THE EFFECTS OF BORON DERIVATIVES AS PRETREATMENT CHEMICALS FOR BIODEGRADATION OF SUNFLOWER STALKS

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ABSTRACT

This study examined the suitability of boron derivatives as pretreatment chemicals with regard to their efficiency in the enzymatic saccharification of sunflower stalks. It was hypothesized that the utilization of boron derivatives might improve enzymatic digestibility. The aim of this study was to examine the effect of NaOH and the boron derivatives sodium borohydride [$NaBH_4$] calcined tincal [$Na_2B_4O_7(1-5)H_2O$], colemanite [$CaB_3O_4(OH)_3H_2O$], boron oxide [B_2O_3], borax decahydrate [$Na_2B_4O_7 10H_2O$], disodium octaborate tetrahydrate [$Na_2B_8O_{13} 4H_2O$] and boric acid [H_3BO_3] on enzymatic saccharification. Results showed that the boron derivatives prevented peeling reactions and preserved more glucan; however, the NaOH removed the highest amounts of xylan and lignin from the structure. Significant xylan removal was observed with H_3BO_3, while B_2O_3 was as effective as $NaBH_4$ and NaOH for selective lignin removal. The highest enzymatic saccharification yield (59,5%) resulted with the 2% NaOH pretreatment, whereas slightly lower yields were achieved with some boron derivatives (1% $NaBH_4$-45%; 2% B_2O_3-40,2%; and 2% H_3BO_3-36,7%).

Keywords: Enzymatic hydrolysis, enzymatic saccharification, glucan, lignin, xylan.

INTRODUCTION

Sunflower seeds are the third largest source of vegetable oil production, the other valuable sources being soybean and palm (Vaithanomsat *et al.* 2009). Turkey is one of the top ten sunflower-producing countries in the world, with nearly 3 % of the world's sunflower production and 6 % of the acreage (Anonymous 2014a). In recent years, Turkish sunflower acreage and crop production have increased from 500000 to 600000 ha and from 800000 to 1,4 million tons, respectively (Anonymous 2014b). The usage of stalks, heads and leaves for medicinal and cosmetic purposes is very limited and, generally, after harvest these materials are left in the fields to be burnt, causing environmental pollution (Sharma *et al.* 2002). The huge volume of these lignocellulose residues (3-7 tons of dry matter/ha) emerging annually (Marechal and Rigal 1999) can become an important low-cost source of materials that can be converted into valuable products such as ethanol. The industrial use of this substrate in bioethanol production may also result in decreasing the soil and air pollution associated with the disposal of the stalks.

Sunflower stalks consist mainly of cellulose, hemicelluloses and lignin in the structure, which is similar to woody biomass. The presence of lignin, and its complex distribution in the cell wall structure, and the crystalline nature of cellulose have motivated researchers to develop several pretreatment techniques which have further improved the effectiveness of enzymatic hydrolysis in reducing the cost of fermentation (Wingren *et al.* 2005).

The main approach of the research dealing with pretreatments is to improve the accessibility of cellulose to hydrolytic enzymes while decreasing hemicellulose and cellulose degradation during the

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application. Carbohydrate degradation and by-products that emerge during the degradation lower the ethanol yield by diminishing the hydrolysis and fermentation processes. This is due to the inhibitory effects of these by-products (Ohgren *et al.* 2007). Thus, the goal of this research is to improve the efficiency of the pretreatments by increasing the conversion rate in order to generate a higher economic benefit.

In this context, the effects of physicochemical pretreatments like hydrothermal (Diaz *et al.* 2011, Eom and Yu 2015) and steam explosion (Ruiz *et al.* 2008, Sharma *et al.* 2002), and of chemical pretreatments like alkali (Soto *et al.* 1994), hydrochloric acid (Bonilla *et al.* 1990), sulfuric acid (Jimenez and Bonilla 1993, Tosun 1997, Ruiz *et al.* 2013) and peracetic acid (Farid *et al.* 1983) have been studied for sunflower stalks. Additionally, Sharma *et al.* (2002) and Ruiz *et al.* (2006) have examined the fermentation and ethanol production from sunflower stalks.

Several pretreatment techniques have been devised to enhance the efficiency of enzymatic saccharification and thus bioethanol production, and chemical pretreatments have already been followed by the environmentally friendly enzymatic hydrolysis. The conventional pretreatment chemical utilized in this context is usually sodium hydroxide (NaOH), as well as other chemicals including peroxide (H_2O_2), sulfuric acid (H_2SO_4) etc. Studies on alternative chemicals like sodium borohydride ($NaBH_4$) are very limited in the literature. Copur *et al.* (2012, 2013a, 2013b) examined the selective capability of $NaBH_4$ as a pretreatment chemical, and they observed it to be as effective as NaOH for wheat straw, corn stalks and hazelnut husks. In addition, as Turkey has 71,3 % of the world boron reserves, it is important to examine boron derivatives as pretreatment chemicals. It was hypothesized that some of the boron derivatives utilized in this study could improve the enzymatic digestibility. Therefore, the aim of this study was to examine the effect of several boron derivatives on enzymatic saccharification. Consequently, sunflower stalks were treated with the conventional pretreatment chemical, NaOH, and the results were compared with sodium borohydride [$NaBH_4$] and other boron derivatives utilized for the first time for this purpose including calcined tincal [$Na_2B_4O_7(1-5)H_2O$], colemanite [$CaB_3O_4(OH)_3H_2O$], boron oxide [B_2O_3], borax decahydrate [$Na_2B_4O_710H_2O$], disodium octaborate tetrahydrate [$Na_2B_8O_{13}4H2O$] and boric acid [H_3BO_3].

