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Pretreatment and Enzymatic Hydrolysis from Water Hyacinth (*Eichhornia crassipes*)

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Abstract

Water hyacinth (*Eichhornia crassipes*) is a noxious aquatic weed which grows fast and is a lignocellulosic material containing cellulose, hemicellulose and lignin. It can be utilized to produce reducing sugar for bioethanol production. This research studied a production of reducing sugar from water hyacinth using physical combined with chemical method. The water hyacinth was milled and dried at 105 °C for 5-6 h, then pretreated with acid (sulfuric acid 2.0, 2.5 and 3.0 % v/v) and alkali (sodium hydroxide 2.0, 2.5 and 3.0 % w/v). After heating in an autoclave at 121°C for 15 min, it was found that using 2.0 % v/v sulfuric acid and 2.0 % w/v sodium hydroxide providing the highest reducing sugar of 15.63 and 2.35 g/L, respectively. Therefore, the sulfuric acid concentration of 2.0 % v/v was the most suitable concentration for pretreated water hyacinth. In addition, enzyme loading and time were studied for the optimization of reducing sugar production. The water hyacinth hydrolysate (sludge) was hydrolyzed with ACCELLERASE1500. The result showed that ACCELLERASE1500 loading at 0.30 ml/g water hyacinth and incubated at 50°C for 48 h produced reducing sugar of 11.95 g/L. This is a first report on enzymatic hydrolysis of water hyacinth by ACCELLERASE1500.

Keywords: water hyacinth, pretreatment, enzymatic hydrolysis

1. Introduction

Increased prices of petroleum fuel due to shortage of fossil fuel reserves and greenhouse effect has stimulated the development of inexpensive production of biofuel. Lignocellulosic material contains mainly of cellulose, hemicelluloses and lignin. Lignocellulose is an interesting raw material for production of bioethanol because of its having large amount and low cost. Water hyacinth (*Eichhornia crassipes*) is one of the abundant waste materials in the world, belonging to the family Pontederiaceae and is native of Brazil. Currently, water hyacinth is a noxious weed in many countries of the world. For Thailand, water hyacinth distributed especial in Chao Phraya River and Tha Chin River. Water hyacinth is a problem to aquatic ecosystem because it grows very fast and obstructs transportation. Attempts carried out to control water hyacinth have resulted in high cost. On the other site, water hyacinth has advantage as they grow in water bodies not being a competition to food crops for arable land. Water hyacinth is a lignocellulosic material that can be used as a potential source for the production of reducing sugars, which can be used for production of ethanol, xylitol, organic acid and other chemicals [1].

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Three major steps for conversion of water hyacinth to bioethanol are pretreatment followed by enzymatic hydrolysis and fermentation. Pretreatment are necessary to improve the digestibility of lignocellulosic biomass because it can break down the complex structure of biomass, decrease the lignin content and crystallinity of cellulose in order to increase accessibility of enzyme to the substrate [2]. Dilute sulfuric acid hydrolysis is one of the most promising pretreatment method because it uses short time, low temperature and pressure. Alkali pretreatment causes swelling, increasing surface area, decreasing degree of polymerization or crystallinity and disrupting the lignin structure [3]. The pretreatment purpose is to prepare the biomass in order to improve the sugars conversion [4].

The main objective of this work was to study conditions on pretreatment in terms of enzymatic hydrolysis yield of reducing sugar, using water hyacinth. The pretreated sample was hydrolyzed using cellulase enzyme from *Trichoderma reesei* (ACCELLERASE1500). It is an enzyme complex for lignocellulosic biomass hydrolysis, which contains endoglucanase activity (2200-2800 CMC unit/g) and beta-glucosidase activity (450-775 pNPG unit/g) to reduce residual cellobiose lead to higher rates of saccharification.

2. Materials and Method

2.1 Preparation of water hyacinth

Fresh water hyacinth with long stem was collected from Phasi Charoen canal, Samut Sakhon province, Thailand. Water hyacinth was thoroughly washed several times with tap water to remove adhering dirt, chopped into small pieces (~2-2.5 cm), blended into small particles and finally dried in a hot air oven at 105°C for 5-6 h. Dried material was stored at room temperature until used [3].

