ENZYMATIC DIGESTIBILITY OF TOMATO, PEPPER, AND EGGPLANT STALKS MIXTURE

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Turkey annually produces 26 million tons of vegetables and is the third-biggest vegetable producer. After harvest, the waste of vegetable stalks lacking of economic value is burnt or left in the fields, causing environmental pollution. The aim of this study was to examine bioethanol production of a mixture of tomato, pepper, and eggplant stalks using an alternative chemical, sodium borohydrate ($NaBH_4$) in a chemical pretreatment step. Both steam-exploded (SE) and dry-milled (DM) stalks were chemically pretreated and enzymatically hydrolyzed in this study. Results showed that SE stalks had better enzymatic digestibility compared to DM. NaOH treatment removed the highest amount of lignin (17.1%; SE, 2%, 90 min) but also glucose (21.5%; SE, 2%, 90 min) from the structure. On the other hand, $NaBH_4$ removed the highest lignin in proportion to glucose for both SE and DM samples. Enzymatically hydrolyzed stalks gave the highest sugar yields of 30.1% (o.d.-dry matter) for the SE sample when it was pretreated with 2% NaOH for 30 min.

Keywords: Environment; Dry-milling; Steam explosion; Pretreatment; Enzymatic hydrolysis

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INTRODUCTION

The environmental impacts associated with using fossil fuels (*e.g.* climate change) and reliance on fuel imports to supply a substantial percentage of energy is of particular concern, leading countries to consider alternative energy resources. The huge volume of annually generated biomass of 140 billion metric tons (Alfro *et al.* 2009) is of interest because this material is renewable, widespread, and cheap. Fifty billion tons of bioethanol may be produced by converting this volume of material into energy.

Lignocellulosic biomass consists of cellulose, hemicellulose, lignin, and a minor amount of extractives and minerals. Cellulose and hemicelluloses together constitute two-thirds of the biomass and comprise carbohydrates that could be processed to produce bioethanol. On the other hand, the three-dimensional, irregular, and cross-linked lignin component gives plants structural support, impermeability, and resistance to microbial attack (Hendriks and Zeeman 2009). The impermeability of lignin limits bioethanol production by diminishing the accessibility of process chemicals and enzymes to the structure and results in a lower hydrolysis rate (Chang and Holtzapple 2000).

Several conversion techniques have been examined to liberate carbohydrate polymers from lignin complexes, to obtain sugar monomers from carbohydrate polymers, and to ferment these monomers into bioethanol. Conversion techniques of physical, chemical, physico-chemical, and biological nature have been applied to enhance the

digestibility of biomass (Yoon *et al.* 1995; Silverstein *et al.* 2007). These techniques are expected to disturb the barriers in the structure and improve the enzymatic hydrolysis of cellulose and hemicelluloses. They also improve the production efficiency by reducing the formation of by-products that are inhibitory to the subsequent enzymatic hydrolysis and fermentation processes. This reduction minimizes the energy and chemical demand and as a result lowers the operating cost (Lynd *et al.* 2008; Mosier *et al.* 2005; Yang and Wyman 2007). Stream explosion and mechanical grinding are physical pretreatment techniques, and in steam explosion, the material is exposed to saturated steam at high temperature and pressure for a period of time and then a rapid reduction to atmospheric pressure, resulting in the breakdown of the matrix that enhances the accessibility of the enzymes/chemicals, and improves the material digestibility (Zimbardi *et al.* 2007). Mechanical grinding reduces the particle size and degree of polymerization while improving the specific surface area available for following treatment (Hendriks and Zeeman 2009). Earlier studies (Fan *et al.* 1982; Chang *et al.* 1981) indicated that milling and grinding processes are energy-intensive and thus expensive among physical pretreatments, but the process yield is almost equal to theoretical (Koullas *et al.* 1992; Millet *et al.* 1976).

Turkey produces almost 26 million tons of vegetables and is the third biggest vegetable producer. Tomato production is relatively common and accounts for 25% of total vegetable production in Turkey. Pepper and eggplant production is also common, and the three vegetables are found together in greenhouses and fields. Vegetable stalks lacking of economic alternatives do not yet have industrial utilization, and they mostly are left in the field or burnt, resulting in environmental pollution and greenhouse gas emission. Bioethanol production from several lignocellulosic materials (Silverstein *et al.* 2007; Zimbardi *et al.* 2007; Koullas *et al.* 1992; Hoije *et al.* 2005; Copur *et al.* 2012) has been examined, but vegetable stalks are unstudied raw material consisting of cellulose and hemicelluloses in the structure. The objective of this work was to evaluate the feasibility of bioethanol production from vegetable stalks (mixture of tomato, eggplant, pepper stalks). This study focused on the use of several pretreatment methods to enhance enzymatic saccharification and the efficiency of bioethanol production. In this study, integrated steam explosion/mechanical grinding and chemical pretreatments were followed with an environmental friendly enzymatic hydrolysis step.