MATERIALS AND METHODS

Materials

Sunflower stalks, obtained from the field in Turkey right after harvest, were cut to suitable sizes (3-5 cm) using a garden chopper. The chopped material was air dried at room temperature and stored in plastic bags. The moisture content was determined (Tappi T 412 om-06) and the material was stored at -5 °C.

Methods

Chemical pretreatments

Solid material (40 g o.d.) was chemically treated with conventional sodium hydroxide (NaOH) as control, and with boron derivatives of sodium borohydride [NaBH$_4$], calcined tincal [Na$_2$B$_4$O$_7$(1-5) H$_2$O], colemanite [CaB$_3$O$_4$(OH)$_3$H$_2$O], boron oxide [B$_2$O$_3$], borax decahydrate [Na$_2$B$_4$O$_7$10H$_2$O], disodium octaborate tetrahydrate [Na$_2$B$_8$O$_{13}$4H$_2$O] and boric acid [H$_3$BO$_3$]. Chemical pretreatments were made for 0,5; 1 and 2 % (w/v) concentrations and 10 % (w/v) solid loading. The pretreatment temperature was 121 °C (15 psi). Pretreatment was made for both 60 and 90 min. Chemically pretreated materials were filtrated and the solid parts were stored in sealed plastic bags at 4 °C. The yield, acid in/soluble lignin and sugar contents were determined in the solid materials and the optimum conditions for chemical pretreatments were determined regarding the ratio of glucan and lignin solubilization. In this study, samples accepted as optimum were further enzymatically hydrolyzed.

Enzymatic hydrolysis

The samples were hydrolyzed using commercial enzyme mixtures (50 % v/v) and the accessibility of the samples for enzymatic conversion was examined. Celluclast 1,5 L; generating the major cellulolytic activities (dosage 10 FPU g^{-1} dw) was supplemented with a beta-glucosidase of Novozym 188 (500 nkat g^{-1} dw) (Novozymes, Bagsvaerd, Denmark). The dry matter consistency in hydrolysis was 2 %. The experiments, in three replicates, were carried out in a volume of 50 ml in 50 mM Na-citrate buffer at pH 5. In addition, NaN$_3$ (0,02 %) was added to prevent contamination during hydrolysis. The temperature was 50 °C and the shaking speed 100 rpm in the incubator (Biosan Environmental Shaker-Incubator ES-20). Samples (1,5 ml) were taken at the beginning (0) and at 6, 24, 48 and 72 h of incubation. Next, the samples were first put into boiling water for 10 min to stop the enzymatic activity and then centrifuged at 10000 rpm for 5 min. The reducing sugars were determined from the supernatant and the results were expressed as percentage of the theoretical hydrolysis yield (formed sugars from theoretical sugars, i.e. polymeric carbohydrates calculated as monosaccharides) and as percentage of formed sugars from the whole dry matter of the hydrolyzed raw material.

Analytical methods

The yield of the samples was determined by gravimetric measurements. The extractives (Tappi T 204 om-88) and ash (Tappi T 211 om-85) contents of the samples were determined. The sugar and lignin contents of the samples were determined according to the Laboratory Analytical Procedures (LAP) from the NREL (Sluiter *et al.* 2004). The sugar contents were determined by utilizing the HPLC (Agilent 1200) system equipped with Shodex SP0810 column (mobile phase: HPLC grade water-0,2 μm filtered and degassed, injection volume: 20 μL, flow rate: 0,6 ml/min, column temperature: 80 °C) and the refractive index detector. The acid-insoluble lignin was obtained by weighing the solid samples. The acid-soluble lignin was analyzed at the adsorption of 320 nm against blank deionized water. The percentage of solids recovered was calculated on an oven dry basis as follows:

$$\text{The percentage of solids recovered} = \frac{W_2}{W_1} x100$$

where W_1 is the dry weight of the sample before pretreatment (g) and W_2 is the dry weight of the treated sample (g).

The reduction in lignin was calculated according to the initial dry weight of lignin in the untreated material (LU) and the dry weight of lignin in the remaining solids after treatment (LP). The percentage of lignin reduction was calculated using the following equation:

The percentage of lignin reduction = $\dfrac{LU - LP}{LU} x100$

In addition, the solubilization of glucan, xylan, galactan and mannan+arabinan in the pretreated samples was calculated in the same manner by substituting the appropriate percentage for glucan, xylan, galactan and mannan+arabinan.

In enzymatic hydrolysis, the reducing sugar content in the hydrolyzates was detected by the DNS method (Liu *et al.* 2011) and calculated by the equation:

$$RSY = \frac{m_{RS}}{m_T} = \frac{nCV}{m_0(1-\omega)} x\ 100\%$$

where RSY is the reducing sugar yield (%), m_{RS} is the content of reducing sugar (g), m_T is the substrate mass (g), m_0 is the substrate weighted material (g), ω is the moisture content (%), C is the concentration of the reducing sugar (g L^{-1}), V is the total volume of hydrolyzate (L) and n is the dilution time.

