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Full Length Research Paper

Preliminary study for bioconversion of water hyacinth (*Eichhornia crassipes*) to bioethanol

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The effect of physical (subcritical water) and chemical (acid and alkali) pretreatment on conversion of lignocellulose (cellulose, hemicellulose) in water hyacinth (WH) was investigated. The highest sugar content in acid pretreated samples was observed in WH treated with 3% H_2SO_4 solution (up to 18.16% w/w). Alkali treatment had nearly no effect on conversion of lignocellulose in WH to sugar. Combinations of acid or alkali pretreatments with enzyme treatment resulted in drastic increase of sugar in samples (up to 31.2 and 22.9 % w/w, respectively). In addition, increasing the applied enzyme concentration from 0.8% w/w (on dry WH basis) to 4% further increased the sugar content in the sample (up to 50.5% w/w). Subcritical water (SCW) (200°C and 10 min) and subsequent enzyme treatment resulted up to 17% w/w sugar in samples. Bioethanol concentration during fermentation (at 30°C) of pretreated sample using *Saccharomyses cerevisae* increased with increasing the fermentation time. After 3 days fermentation, up to 60% of sugar in the sample was converted in ethanol.

Key words: Bioethanol, subcritical water, chemical methods, water hyacinth.

INTRODUCTION

As the finite nature of the world's fossil fuel resources becomes more apparent, focus must be shifted to other forms of renewable energy sources. Fuel alcohol is now considered as a suitable alternative or supplement to fossil fuel for transportation. The production and combustion of ethanol do not contribute to the total amount of carbon dioxide in the atmosphere. The emission and toxicity of ethanol is lower than those of petroleum (Yoosin and Sorapipatana, 2007). Fast growing plants are potential sources for producing a useable grade of ethanol or biogas for energy production. Potential plants use as sustainable energy sources include trees, certain grasses, crops such as corn and sugarcane, and aquatics such as the water hyacinth (WH) and algae. Also, the harvest frequency for aquatics tends to be in the order of days, whereas the frequency for trees and crops are in the order of months and years. WH is considered as an attractive raw material for the production of fuel ethanol, because of its availability in large quantities at low cost (Balat et al., 2007). WH grows extremely rapidly and produces almost 2 tons of biomass per acre and population doubles every 5 to 15 days (Craft et al., 2003). WH is low in lignin content (10%) and contains high amounts of cellulose (20%) and hemicellulose (33%) (Bolenz et al., 1990; Poddar et al., 1991; Gressel, 2008).

One of the major difficulties in the production of bioethanol from lignocellulosic material is low solubilization of cellulose and hemicelluloses during the hydrolysis process to produce sugar. In lignocellulosic material, the cellulose, hemicelluloses and lignin are linked and formed a strong structure which is difficult to be processed in raw conditions (Harun and Dayang 2011). Thus, pretreatment is required in order to render those components for further exploitation leading to improvement of the hydrolysis process. Biomass pretreatment methods can be classified into different categories, that is, physical, chemical, biological and combination of these methods (Silverstein et al., 2007).

Acid treatments (using sulfuric, hydrochloric, nitric and peracetic acids) normally aim for high yields of sugar from lignocellulosic biomass (Um et al., 2002). Dilute acid pretreatments were used for converting lignocellulosic

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biomass to soluble sugars, followed by enzyme catalyzed hydrolysis of the cellulosic fraction to glucose (Um et al., 2002).

The characteristic of alkaline pretreatment is that it can remove the lignin without having large effects on other components (McMillan, 1997). NaOH treatment induces swelling of lignocellulosic biomass, leading to an increase in internal surface area, a decrease in the degree of crystallinity, and disruption of the lignin structure (Li et al., 2004). Subcritical water, as a physical pretreatment method can be used to break down organic materials at temperatures ranging from 200 to 374°C with pressurization. It can be used to release glucose from cellulose (Williams, 2006).

The objective of this study was to apply chemical (acid alkali) and physical methods (subcritical water) combined with enzymatic hydrolysis as treatment methods for converting cellulose and hemicellulose to sugar and subsequent bioethanol production using S*accharomyses cerevisae* yeast.

MATERIALS AND METHODS

Fresh WH with long stems was collected from a natural pond at Salaya, Nakornpathom province (Thailand). The WH was thoroughly washed several times with tap water to remove adhering dirt, chopped into small pieces (~1 to 2 cm) and finally dried in a hot air oven at 70°C with air circulation for 6 h. The dried material was stored at room temperature until used.