2.2 Pretreatment

The following methods were used for pretreatment of water hyacinth

2.2.1 Dilute acid pretreatment

The dilute sulfuric acid solutions at concentration of 0.0, 2.0, 2.5 and 3.0 % (v/v) were added in flasks containing 10 g sample of water hyacinth (1:10 ratio). The mixture was autoclaved at 121°C for 15 min and cooled down to room temperature. The hydrolysate was filtered with two layers of cheesecloth. The sludge was washed several times with tap water to neutralize the pH followed by a final rinse in distilled water [5]. After that, sludge was dried at 60°C in a hot air oven for 48 h and used for composition analysis. The hydrolysate (liquid) was used to analyze the reducing sugar by DNS method.

2.2.2 Alkali pretreatment

The sodium hydroxide solutions at concentration of 0.0, 2.0, 2.5 and 3.0 % (w/v) were added in flasks containing 10 g sample of water hyacinth (1:10 ratio). The mixture was autoclaved at 121°C for 15 min and cooled down to room temperature. The hydrolysate was filtered with two layers of cheesecloth. The sludge was washed several times with tap water to neutralize the pH followed by a final rinse in distilled water [5]. After that, sludge was dried at 60°C in a hot air oven for 48 h and used for composition analysis. The hydrolysate (liquid) was used to analyze the reducing sugar by DNS method.

2.3 Water hyacinth hydrolysate (sludge) composition analysis

The composition of pretreated water hyacinth (cellulose, hemicellulose and lignin) was estimated by the method of Goering and Van Soest [6].

2.4 Enzymatic hydrolysis of pretreated water hyacinth hydrolysate (sludge)

2.4.1 Optimization of cellulase concentration and hydrolysis time

Two grams water hyacinths (2% loading) were pretreated using 2.0% (v/v) sulfuric acid. After that, the addition of cellulase (ACCELLERASE1500 in 0.05 M acetate buffer, pH 5.0) with 0.05, 0.10, 0.15, 0.20, 0.25 and 0.30 ml/g water hyacinth each in triplicate. These flasks were incubated in a water bath at 50°C for 48 h. Then, samples were taken at 0, 1, 2, 3, 4, 12, 24 and 48 h of incubation. A sample was centrifuged at 18000×g for 15 min. The supernatants with suitable dilution were used for the determination of reducing sugar.

2.5 Reducing sugar measurement

Reducing sugar concentrations were determined using the dinitrosalicylic (DNS) acid method [7].

2.6 Statistical analysis

The experiments were analyzed using analysis of variance (ANOVA) and Duncan's multiple range test (DMRT) to determine significant differences ($p \leq 0.05$) among group means. The statistical program SPSS, version 17.0 was used for the statistical analysis.

3. Results and Discussion

3.1 Effect of dilute sulfuric acid pretreatment

Acid pretreatment is a very effective and recognized process to obtain a structure suitable for enzymatic hydrolysis, which made the cellulose accessible to hydrolyze for conversion to glucose [8]. Especially, using sulfuric acid has been widely studied because of high catabolic activity and fast method. Table 1 presented the data obtained from sulfuric acid pretreatment at concentrations of 0.0, 2.0, 2.5 and 3.0 % (v/v). The maximum reducing sugar of 15.63 ± 0.11 g/L was obtained from 2.0 % (v/v) sulfuric acid pretreatment. It was higher than 0.0, 2.5 and 3.0 % (v/v). The results were in agreement with previous report on sulfuric acid pretreatment of water hyacinth at 121 °C and 20 min, which 2% (v/v) sulfuric acid gave maximum reducing sugar [9]. However, pretreatment with dilute acid at the high concentration, temperature and pressure could cause toxic byproducts, such as furfural, hydroxymethyl furfural, acetic acid, formic acid and levulinic acid [3]. Furfural and hydroxymethyl furfural were formed by the decomposition of pentoses and hexoses [10]. Acetic, formic and levulinic acids were released because the hydrolysis of the acetyl groups linked to the sugar or other linkage present in hemicellulosic backbone [11]. These toxics affected microbial cell metabolism during fermentation. Furthermore, these toxics caused DNA breakdown, which resulted in the inhibition of RNA and protein synthesis [12]. However, the inhibition effect of these toxics depends on the concentration of inhibitors in the hydrolysate [13]. Nigam found that a furfural concentration of 1.5 g/L effect on growth and respiration of *P. stipitis* [14]. In addition, Delgenes et al. showed that the growth of *P. stipitis* was reduced by 43%, 70% and 100% in presence of hydroxymethyl furfural concentration of 0.5, 0.75 and 1.5 g/L, respectively [15]. Toxics removal by overliming has widely recommended but there is the disadvantage of siginificant loss of sugar in the form of gypsum [3] and removal of these toxic cause increase costs. Furthermore, acid pretreatment depends on parameters, such as acid type, concentration, ratio of substrate to acid and temperature.