RESULTS AND DISCUSSION

Characterization of sunflower stalks

The composition (% o.d. stalks) of the sunflower stalks used in this study consisted mainly of glucose, at 32,8 % (±0,40). The other sugars of xylose, galactose and mannose+arabinose were 16,7 % (±0,12); 1,44 % (±0,22) and 1,89 % (±0,12); respectively. The total lignin (acid insoluble+acid soluble), extractives and ash content were 16,6 % (±0,45); 15,3 % (±0,14) and 7,10 % (±0,85); respectively.

The composition of sunflower stalks as reported earlier in the literature (Akpinar *et al.* 2011, López *et al.* 2010, Antonopoulou *et al.* 2015) revealed similar results. The only observed difference was the lignin content, and the lower lignin content in this study may be attributed to the differences in plant variety, climate, water availability, as well as other factors.

Effect of chemical pretreatments

The stalks were pretreated with chemicals and the recovered solids are shown in Table 1. Chemical pretreatments lowered the yields, and this could mainly be explained by the removal of lignin, hemicellulose and other soluble materials from the structure. Stalks treated with 2 % $CaB_3O_4(OH)_3H_2O$ and NaOH for 90 min treatment times resulted in the highest (93,3 % o.d. stalk) and the lowest (64,8 % o.d. stalk) solid recoveries, respectively. The different pretreatment chemical factors (8), chemical concentrations (3) and pretreatment times (2) and their interactions on glucan, xylan, galactan, mannan+arabinan and lignin are shown in Tables 2 and 3.

Table 1. Solids recovery after pretreatments.

Time (min); Concentration (%)	Solids recovered after chemical pretreatments (%)[a,b]							
	NaOH	NaBH$_4$	Na$_2$B$_4$O$_7$(1-5) H$_2$O	CaB$_3$O$_4$(OH)$_3$ H$_2$O	B$_2$O$_3$	Na$_2$B$_4$O$_7$ 10H$_2$O	Na$_2$B$_8$O$_{13}$ 4H$_2$O	H$_3$BO$_3$
60; 0,5	85,8±0,33	86,8±0,23	86,9±0,38	87,4±0,18	87,2±0,47	88,5±0,58	90,8±0,13	85,4±0,27
60;1	84,0±0,37	86,6±0,17	87,2±0,48	90,3±1,07	87,2±0,65	85,9±0,30	89,2±0,45	85,3±0,29
60;2	65,2±0,02	83,8±0,35	86,7±0,73	90,8±0,33	87,9±0,33	88,1±0,22	88,9±0,15	86,4±0,36
90;0,5	83,9±0,21	86,4±0,12	87,1±0,41	90,4±0,48	85,8±0,51	88,1±0,11	88,6±1,01	88,8±0,51
90;1	81,9±0,37	86,0±0,35	88,3±0,21	91,0±0,17	85,9±0,42	86,2±097	84,6±0,67	90,5±0,19
90;2	64,8±0,32	87,8±0,19	84,4±0,16	93,3±0,38	86,8±0,24	87,8±0,09	89,5±0,56	89,6±0,33

[a]Percentages calculated from value on a dry-weight basis.
[b]Data are averages of three replicates.

Table 2. The effects of chemicals, concentrations and time on glucan, xylan, galactan, mannan+arabinan and lignin (Duncan and T test).

Factor	Treatment	Glucan, %	Xylan, %	Galactan, %	Mannan+Arabinan, %	Lignin, %
Chemical	NaOH	27,6 (ab)	13,6 (b)	0,33 (b)	1,02 (a)	18,7 (e)
	NaBH$_4$	33,9 (c)	17,3 (e)	0,50 (c)	1,04 (ab)	15,5 (a)
	Na$_2$B$_4$O$_7$(1-5)H$_2$O	27,4 (a)	11,4 (a)	0,26 (ab)	1,38 (c)	17,8 (de)
	CaB$_3$O$_4$(OH)$_3$H$_2$O	27,7 (ab)	14,9 (c)	0,29 (ab)	1,43 (c)	16,1 (ab)
	B$_2$O$_3$	28,8 (b)	16,3 (de)	0,31 (ab)	1,54 (c)	16,5 (abc)
	Na$_2$B$_4$O$_7$10H$_2$O	27,8 (ab)	16,1 (d)	0,29 (ab)	1,25 (abc)	17,1 (bcd)
	Na$_2$B$_8$O$_{13}$4H$_2$O	29,0 (b)	15,9 (d)	0,17 (a)	1,25 (abc)	16,9 (bcd)
	H$_3$BO$_3$	27,6 (ab)	14,0 (bc)	0,30 (ab)	1,33 (bc)	17,3 (cd)
Concentration	0,5 %	29,2 (a)	15,5 (b)	0,42 (a)	1,45 (a)	17,6 (a)
	1 %	28,5 (a)	14,8 (a)	0,32 (b)	1,39 (a)	16,8 (b)
	2 %	28,5 (a)	14,6 (a)	0,18 (c)	1,00 (b)	16,5 (b)
Time	60 min	30,5 (a)	16,0 (a)	0,29 (a)	1,44 (a)	17,5 (a)
	90 min	26,9 (a)	13,9 (b)	0,32 (a)	1,12 (b)	16,5 (a)

Means within each column and factor followed by the same letter are not significantly different by Duncan test (p < 0,05) for chemicals and concentrations.

Means within each column and factor followed by the same letter are not significantly different by T test (p < 0,05) for time.