MATERIALS AND METHODS

Raw Material

Vegetable stalks (mixture of tomato, pepper, and eggplant stalks) were obtained from the field right after harvest in Antalya province in Turkey. The leaves were stripped from the stalks, and the stalks were chopped to chip size (3 cm) using dividing shear. Then the material was air dried at room temperature.

After determining the moisture content (TAPPI T 412 om-06), each having equal oven dry weight, tomato, pepper, and eggplant stalks were mixed and then they were stored in plastic bags at -5 °C.

Pretreatments

To examine the effect of steam explosion and dry-milling, vegetable stalks were both steam-exploded and dry-milled in this study. Steam-explosion pretreatment was carried out in a batch unit consisting of 20 L reaction vessel (manufactured by Derya Krom Industry/Turkey). A total of 1000 g (o.d.) of stalks was put into the reaction vessel, and the saturated steam was exposed at 198 to 200 °C and 15 bar for 5 min (15 psi). Then the ball valve at the bottom of the vessel was suddenly opened, and the materials were allowed to blow out into the underlying vessel at atmospheric pressure. After the process, materials were separated into solid and liquid fractions by filtration (200 mesh wire screen). Mechanical grinding was made using a laboratory mill (Altundal milling). The moisture content of the milled material was determined, and it was put into a plastic bag for further processes.

Steam-exploded and dry-milled materials (40 g o.d.) were chemically pretreated with NaOH, H_2SO_4, and $NaBH_4$. Treatments were made in an autoclave (Nuve OT 4060V) at a 2% (w/v) concentration and 10% (w/v) solid loading. Duplicate samples were processed at 121 °C (15 psi) for residence times of 30 and 90 min. The solid residues were filtrated, and the solid part was stored in sealed plastic bags at 4 °C for the enzymatic hydrolysis.

Enzymatic Hydrolysis

Chemically pretreated and untreated (for control) samples (1 g. o.d.) were enzymatically hydrolyzed using a mixture (50% v/v) of Celluclast 1.5 L (700 U/g) and Novozym 188 (250 U/g) at 2% solid loading in 50 mL of 50 mM sodium acetate buffer at pH 5.0. The enzyme reaction was accomplished in a rotary shaker (Biosan Environmental Shaker-Incubator ES-20) at 42 ºC for 100 rpm. 1.5 mL of samples was taken at the start and after 6, 24, 48, and 72 hours. Taken samples were first put into boiling water for 10 min to stop the enzymatic activity and then centrifuged at 10,000 rpm for 5 min using a Nuve NF 800 Centrifuge. The samples were stored at 4 °C until HPLC analysis.

Analytical Methods

The chemical compositions of the samples were determined by appropriate methods of hot and cold water solubility (TAPPI T 207 om-88), 1% NaOH solubility (TAPPI T 212 om-88), extractives content (TAPPI T 204 om 88), ash (TAPPI T 211 om-85), and holocellulose (Wise and Karl 1962). The yields were determined by gravimetric measurements.

The sugar and the lignin contents were determined by Laboratory Analytical Procedures (LAP) from the National Renewable Energy Laboratory (NREL) (Sluiter *et al.* 2008). The sugars contents were studied using HPLC (Agilent 1200 system) equipped with Shodex 1011 column (mobile phase: 5 mM H_2SO_4, flow rate: 0.5 mL min^{-1}, column temperature: 60 °C) and refractive index detector. The acid-insoluble and soluble lignin were evaluated by weighing and the adsorption at 320 nm against deionized water blank, respectively.

The reduction in lignin for all pretreatments was calculated based on the initial dry-weight of lignin in the untreated sample (LU) and the dry-weight of lignin in the

remaining solids after pretreatment (LP). In addition, the percentage of weight loss was calculated on an oven-dry basis as follows,

$$\text{Weight loss (\%)} = [100 - (W_2 \div W_1) \times 100] \tag{1}$$

where W_1 is the dry weight of whole biomass before pretreatment (g), and W_2 is the dry weight of the pretreated sample (g).

