STUDY OF ENZYMATIC HYDROLYSIS OF MILD PRETREATED LIGNOCELLULOSIC BIOMASSES

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The effect of mild acidic and alkaline pretreatments of various plant biomasses on their enzymatic hydrolysis has been studied. The yield of reducing sugars and utilization rate of the biomass were used as reliable characteristics of enzymatic digestibility of the biomasses. The experiments showed that alkaline pretreatment was more efficient than acidic pretreatment. As a result of alkaline pretreatment, a more efficient delignification of the biomasses and considerable improvement of the digestibility parameters were observed. It was found that residual lignin content in the biomass after alkaline pretreatment was related to initial lignin content in untreated biomass. Moreover, residual lignin showed an evident negative effect on enzymatic hydrolysis of pretreated biomass samples, and its removal contributed to higher enzymatic digestibility. It is more preferred to select a mild alkaline pretreatment for biomass that has low content of initial lignin. Such treatment yielded highly delignified biomass with increased percentage of cellulose fraction, which enhanced digestibility at low enzyme loading with a relatively short hydrolysis time.

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INTRODUCTION

An important area of modern biotechnology is production of fermentable sugars from non-edible lignocellulosic biomass resources. These biomasses can involve natural herbaceous plants (e.g. switchgrass, Bermuda grass, *Miscanthus*, etc.), residue of agricultural crops (e.g. stalks, husks, cobs, etc.), forest residues (e.g. sawdust, twigs, etc.), or industrial and municipal lignocellulosic wastes (e.g. paper, cardboard, etc.). Agriculture, forestry, pulp and paper industry, as well as cities create vast amounts of lingocellulosic residues and wastes. Moreover, some natural grasses and shrubs are not utilized yet and can be used as appropriate sources for sugars production. Lignocellulosic materials are related to abundant, renewable, and inexpensive natural sources. The estimated annual production of plant biomasses in the US is more than 1.3 billion tons per year (Perlack et al. 2005).

Obtaining sugars from the lignocellulosic biomass is usually achieved by enzymatic hydrolysis. However, the lignified biomass exhibits resistance to biological cleavage because of the compact structure of the plant material and barrier properties of lignin (Cowling 1975; Himmel et al. 2007; Mooney et al. 1998). In the plant cell walls, the cellulose fibrils form a skeleton that is surrounded by hemicellulose and lignin layers (Fengel 1971). Natural lignocellulosic biomass consists of about 25 to 50% cellulose, 20

to 40% hemicelluloses, and 5 to 35 % lignin (Fengel et al. 1971, 1984; Saha 2003; Sun et al. 2002).

Hemicelluloses are branched, amorphous, and hydrophilic polysaccharides. In addition to physical bonding of cellulose, hemicelluloses also form ester bonds with lignin. Thus, hemicelluloses fulfill the function of "glue" between hydrophilic cellulose and hydrophobic lignin. Swollen hemicelluloses probably are not a barrier for cellulolytic enzymes. However these polysaccharides adsorb molecules of cellulases, and due to the unproductive binding, the hydrolysis of cellulose is delayed (Kim et al. 2003; Zhu 2005).

Lignin is a rigid aromatic, amorphous, and hydrophobic polymer stable to cellulolytic enzymes. In the plant cell wall, lignin layers surrounding the hydrophilic cellulose fibrils protect cellulose from enzymatic attack (Mooney et al. 1998; Pan et al. 2005; Zhu 2005).

In order to improve enzymatic hydrolysis the lignocellulosic biomass should be pretreated. Main objectives of the pretreatment are to loosen the physical structure of the biomass and eliminate non-cellulosic components (lignin and hemicelluloses) in order to increase accessibility and the percentage of cellulose fraction (Petersen et al. 2009; Mosier et al. 2005; Zhu 2005). Hemicelluloses are unstable to chemical treatments and can be easily removed even under mild conditions. However, lignin is resistant to chemicals. Its extraction from the biomass demands harsher pretreatment conditions.