Table 3. The interactions of chemicals, time and concentrations on glucan, xylan, galactan, mannan+arabinan and lignin (ANOVA).

Source		Type III Sum of Squares	df	Mean Square	F	Sig.
Glucan	Chemical	402,2	7	57,5	25,2	*
	Concentration	11,0	2	5,52	2,42	**
	Time	309,6	1	309,6	135,6	*
	Chemical * Concentration	136,3	14	9,74	4,27	*
	Chemical * Time	583,6	7	83,4	36,5	*
	Concentration * Time	1,45	2	0,73	0,32	NS
	Chemical * Concentration * Time	9,61	14	0,69	0,30	NS
Xylan	Chemical	296,4	7	42,3	29,7	*
	Concentration	14,8	2	7,40	5,20	**
	Time	107,2	1	107,2	75,3	*
	Chemical * Concentration	54,6	14	3,90	2,74	**
	Chemical * Time	105,6	7	15,1	10,6	*
	Concentration * Time	1,38	2	0,69	0,48	NS
	Chemical * Concentration * Time	3,99	14	0,29	0,20	NS
Galactan	Chemical	0,72	7	0,10	3,69	**
	Concentration	0,89	2	0,44	16,0	*
	Time	0,34	1	0,03	1,22	NS
	Chemical * Concentration	0,49	14	0,04	1,26	NS
	Chemical * Time	0,80	7	0,12	4,14	**
	Concentration * Time	0,01	2	0,01	0,04	NS
	Chemical * Concentration * Time	0,49	14	0,04	1,27	NS
Mannan + Arabinan	Chemical	2,79	7	0,40	3,32	**
	Concentration	3,74	2	1,87	15,6	*
	Time	2,38	1	2,38	19,8	*
	Chemical * Concentration	5,10	14	0,37	3,04	**
	Chemical * Time	3,24	7	0,46	3,87	**
	Concentration * Time	0,06	2	0,03	0,26	NS
	Chemical * Concentration * Time	1,71	14	0,12	1,02	NS
Lignin	Chemical	84,0	7	12,0	7,63	*
	Concentration	21,8	2	10,9	6,94	**
	Time	26,9	1	26,9	17,1	*
	Chemical * Concentration	45,4	14	3,24	2,06	***
	Chemical * Time	7,99	7	1,14	0,73	NS
	Concentration * Time	0,74	2	0,37	0,24	NS
	Chemical * Concentration * Time	5,10	14	0,37	0,23	NS

P= Significance level.

NS = Non significant for ANOVA.

* Significant at 0,001 for ANOVA.

** Significant at 0,01 for ANOVA.

*** Significant at 0,05 for ANOVA.

Effects of chemical pretreatment on glucan content

Using NaOH as the pretreatment agent degrades the H-bonds in cellulose and hemicellulose, and in addition breaks ester linkages between lignin and xylan (Sun *et al.* 2005). This consequently results in swelling that causes hemicelluloses and lignin dissolution (Chen and Sharma-Shivappa 2007). The NaOH pretreatment showed that higher application times and alkali concentrations dissolved more material from the structure and resulted in a lower yield (Table 1). The glucan content of the NaOH pretreated stalks ranged from 17,7 % (0,5 % conc., 90 min) to 40,6 % (2 % conc., 60 min). On the other

hand, dissolved glucan was found to be between 11,3 % (0,5 % conc., 60 min) and 55,4 % (2 % conc., 90 min) (Figure 1a). Results showed that, compared to alkali concentration, residency time was more effective in glucan solubilization. Silverstein *et al.* (2007) reported nearly 21 % glucan solubilization for cotton stalks when treated with 2 % NaOH at 121 °C for 60 min. On the other hand, Monlau *et al.* (2013) observed a much lower (< 8 %) glucan solubilization when sunflower stalks were pretreated with 4 % NaOH (55 °C for 24 h or 170 °C for 1 h). The unpredicted glucan dissolution in this study could be due to the pretreatment conditions, raw material characteristics (region, crop maturity, harvest times, plant parts), etc.

The pulping additive $NaBH_4$ is conventionally used to improve the pulping selectivity of the kraft method. The chemical acts as a catalyst and results in selective delignification (Copur and Tozluoglu 2007). It improves the pulping yield by preventing carbohydrate degradation (Copur *et al.* 2012). On the other hand, there is limited knowledge available on of the use of $NaBH_4$ as a pretreatment chemical in bioethanol production. The effects of $NaBH_4$ used as a pretreatment chemical for wheat straw, corn stalks and hazelnut husks have been studied (Copur *et al.* 2012, Copur *et al.* 2013a, Copur *et al.* 2013b) and it has been shown to be as effective as NaOH for chemical pretreatment. The glucan solubilization during $NaBH_4$ pretreatment is presented in Figure 1b and the solids recovered after pretreatment are given in Table 1. Results showed that higher residency times and treatment concentrations diminished the yield. The glucan content of $NaBH_4$ pretreated samples ranged from 32,3 % (2 % $NaBH_4$, 90 min) to 35,3 % (0,5 % $NaBH_4$, 60 min) (o.d. stalk) and the glucan solubilization was found to be as high as 13,5 % (2 % $NaBH_4$, 90 min) (Figure 1b). Copur *et al.* (2012 and 2013b) reported 7,27 % and 31,6 % glucan solubilization, respectively, when wheat straw and hazelnut husks were pretreated with 2 % and 4 % $NaBH_4$ at 121 °C for 30 min.
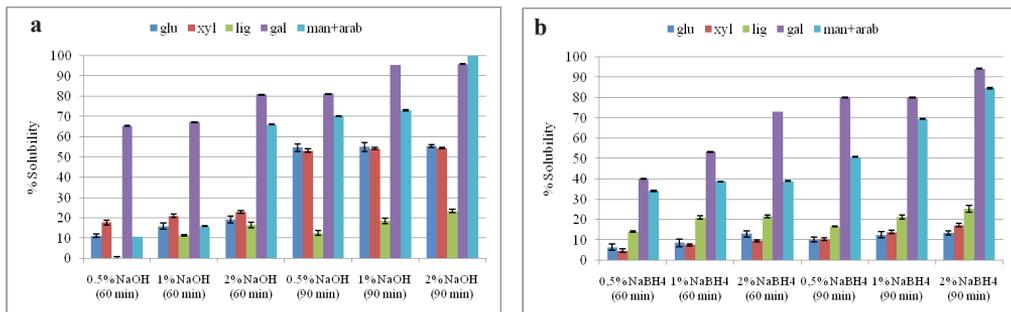