The percentage of lignin reduction was calculated with the following equation,

$$\text{Lignin reduction (\%)} = [(LU - LP) \div LU)] \times 100 \tag{2}$$

where LP is dry-weight of lignin in the pretreated sample, and LU is the dry-weight of lignin in untreated biomass. The solubilization of xylan and glucan during pretreatment was also calculated in the same manner by substituting the appropriate percentages for xylan and glucan.

The reducing sugar (at 540 nm) was determined by the DNS method (Sluiter *et al.* 2008). The obtained data were statistically analyzed by the SPSS 16.0 packet program. ANOVA was used to identify significant differences, and the differences within groups were identified by the Tukey test.

RESULTS AND DISCUSSION

Characterization of Raw Material

Table 1 summarizes the chemical compositions of tomato, pepper, and eggplant stalks, mixtures of raw and steam-exploded stalks, and literature values for wheat straw and hard/softwood. Results showed that pepper stalks (2.49%) had lower extractives content compared to tomato and eggplants (10.4 to 10.5%), and the amount was almost similar to hard/softwood (2 to 8%). Tomato stalks had the highest solubility (hot, cold and 1% NaOH), and the solubility diminished from eggplant to pepper stalks. Results showed that vegetable stalks dissolved more compared to hard/softwood. The holocellulose contents of the vegetable stalks (61.7 to 78.7%) were in the range of hard/softwood (63 to 78%). Tomato stalks (17.9%) had the lowest lignin content compared to wheat straw and wood species, and the lignin content of pepper and eggplant stalks was higher than wheat straw but within the range of hard/softwood. The glucose fractions based on the HPLC results were 45.1%, 29.9%, and 30.1% (o.d. straw) for pepper, tomato, and eggplant, respectively. Results indicated that pepper stalks (45.1%) had the highest glucose content, which was greater than that of wheat straw (29.6%). The major hemicellulose constituent, xylose, was the highest in pepper stalks (26.1%). Compared to wheat straw (17.5%), tomato and eggplant stalks (20.7 to 20.8%) had slightly higher xylose content. Arabinose was only a small portion of the stalks (1.10 to 2.90 %), and mannose and galactose were not determined in this study. The difference between holocellulose content and the total sugars determined in HPLC could be explained by the sugar degradation due to intense sulfuric acid hydrolysis in the HPLC process (Badger 2002). It should be pointed out that tomato stalks had greater ash content

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than wheat straw. In addition, pepper and tomato stalks had more ash compared to hard/softwood. Consequently, the high sugar content (approximately 60 to 70%) of vegetable stalk indicated that they could be appropriate lignocellulosic substrates for ethanol production.

The Effect of Steam Explosion

The mixed raw stalks were steam exploded to enhance the enzymatic digestibility. The chemical compositions of the mixed untreated and steam-exploded stalks are also presented in Table 1. Solid material recovered after steam explosion was 85.2% (w/w), and the dissolved material (14.8%) in steam explosion was in the liquid part. After steam explosion, 92.3% of glucan was recovered in solid material. The material loss could mainly be explained by the removal of hemicelluloses, and a significant decrease (12.6% relative difference) in xylose was observed (Table 1). On the other hand, glucose (8.98% relative difference) and lignin (31.5% relative difference) portions were relatively increased in steam explosion, and this finding could be due to the removal of hemicelluloses from the structure. Steam explosion conditions conducted in this study degraded and released low amounts of glucose but solubilized and removed high amounts of hemicellulose. Intensive steam explosion conditions may remove more xylan and lignin from the structure, but this resulted in lower glucan recovery (Martin *et al.* 2008).

Table 1. Chemical Composition of Vegetable Stalks (Each, Mixture and pretreated), Wheat Straw and Hard/Softwood

