STUDY OF ENZYMATIC HYDROLYSIS OF PRETREATED BIOMASS AT INCREASED SOLIDS LOADING

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The effect of biomass loading from 50 to 200 g/L on enzymatic hydrolysis was studied using switchgrass samples pretreated by dilute acid and hypochlorite-alkaline methods. It was confirmed that an increase of initial loading of the pretreated biomass leads to a decrease of enzymatic digestibility, probably due to difficulty of mass transfer of cellulolytic enzymes in the high-viscous substrate slurry and also because of an inhibiting effect of the formed sugars. An additional sharp problem connected with enzymatic hydrolysis at the high-solids loading is absorption and retention of liquid hydrolysate by residual non-hydrolyzed biomass that causes diminution of the available volume (V_a) of the sugar solution and decreases productivity of the saccharification process. To optimize the high-solids enzymatic hydrolysis, the maximal amount of the formed sugars was determined $A_m = C_m \times V_{a,m}$, where C_m is maximal concentration of the sugar solution and $V_{a,m}$ is maximal available volume. Such an approach makes it possible to find the optimal conditions for the hydrolysis: optimal biomass loading and hydrolysis time. After enzymatic hydrolysis at these optimal conditions, the low-lignified biomass pretreated by hypochlorite-alkaline method displayed much more available volume of sugar solution and higher digestibility characteristics than the cellolignin obtained by acidic pretreatment of the initial biomass sample.

Keywords: Switchgrass; Pretreated biomass; Biomass loading; Enzymatic hydrolysis; Available volume of hydrolyzate; Amount of sugar; Optimal hydrolysis conditions; Maximal digestibility characteristics

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INTRODUCTION

The main method currently in use for production of fermentable sugars from cellulose and pretreated lignocellulosic biomasses is enzymatic hydrolysis. The hydrolysis of cellulose-based substrates by cellulolytic enzymes has been studied intensively over the few past decades (Draude *et al.* 2001; Hendrikis and Zeeman 2009; Ioelovich *et al.* 2011, 2012; Wada *et al.* 2010; Wang *et al.* 2011; Xu *et al.* 2011, 2012; Yang *et al.* 2006; *etc.*). Despite intensive investigations, the enzymatic hydrolysis step remains a major bottleneck in the process of bioconversion of the cellulose-based substrates into fermentable sugars and further into final bioproducts (*e.g.* bioethanol). Conventional laboratory enzymatic hydrolysis of lignocellulosic materials is typically carried out at a substrate solids content of ≤ 5 wt.% (Ioelovich *et al.* 2011, 2012; Wang *et al.* 2011; Xu *et al*. 2011, 2012). However, at the decreased biomass loading, both the

concentration of obtained sugars in the hydrolysate and the concentration of final bioproducts are low, leading to low productivity and high production cost.

Enzymatic hydrolysis at the increased biomass loading is a key to scale up this process to pilot industrial production (Di Risio *et al.* 2011). The use of a high biomass loading throughout its conversion into fermentable sugars and bioproducts is important for techno-economical reasons. This approach makes it possible to achieve a high concentration of the sugars and bioproducts, which can bring significant economic savings to the bioconversion process, such as reducing capital, decreasing operating cost for hydrolysis and fermentation, and minimizing energy consumption for distillation/evaporation and other downstream processes (Modenbach and Nokes 2012; Mohagheghi *et al.*, 1992; Wingren *et al.* 2003). It was shown for example that the energy required for distillation of the bioethanol can be significantly reduced if the fermentation broth contains higher than 4 wt% ethanol (Zacchi and Axelsson 1989). To reach this ethanol level, a sugar concentration must be at least 80 g/L, and moreover, an initial loading the steam exploded or acid pretreated lignocellulosic biomass should be approximately 20 wt% (Larsen *et al.* 2008).