Pretreatment determines the structure, chemical composition, and hydrolysis rate of the pretreated biomass. The effective pretreatment should be inexpensive and should provide more accessible and cellulose-rich biomass with high enzymatic digestibility. Various pretreatment methods of lignocellulosic biomasses have been proposed, including steam explosion, acidic treatment, alkaline extraction, ammonia treatment, and oxidation (Draude et al. 2001; Galbe et al. 2007; Hendiks et al. 2009; Hsu 1996; Selig et al. 2009; Wang et al. 2011; Wu et al. 2010; Xu et at. 2012; Yu et al. 2011).

Currently available industrial reactors cannot heat huge amount of the biomass within very short times (1 to 5 min) up to high temperatures (200 to 250 $^{\circ}$C). Therefore the steam explosion (SE) method cannot be scaled up to industrial production. An additional problem of the high-temperature SE-method is the transformation of hemicelluloses and celluloses into aromatic aldehydes - furfural and hydroxymethylfurfural, which may inhibit hydrolysis of enzymes and fermentation of yeasts. Besides, as a result of condensation of furfural-based substances so called pseudolignin can be formed, increasing the total lignin content in the steam-exploded biomass (Miranda et al. 1979; Sannigrahi et al. 2011).

Pretreatment of lignocellulosic biomass with diluted (0.5 to 1 wt.%) mineral acids, mainly with sulfuric acid, at high temperatures (160 to 190 $^{\circ}$C) for 30 to 60 min has been realized in industry. However this method demands high energy consumption. Moreover, as a result of high-temperature acidic treatment, the percentage of lignin fraction was increased in the pretreated biomass, and pseudolignin was generated along with aromatic aldehydes and some other inhibitors (Pingali et al. 2010; Sannigrahi et al. 2011). To prevent the formation of pseudolignin and inhibitors, the temperature of the acidic pretreatment should be decreased. For example, it was proposed to pretreat biomass samples with the temperature range 90 to 100 $^{\circ}$C using 3 to 5 wt.% sulfuric acid (Galbe et al. 2007; Gong et al. 1993).

Pretreatment with lime and sodium hydroxide is considered as a cheap and efficient method, especially for some herbaceous plants (Keshwani et al. 2009; Xu et al. 2011). However, pretreatment of the initial biomass with ammonia is difficult to implement currently in the industry due to the danger of release of this harmful reagent.

Oxidative pretreatment of plant materials with various oxidants (ozone, peracetic acid, peroxides, etc.) is efficient but highly expensive to use at an industrial scale. Moreover, inhibitory by-products can be formed during oxidation processes (Hendiks et al. 2009).

Among various methods, pretreatments of plant biomasses with diluted (1 to 3 wt. %) acids, and alkalis at moderate temperatures (50 to 120 ^{o}C) have been intensively studied due to their industrial feasibility, relatively low capital investment, and low consumption of chemicals and energy (Galbe et al. 2007; Gong et al. 1993; Keshwani et al. 2009; Wang et al. 2011; Xu et al. 2011). However, the overall effect of mild pretreatment of the biomass on enzymatic digestibility can depend on chemical composition of the initial lignocellulosic materials, in particular on structure and content of lignin, which is difficult to extract.

As it follows from results of delignification of various softwood and hardwood samples (Laine et al. 2004; Saltberg et al. 2009; Shackford 2003; Olm et al. 2009), the content of the residual lignin in partially delignified biomasses is caused mainly by content of the initial lignin, while structural peculiarities of the lignins have lower importance. In this connection it can be expected that at the same pretreatment conditions a lower level of lignin in the initial biomass will yield a lower content of residual lignin and higher enzymatic digestibility. To verify this supposition, the effect of mild acidic and alkaline pretreatments of plant biomasses with different lignin content on the enzymatic hydrolysis were studied, with results reported in the present paper.

