Evaluation of *Eichhornia crassipes* as an Alternative Raw Material for Reducing Sugars Production

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Water hyacinth was analyzed to determine its hemicellulose/lignin content, evaluating the conditions for the saccharification process with commercial microbial enzymes. Plant material, including leaves and stalks, was pretreated at several temperatures (100, 110, and 120 °C) with different sulfuric acid concentrations (0.5, 1.0, 1.5, 2.0, 2.5, and 3%) and residence times (0, 15, 30, 45, 60, 90, and 120 min). Total reducing sugars were measured by the dinitrosalicylic acid method. The optimum conditions that maximized the yield of reducing sugars included a pretreatment with 2% (v/v) sulfuric acid at 110 °C for 90 min. The optimum conditions for enzymatic saccharification used the commercial enzyme Celluclast at 50 °C for 24 h of hydrolysis. The maximum yield was 0.54 g of fermentable sugars per gram of biomass. Data demonstrated that *E. crassipes* is suitable as a raw material for products such as bioethanol; however, further fermentation studies are required.

Keywords: Bioethanol; Lignocellulose; Pretreatment; Reducing sugar; Water hyacinth

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INTRODUCTION

Water hyacinth, a freshwater aquatic plant, is native to Brazil. It is an ornamental plant found in different countries around the world. *Eichhornia crassipes* is used in traditional medicine and to remove heavy metals from water bodies (Ganguly *et al*. 2012).

E. crassipes is considered a weed and sometimes even a plague around the world; however it can potentially be a resource due its high carbohydrate content (18% cellulose, 50% hemicellulose). *E. crassipes* may be an excellent source of sugars, which then can be fermented to produce ethanol. The bioethanol production is achieved through three steps: pretreatment to liberate cellulose and hydrolyze hemicellulose; hydrolysis of both cellulose and hemicellulose to obtain free sugars, and then fermentation to convert the sugars to alcohol (Singh and Bishnoi 2013).

In Mexico, utilization of new lignocellulosic materials for biofuel production is of vital importance to the future of bioethanol. The use of lignocellulosic materials for ethanol production is favored by abundance and low cost. *E. crassipes* grows very fast under the climatic conditions present in some regions in Mexico (Kumar and Wyman 2009).

It has been reported (Nigam 2002) that using dilute acid pretreatment is generally more effective than alkali pretreatment. Pretreatment solubilizes hemicellulose, and at the same time it reduces cellulose crystallinity and removes lignin from biomass. Comminution, acid or alkali pretreatment, ammonia fiber explosion (AFEX), and steam explosion are some techniques that have been used to solubilize hemicellulose from lignocellulosic biomass in preparation for its fermentation to sugar (Kumar and Wyman 2009). Singh and Bishnoi (2013) obtained higher sugar yield using alkali pretreatment (68%). Here, some previous analysis showed higher yield using high acid concentrations for pretreatment than hydroxide sodium pretreatment. The aim of this research was to evaluate the use of dilute sulfuric acid pretreatment followed by enzymatic saccharification to optimize the yield of fermentable sugars from *E. crassipes*.

EXPERIMENTAL

Substrate Preparation

Water hyacinth plants were collected from Chapultepec Lake, located in D. F., Mexico (19°25'22'' Latitude, 99°11'18'' Longitude), and the Tunal River, located in Durango, Mexico (26° 48' – 22° 19' Latitude, 102° 28' – 107° 11' Longitude). Harvesting took place in 2010 between the months of September and November. Metropolitan Universidad of México, Campus Iztapalapa, donated a sample from Chapultepec. The plant material was washed to remove adhering dirt, cut into small pieces, dried, milled, and passed through a number 40 sieve. The prepared sample was packed in sealed plastic bags and stored at room temperature.

Raw Material Characterization

The moisture content of the biomass was measured by drying the sample on trays for seven days at room temperature. The carbohydrates were obtained by sulfuric acid hydrolysis (Ruiz and Ehrman 1996). Ash content was determined using the Technical Association of the Pulp and Paper Industry (TAPPI) method (Ehrman 1994). The total reducing sugars were determined by the dinitrosalicylic acid (DNS) method with glucose as the sugar standard (Miller 1959). Lignin content was measured using the TAPPI method. The Bradford method (1976) was performed to determine protein content. All tests were done in triplicate.