Acid pretreatment

Ten gram (10 g) of the sample (dried WH) were mixed with 1, 3, 5, 7 and 9% (w/w) of sulfuric acid solution to a final volume of 100 ml. The samples were poured in a pyrex bottle and the cap was closed. After that the mixture was autoclaved at 121°C for 15 min and further cooled down to room temperature. The hydrolysate was filtered using cheese clothes to remove the solid parts (not digested) of the material. The filtrate was collected and subjected to analysis of sugar content.

Alkali pretreatment

Ten gram (10 g) of the sample were mixed with 1, 2, 3, 4 and 5% w/w of sodium hydroxide solution to a final volume of 100 ml. The mixture was heated in a water bath at 85°C for 1 h and further cooled down to room temperature. The hydrolyzed sample was filtered using cheese clothes to remove the solid parts (not digested) of the material. The filtrate was collected and subjected to analysis of sugar content.

Subcritical water (SCW) pretreatment

Two gram (2 g) of the sample were placed in a SCW vessel (high-pressure tube with 1.8 cm OD and 20 cm length). Twenty milliter (20 ml) distilled water were added into the vessel. The vessel was closed and immersed in a pre heated oil bath for 10 min at constant temperatures of 160 and 200°C. The pressure during treatment was observed using an analog manometer. After SCW treatment, the vessel was immediately immersed in a cooled water bath (≈30°C) and further cooled in an ice bath. After that the vessel was opened and the content was poured into a 100 ml beaker. The SCW treated sample was subjected to enzyme treatment (0.8% v/w enzyme on dry WH weight basis) at 55°C and 12 h followed by sugar measurement. The pressure increase during SCW treatment was about 5 and 17 bar at 160 and 200°C, respectively.

Enzymatic hydrolysis of acid and alkali pretreated samples

The acid (3% sulfuric acid concentration) or alkali (1% NaOH) pretreated samples were neutralized using concentrated alkali or acid to pH=4.3 ± 02, respectively. After that 0.8% w/w (on dry WH basis) enzyme mixture was added into the samples. The enzyme mixture used in this study consisted of 5 different enzymes [Crystalzyme 200 XL (Novozyme, Denmark), Celluclast 1.5 L FG (Novozyme, Denmark), Alcalase 2.5 L DX (Novozyme, Denmark), Validase ANC-L (Valley enzyme, USA) and Xylanase (Dr. Luca, Germany)]. These 5 enzymes were used in 1:1:1:1:1 ratio in the enzyme mixture. The pH and temperature optima of these enzymes were between 3.5 to 5.5 and 45 to 65°C respectively. The volume of the samples containing enzyme was adjusted to 100 ml without adding buffer and incubated in a water bath at 55°C for 12 h. After that the enzyme treated samples were filtered through cheesecloth. The filtrate was collected and subjected to analysis of sugar content. The remaining pulp was subjected for the second enzyme treatment as described above. After the second enzyme treatment, the sample was again filtered using cheesecloth. The liquid phase of second and first enzyme treatment was subjected to sugar measurement.

Fermentation and bioethanol production

The acid pretreated (3% acid) and subsequent enzyme (0.8% w/w enzyme on dry WH basis) sample was chosen for fermentation experiments. About 0.2 g dried yeast granular (*S. cerevisiae,* AB enzyme, Germany) was added into a 50 ml pretreated sample at sterile condition. The initial yeast count in fermentation samples were 2 to 8 x 10^8 CFU. Fermentation was carried out at 30°C without agitation and in darkness for a maximum of 6 days. At the beginning and every 3 days, approximately 15 ml of sample was taken for measurement of ethanol content as well as for determining the remaining sugar in the sample.

Analytical methods

Water content measurement was carried out using the gravimetric method. Five gram (5 g) of fine ground sample was dried at 103 ± 5°C for 2 h in a hot air oven and the weight loss was calculated as the percentage of water content. The Luff-Schoorl method was applied for the measurement of total reducing sugar content (glucose, Xylose). The total sugar in the sample per 100 g dried WH was calculated and reported as percentage conversion of cellulose and hemicelluloses to sugar (ICUMSA, 2009).The amount of bioethanol production during fermentation was measured using gas chromatography (GC) method (AOAC official method 984.14). Defined ethanol solution with exact ethanol content was subjected to GC and the standard curve was plotted. A GC equipment (Hewlet Packard, Model 4890) with FID detector and column HP20 M was applied. The detector temperature, oven temperature and injection temperature were 200, 80 and 250°C, respectively. As carrier gas, N_2 gas was used. Standard ethanol solution of 0.5 to 2.5% v/w was applied for plotting the standard curve (AOAC official method 984.14). To avoid complications during ethanol measure-