Chemical composition (%, o.d)	Tomato stalks	Pepper stalks	Eggplant stalks	Wheat straw**	Hard/ Softwood ***	Mixture untreated stalks	Mixture pretreated (SE) stalks
Extractives	10.4±0.70*	2.49±0.40	10.5±0.50	19.1	2-8	10.0±0.00	15.4±1.40
Hot water solubility	27.0±0.22	9.22±0.24	17.9±0.18	-	2-7	-	-
Cold water solubility	29.1±0.50	8.87±0.02	22.1±0.08	-	2-6	-	-
1 % NaOH solubility	52.3±0.53	29.4±0.20	43.8±0.23	-	9-20	-	-
Holocellulose	61.7±0.75	78.7±0.14	66.4±0.62	50.5	63-78	63.4±0.66	52.4±0.49
Acid insoluble lignin (AIL)	16.0±0.90	27.8±0.20	29.7±0.96	23.8	25-35	23.8±0.71	31.3±0.00
Acid soluble lignin (ASL)	1.98±0.79	0.60±0.02	0.78±0.03	3.80	-	0.82±0.04	1.02±0.26
Glucose	29.9±1.83	45.1±1.52	30.1±1.26	29.6	-	32.3±0.57	35.2±1.25
Xylose	20.8±0.78	26.1±0.59	20.7±0.25	17.5	-	13.4±0.49	11.9±0.50
Arabinose	1.9±0.43	2.90±0.08	1.10±0.07	2.10	-	1.40±0.07	0.36±0.05
Ash	13.4±0.08	5.80±0.02	7.24±0.08	9.00	0.35	10.8±0.16	12.0±0.14

*Values are the standard deviations of duplicates
**Francisco *et al.* 2009
***Fengel and Wegener 1984

The Effect of Chemical Pretreatment

In this study, steam-exploded samples were further pretreated with common (NaOH and H_2SO_4) and alternative ($NaBH_4$) chemicals to render hemicelluloses and

make the material more amendable to enzymatic treatment. In addition, to examine the effect of dry-milling of mixed stalks, untreated dry-milled stalks were also chemically treated, and the removal yield in chemical composition is given in Table 2. Samples were chemically pretreated for treatment times of 30 and 90 min. As expected, treatment time played a significant role in material removal, and higher treatment time removed more material from the structure for all samples. When steam-exploded and dry-milled samples were compared, results indicated that steam explosion was more effective and it removed more lignin, but also more glucan from the structure, resulting in a more porous structure for enzymatic process ($p<0.05$).

**Table 2.** Component Removal in Steam-Exploded and Dry-Milled Stalks for Each Chemical Pretreatment

Pretreatment	Time, min	Removal yield, % (o.d. stalks)						
		Weight loss	Lignin	Cellobiose	Glucose	Xylose	Arabinose	Total sugar
SE+NaBH$_4$	30	7.70	6.55	0.07	3.12	1.05	0.00	4.24
	90	22.5	9.67	0.00	8.55	2.86	0.00	11.4
SE+NaOH	30	22.8	7.98	0.78	8.80	2.39	0.00	12.0
	90	50.3	17.1	1.50	21.5	5.18	0.00	28.2
SE+H$_2$SO$_4$	30	24.4	0.47	0.78	10.7	3.17	0.00	14.7
	90	41.0	3.86	1.11	17.6	5.45	0.00	24.2
DM+NaBH$_4$	30	3.54	4.73	0.00	1.15	0.59	0.00	1.70
	90	11.7	3.85	0.40	4.33	2.14	0.01	6.87
DM+NaOH	30	12.5	6.40	0.42	4.54	1.76	0.02	6.72
	90	25.7	6.47	0.68	10.5	3.32	0.01	14.4
DM+H$_2$SO$_4$	30	17.0	0.70	0.65	7.34	2.94	0.03	11.8
	90	23.7	1.71	0.60	10.1	3.65	0.00	14.1

SE- Steam explosion; DM – Dry-milled

The used chemicals (NaBH$_4$, NaOH, and H$_2$SO$_4$) in this study showed dissimilar mechanisms and removed varying amount and type of materials from the structure (Fig. 1). The highest material loss of almost 50% and 25% of the material was observed when the samples were treated with NaOH (90 min) for both steam-exploded and dry-milled samples, respectively (Table 2). Results on the other hand indicated that the lowest material solubility of 7.70% and 3.54% was obtained when samples were treated with NaBH$_4$ (30 min). The highest xylan (5.18%) solubilization and lignin (17.1%) reduction but also glucan (21.5%) solubilization was observed for NaOH for the treatment time of 90 min. 30 min NaOH treated sample had 38.6%, 10.5%, and 31.5% glucan, xylan, and lignin, respectively. Results showed that NaOH treatment is an effective way to remove lignin from the structure. Lignin, a three-dimensional complex polymer covering up cellulose and hemicelluloses, limits the accessibility of hydrolytic enzymes to carbo-hydrates and must be removed from the structure for effective biomass digestibility. NaOH breaks the ester linkages between lignin and xylan and causes deprotonation of lignin phenolic groups. Glucan solubilization could be explained by the distribution of cellulose and hemicellulose bonds due to swelling and partial hemicellulose solubilization (Chen and Sharma-Shivappa 2007). In addition, xylan solubilization could be explained by its amorphous, low molecular weight, heterogeneous, and branched structure.

