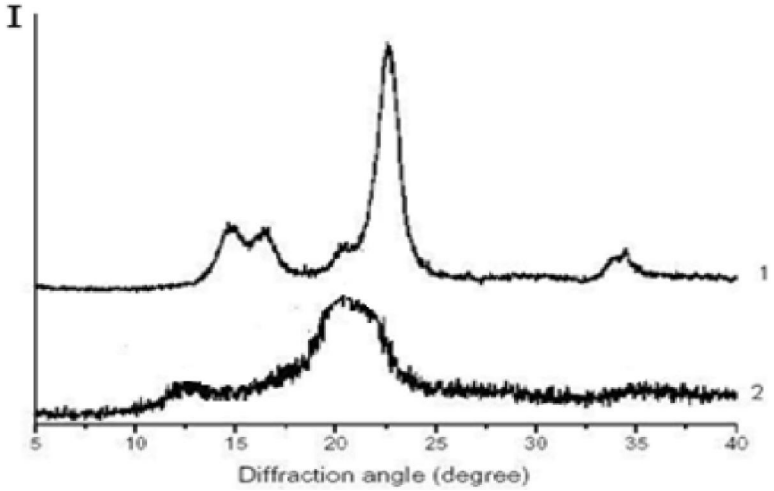

Figure 1 : X-ray diffractograms of acid-hydrolyzed cotton (1) and regenerated cellulose (2)

lose samples were characterized by high volume of pores, 1.5-2 cm^3/g, while drying of the wet samples led to falling in the V_p-value (Figure 2).

Kinetics of enzymatic hydrolysis of cellulose materials

Kinetics of the enzymatic hydrolysis of cellulose samples was characterized by the decrease in hydrolysis rate over time (Figure 3). Drying of the undried samples caused a decline of the conversion degree due to porosity decrease (Figure 4). At a certain time of hydrolysis, the conversion degree was higher for the cellulose sample having more decrystallized and more porous structure, e.g. for the wet RC.

To linearize the experimental kinetic curves, a logarithmic form of AKE-equation was used:

$$\ln F = \ln K + n \ln t \quad (5)$$

where $F = -\ln(1-\alpha)$

The verification confirmed that experimental kinetics can be linearized really in coordinates of the eq. (5), as shown for example in Figure 5, 6.

The parameters of AKE-equation, coefficient n and effective rate constant K, for the investigated cellulose samples are presented in TABLE 2. These parameters permit to calculate the conversion degree of cellulose during the enzymatic hydrolysis by the equation (6):

$$\alpha = 1\text{-AntiLn}(-Kt^n) \quad (6)$$

As can see from the example presented in Figure 7, the calculated results coincide with the experimental points, which confirm the adequacy of AKE-equation.

Coefficient n in AKE-equation for highly crystalline cellulose samples is 0.5 that evidences on the diffusion mechanism of the enzymatic hydrolysis

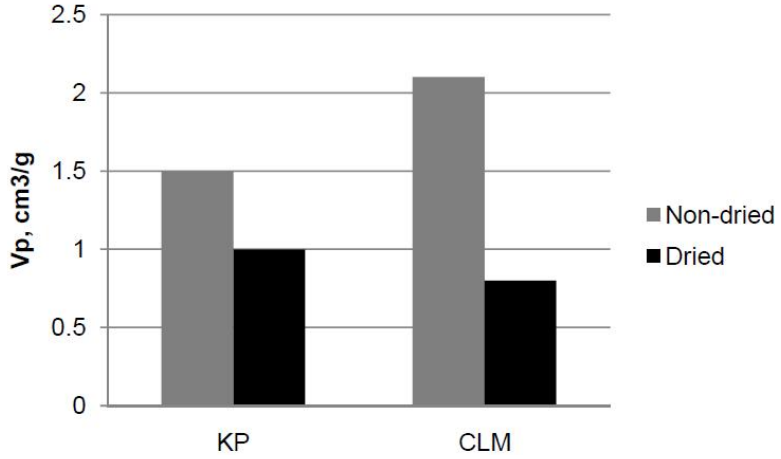

Figure 2 : Pore volume for not dried and dried cellulos samples: kraft pulp (KP) and mercerized cotton linter (CLM)