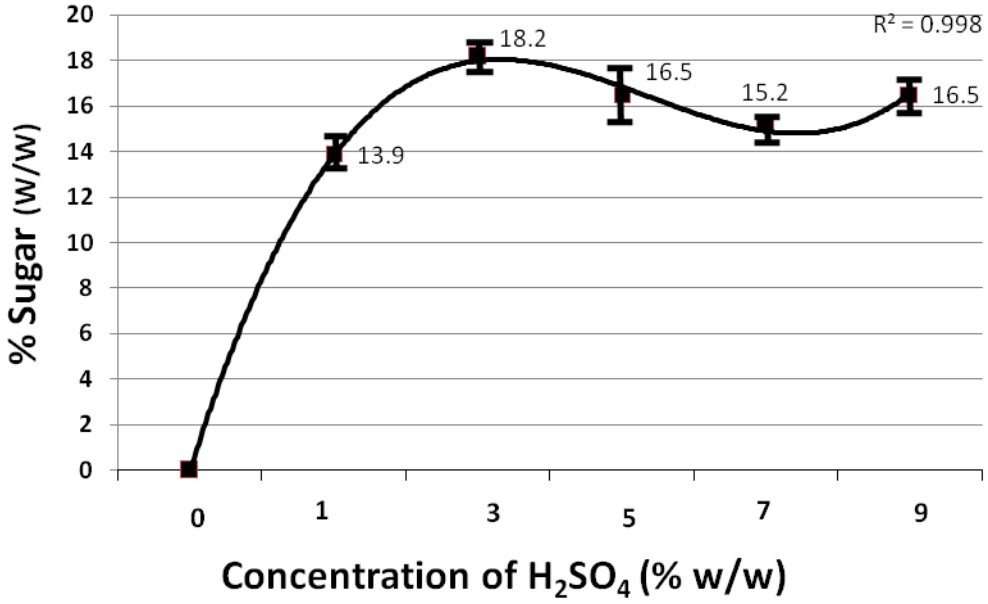

Figure 1. Sugar content in acid pretreated water hyacinth (at 121°C, 15 min).

ment using GC (avoid burning remaining sugar in fermented sample inside GC column), 10 ml fermented WH was placed in a ball flask and 30 ml distilled water was added. The ball flask was fixed in an electrical heater and ethanol was distilled in another conical flask over cooling reflux until approximately 20 to 30 ml of distillate and collected in the ball flask. The distilled sample was injected (1 µL) in GC. The amount of ethanol in the fermented sample was calculated using the standard curve.

The total yeast count in the fermentation broth was determined by the plate count method using potato dextrose agar (PDA). The sample was diluted with sterile distilled water and the corresponding dilutions were added to PDA plates. The plates were incubated at 37°C for 72 h and the count of the yeast colonies was expressed as CFU/ml.

Statistical methods

Each pretreatment experiment (acid, alkali and subcritical water) was replicated at least two times and each analytical experiment was carried out three times. The experimental results were means based on data. Standard deviations were shown by error bars in figures.

RESULTS AND DISCUSSION

The effect of acid concentration on sugar content of samples is shown in Figure 1.Treatment of WH at 121 °C showed that no sugar could be released from WH if no acid was added to the sample. With an increase in acid concentration, up to 3% the sugar content in the sample increased up to 18% (on the basis of dried sample). Higher acid concentrations have negative effects on sugar contents of samples. This is may be due to hydrolysis of sugar molecules in furfural and hydroxy methyl furfural at high acid concentrations and elevated temperatures (121°C). Masami et al. (2008) investigated the optimal conditions for acid hydrolysis of water hyacinth. They found that the best conditions for WH hydrolysis were 1% sulfuric acid at 121°C for 1 h. This result confirmed our finding that autoclavation of WH at 121°C using acid is suitable method for conversion of ligno-celluloses to sugar.

Effect of alkali treatment

In contrast to the acid treatment, the alkali treatment at concentrations up to 5% had only a slight effect on sugar release from WH (Figure 2). The highest sugar content of 0.63% w/w (on dry WH basis) could be achieved after treatment with 1% alkali. That is far less sugar compared to sugar release by acid treatment (18% w/w). Further increase of the alkali concentration has a reverse effect on sugar content of alkali treated samples.

Ahn et al. (2012) suggested that combined pretreatment using 7% (w/v) NaOH at 100°C and 2% (w/v) H_2O_2 was a suitable chemical pretreatment method for WH. Enzymatic treatment of chemical pretreated WH increased the conversion of lignocelluloses to sugar. The ethanol concentration after fermentation using *S. cerevisae* KCTC7928 was 0.35 g ethanol/g biomass.