Figure 1. Solubilization of glucan, xylan, galactan, mannan+arabinan and lignin in (a) NaOH and (b) $NaBH_4$ pretreated samples as a function of residence time and concentration.

The glucan content of the $Na_2B_4O_7(1-5)H_2O$, $CaB_3O_4(OH)_3H_2O$, B_2O_3, $Na_2B_4O_7 10H_2O$, $Na_2B_8O_{13} 4H_2O$ and H_3BO_3 pretreated samples ranged from 24,7 % (1 % conc., 90 min) to 30,7 % (0,5 % conc., 60 min); 26,3 % (2 % conc., 90 min) to 29,7 % (0,5 % conc., 60 min), 27 % (2 % conc., 90 min) to 30,6 % (0,5 % conc., 60 min); 25,5 % (2 % conc., 90 min) to 29 % (0,5 % conc., 60 min), 27 % (2 % conc., 90 min) to 29,9 % (0,5 % conc., 60 min) and 26 % (2 % conc., 90 min) to 29 % (0,5 % conc., 60 min), respectively. The highest glucan solubilization was found to be 33,6 % when the stalks were pretreated with $Na_2B_4O_7(1-5)H_2O$ (2 % conc., 90 min), while the lowest was 25,9 % with $CaB_3O_4(OH)_3H_2O$ (1 % conc., 90 min) (Figure 2a-f).
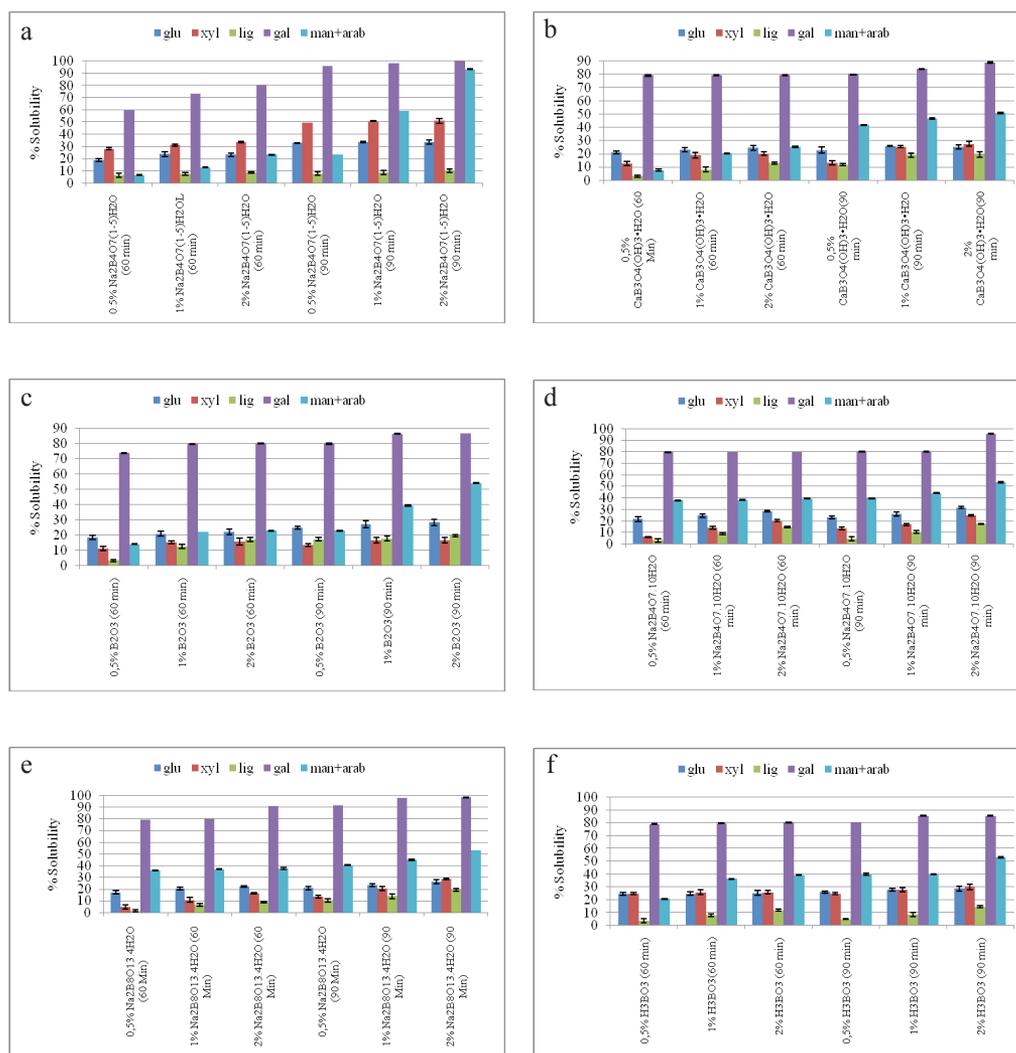
Figure 2. Solubilization of pretreated samples as a function of residence time and concentration: glucan, xylan, galactan, mannan+arabinan and lignin in (a) $Na_2B_4O_7(1\text{-}5)H_2O$, (b) $CaB_3O_4(OH)_3H_2O$, (c) B_2O_3, (d) $Na_2B_4O_710H_2O$, (e) $Na_2B_8O_{13}4H_2O$ and (f) H_3BO_3.