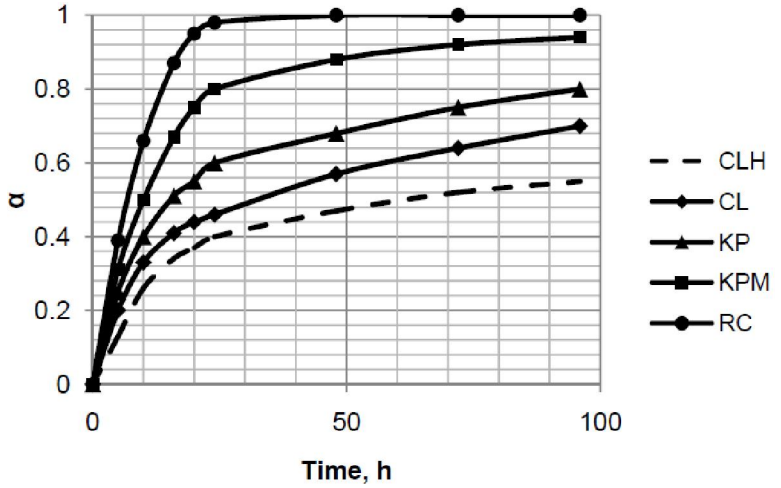

Figure 3 : Kinetics of the enzymatic hydrolysis of undried cellulose samples

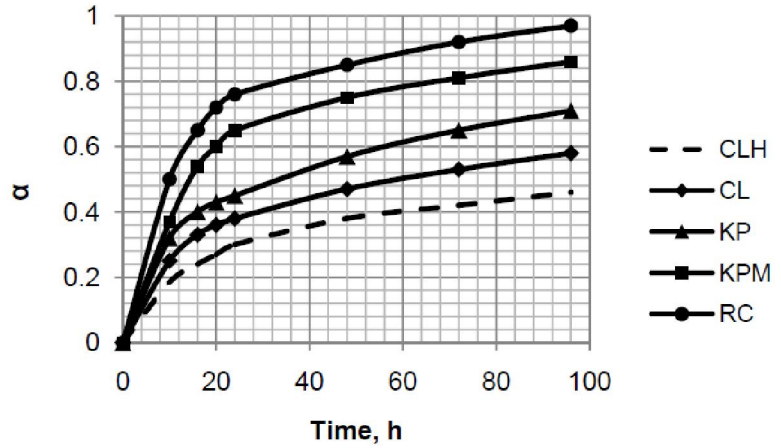

Figure 4 : Kinetics of the enzymatic hydrolysis of dried cellulose samples

(TABLE 2, Figure 8). With the decrease of crystallinity degree of cellulose, the coefficient n increases and reaches 1 for completely amorphous cellulose in a wet state that indicates on the reaction of first-order.

Intermediate n-value from 0.5 to 1 for the other samples shows that the enzymatic hydrolysis is limited by diffusion of the large enzyme molecules into cellulose substrates. Drying of cellulose samples decreases volume of pores and therefore amplifies

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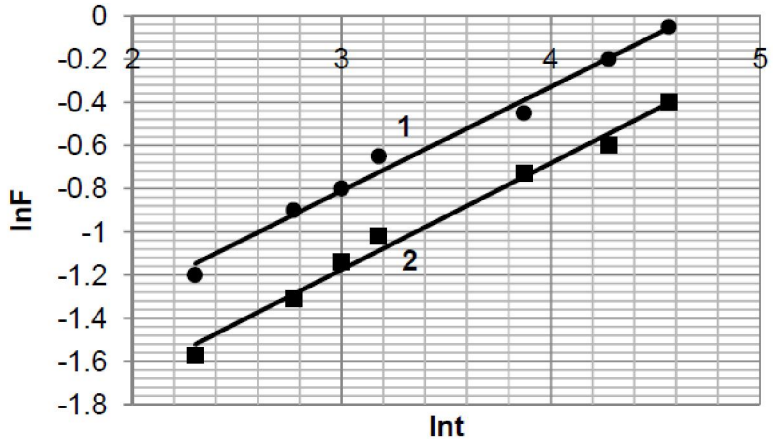