Dilute Acid Pretreatment

First, 1 g of sample was mixed with 9 mL of dilute sulfuric acid at different concentrations (0.5, 1, 1.5, 2, 2.5, and 3% [v/v]), and then heated by autoclaving at 100, 110, and 120 °C for different lengths of residence time (0, 15, 30, 45, 60, 90, and 120 min). The pretreated biomass was then neutralized with calcium hydroxide (3 N) addition and filtered with a membrane filter (0.45 μm). The solids residues after pretreatment were washed with water, dried, and stored in preparation for enzymatic saccharification.

Enzymatic Hydrolysis

The solid residues of the acid pretreatment were recovered and hydrolyzed using an enzymatic commercial concentrate. Experimental conditions were varied using the Plackett-Burman statistical design to evaluate seven independent variables, with two variation levels for each one. The variables were enzyme concentration (25 and 30

FPU/g), pH (4.6 and 5.0), polyoxyethylene-20-sorbitan monooleate (Tween-80®) (0.001 and 0.003% [w/v]), enzyme type (Celluclast® 1.5 L from Novozymes [Bagsvaerd, Denmark] and Powercell from Prozyn [Sao Paulo, Brazil]), temperature (46 and 50 °C), hydrolysis time (12 and 24 h), and substrate mass (0.5 and 1 g). The supernatant was evaluated for reducing sugar content by the dinitrosalicylic acid (DNS) method (Miller, 1959). All tests were done in triplicate.

Statistical Analysis

Experimental results were statistically analyzed by one-way ANOVA using the software STATSOFT STATISTICA, version 7.0. The Tukey test ($p < 0.05$) was used to determine the effects of pretreatment and residence time on the yield of reducing sugars. The yields of sugars obtained experimentally during enzymatic analysis were subjected to statistical analysis using the Plackett-Burman design.

RESULTS AND DISCUSSION

Chemical Composition of Water Hyacinth

The chemical composition of water hyacinth plants harvested for this study was compared to values reported for plants from other locations and water sources. The samples obtained from the Chapultepec Lake and the Tunal River yielded 5.6 and 4.4% w/w total solids (TS), respectively. Table 1 shows the chemical composition for the raw material used in this work.

The samples obtained in this study exhibited hemicellulose levels lower than the reported 53% for the same species by Groote *et al.* (2003), but similar to the 48% reported by Nigam (2002). In general, *E. crassipes* contains high levels of hemicellulose relative to cellulose, perhaps because aquatic plants do not require much cellulose to hold their own weight.

The high ash content of water hyacinth makes it a good source of minerals. The samples from Chapultepec Lake and Tunal River yielded 19.1% and 22.9% ash, respectively. Both of these levels are greater than the 15.1% ash content of samples studied by Nigam (2002). The high values we measured in our samples are probably due to contamination of their water sources.

E. crassipes is thus a potential source of cellulose and hemicellulose that can be transformed into useful products.

Table 1. Compositions of *Eichhornia crassipes* from D. F. and Durango

Constituents	Chapultepec Lake (% w/w)	Tunal River (% w/w)
Total Solids (TS)	5.6±0.7	4.4±0.1
Moisture	95.3±0.2	95.8±0.2
Ash (as % of TS)	19.1±0.8	22.9±0.9
Hemicellulose (as % of TS)	50.1±0.9	49.3±0.2
Cellulose (as % of TS)	16.8±0.9	18.3±0.5
Lignin (as % of TS)	3.8±0.5	5.3±0.3
Protein (as % of TS)	3.6±0.1	3.8±0.2

Note: Averages ± Standard Deviation are shown.

Table 2 summarizes data for different lignocellulosic materials used for ethanol production and their lignin contents. Most have ≥ 15 % lignin. Compared to these other

materials, the *E. crassipes* samples reported here have lower lignin content (5.3% dry weight). This suggests that *E. crassipes* may be easier to hydrolyze than other feedstocks, making it an attractive potential raw material for ethanol production.

Table 2. Lignin Content from Different Raw Material Used for Ethanol Production

Raw Material	Lignin (% w/w)	Reference
Water Hyacinth[1]	5.3	This Work
Water Hyacinth[2]	3.8	This Work
Maize	30	Chabbert *et al*. 1994
Wheat Straw	18	Dale *et al.* 1996

[1]The Tunal River, Durango, Mexico; [2]Chapultepec Lake, D.F., Mexico.