Effects of chemical pretreatment on xylan content

The xylan content of NaOH pretreated solids ranged from 9,30 % (0,5 % conc., 90 min) to 19,7 % (2 % conc., 60 min). Results (Figure 1a) indicated that the pretreatments removed xylan from the structure, and harsher pretreatment conditions dissolved more xylan. Xylan, which is an amorphous, low molecular weight, heterogeneous and branched polysaccharide, is more susceptible to alkaline attacks. The NaOH pretreatment dissolved from 17,8 % (0,5 % conc., 60 min) to 54,6 % (2 % conc., 90 min) of the xylan. These results were comparable to the findings of Antonopoulou *et al.* (2015), who achieved xylan removal of 30,2; 34,8 and 44,9 % with sunflower stalks pretreated, respectively, with 2, 10 and 20 % NaOH at 80 °C for 24 h. In addition, Monlau *et al.* (2013) reported 26 and 58 % xylan removal when sunflower stalks were pretreated with 4 % NaOH at 55 and 170 °C, respectively, for 24 h, while Wang *et al.* (2010) obtained 60,5 % xylan solubilization for bermuda grass (3 % NaOH, 121 °C, 90 min). On the other hand, slightly lower xylan solubility was reported by Chen and Sharma-Shivappa (2007) for barley straw (40 %), triticale straw (35 %) and wheat straw (35 %) when the

materials were pretreated with 2 % NaOH at 121 °C for 60 min.

When the $NaBH_4$ pretreatment is take into account, the xylan content of the stalks ranged from 15,7 % (2 % conc., 90 min) to 18,3 % (0,5 % conc., 60 min), whereas xylan removal ranged from 4,88 % (0,5 % conc., 60 min) to 17,4 % (2 % conc., 90 min) (Figure 1b). Copur *et al.* (2012 and 2013b) reported almost 58,7 % and 66,2 % xylan solubilization for wheat straw and hazelnut husks when the materials were pretreated with 4 % $NaBH_4$ at 121 °C for 90 min and 30 min, respectively. The lower xylan solubility in the present study could be due to differing raw material characteristics.

On the other hand, the samples pretreated with $Na_2B_4O_7(1-5)H_2O$, $CaB_3O_4(OH)_3H_2O$, B_2O_3, $Na_2B_4O_710H_2O$, $Na_2B_8O_{13}4H_2O$ and H_3BO_3 resulted in 9,33 % (1 % conc., 90 min) to 13,8 % (0,5 % conc., 60 min), 13 % (2 % conc., 90 min) to 16,7 % (0,5 % conc., 60 min), 16 % (2 % conc., 90 min) to 17 % (0,5 % conc., 60 min); 14,3 % (2 % conc., 90 min) to 17,7 % (0,5 % conc., 60 min); 13,3 % (2 % conc., 90 min) to 17,5 % (0,5 % conc., 60 min) and 13 % (2 % conc., 90 min) to 14,7 % (0,5 % conc., 60 min) of xylan in the structure, respectively. These results indicated that H_3BO_3 removed a significant amount (30,3 %) of xylan from the structure when the stalks were pretreated with a 2 % chemical concentration for 90 min (Figure 2a-f).

Effects of chemical pretreatment on lignin content

Lignin, a three-dimensional, complex aromatic polymer, surrounds the carbohydrates (cellulose and hemicellulose) in woody biomass (Fan *et al.* 1987). However, lignin limits the accessibility of hydrolytic enzymes to the carbohydrates and some of its decrease or removal is crucial in order to improve the digestibility. The NaOH pretreatment removed the lignin from the stalk structure. The amount of lignin in the solids after the NaOH pretreatment ranged from 16,5 % (1 % conc., 90 min) to 21,5 % (2 % conc., 60 min). An increased alkali concentration removed greater amounts of lignin, with the highest lignin removal in this study being 23,5 % (2 % conc., 90 min) (Figure 1a).