Figure 5 : Linearized kinetics of the enzymatic hydrolysis of undried (1) and dried (2) samples of hydrolyzed cotton linter (CLH)

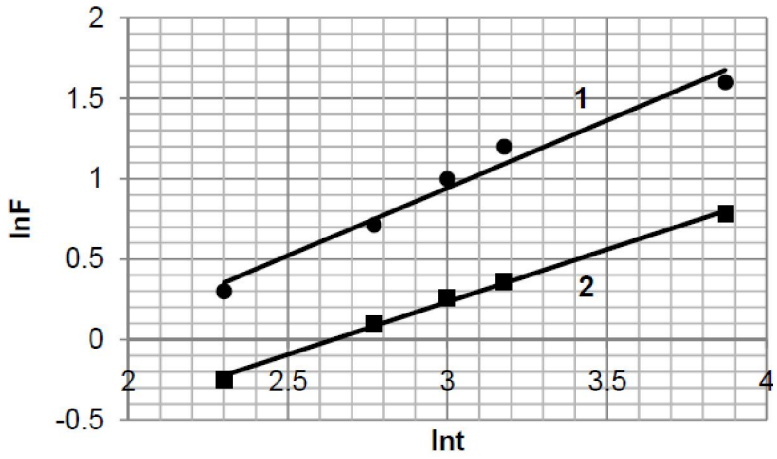

Figure 6 : Linearized kinetics of the enzymatic hydrolysis of undried (1) and dried (2) samples of regenerated cellulose (RC)

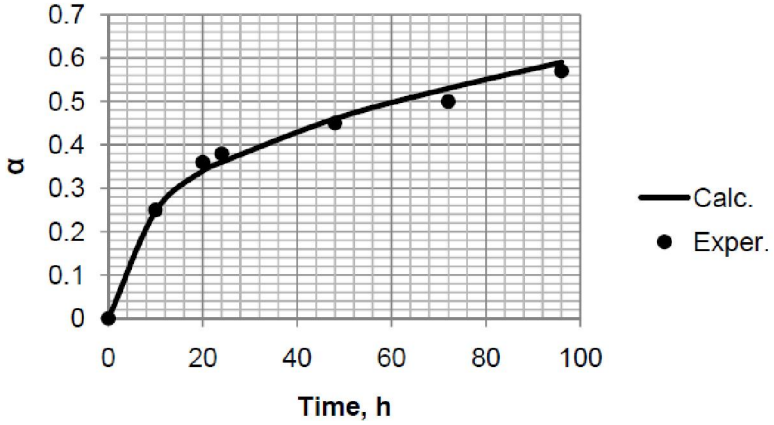

Figure 7 : Calculated and experimental kinetics of enzymatic hydrolysis of dried cotton linter (CL)

the contribution of diffusion to integral hydrolysis process.

Effective rate constant, K, of enzymatic hydrolysis increases with decreasing of crystallinity degree of cellulose. Moreover, K-value for the wet sample is higher than for the dry sample (Figure 9).