Removing hemicelluloses from the structure is expected to increase the porosity of the biomass structure and expected to enhance the enzymatic digestibility. In terms of hemicelluloses removal, H_2SO_4 might be an effective pretreatment alternative. Compared to lignin reduction, results showed that H_2SO_4 treated samples dissolved a lot more xylan from the structure for both steam-exploded and dry-milled samples (Fig. 1). Although preservation of the cellulose portion is desirable, higher glucan solubility was observed with H_2SO_4-treated samples (Fig. 1).
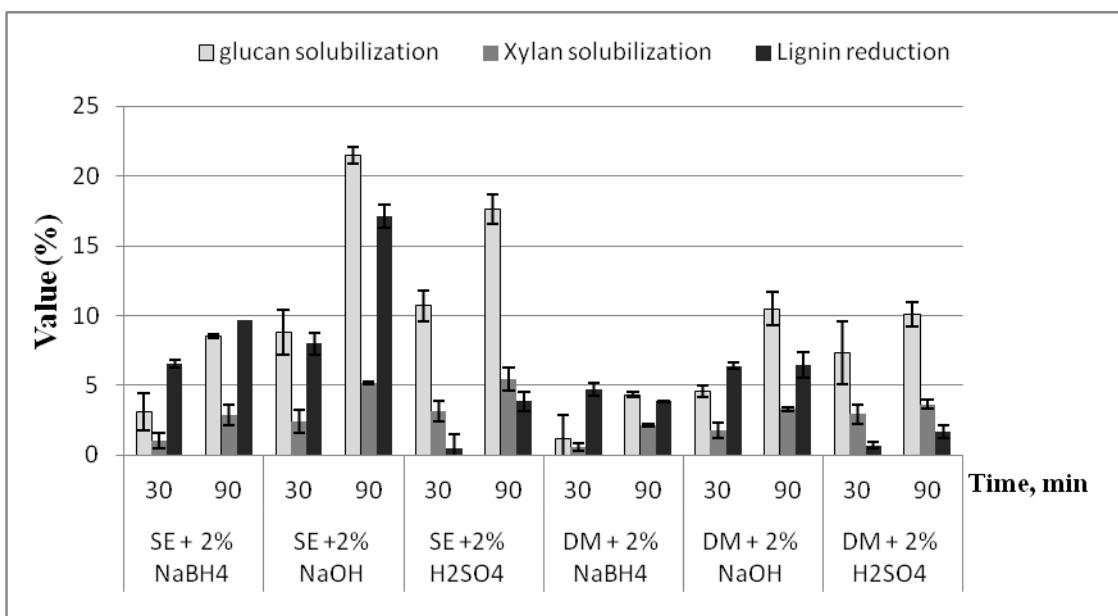
Fig. 1. Glucan and xylan solubilization and lignin reduction of sodium borohydrate (NaBH$_4$), sodium hydroxide (NaOH), and sulfuric acid pretreated samples for steam-exploded (SE) and dry-milled (DM) samples as a function of residency time and chemical concentration (2%)

NaBH$_4$ is a pulping additive utilized to improve pulping selectivity (Copur and Tozluoglu 2007) by preventing peeling reactions and hemicelluloses degradation (Hoje *et al.* 2005). The aim of using NaBH$_4$ in this study was to preserve more glucan and to degrade lignin more selectively (Çöpür *et al.* 2012). Results showed that, as expected, NaBH$_4$ preserved the highest glucan and xylan. The lowest glucan and xylan solubility compared to the other treatment chemicals was observed when the samples were treated with NaBH$_4$ ($p<0.05$) (Fig. 1). In addition, lignin reduction was moderate in terms of glucan to xylan solubility (Fig. 1).

Enzymatic Hydrolysis

It could be stated that removal of lignin is important for better enzymatic digestibility, and samples were selected for further enzymatic hydrolysis based on the glucan to lignin ratio. Among steam-exploded samples, the highest glucan to lignin ratio of 1.45 was observed following NaOH (30 min) treatment. Results showed that H_2SO_4-treated samples had the lowest glucan to lignin ratio.