Effect of combined acid treatment and enzymatic hydrolysis

Acid pretreatment and subsequent enzyme treatment increased the total lignocellulose conversion to sugar drastically (Figure 3). Up to 30% sugar (on dry WH basis) could be observed after acid pretreatment (3% w/w acid)

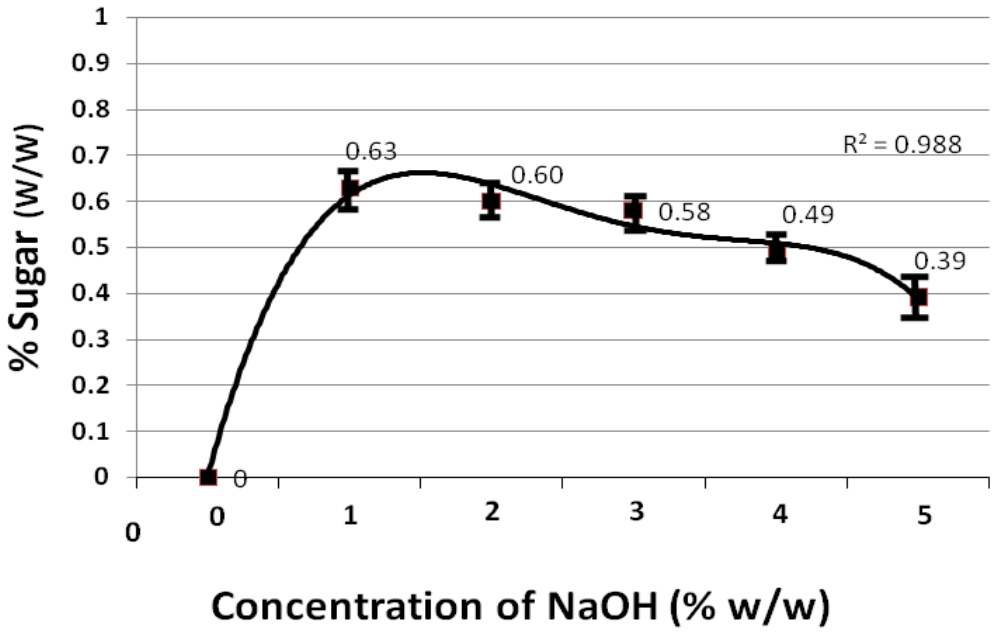

Figure 2. Sugar content in alkali pretreated water hyacinth (85°C, 1 h).

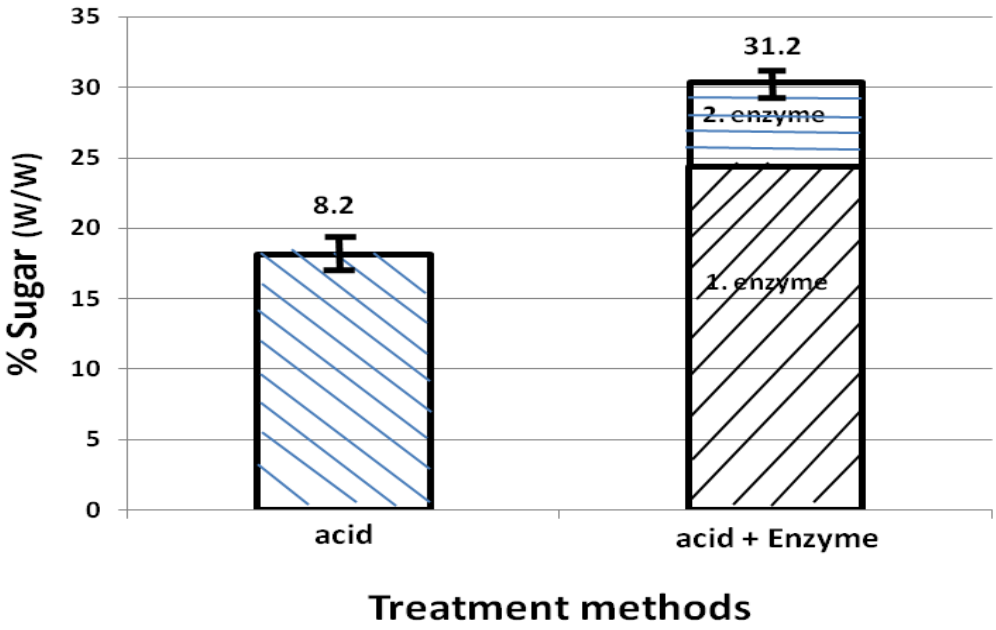

Figure 3. Conversion of lignocelluloses to sugar using acid or combined acid and enzyme treatment.

followed by enzyme treatment (0.8% w/w enzyme on dry WH basis).