EXPERIMENTAL

Materials

The four plant materials – poplar (PO), switchgrass (SG), corn cobs (CC), and rice straw (RS), were chosen and used as initial biomass samples. Poplar chips were supplied from ZeaChem Inc. (USA); switchgrass pellets from Nott Farms (Canada); and rice straw from Shenzhen Fengyuan Trading Co. (China).

The corn cobs sample was prepared from corn ears bought on the free market. The corn ears were cooked in water for 1h. Then corn grains were removed, while the wet corn cobs were dried at 105 ^{o}C up to constant weight.

The all initial samples were cut, knife-milled, and screened through a sieve of 2 mm.

Pretreatment

The biomass samples were pretreated with 3 wt.% sulfuric acid and 2 wt.% sodium hydroxide at moderate temperatures. About 100 g samples were put in lab glasses; then reagent solution was added to a liquor/solid ratio of about 7. The glasses containing biomass and reagent were placed into an oil bath with temperature 110 ^{o}C and

treated at stirring for 1h. The pretreated biomasses were washed up to neutral pH and squeezed on vacuum glass-filter up to a final solids content of 20 to 30 wt.%.

Chemical Analysis

The chemical composition of initial and pretreatment biomasses was determined by conventional methods of chemical analysis (Fengel et al. 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 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 my 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 at determination of percentage of the components was in the range ± 1 %.

Enzymatic Hydrolysis

The 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 5 CBU per 1 g of solid sample. Hydrolysis of the samples was carried out in 50-mL polypropylene tubes. The samples containing 1 g of the solid matter and 10 ml of 50 mM/L acetate buffer (pH=4.8) were put into the tubes. Then cellulase and β–glucosidase were added. Further, an additional amount of the buffer was supplemented to obtain total volume of the liquid phase 20 mL and concentration of the biomass sample 50 g/L. The tubes closed with covers were placed in a shaker incubator at 50 $^{\circ}$C and shaken at 180 rpm for 48 h. Finally, the tubes were centrifuged in order to separate the residual biomass and sugar solution. Concentration of the reducing sugars (**RS**) in the solution after enzymatic hydrolysis of the biomass samples was tested by the conventional **DNS**-assay using glucose for calibration (Miller 1959). Weight loss (**WL**, *g*) of the biomass samples at the hydrolysis also was studied.

From the experimental results the following characteristics of enzymatic digestibility were calculated,

Yield of Reducing Sugars (%), $\quad$ **YS** *= 100 (RS/B)* $\quad$ (1)

Utilization Rate of Biomass (%), $\quad$ **UR** *= 100 (WL/P$_o$)* $\quad$ (2)

where **RS** is the concentration of reducing sugars in solution (g/L) after enzymatic hydrolysis of the biomass; **B** is the initial concentration of the biomass (50 g/L); and **P$_o$** is the initial weight of the biomass sample (1 g).

Three samples of the same biomass type were hydrolyzed simultaneously to obtain accurate results. The standard deviation at determination of the digestibility characteristics was in the range ± 2%.

RESULTS AND DISCUSSION

Contents of three main components – cellulose, hemicelluloses, and lignin – in the initial biomasses are shown in Table 1. The other components of the initial biomass samples were extractives, ash, and protein. The chosen biomass samples contained 35 to 44% cellulose, 25 to 38% of hemicelluloses, and 7 to 23 % of Klason lignin. The higher content of lignin (23%) and cellulose (44%) was observed for poplar biomass. Switch-grass contained an intermediate content of lignin (18%) and cellulose (37%). The low lignified corn cobs and rice straw contained 7 to 10% lignin and 35 to 36% cellulose. Besides, the corn cobs had the highest content of hemicelluloses, 38%.