The AKE-equation allows calculating also the concentration (C_g) of reducing sugar – glucose, formed after a certain hydrolysis time, using the following equation:

TABLE 2 : Kinetic parameters of enzymatic hydrolysis of cellulose samples

Samples	X	Undried		Dried	
		n	K	n	K
CLH	0.77	0.50	0.10	0.50	0.06
AV	0.75	0.50	0.10	0.50	0.06
FP	0.71	0.55	0.10	0.50	0.08
CL	0.69	0.55	0.11	0.50	0.09
KP	0.65	0.55	0.14	0.55	0.10
SFI	0.63	0.6	0.14	0.55	0.11
AVM	0.57	0.65	0.14	0.55	0.12
CLM	0.55	0.65	0.14	0.60	0.13
KPM	0.53	0.70	0.15	0.60	0.13
LCC	0.38	0.75	0.20	0.60	0.15
RC	0.25	0.82	0.25	0.65	0.18
Am	0	1	0.33	0.70	0.25

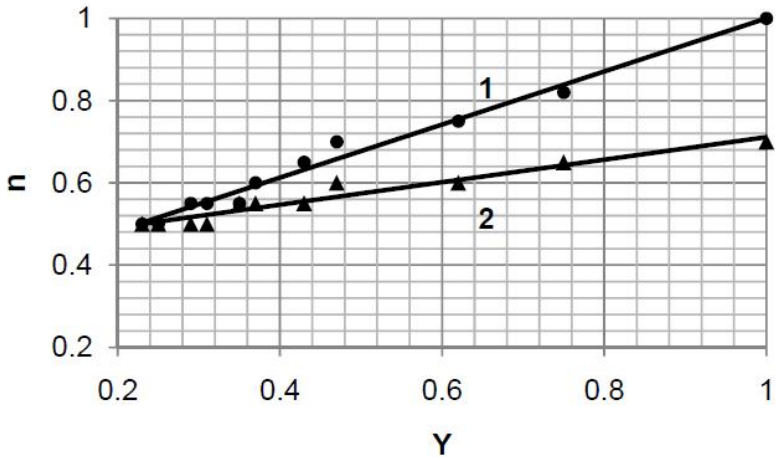

Figure 8 : Dependence of coefficient (n) on the content of amorphous domains of cellulose (Y) for undried (1) and dried (2) samples

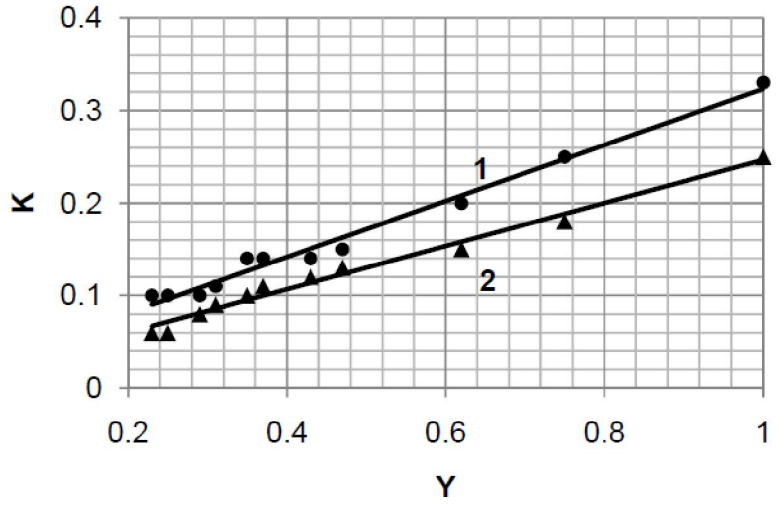

Figure 9 : Dependence of effective rate constant (K) on the content of amorphous domains of cellulose (Y) for undried (1) dried (2) samples