Effect of combined alkali treatment and enzymatic hydrolysis

Alkali pretreatment followed by enzyme treatment was an effective method to increase the sugar content in samples. In fact, most sugar (less than 1% sugar) could be released during only alkali treatment, but if the alkali pretreated sample is additionally treated with enzyme, the sugar content will be increased drastically. Up to 22.9% sugar could be measured in the case of combined alkali and enzyme treatment (Figure 4).It is important to note that enzyme treatment without chemical pretreatment has only very little (approximately 3% sugar release) effect on

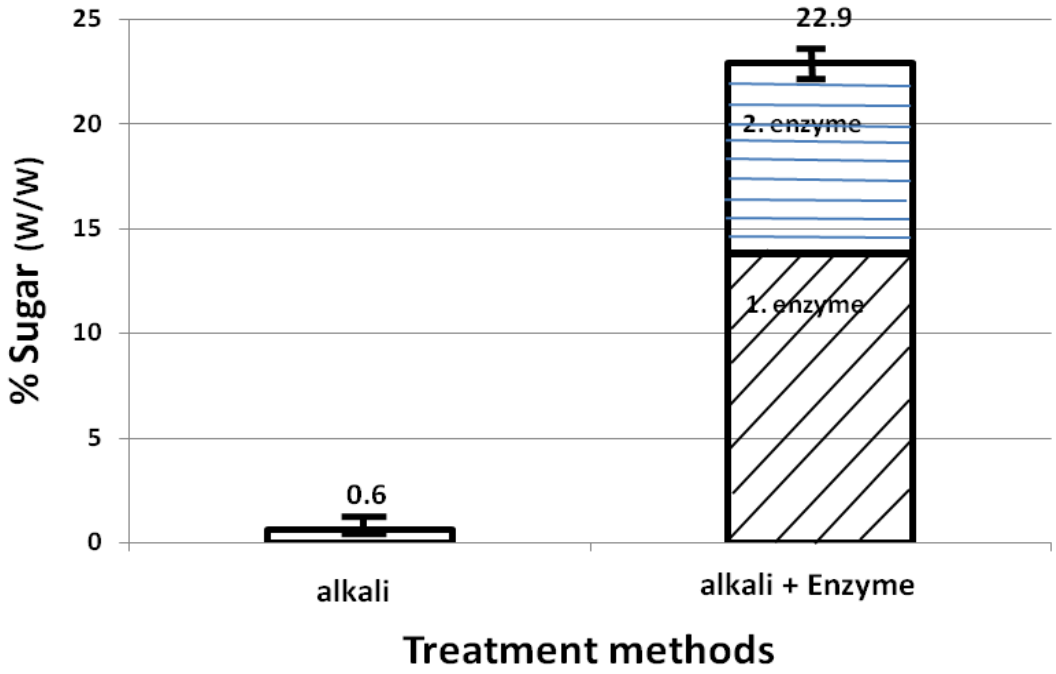

Figure 4. Conversion of lignocelluloses to sugar using alkali or combined alkali and enzyme treatment.