Table 1. Chemical Composition of the Initial Untreated Biomass Samples

Biomass	Cellulose, %	Hemicelluloses, %	Lignin, %
Poplar	44	27	23
Switchgrass	37	28	18
Corn cobs	35	38	10
Rice straw	36	25	7

Study of the enzymatic hydrolysis of the initial biomass samples showed that initial biomass samples cannot be considered as appropriate feedstocks for enzymatic hydrolysis due to insufficient yield of reducing sugars, 17 to 24% and low utilization rate of the biomass, 15 to 22%; moreover increasing lignin content worsened enzymatic digestibility of the samples (Fig. 1). Besides, the main part of the valuable plant material, from 78 to 85%, remained intact and provided no value. The limited enzymatic digestibility of the initial plant material can be connected probably with low cellulose content, as well as with low accessibility due to dense structure of the biomass and barrier properties of lignin (Cowling 1975; Mooney et al. 1998; Zhu 2005).
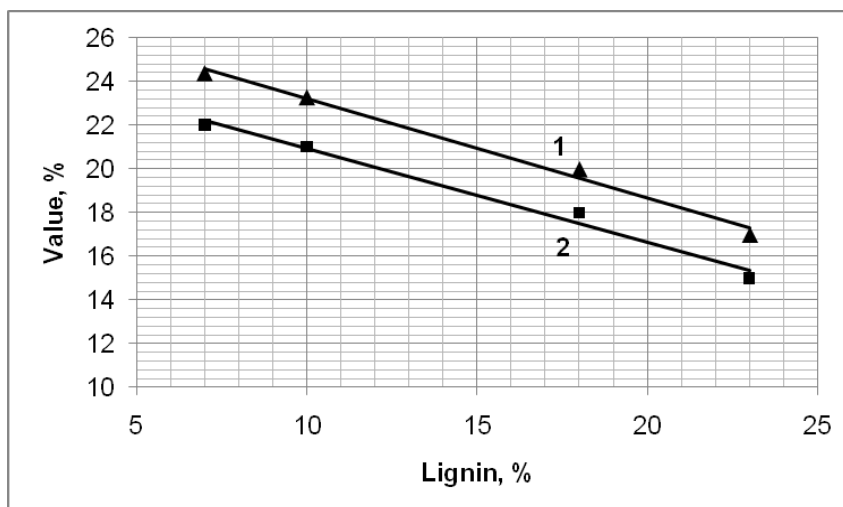

Fig. 1. Dependence yield of reducing sugars (1) and utilization rate (2) at enzymatic hydrolysis of the initial biomass samples on content of lignin

Difference in content of hemicelluloses (Hemi, %) had no appreciable effect on the digestibility of the initial plant materials, as shown by the fact that the squared correlation coefficients for relationships, YS=f(Hemi) and UR=f(Hemi), were low, R^2=0.05-0.06 only.

To enhance the enzymatic digestibility of the used biomasses they were pretreated with diluted solutions of acid and alkali at the mild conditions. The distinctive feature of the acidic pretreatment was removing the main part of hemicelluloses, loosening of biomass structure, and forming cellolignin with increased content of cellulose and lignin (Table 2, Fig. 2, 3).
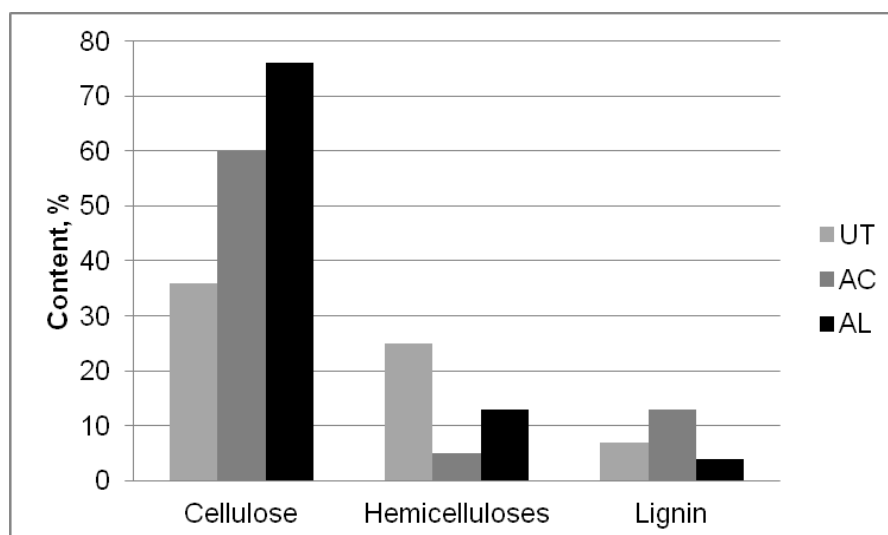
Table 2. Percentage of Cellulose, Hemicelluloses (Hemi) and Lignin in the Biomass Samples: Untreated *(UN)* and Pretreated with Acid *(AC)* and Alkali *(AL)*