$$C_g = C_m\,[1\text{-AntiLn}(-Kt^n)] \quad (7)$$

The example of Figure 10 shows that the calcu-

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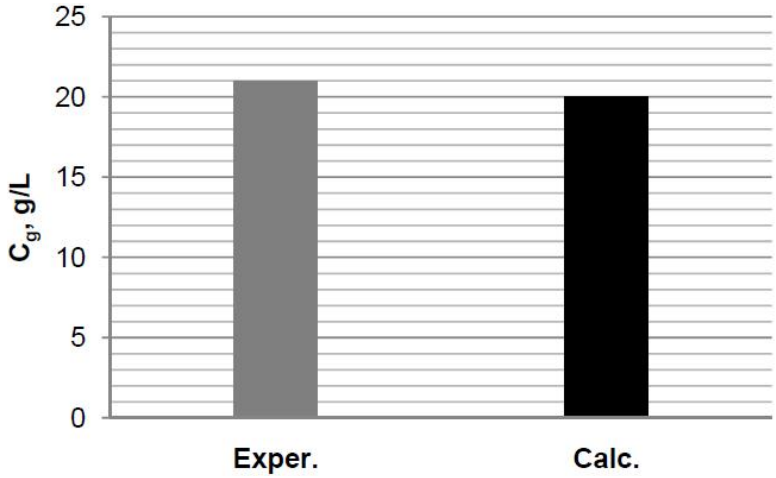

Figure 10 : Experimental and calculated concentrations of glucose after enzymatic hydrolysis of the dried cotton liner for 24 h

lated glucose concentration is approximately the same as the experimentally determined concentration of the sugar.

CONCLUSIONS

The equation of Avrami-Kolmogarov-Erofeev (AKE) was used for kinetic analysis of the enzymatic hydrolysis of cellulose samples with different structural characteristics. It was shown that AKE-equation adequately describes the experimental kinetic curves. For highly crystalline microcrystalline cellulose coefficient n in the AKE-equation is 0.5, which is typical for diffusion mechanism of the process. With decreasing of crystallinity degree of cellulose samples coefficient n increases and for completely amorphous cellulose in a wet state n-value achieves 1 that indicates on the first-order reaction. Intermediate n-value from 0.5 to 1 for the other samples shows that the enzymatic hydrolysis is limited by diffusion of the large enzyme molecules into the cellulose substrates. Drying of cellulose samples decreases volume of pores and therefore increases contribution of the diffusion to integral enzymatic hydrolysis process. Effective rate constant K of enzymatic hydrolysis also increases with decreasing of crystallinity of the cellulose sample. Besides, the K-value for the wet sample was higher than for the dry sample. The use of parameters of AKE-equation allows predicting the kinetics of cellulose conversion into glucose during enzymatic hydrolysis.

REFERENCES

[1] T.Jeoh, C.I.Ishizawa, M.F.Davis, M.E.Himmel, W.S.Adney, D.K.Johnson; Cellulase digestibility of pretreated biomass is limited by cellulose accessibility, Biotechnol.Bioeng., **98**, 112-122 **(2007)**.

[2] A.Hendrikis, G.Zeeman; Pretreatments to enhance the digestibility of lignocellulosic biomass, Biores.Technol., **100**, 10-18 **(2009)**.

[3] M.Wada, I.Masakazu, K.Tokuyasu; Enzymatic hydrolysis of cellulose I is greatly accelerated via its conversion to the cellulose II form, Polym.Degradation and Stability, **95**, 543-548 **(2010)**.

[4] L.T.Fan, Y.H. Lee, D.H.Beardmore; Mechanism of the enzymatic hydrolysis of cellulose: Effects of major structural features of cellulose on enzymatic hydrolysis, *Biotechnol.Bioeng.*, **22,** 177-199 **(1980)**.

[5] L.T.Fan, Y.H.Lee, D.H.Beardmore; The influence of major structural features of cellulose on rate of enzymatic hydrolysis, *Biotechnol.Bioeng.*, **23,** 419-424 **(1981)**.

[6] S.Park, J.O.Bake, M.E.Himmel, P.A.Parilla, D.K.Johnson; Cellulose crystallinity index: measurement techniques and their impact on interpreting cellulase performance, *Biotechnol.for Biofuels,* **3, 1**-10 **(2010)**.

[7] M.Ioelovich, E.Morag; Effect of cellulose structure on enzymatic hydrolysis, Bioresources, **6**, 2818-2834 **(2011)**.