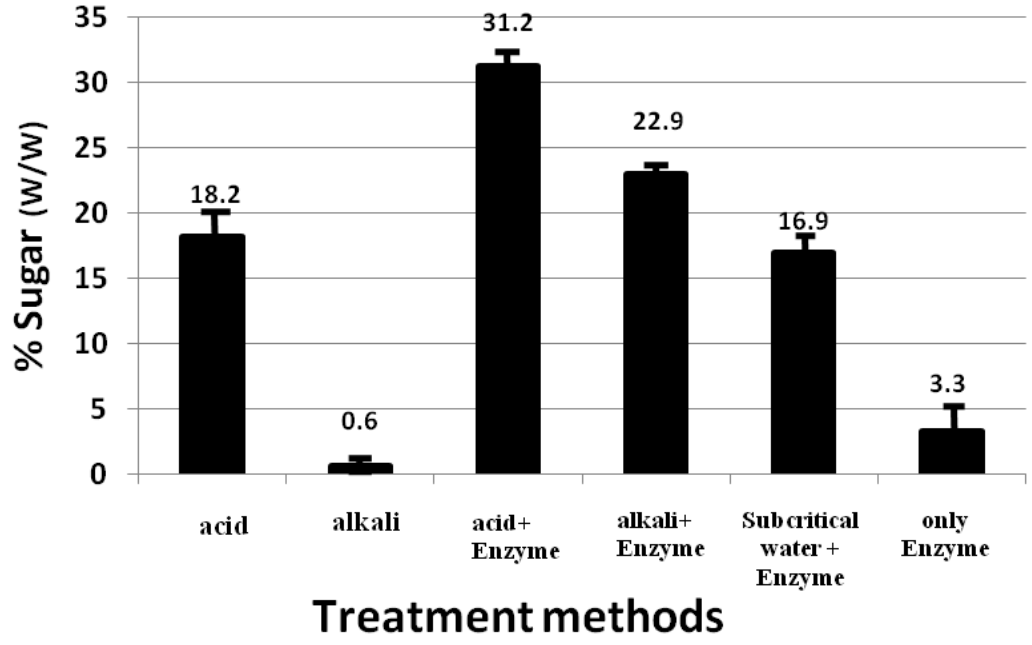

Figure 5. Effect of different pretreatment methods on conversion of lignocellulose to sugar.

the release of sugar from lignocellulose material in WH (Figure 5). This made the importance of the combined method of chemical and enzyme treatment clear. Unfortunately, because of high price of technical enzymes, the enzyme treatment for conversion of lignocelluloses to sugar is very expensive and not economical for large scale processing. It is also necessary to develop on-site enzyme production to decrease the processing cost and to make the large scale production of bioethanol from waste woody materials economical. In our laboratory, we implemented the on-site cellulose enzyme production in a laboratory scale. The produced cellulose enzyme

Table 1. Sugar content in water hyacinth using subcritical water pretreatment followed by enzyme treatment.

Treatment	Sugar content % w/w (on dry water hyacinth basis)
Subcritical water at 160°C, 10 min + enzyme treatment (0.8% enzyme)	12.04 ± 0.76
Subcritical water at 200°C, 10 min + enzyme treatment (0.8% enzyme)	16.96 ± 0.95
Only enzyme treatment (0.8% enzyme) (no pretreatment)	3.29 ± 0.12

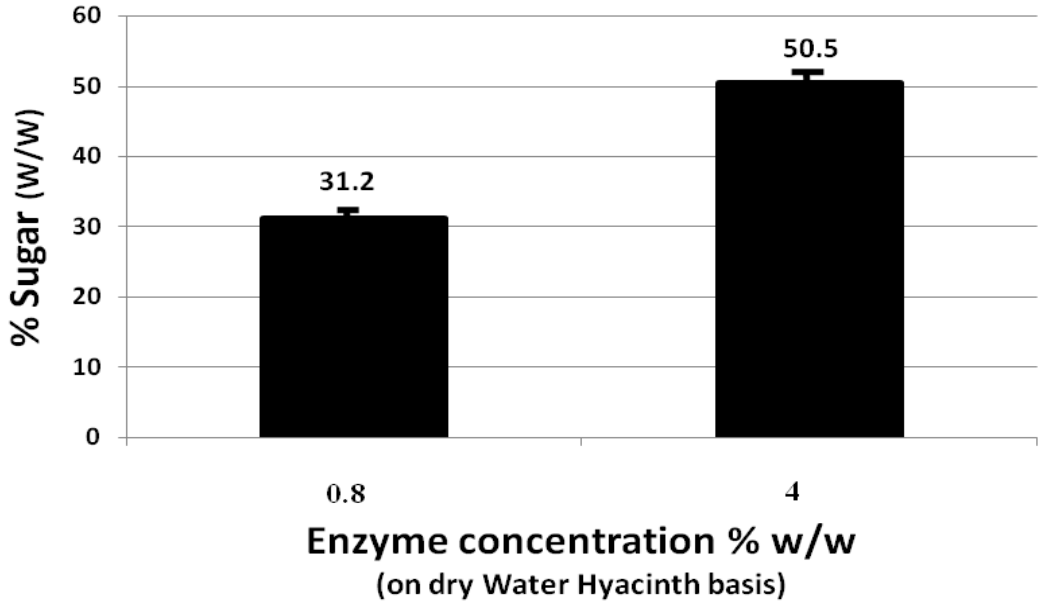

Figure 6. Effect of enzyme concentration on conversion of lignocelluloses to sugar.

showed good performance for conversion of sugar cane bagasse to sugar (data not shown).