Table 1 Effect of pretreated water hyacinth using sulfuric acid for reducing sugar production

Sulfuric acid concentration % (v/v)	Reducing sugar (g/L)
0 .0	0.86 ± 0.03^{b}
2.0	15.63 ± 0.11^{a}
2.5	14.92 ± 0.56^{b}
3.0	14.52 ± 0.47^{b}

Mean in the same rows with different letters differ significantly ($p \leq 0.05$). Values are expressed as mean $\pm$ standard error of triplicate analyses.

3.2 Effect of sodium hydroxide pretreatment

Alkaline pretreatment methods gained attention because the process was cheap and used less energy. The sodium hydroxide concentrations were varied from 0.0, 2.0, 2.5 and 3.0 % (w/v) for pretreatment water hyacinth. As shown in Table 2, 2.0 % (w/v) sodium hydroxide produced the highest amount of reducing sugar (2.35 ± 0.34 g/L). It was not statistically significant, compared with 2.5 and 3.0 % (w/v) of sodium hydroxide. Pretreatment with sodium hydroxide caused swelling in internal surface area of lignocellulosic materials. In addition, it decreased degree of polymerization, crystallinity and disruption of lignin structure [3]. However, sodium hydroxide pretreatment provided reducing sugar less than sulfuric acid pretreatment.

Table 2 Effect of pretreated water hyacinth using sodium hydroxide for reducing sugar production

Sodium hydroxide concentration % (w/v)	Reducing sugar (g/L)
0 .0	0.86 ± 0.03^{b}
2.0	2.35 ± 0.34^{a}
2.5	2.19 ± 0.10^{a}
3.0	2.23 ± 0.43^{a}

Mean in the same rows with different letters differ significantly ($p \leq 0.05$). Values are expressed as mean $\pm$ standard error of triplicate analyses.

3.3 Composition of water hyacinth hydrolysate (sludge)

The water hyacinth hydrolysate (sludge) pretreated with sulfuric acid concentrations of 0.0, 2.0, 2.5 and 3.0 % (v/v) were tested for chemical composition (cellulose, hemicellulose and lignin) by the method of Goering and Van Soest [6]. The untreated water hyacinth contained 25.42 ± 0.78 % cellulose, 10.04 ± 5.11 % hemicellulose and 3.28 ± 0.67 % lignin, as shown in figure 1. The lignocellulosic content reported by Singh and Bishnoi [5] were difference to this study (cellulose 19.2 %, hemicellulose 40.0 % and lignin 4.8 %). The difference of the compositions maybe caused from many factors, such as country, season, geography, nutrients in water and time of harvesting. The pretreated samples with 2.0 % (v/v) sulfuric acid showed the highest amount of cellulose (15.36 ± 2.43 % dry matter). The lignin content 1.81 ± 0.45 % dry matter. Dilute acid pretreatment did not remove lignin from the substrate but only modified the lignin linkage [16]. In addition, it also resulted in the loss of hemicelluloses. Then, it led to loss of sugars. Similar result was shown by Singh *et al*. [16]. They showed that dilute acid pretreatment remove hemicelluloses.