[8] M.Hall, P.Bansal, J.Lee, M.Realff, A.Bommarius; Cellulose crystallinity – a key predictor of the enzymatic hydrolysis rate, FEBS Journal, **277**, 1571-1582

(2010).

[9] A.P.Dadi, S.Varanasi, A.A.Schall; Enhancement of cellulose saccharification kinetics using an ionic liquid pretreatment step, *Biotechnol.Bioeng.*, **95,** 904-910 **(2006)**.

[10] Y.Zhang, J.B.Cui, L.R.Lynd, L.R.Kuang; A transition from cellulose swelling to cellulose dissolution by o-phosphoric acid: evidence from enzymatic hydrolysis and supramolecular structure, *Biomacromolecules*, **7,** 644-648 **(2006)**.

[11] F.M.Gama, M.Mota; Enzymatic hydrolysis of cellulose: relationship between kinetics and physicochemical parameters, Biocatalysis Biotranform., **15**, 221-236 **(1997)**.

[12] J.B.Kristensen, C.Felby, H.Jørgensen; Yield-determining factors in high-solids enzymatic hydrolysis of lignocelluloses, Biotechnol.for Biofuels, **2**, 1-10 **(2009)**.

[13] K.Ohmine, H.Ooshima, Y.Harano; Kinetic study on enzymatic hydrolysis of cellulose by cellulase from *Trichoderma viride*, Biotechnol.and Bioeng., **25**, 2041- 2053 **(1983)**.

[14] A.K.Sarkar, J.N.Etters; Enzymatic hydrolysis of cotton fibers: modeling using an empirical equation, J.Cotton Sci., **8**, 254–260 **(2004)**.

[15] Z.Wang, J.Xu, J.J.Cheng; Modeling biochemical conversion of lignocellulosic materials for sugar production – a review, Bioresources, **6**, 5282-5306 **(2011)**.

[16] D.Baley; Enzymatic kinetics of cellulose hydrolysis, Biochem.J., **262**, 1001-1002 **(1989)**.

[17] G.Radeva, I.Valchev, S.Petrin, E.Valcheva, P.Tsekova; " Kinetic study of the enzyme conversion of steam exploded *Paulownia tomentosa* to glucose, Bioresources, **7**, 412-421 **(2012)**.

[18] J.N.Etters; Diffusion equation made easy, Textile Chemist Colorist, **12**, 140-145 **(1980)**.

[19] M.Kurakake, T.Shirasawa, H.Ooshima, A.O.Converse, J.Kato; An extension of the Harano-Ooshima rate expression for enzymatic hydrolysis of cellulose to account for changes in the amount of adsorbed cellulase Appl.Biochem.Biotechnol., **50**, 231-241 **(1995)**.

[20] H.Ooshima, K.Ohmine, Y.Ishitani, Y.Harano; Applicability of an empirical rate expression to enzymatic hydrolysis of cellulosic materials, Biotechnol.Letter, **4**, 729-734 **(1982)**.

[21] M.Avrami; Kinetics of phase change: Transformation – time relations for random distribution of nuclei, J.Chem.Phys., **8**, 212-224 **(1940)**.

[22] V.M.Gorbachev; To the problem of applying the equation of Kolmogorov, Erofeev, Kazeev, Avrami and Mampel to the kinetics of non-isothermal transformations, J.Thermal Analysis and Calorimetry, **20**, 483-485 **(1981)**.

[23] M.A.Zilbergleit, B.C.Symchovich, V.M.Reznikov; Study of wood delignification process using water solutions of acetic acid, Wood Chemistry, **6**, 28–34 **(1987)**.

[24] G.Jayme, G.Hahn; A simple method of WRV-measuring for celluloses, Papier, **14**, 138- 139 **(1960)**.

[25] M.Ioelovich; Accessibility and crystallinity of cellulose, Bioresources, **4**, 1168-1177 **(2009)**.

[26] M.Ioelovich, A.Leykin, O.Figovsky; Study of cellulose paracrystallinity, Bioresources, **5**, 1393-1407 **(2010)**.