Unfortunately, enzymatic hydrolysis at the increased biomass solids level has a number of shortcomings. Since the viscosity of the biomass system increases abruptly at increased loadings, the high-solids process results in insufficiently uniform mixing and limited mass transfer of the enzymes (Di Risio *et al.* 2011; Modenbach and Nokes 2012; Qin 2010). Besides, it has been shown that the rise of the biomass percentage leads to reduced conversion degree due to inhibition of the enzymes by the increased concentration of the sugars (Cara *et al.* 2007; Hodge *et al.* 2008; Jorgensen *et al.* 2007; Kristensen *et al.* 2009; Roshe *et al.* 2009; Qin 2010).

To provide an increased biomass loading without difficulties in mixing and mass transfer, the fed batch method of enzymatic hydrolysis was proposed (Qin, 2010; Zhang *et al.* 2012). However, this method has its own drawbacks, namely complicated performance, gradual accumulation of non-hydrolyzed biomass, and a decrease in conversion degree with increasing numbers of feed portions.

Moreover, there exists an additional serious problem connected with the enzymatic hydrolysis at the high-solids loading that has not been discussed yet, namely, the diminution of the available volume of the sugar solution (hydrolyzate) owing to its absorption and retention by residual non-hydrolyzed biomass. If the biomass loading is low ($\leq$ 5 wt. %), then the substrate system has a fluid character, and after enzymatic hydrolysis, an increased available volume of the hydrolysate can be obtained. With an increase of the biomass loading, the content of the residual biomass and retained volume of the liquid phase rises, while its available volume decreases. The high-loaded biomass system (usually 15 to 30 wt. %) has a solid-like nature due to the relatively low amount of free liquid (Hodge *et al.* 2008; Kristensen 2008; Rosgaard *et al.* 2007). During enzymatic hydrolysis, a gradual liquefaction of the solid system is observed; however, due to increased content of the residual biomass, it absorbs most of the volume of the liquid hydrolyzate, and, therefore, the final available volume of the sugar solution becomes so low that its use for the further fermentation into final bioproducts will not be economically effective.

The main purpose of this paper was to investigate the enzymatic hydrolysis at the increased content of pretreated lignocellulosic materials and determine the optimal biomass loading and duration for the hydrolysis process, in order to achieve the maximal available volume of the sugar solution and maximal amount of the fermentable sugars.

EXPERIMENTAL

Materials

Switchgrass pellets (about 20 to 25 x 2 to 3 mm) of Nott Farms (Canada) were used as the initial material.

Pretreatment

The initial material was pretreated using the dilute acid and hypochlorite-alkaline methods. Acidic pretreatment was carried out with boiling 3 wt% sulfuric acid for 1h at the liquor to solids weight ratio (LSR) of about 10. Hypochlorite-alkaline pretreatment was a two-stage process. In the first stage, the initial material was treated with 6 wt% sodium hypochlorite at room temperature, with an LSR of approximately 10, under stirring for 1h, and then the material was washed and squeezed using a vacuum glass-filter. In the second stage, the hypochlorite-treated biomass was extracted by boiling with 2 wt% sodium hydroxide at an LSR value of about 10 under stirring for 1 h.

The pretreated biomass was washed up to neutral pH, squeezed on the vacuum glass-filter, and evaporated in a drying chamber at 50 to 60 $^{\circ}$C up to a final solids content of about 50 wt%.

Chemical Analysis

The chemical composition of initial and pretreatment samples was determined by conventional methods of chemical analysis (Fengel and Wegener 1984; Obolenskaya *et al.* 1991; Rowell 2005). The content of holocellulose was measured after delignification of the biomass with sodium chlorite. The obtained holocellulose sample was hydrolyzed with boiling 1.5% hydrochloric acid for 2 h. The content of cellulose was calculated from the dry residue that remained after hydrolysis of the holocellulose, while the content of hemicelluloses was measured from weight loss of the hydrolyzed holocellulose sample. Lignin Klason was analyzed by means of standard TAPPI procedure T222 (TAPPI Standard 2002). Three of the same samples were tested to calculate an average value and standard deviation. The standard deviation of the analysis was in the range ± 1 wt %.