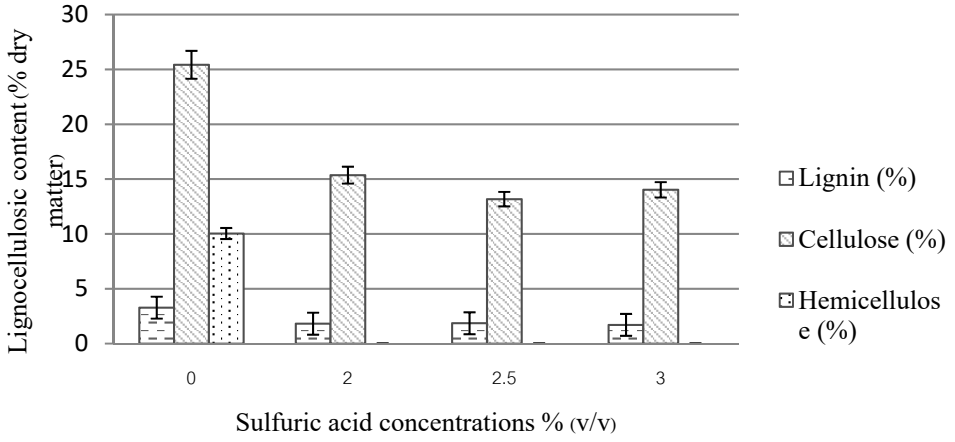

Figure 1 Lignocellulosic components of water hyacinth hydrolysate (sludge) using different sulfuric acid concentrations

The pretreated water hyacinth with 2.0 % (w/v) sodium hydroxide showed the highest content of cellulose (17.58 $\pm$ 1.19 % dry matter), as shown in figure 2. The lignin content was 0.70 $\pm$ 0.16 % dry matter. The lignin concentration would be decreased because of its solubilization in alkali aqueous solution [5] and the ester bonds of the cross-linkage in lignin and xylan were broken. Alkali pretreatment has been considered as an efficient pretreatment method for removing lignin from lignocellulosic biomass [17]. Similar result was shown by Singh *et al.* [16]. They showed that alkaline pretreatment reduced lignin in biomass, increased the surface aea and then water molecules penetrated into inner layers and broke the bonds between lignin and hemicelluloses.

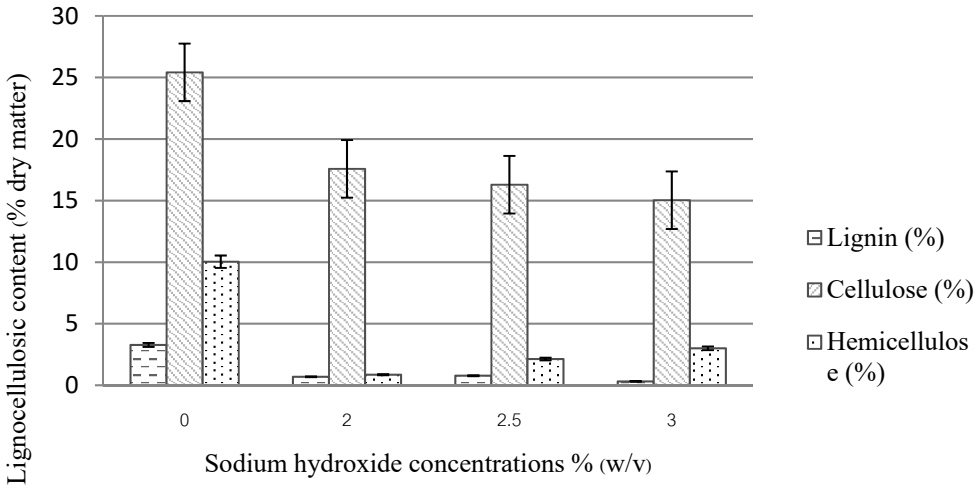

Figure 2 Lignocellulosic components of water hyacinth hydrolysate (sludge) using different sodium hydroxide concentrations