Effect of SCW treatment combined with enzymatic hydrolysis

Because of not adding acid or other chemicals during SCW pretreatment, this method could be considered as an environmentally friendly pretreatment method. SCW treatment and subsequent enzyme treatment led to release of sugar from WH (Table 1). Up to 12% sugar (on the basis of dry water hyacinth) could be produced after SCW treatment at 160°C and 10 min pretreatment combined with enzyme (0.8% w/w) treatment. Further increasing of sugar in sample up to 17% could be achieved if the temperature during SCW treatment rises to 200°C. This makes the importance of treatment temperature (and pressure) during SCW pretreatment obvious (Table 1). The comparison between different pretreatment methods on conversion of lignocellulose material to sugar is shown in Figure 5. The highest sugar content could be observed in the case of acid pretreated sample combined with subsequent enzyme treatment (up to 31.2% on dry WH basis) followed by alkali pretreated sample and subsequent enzyme treatment (about 23% on dry WH basis). In general, combination of pretreatment with subsequent enzyme treatment have a positive effect on increasing the sugar content in samples whereas enzyme treatment without any pretreatment only slightly increased the sugar content of sample (3.3% sugar on dry WH basis) (Figure 5).

Effect of enzyme concentration

The effect of enzyme concentration during enzyme treatment of acid pretreated samples and subsequent enzyme treatment of WH is shown in Figure 6. Increasing the enzyme concentration from 0.8% (on dry WH weight

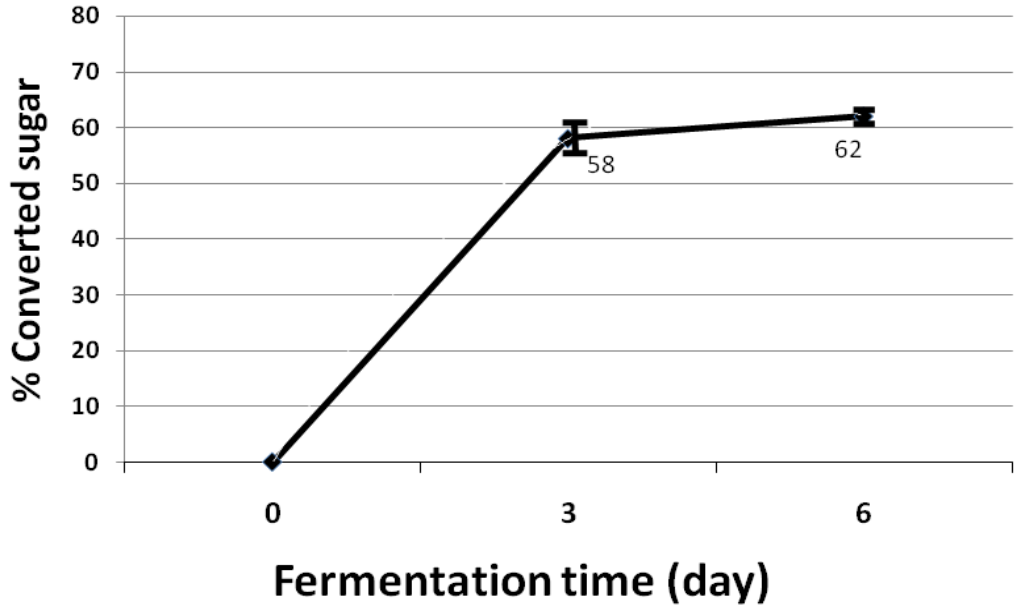

Figure 7. Converted sugar to ethanol during fermentation of broth (as % on the basis of total sugar in the fermentation broth

basis) to 4% increased the conversion of lignocellulose in the pulp to sugar from 31.2 to 50.5%.

Fermentation of water hyacinth

Figure 7 shows the effect of fermentation time on conversion of sugar to ethanol during fermentation of sample pretreated with combined acid and enzyme. With an increase in the fermentation time, the remaining sugar decreased continuously. This was especially obvious during the first 3 days of fermentation. Expanding the fermentation time from 3 to 6 days had only a slight effect on converting the sugar to ethanol. As shown in Figure 7, up to 58 to 62% of total reducing sugar (glucose and xylose) in the sample was converted to ethanol during the 6 days fermentation time. After 6 days fermentation, about 40% sugar was inside the sample that could not be converted to ethanol. The remaining sugar in the fermentation broth is perhaps xylose. WH contains high amounts of hemicellulose. The hemicelluloses contain, among other monosacharides, mainly xylose as monosaccharide.