Enzymatic Hydrolysis

The pretreated biomass samples were hydrolyzed with a mixture of commercial cellulolytic enzyme (cellulase) NS50013 and β–glucosidase NS50010 (Novozymes A/S, Bagsvaerd, Denmark). The loading of cellulase was 5 FPU per 1 g of solid sample, and of β–glucosidase was 7 CBU per 1 g of solid sample. Enzymatic hydrolysis of the samples was carried out in 2-L fermentor "Biostat A Plus" of Sartorius AG (Germany). The wet biomass sample containing 50 to 200 g of the solid matter was put into fermentor. The required volumes of the enzymes (cellulase and β–glucosidase) were

mixed with the 0.05 M acetate buffer (pH = 4.8) and then poured out into the fermentor to obtain the total volume of the liquid medium V_o = 1L. The fermentor was closed with a cover and its contents were heated up to 50 ^oC at 60 rpm stirring. The enzymatic hydrolysis of the pretreated biomass samples was carried out during various times, from some hours up to 5 days.

After hydrolysis, the residual biomass and sugar solution were separated by centrifuge "Sorvall RC-5 C" at acceleration about 4000 G for 10 min, and the relative available volume of the sugar solutions was calculated,

$$V_a = V_s/V_o \quad (1)$$

Then, the relative retained volume of the hydrolyzate will be:

$$V_r = 1\text{-}\ (V_s/V_o) \quad (2)$$

where V_s is volume of the separated sugar solution – hydrolyzate (L); V_o = 1L is initial volume of the liquid medium.

The residual biomass was washed by water and ethanol, dried at 105 ^oC, and weighed in order to determine the weight of the residual biomass and also weight loss of the hydrolyzed sample.

Two samples of the same biomass type were hydrolyzed simultaneously in the two *"Biostat A Plus"* fermentors at the equal biomass loading and hydrolysis time. The standard deviation at the weight determination was ± 2 wt%, while at the volume determination ± 5 vol%.

From the experimental results, the digestibility parameters were calculated as follows,

Conversion Degree of Biomass (%), $\boldsymbol{CD} = 100\ (WL/P_o)$ (3)

Real Yield of Reducing Sugars (%), $\boldsymbol{Y_r} = 100\ [(C\ V_a)/BL]$ (4)

where ***WL*** is weight loss of the hydrolyzed sample (g); $\boldsymbol{P_o}$ is the initial dry weight of the biomass; ***C*** is the concentration of reducing sugars in solution (g/L) after enzymatic hydrolysis; $\boldsymbol{V_a}$ is relative available volume of the sugar solution (L/L); and ***BL*** is the initial biomass loading (g/L).

Sugar Assay

Concentration of reducing sugars (***C***) in the solution after enzymatic hydrolysis of the pretreated biomass samples was tested by means of the conventional ***DNS***-assay using glucose for calibration (Ghose 1987; Gusakov *et al.* 2011; Miller 1959; Wang *et al.* 2011). Three testings of the same hydrolyzate were performed. The standard deviation at determination of the *RS*-concentration was ± 2 w/v %.

Sugar composition of the hydrolyzates was determined by the HPLC-method. The used "Rezex RPM Pb" column had a temperature at 80 ^oC. The flow rate of the mobile

phase, doubly distilled water, was 0.6 mL/min. The injected volume of the sugar solutions was 10 μL.

RESULTS AND DISCUSSION

The contents of the three main components – cellulose, hemicelluloses, and lignin, in the initial switchgrass (SG_o) and samples pretreated by acid (SG-AC) and hypochlorite-alkaline method (SG-HA), are shown in Table 1. The other components of the samples were extractives, ash, and protein. The untreated sample contained about 37 wt% of cellulose, 28 wt% of hemicelluloses, and 18 wt% of Klason lignin. After acidic pretreatment of the initial sample, the main part of hemicelluloses was removed, and the obtained cellolignin sample was characterized by increased content of both cellulose and lignin. Hypochlorite-alkaline pretreatment caused a sharp enhancement in cellulose content from 37 to 91%, and low content of other non-cellulosic components.