|	Poplar			Switchgrass			Corn cobs			Rice straw		
	UT	*AC*	*AL*	*UT*	*AC*	*AL*	*UT*	*AC*	*AL*	*UT*	*AC*	*AL*
Cellulose	44	58	62	37	55	65	35	58	71	35	60	76
Hemi	27	6	14	28	7	16	38	10	15	25	5	13
Lignin	23	31	16	18	26	12	10	18	6	7	13	4
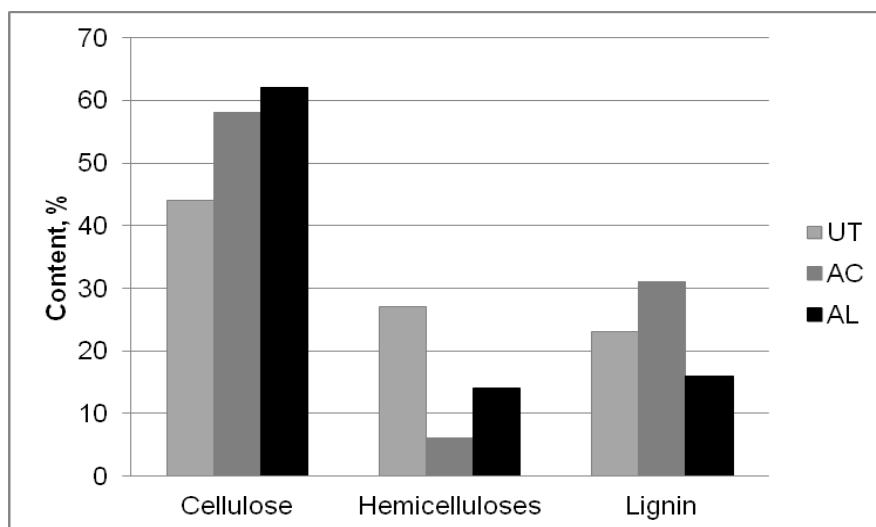
Fig. 2. Percentage of cellulose, hemicelluloses and lignin in untreated (UT), acid (AC) and alkali (AL) pretreated poplar

These structural changes have an ambiguous effect on enzymatic hydrolysis of the acid pretreated biomass: on the one hand loosening of structure and increase of cellulose content could promote achieving higher digestibility, but on the other hand increased content of lignin should hinder the enzymatic hydrolysis.

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Fig. 3. Percentage of cellulose, hemicelluloses and lignin in untreated (UT), acid (AC) and alkali (AL) pretreated rice straw