This pentose sugar could not be digested using *S. cerevisae* and remained unchanged during fermentation. To convert xylose to ethanol, it is necessary to use a xylose fermenting microorganism. Nigam (2002) investigated the bioethanol production from acid hydrolyzed WH using *Pichia stipitis* NRRL y-7124. He found that the bioethanol concentration was not high enough. He suggested that sugar in samples will be converted partially to acetic acid and decrease the ethanol yield. In contrast, Isarankura-Na-Ayudhya et al. (2007) successfully fermented sugar in acid pretreated WH in bioethanol using xylose fermenting *Candida shehatae* yeast. The maximum ethanol yield was 0.19 g/g dried water hyacinth.

The measurement of ethanol using the GC method indicated that after 3 days fermentation up to 1.5% v/w ethanol could be achieved (Figure 8). The ethanol concentration of samples decreased slightly during 3 to 6 fermentation days. This is because of some evaporation of ethanol through the cotton on top of the fermentation flasks during the long fermentation time.

Conclusions

The effect of chemical pretreatment methods such as acid and alkali treatments on conversion of polysaccharides (cellulose, hemicellulose) in WH was investigated. The highest sugar content could be observed in WH treated with 3% H_2SO_4 solution [up to 18.16% sugar (on dry WH basis)]. Subsequent enzymatic treatment of acid pretreated WH resulted in a drastic increase of sugar in samples. Up to 31.2% conversion of lignocellulose to sugar could be achieved after a combination process with acid and enzyme treatment. In addition, increasing the applied enzyme concentration from 0.8% w/w (on dry WH basis) to 4% increased further the sugar content in samples. Up to 50.5% lignocellulose conversion to sugar could be observed if the acid pretreated sample was treated with 4% enzymes. SCW as a physical method is a useful method for the pretreatment of WH before enzyme treatment. Because of not adding acid or other chemicals during SCW pretreatment, this method could

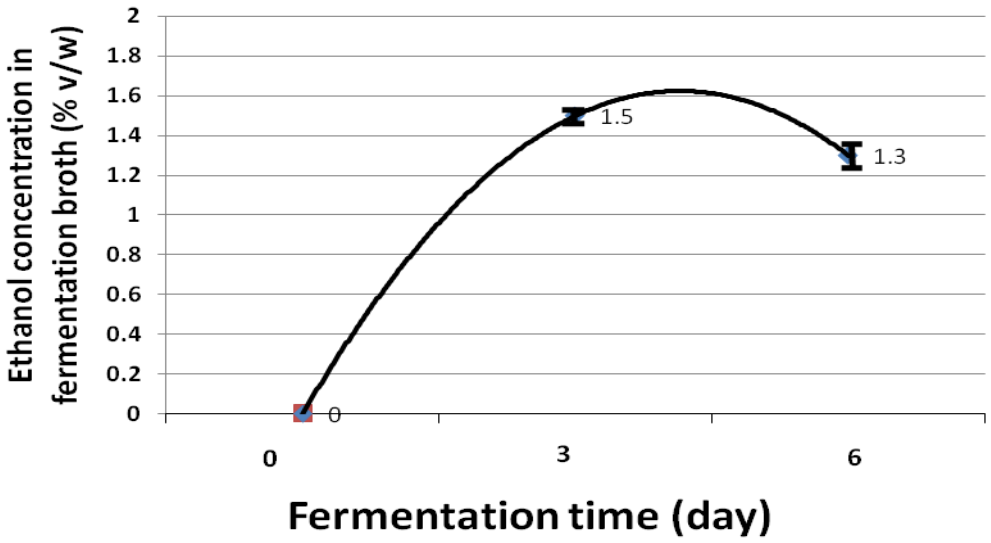

Figure 8. Amount of ethanol in fermentation broth during fermentation time of 6 days (as ml ethanol in 100 g broth).

be considered as an environmentally friendly pretreatment. Up to 17% conversion of lignocellulose to sugar could be measured if the sample was treated at 200°C and 10 min followed by enzyme treatment (0.8% enzyme).

Bioethanol concentration during fermentation of pretreated and enzymatic treated sample increased with increasing the fermentation time. After 3 days fermentation, up to 1.5% ethanol in fermented samples could be measured. Longer fermentation times had less effect on ethanol concentration. Up to 60% of sugar in samples will be converted to ethanol using yeast *S. cerevisae* during 6 days fermentation at room temperature. The remaining 40% sugar is perhaps xylose that cannot be converted to bioethanol. Application of a mixed culture such as a mixture of *S. cerevisae* and *C. shehatae* is maybe useful to convert both glucose and xylose to bioethanol and increasing the bioethanol concentration in sample. To decrease the total processing cost during conversion of WH to sugar it is necessary to apply on-site produced enzymes. Directly using the fresh WH for acid pretreatment instead of dried, a partial energy saving will be achieved.

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