Table 1. Percentage of Cellulose, Hemicelluloses, and Lignin in the Initial Switchgrass *(SG_o)* and Samples Pretreated by Acid *(SG-AC)* and Hypochlorite-Alkaline Method *(SG-HA)*

Components	SG_o	SG-AC	SG-HA
Cellulose	37	55	91
Hemicelluloses	28	7	5
Lignin	18	28	2
Recovery	-	68	42

Study of enzymatic hydrolysis of the pretreated switchgrass samples having different initial biomass loading (*BL*) showed that increasing of *BL* resulted in a decrease in the conversion degree (Figs. 1 and 2). This effect conforms to results obtained by other researchers (Di Risio *et al.* 2011; Kristensen 2008; Kristensen *et al.* 2009; Qin 2010).

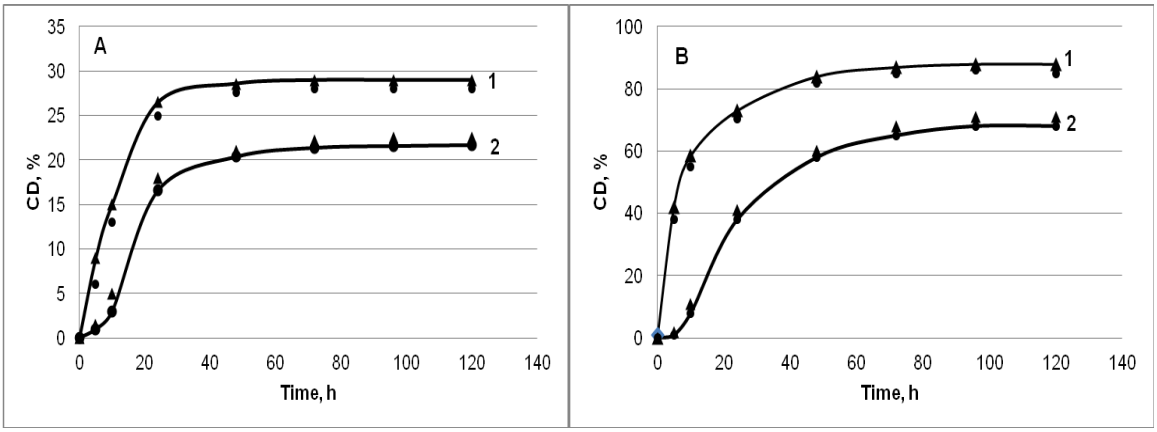

Fig. 1. Conversion degree of the pretreated biomass samples *SG-AC* (A) and *SG-HA* (B) having biomass loading 50 (1) and 200 g/L (2) during the enzymatic hydrolysis

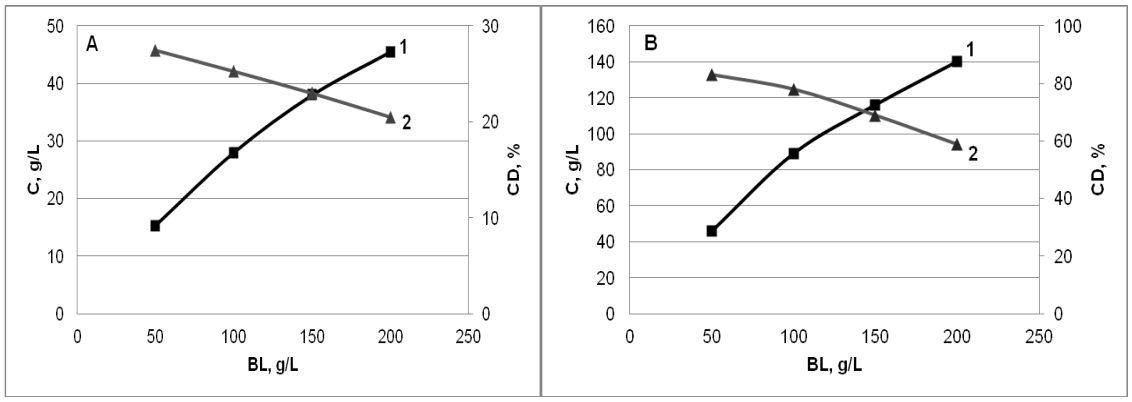

Fig. 2. Dependence of sugar concentration (1) and conversion degree (2) on the biomass loading for the pretreated samples *SG-AC* (A) and *SG-HA* (B) after enzymatic hydrolysis during 48 h