The final result showed some improvement of the enzymatic digestibility of the acid pretreated biomasses in comparison with untreated samples (Fig. 4, 5). However, the digestibility characteristics were insufficient: the utilization rate of biomass was in the range 30 to 42%, and the yield of reducing sugars was in the range 40 to 50%.
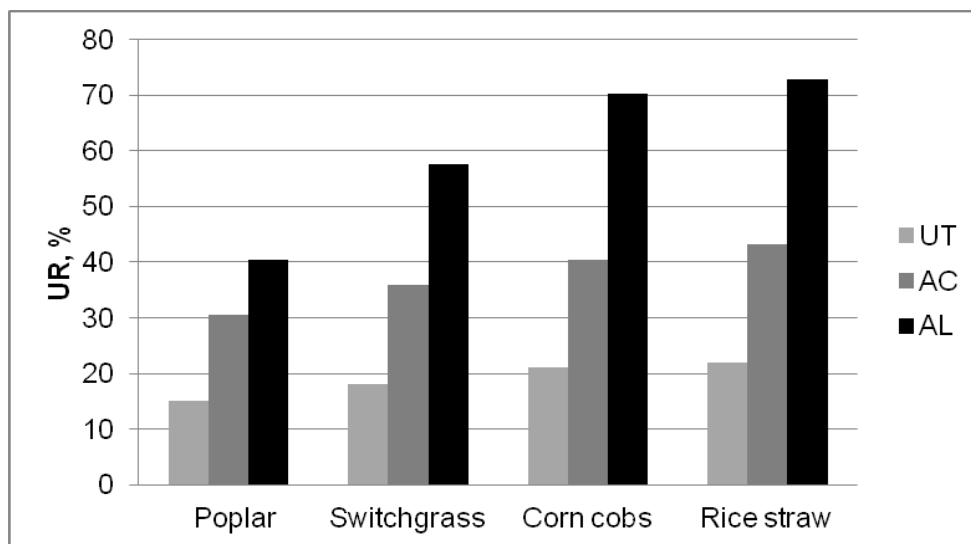
Fig. 4. Utilization rate of biomass (UR) after enzymatic hydrolysis of untreated (UT), acid (AC) and alkali (AL) pretreated biomass samples

In contrast to acidic pretreatment, alkali pretreatment of the biomass caused decreases of both of hemicelluloses and lignin (see Table 2, Fig. 2, 3). Simultaneously, increasing cellulose content was observed, which should promote the enzymatic digestibility of the pretreated biomass. Indeed, the experiments showed that pretreatment

of the biomass samples with diluted alkali solution caused considerable rise of the utilization rate and yield of sugars after enzymatic hydrolysis of alkali pretreated biomasses in comparison with both untreated and acid pretreated samples (Fig. 4, 5). This result is in agreement with the findings of other researchers (Xu et al. 2011).
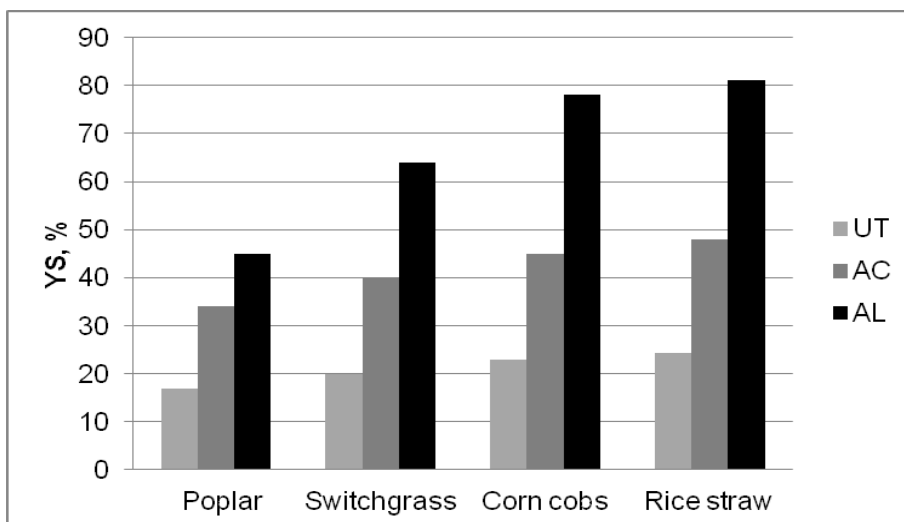
Fig. 5. Yield of reducing sugars (YS) after enzymatic hydrolysis of untreated (UT), acid (AC) and alkali (AL) pretreated biomass samples