3.4 Effects of enzymatic hydrolysis on water hyacinth hydrolysate (sludge)

The dilute sulfuric acid pretreatment (2.0 % (v/v)) was selected to carry on for the enzymatic hydrolysis because of its contained high cellulose (15.36 ± 2.43%) and low lignin (1.81 ± 0.45%). Lignin was a major obstrucle [18] and limited the rate of enzymatic hydrolysis of water hyacinth by acting as a shield. The lignin and hemicelluloses made enzyme access to cellulose difficultly, therefore reducing the efficiency of enzymatic hydrolysis could occurred [19]. The amount of reducing sugar was investigate when samples were pretreated with different cellulase (ACCELLERASE1500) concentrations (0.05, 0.10, 0.15, 0.20, 0.25 and 0.30 ml/g water hyacinth) and different incubation time (0, 1, 2, 3, 4, 12, 24 and 48 h) at 50°C. ACCELLERASE1500 is an enzyme complex specifically for the lignocellulosic biomass and renewable fuels, which capable of efficiently hydrolyzing lignocelluloses biomass into fermentable monosaccharides and contains high activity of beta-glucosidase to conversion of cellobiose to glucose lead to higher rates of saccharification. The result showed that enzyme concentration of 0.30 ml/g water hyacinth and incubation time of 48 h produced the highest reducing sugar concentration of 11.95 ± 0.22 g/L (Table 3). However, the efficiency of cellulose hydrolysis of pretreated substrate has been depended on several process parameters, such as enzyme loading, biomass loading, incubation time and surfactant concentration [20].

Table 3 Effect of enzymatic hydrolysis using different enzyme concentrations and incubation time for reducing sugar production

Incubation time (h)	Reducing sugar (g/L)					
	Enzyme concentration (ml/g water hyacinth)					
	0.05	0.10	0.15	0.20	0.25	0.30
0	0.38 ± 0.03^{h}	0.36 ± 0.07^{i}	0.45 ± 0.13^{h}	0.48 ± 0.11^{i}	0.41 ± 0.10^{f}	0.49 ± 0.08^{i}
1	0.76 ± 0.16^{g}	0.90 ± 0.10^{h}	1.34 ± 0.04^{g}	1.43 ± 0.05^{h}	1.56 ± 0.19^{f}	1.78 ± 0.26^{h}
2	0.93 ± 0.04^{g}	1.46 ± 0.15^{g}	2.00 ± 0.12^{f}	2.63 ± 0.06^{g}	2.86 ± 0.07^{e}	3.33 ± 0.23^{g}
3	1.39 ± 0.07^{f}	2.18 ± 0.20^{f}	2.93 ± 0.20^{e}	3.72 ± 0.07^{f}	3.83 ± 0.54^{de}	4.63 ± 0.27^{f}
4	1.74 ± 0.08^{e}	2.70 ± 0.11^{e}	3.72 ± 0.15^{d}	4.47 ± 0.14^{e}	4.62 ± 0.50^{cd}	5.75 ± 0.12^{e}
5	2.03 ± 0.05^{d}	3.29 ± 0.11^{d}	4.09 ± 0.18^{d}	5.12 ± 0.09^{d}	5.25 ± 0.70^{c}	6.49 ± 0.02^{d}
12	3.28 ± 0.06^{c}	4.97 ± 0.07^{c}	6.32 ± 0.12^{c}	7.79 ± 0.20^{c}	7.87 ± 0.84^{b}	9.28 ± 0.14^{c}
24	5.14 ± 0.13^{b}	7.61 ± 0.01^{b}	9.30 ± 0.30^{b}	10.01 ± 0.06^{b}	9.88 ± 1.16^{a}	10.95 ± 0.22^{b}
48	7.26 ± 0.16^{a}	9.40 ± 0.21^{a}	10.58 ± 0.28^{a}	11.27 ± 0.28^{a}	11.01 ± 1.11^{a}	11.95 ± 0.22^{a}

Mean in the same rows with different letters differ significantly ($p\leq0.05$). Values are expressed as mean ± standard error of triplicate analyses.

4. Conclusions

Water hyacinth can be utilized to produce ethanol due to its high levels of cellulose and hemicellulose. The pretreatment method for water hyacinth using acid and alkali hydrolysis convert cellulose and hemicellulose into fermentable sugars was investigated. The water hyacinth was hydrolyzed by 2.0-3.0 % (v/v) sulfuric acid and 2.0-3.0 % (w/v) sodium hydroxide at temperature of 121°C for 15 min. It was found that the maximum reducing sugar was obtained at 2.0 % (v/v) sulfuric acid. The optimum conditions for the enzymatic hydrolysis of water hyacinth hydrolysate

(sludge) were investigated. Cellulase (ACCELLERASE1500) loading at 0.30 ml/g water hyacinth and incubating at 50°C for 48 h produced the highest reducing sugar concentration of 11.95 g/L.

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