In this study, the highest sugar yield of steam-exploded and NaOH treated sample (30 min) increased from 23.1% to 30.1% (Fig. 2). The highest sugar yield and increase in

sugar yield observed in this study was lower compared to literature findings. The sugar yield from the 2% NaOH-pretreated Bermuda grass increased from almost 10% to 35% (Wang *et al.* 2010), and the increase was from 20.2% to 46.7% for corn stover (He *et al.* 2010). The lower sugar yield in this study can be explained by the nature of the vegetable stalks mixture. On the other hand, $NaBH_4$-treated samples had moderate yield, but H_2SO_4 treated samples resulted in the lowest for the 30 min $NaBH_4$ treated sample, but among dry-milled samples sugar yields. Large differences in reducing sugars during enzymatic hydrolysis may be due to the amount of lignin and xylan in the samples after pretreatments. The untreated material had approximately two times more lignin compared to the amount of xylan, and results of enzymatic hydrolysation showed that reduction in lignin in the structure may have more impact.
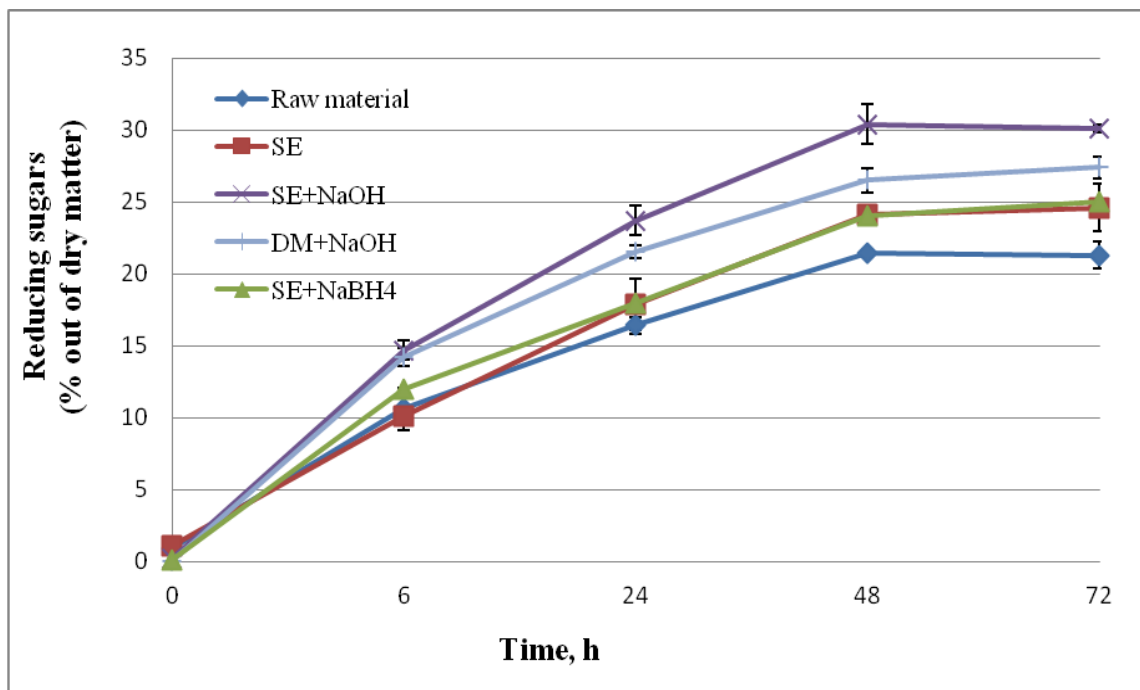

Fig. 2. Reducing sugar contents of pretreated samples after enzymatic hydrolysis

CONCLUSIONS

The high content of polysaccharides (approximately 65%) in vegetable stalks indicates that these materials might be valuable recourses for ethanol production. Steam explosion diminished the holocellulose content and xylan contents indicated that it dissolved mostly hemicelluloses from the structure. In addition, a moderate amount of glucan removal was also observed (4.78%) in steam explosion. Results showed that steam explosion was cheaper than dry-milling and resulted in better lignin removal and also preserved more glucan in the structure. The highest lignin removal was observed when samples were treated with NaOH; along with this delignification, some undesirable removal of glucan was observed as well. On the other hand, $NaBH_4$ preserved more glucan and removed some lignin from the structure. After enzymatic hydrolysis, results

indicated that the highest reducing sugar of 30.1% (% out of dry matter) was obtained when a steam-exploded sample was treated with NaOH.

ACKNOWLEDGEMENTS

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