To compare the effect of residual lignin remaining in the biomass samples after mild acidic and alkaline pretreatments on enzymatic digestibility of the pretreated biomasses, a correlation analysis was carried out in order to calculate regression equation and squared correlation coefficient (R^2). The analysis showed that content of residual lignin (**RL,** %) had an essentially negative effect on the yield of sugars:

$$YS = 82 - 1.75\,RL \quad (R^2 = 0.83) \tag{3}$$

Increased content of residual lignin hindered conversion of the pretreated biomasses into sugars by enzymatic hydrolysis; and vice versa, lowering of content of residual lignin facilitated enzymatic digestibility. This conclusion conforms to results obtained also by some other researchers (Selig et al. 2009; Sun et al. 2011).

Experimental results showed that at the same pretreatment conditions, the content of residual lignin (*RL*) remaining in the biomass after alkaline pretreatment depended on the content of initial lignin (*IL*) in untreated plant samples (Fig. 6).

From structural investigations it is known that various lignins comprise of two major units – guaiacyl (*G*), and syringyl (*S*) (Obst 1982). Lignins of softwoods contain mainly *G*-units. As against, lignins of hardwoods and also herbaceous plants contain comparable molar contents both *S*- and *G*-units (Grabber et al. 2004; Davison et al. 2006).

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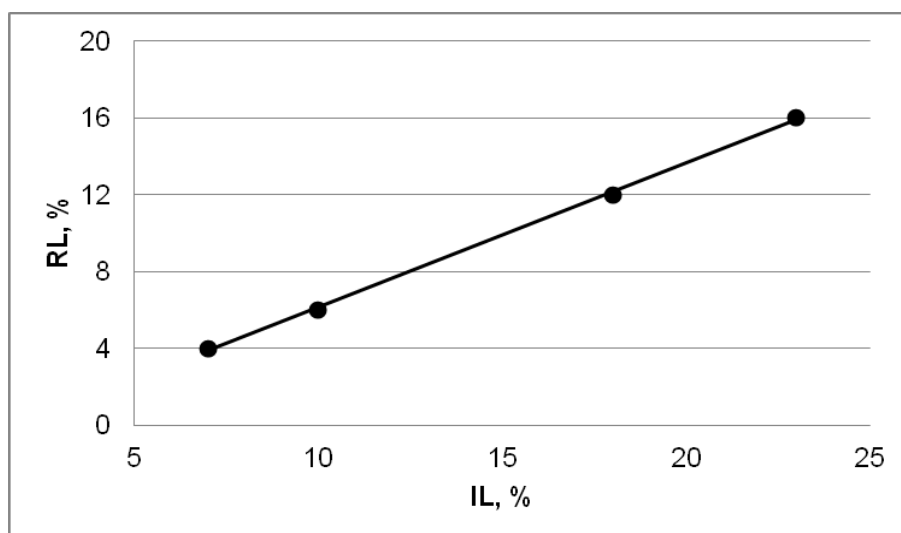
Fig. 6. Correlation between content of initial lignin (IL) and residual lignin (RS) remaining in alkali pretreated biomass samples at the same treatment conditions