Though they showed lower conversion, the samples having the increased biomass loading after enzymatic hydrolysis gave higher concentrations of the reducing sugars (Fig. 3). Testing of sugar compositions of various hydrolysates by the HPLC method showed that the main sugar formed after enzymatic saccharification of the pretreated samples was glucose (96 to 98%), while the content of other sugars, cellobiose, and xylose, was small (2 to 4%).

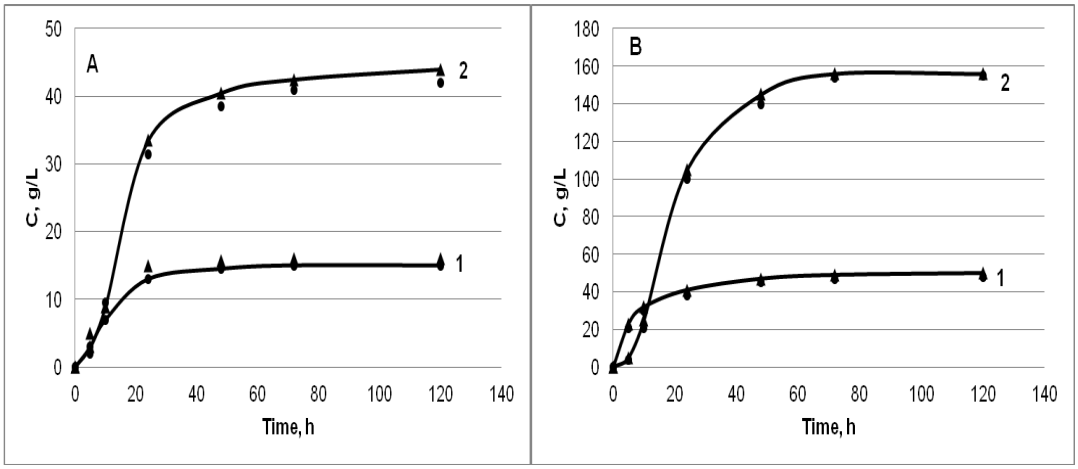

Fig. 3. Concentration of the reducing sugars after enzymatic hydrolysis of the pretreated biomass samples *SG-AC* (A) and *SG-HA* (B) having biomass loading 50 (1) and 200 g/L (2)

At increased biomass loading (*BL*>100 g/L), the used samples had a solid-like character due to lack of free liquid. As known, in such high-solids biomass samples, molecules of cellulases are immobilized into the porous structure of the fibers and have limited mobility, which delays digestibility of the biomass during the initial stage of the enzymatic hydrolysis (Di Risio *et al.* 2011; Qin 2010). However even after the prolonged hydrolysis of the solid samples, when liquefaction of the biomass system was observed, the conversion degree did not reach the level of the fluid biomass corresponding to the decreased *BL* (Figs. 1 and 2). This effect for the final hydrolysis stage is caused probably

with inhibition of the cellulases by increased concentration of the formed sugars, mainly glucose (Figs. 2 and 3), as has been discussed in various papers (Hodge *et al.* 2008; Kristensen 2008, 2009; Qin 2010).

Detailed investigations of enzymatic hydrolysis of the pretreated biomass at the increased loading disclosed an additional effect, namely the retention of hydrolyzate by the residual non-hydrolyzed residual part of the biomass, *RB* (Fig. 4). As a result, there was a reduction in the available volume of sugar solution, especially in the case of the high-solids enzymatic hydrolysis.