Comparable lignin removal (20 %) was observed by Antonopoulou *et al.* (2015) while pretreating sunflower stalks with 2 % NaOH at 80 °C for 24 h. In addition, Monlau *et al.* (2013), by pretreating sunflower stalks with 4 % NaOH at 55 and 170 °C for 24h. observed nearly the same lignin removal (22 and 36 %, respectively). Gaspar *et al.* (2005) and Wang *et al.* (2010) reported 95 % lignin removal for corn fiber (2,5 % conc.,121 °C, 60 min) and 86 % removal for bermuda grass (3 % conc., 121 °C, 90 min), respectively. The higher lignin removal (95 %) for corn stover reported by Varga *et al.* (2002) might be attributed to the higher NaOH concentration (10 %). In the present study, the NaOH concentration was limited to 2 %. The results indicated that lignin removal depends on pretreatment conditions and the characteristics of the raw material.

The lignin content of the $NaBH_4$ treated samples ranged from 14,1 % (2 % conc., 90 min) to 16,6 % (0,5 % conc., 60 min) and lignin removal ranged from 14,2 % (0,5 % conc., 60 min) to 25,4 % (2 % conc., 90 min) (Figure 1b). Copur *et al.* (2012 and 2013b) reported almost 58,4 and 49,1 % delignification, respectively, for wheat straw and hazelnut husks (4 % $NaBH_4$, 121 °C, 90 min). Similar lignin removal (23,5 %) was observed in this study when the sunflower stalks were pretreated with 2 % NaOH for 90 min. More lignin, as compared to xylan, was removed from the sunflower stalks treated with $NaBH_4$. It is known that the removal of lignin is considerably important to improve enzymatic digestibility.

The lignin content of the samples treated with $Na_2B_4O_7(1-5)H_2O$, $CaB_3O_4(OH)_3H_2O$, B_2O_3, $Na_2B_4O_710H_2O$, $Na_2B_8O_{13}4H_2O$ and H_3BO_3 ranged from 17,4 % (1 % conc., 90 min) to 18,1 % (0,5 % conc., 60 min); 14,3 % (2 % conc., 90 min) to 18,4 % (0,5 % conc., 60 min); 15,5 % (2 % conc., 90 min) to 18,6 % (0,5 % conc., 60 min); 15,6 % (2 % conc., 90 min) to 18,2 % (0,5 % conc., 60 min), 15 % (2 % conc., 90 min) to 18 % (0,5 % conc., 60 min) and 15,8 % (2 % conc., 90 min) to 18,7 % (0,5 % conc., 60 min), respectively. The minimum (10,1 %) and maximum (19,8 %) lignin removal was found when stalks were treated with $Na_2B_4O_7(1-5)H_2O$ and B_2O_3, respectively (2 % conc., 90 min) (Figure 2a-f).

Optimum pretreatment application

Results showed that, compared to NaOH, all boron derivatives prevented peeling reactions and preserved more glucan in the structure. Among the chemicals used in pretreatments, NaOH removed the highest xylan and lignin from the structure. On the other hand, significant xylan removal was observed when the sunflower stalks were pretreated with H_3BO_3. It was found that B_2O_3 was as effective as $NaBH_4$ and NaOH for selective lignin removal. Moreover, it is known (Copur *et al.* 2013a, 2013b) that effective enzymatic hydrolysis is possible when conjoint removal of xylan and lignin is in progress.

The optimum pretreatment conditions were determined based on the ratio of the glucan and lignin removal during pretreatment applications. The optimum ratios were 0,80; 2,44; 0,37; 0,78; 0,78; 0,51; 0,73 and 0,51 when the samples were pretreated with 2 % NaOH (60 min), 1 % $NaBH_4$ (60 min), 2 % $Na_2B_4O_7(1\text{-}5)H_2O$ (60 min), 2 % $CaB_3O_4(OH)_3H_2O$ (90 min), 2 % B_2O_3 (60 min), 2 % $Na_2B_4O_710H_2O$ (60 min), 2 % $Na_2B_8O_{13}4H_2O$ (90 min) and 2 % H_3BO_3 (90 min), respectively. Results indicated that extremely selective delignification was possible when stalks were pretreated with $NaBH_4$.

Enzymatic hydrolysis

In this study, the samples pretreated under optimum conditions were enzymatically hydrolyzed. The reducing sugar contents were 58,8±0,01 %; 73,3±0,01 %; 70,8±0,14 %; 66,9±0,01 %; 67,8±0,05 %; 68,6±0,01 %; 67,3±0,07 %; 62,4±0,01 % and 72,2±0,01 % for the untreated and pretreated samples (2 % NaOH, 60 min; 1 % $NaBH_4$, 60 min; 2 % $Na_2B_4O_7(1\text{-}5)H_2O$; 60 min; 2 % $CaB_3O_4(OH)_3H_2O$, 90 min; 2 % B_2O_3, 60 min; 2 % $Na_2B_4O_710H_2O$, 60 min; 2 % $Na_2B_8O_{13}4H_2O$, 90 min; and 2 % H_3BO_3, 90 min), respectively. The reducing sugars observed for samples of 2 % B_2O_3 (60 min), 2 % H_3BO_3 (90 min), 1 % $NaBH_4$(60 min) and 2 % NaOH (60 min) were nearly similar. This finding indicated that the abovementioned boron derivatives as well as NaOH could improve the enzymatic digestibility of the pretreated samples.

The effects of enzymatic hydrolysis and incubation periods on the hydrolysis of sunflower stalks were examined and the results are presented in Figure 3. The saccharification rate up to 24 h was higher, and then, continuing to 72 h, the rate slowed down. This behavior might be due to the diminishing enzyme adsorption during saccharification, the changing of the cellulose structure to a less digestible form and the inhibiting enzymes resulting from the products of hydrolysis (Lee and Fan 1982). The slow rate of saccharification after 72 h has also been reported earlier (Sreenath *et al.* 1999).