The correlation *RL=F(IL)* can be established for various used plant materials because both hardwood (poplar) and investigated herbaceous plants contain the similar S-G type of lignins. Therefore at the identical pretreatment conditions, just the content of initial lignin, rather than structural details of the lignins of different plant samples, plays the major role in alkaline pretreatment process and determines content of residual lignin. After mild alkaline pretreatment, similar proportions of the initial lignin (about 0.35) were extracted from all the used plant materials; thus, the percentage of remaining residual lignin for the more lignified hardwood sample (e.g. poplar) will be higher than for the lower lignified sample, i.e. the herbaceous plant (e.g. rice straw).
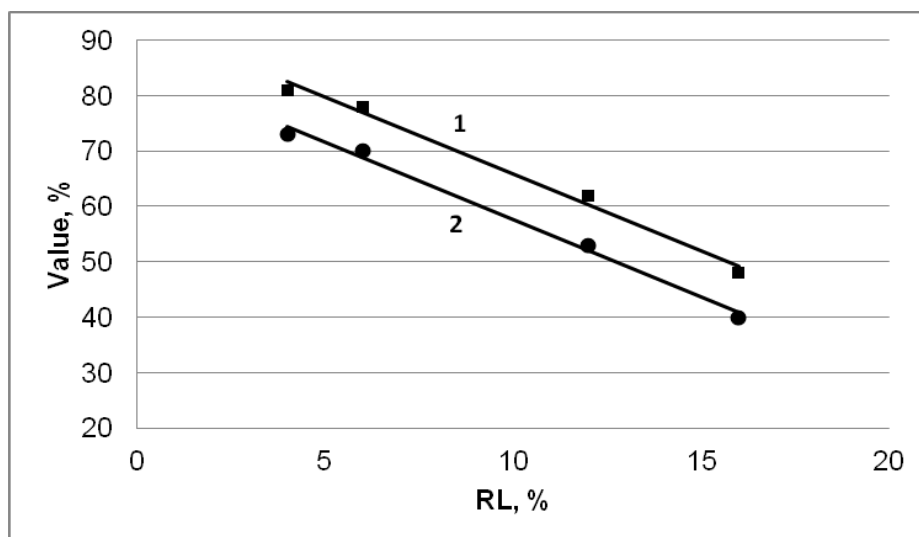
Fig. 7. Dependence of yield of reducing sugars (1) and utilization rate (2) of alkali pretreated biomass samples at enzymatic hydrolysis on content of residual lignin (RL)

As follows from the above mentioned regression analysis, the residual lignin also in the alkali-pretreated biomass has a negative influence on enzymatic hydrolysis, and therefore decrease of its content promotes improvement of the digestibility characteristics (Fig. 7). As follows from the experiments, the content of residual lignin in various biomass samples at the same pretreatment conditions decreased in the following order: Poplar > Switchgrass > Corn cobs > Rice straw; this facilitates increasing the enzymatic digestibility of the alkali pretreated biomass in the same order.

Thus, to obtain a highly hydrolysable pretreated biomass sample it is preferable to select and use such biomass type that has the lowest content of initial lignin. For example, the low lignified rice straw (IL= 7%) after the mild alkaline pretreatment yielded a low content of residual lignin (RL=4%). It also exhibited sufficiently high characteristics of enzymatic digestibility (UR=74% and YS=82%) at the low enzyme loading (5 FPU/g) and with a relatively short hydrolysis time (48 h).

CONCLUSIONS

The effects of acidic and alkaline pretreatments under mild conditions of various plant biomasses (poplar, switchgrass, corn cob, and rice straw) on efficiency of enzymatic hydrolysis were studied. It was shown that two reliable characteristics to determine the enzymatic digestibility of different biomass types are yield of reducing sugars and utilization rate of the biomass.

With acidic pretreatment, the samples showed some improvement of enzymatic hydrolysis; however the obtained characteristics of enzymatic digestibility were not satisfactory. Alkaline pretreatment was more efficient and resulted in considerable increase of all digestibility characteristics in comparison with acid pretreatment.

The content of residual lignin remaining in the biomass after alkaline pretreatment depended on the content of initial lignin. The residual lignin had a negative effect on enzymatic hydrolysis of pretreated biomass, and its removal led to increasing enzymatic digestibility.

It is more preferred to select mild alkaline pretreatment just for the plant material that has low content of initial lignin. Such a combination yielded highly delignified biomass with increased percentage of cellulose fraction, which enhanced digestibility at low enzyme loading with a relative short hydrolysis time.

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