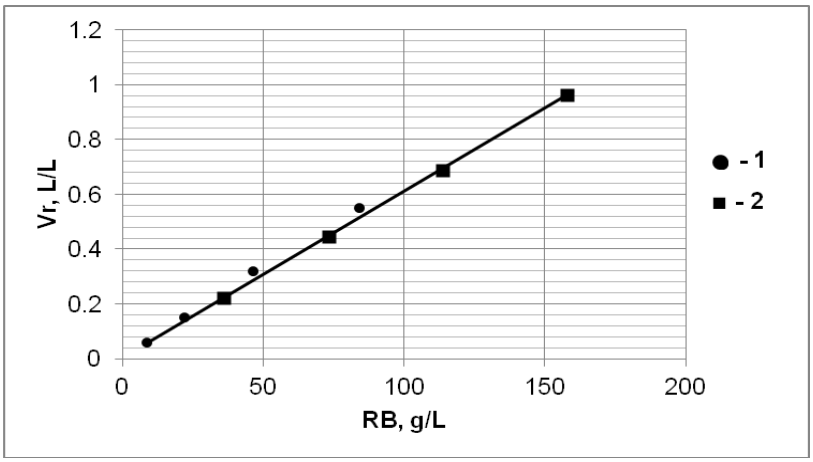

Fig. 4. Dependence of the relative retained volume of sugar solution on content of the non-hydrolyzed residual biomass for *SG-HA* (1) and *SG-AC* (2) samples after 48 h of enzymatic hydrolysis

As shown in Fig. 5, enhancement of the initial biomass loading leads to considerable diminution of available volume of the hydrolyzate. This negative occurance reduces productivity of the saccharification process; moreover, use of the low volume of the sugar solution for the further fermentation decreases the volume of final bioproducts.

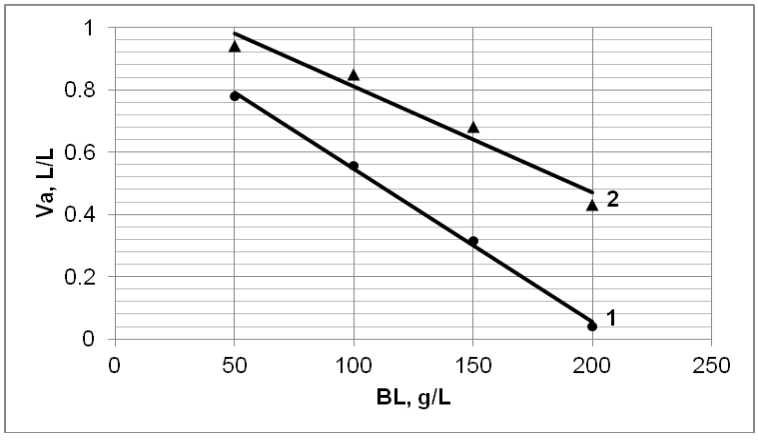

Fig. 5. Dependence of the relative available volume of sugar solution on the biomass loading for *SG-AC* (1) and *SG-HA* (2) samples after 48 h of enzymatic hydrolysis

To find the optimal conditions of enzymatic hydrolysis, and namely the optimal initial loading of biomass and hydrolysis time, the sugar amount (*A*, g per 1L of the initial liquid medium) was determined,

$$A = C\,V_a \quad (5)$$

where C is concentration of the sugar solution – hydrolyzate (g/L), and V_a is its relative available volume (L/L).

At a certain hydrolysis time, as the biomass loading is increased, the concentration of the hydrolyzate increases, while the available volume declines; as a result, the function $A=F(BL)$ shows a maximum (Fig. 6).

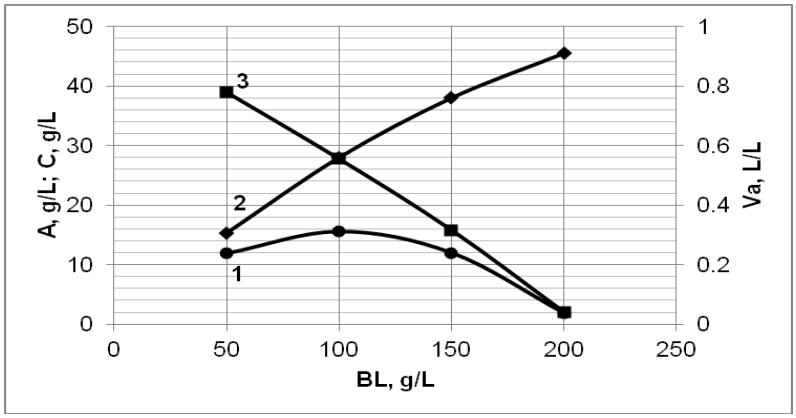

Fig. 6. Dependence of the sugar amount (1), sugar concentration (2), and relative available volume (3) on the initial biomass loading after enzymatic hydrolysis of the acid-pretreated biomass *SG-AC* for 48 h