The untreated samples gave a hydrolysis yield of 26,6 % when enzymatic application progressed for 72h. This value was significantly higher compared to that of 18 % observed by Ruiz *et al.* (2008). The only difference between the studies was in the substrate concentration, which was 10 % (w/v) compared to the 2 % (w/v) employed in this study. It is known that a decrease in the substrate concentration accelerates the rate of hydrolysis. Substrate concentrations of over 0,05-0,075 g/ml may cause substrate inhibition (Soto *et al.* 1994). In addition, enzymatic hydrolysis may be decreased as a result of stirring problems, aqueous movable phase reduction and end product inhibition (Sharma *et al.* 2002).

In this study, the 2% NaOH (60 min) pretreatment resulted in the highest enzymatic saccharification yield (59,5 %) among the samples. The boron derivatives gave slightly lower values (1 % $NaBH_4$, 45 %; 2 % B_2O_3, 40,2 %; and 2 % H_3BO_3, 36,7 %). Sharma *et al.* (2002) observed an enzymatic saccharification yield of 57,8 % for steam exploded and chemically pretreated sunflower stalks. On the other hand, Ruiz *et al.* (2008 and 2013) obtained enzymatic hydrolysis yields of 67,8 and 65 % of with steam exploded and 1,3 % sulfuric acid pretreated sunflower stalks, respectively. These variations in the enzymatic hydrolysis yield could be explained by the diverse process conditions applied in the studies.

It could be mentioned that enzymatic hydrolysis yield is a function of the lignin and xylan contents of the stalk. In this study, the $Na_2B_4O_7(1\text{-}5)H_2O$ pretreated sample had the highest lignin content (17,7 %); however, the lowest lignin content was observed when the sample was pretreated with $NaBH_4$ (15,3%). Consequently, the $Na_2B_4O_7(1\text{-}5)H_2O$ pretreated sample had 1,16 times more lignin and an enzymatic yield 1,52 times lower compared to samples treated with $NaBH_4$.

The effects of boron derivatives as..: Tozluoğlu

Maderas. Ciencia y tecnología 18(4): 663 - 676, 2016

In addition, regarding the xylan content of the samples, those pretreated with $Na_2B_4O_7 10H_2O$ and H_3BO_3 had 15,1 and 13 % xylan content, respectively. Therefore, the $Na_2B_4O_7 10H_2O$ pretreated sample had 1,16 times more xylan in the structure compared to the H_3BO_3 pretreated sample, and consequently, $Na_2B_4O_7 10H_2O$ gave an enzymatic yield that was 1,13 times lower. These results indicated that xylan as well as lignin removal had a significant effect on the enzymatic digestibility. Similar findings were reported earlier by Lu *et al.* (2002), and Yang and Wyman (2004), who concluded that lignin in the structure obstructed the hydrolysis and attributed this to the protein adsorption of lignin in aqueous solutions.
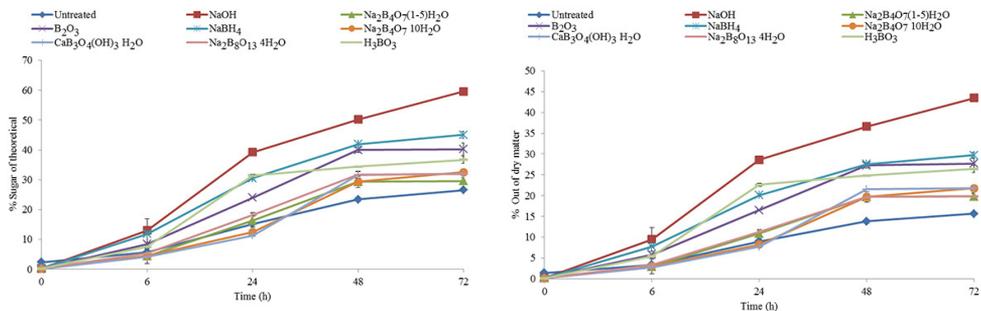

Figure 3. Enzymatic hydrolysis of pretreated sunflower stalks, expressed as % of total carbohydrates.

CONCLUSIONS

Sunflower stalks were treated with NaOH, $NaBH_4$ and the boron derivatives $Na_2B_4O_7(1-5)H_2O$, $CaB_3O_4(OH)_3H_2O$, B_2O_3, $Na_2B_4O_7 10H_2O$, $Na_2B_8O_{13}4H_2O$ and H_3BO_3. Results showed that the boron derivatives prevented peeling reactions and preserved more glucan; however, the NaOH removed the highest amounts of xylan and lignin from the structure. The significant xylan removal observed with H_3BO_3 and B_2O_3 was as effective as $NaBH_4$ and NaOH for selective lignin removal, although the highest enzymatic saccharification yield was 59,5 % for the 2 % NaOH pretreated sample. In addition, some boron derivatives gave slightly lower yields (1 % $NaBH_4$-45 %; 2 % B_2O_3-40,2 %; and 2 % H_3BO_3-36,7 %) compared to NaOH. Although the price is disadvantage compared to NaOH, the potential results of using of boron derivatives might be worth the cost.

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Maderas. Ciencia y tecnología 18(4): 663 - 676, 2016

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