Prolongation of the hydrolysis process promotes a rise of the maximum *A*-value (*e.g.* Fig.7).

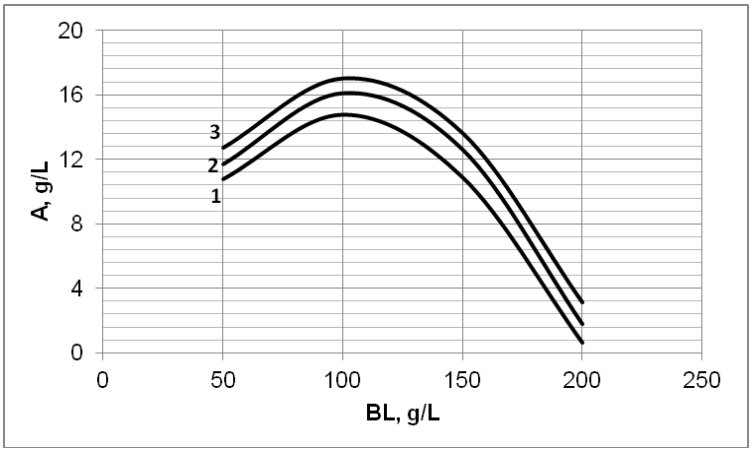

Fig. 7. Dependence of the sugar amount on the initial biomass loading after enzymatic hydrolysis of the acid-pretreated biomass *SG-AC* for 24 h (1), 48 h (2), and 72-120 h (3)

Determination of the maximal sugar amount, A_m, makes it possible to find the optimal biomass loading (BL_o) and time (t_o) of enzymatic hydrolysis of the pretreated biomass samples, as well as maximal enzymatic digestibility (Table 2).

Table 2. Optimal Conditions of Enzymatic Hydrolysis and Maximal Digestibility Characteristics of the Pretreated Samples

Features	*Values for the samples*	
	SG-AC	***SG-HA***
Optimal Conditions		
Biomass loading (BL_o), g/L	100	150
Hydrolysis time (t_o), h	72	90
Maximal Digestibility Characteristics		
Conversion degree (CD_m), %	27	78
Concentration of hydrolyzate (C_m), g/L	30	130
Available volume of the hydrolyzate ($V_{a,m}$), vol. %	56	77
Amount of sugars (A_m), g/L	17	100
Real yield of sugars ($Y_{r,m}$), %	17	67

The obtained results showed that after enzymatic hydrolysis at the optimal biomass loading and time, the low-lignified biomass pretreated by hypochlorite-alkaline method displayed considerably higher digestibility than the cellolignin obtained by acidic pretreatment of the initial sample (Table 2).

CONCLUSIONS

1. Enzymatic hydrolysis of the pretreated switchgrass samples was studied with various initial biomass loadings from 50 to 200 g/L. It was confirmed that increasing the biomass loading reduced the enzymatic digestibility.
2. An additional serious problem was found regarding the enzymatic hydrolysis at high-solids loading, namely the diminution of the available volume of the sugar solution owing to its absorption and retention by residual non-hydrolyzed biomass; this effect decreases productivity of the saccharification process.
3. The optimal biomass loading and hydrolysis time giving the maximal sugar amount were determined. After enzymatic hydrolysis at these optimal conditions, the low-lignified biomass pretreated by hypochlorite-alkaline method displayed considerable more available volume of sugar solution and higher digestibility than the cellolignin obtained by acidic pretreatment of the initial switchgrass sample.

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