Dilute Acid Pretreatment

Figure 1 illustrates the effects of temperature and residence time on the yield of reducing sugars during the pretreatment step. The yield increased with increasing residence time, with a maximum yield of 0.54 g of reducing sugars per gram of water hyacinth obtained by pretreatment at 110 °C for 90 min (p=0.0002). Masami *et al*. (2008) reported an optimum pretreatment temperature of 120 °C and optimum residence time of 1 h for pretreatment of water hyacinth using 1% (v/v) sulfuric acid. Likewise, Satyanagalakshmi *et al*. (2011) reported optimum pretreatment conditions of 121 °C for 60 min using 2% (v/v) sulfuric acid for a maximum yield of 0.327 g reducing sugars per gram pretreated water hyacinth biomass. Differences in the reducing sugars yield could be a result of the material used in this work, which had less lignin content than other reported materials. This is based on an understanding that lignin impedes hemicellulose disruption.

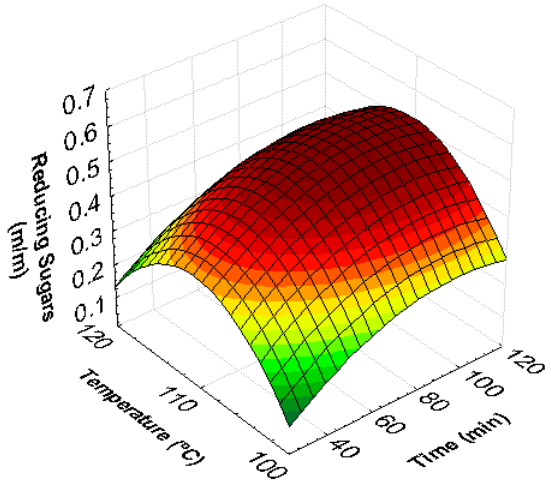

Fig. 1. 3D surface plot showing the influence of temperature and time on reducing sugars yield during acid pretreatment of *E. crassipes*

Figure 2 illustrates the effects of acid concentration and time on the yield of reducing sugars during pretreatment. The yield increased when both acid concentration and time were increased simultaneously, reaching a maximum value of 0.4 g reducing sugars per gram starting material for pretreatment with 2% (v/v) sulfuric acid at 110 °C for 90 min.

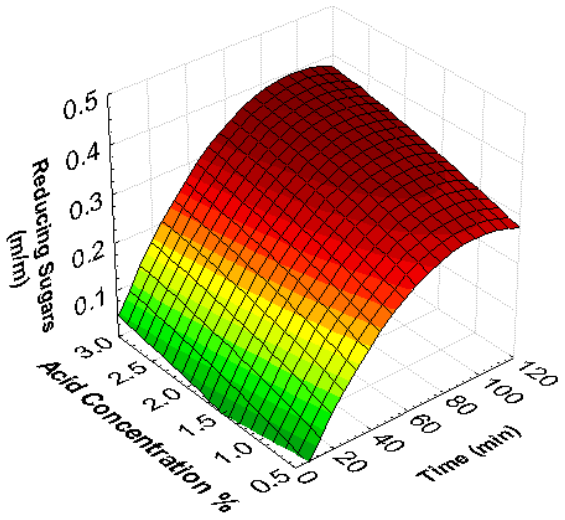

Fig. 2. 3D surface plot showing the influence of acid concentration and time on reducing sugars yield during acid pretreatment of *E. crassipes*

Acid concentrations of 2.0, 2.5, and 3% (v/v) did not show a significant difference (p=0.3) while the residence time increased. This is in contrast to a report by Nigam (2002), where acid concentrations greater than 1% greatly decreased the yield of reducing sugars (Nigam 2002). Awasthi *et al*. (2013) reported 12.63 mg of reducing sugar from water hyacinth using 4% sulfuric acid. This yield is much lower than reported in this work, and the acid concentration is much greater. Adding a lower acid concentration inhibits further reaction and decreases the reducing sugars yield. Most studies report between 51% and 71% saccharification from various raw materials (Aswathy *et al*. 2010). In this work, 80% percent saccharification was obtained.

Figure 3 illustrates the relationship between temperature and sulfuric acid concentration on the yield of reducing sugars during pretreatment step. The maximum yield of 0.41 g reducing sugars per gram starting material was obtained at 110 °C using 2% (v/v) sulfuric acid. This is comparable to the 0.51 g/g reported for water hyacinth collected in India with 3.0% (v/v) sulfuric acid for 90 min (Nigam 2002).

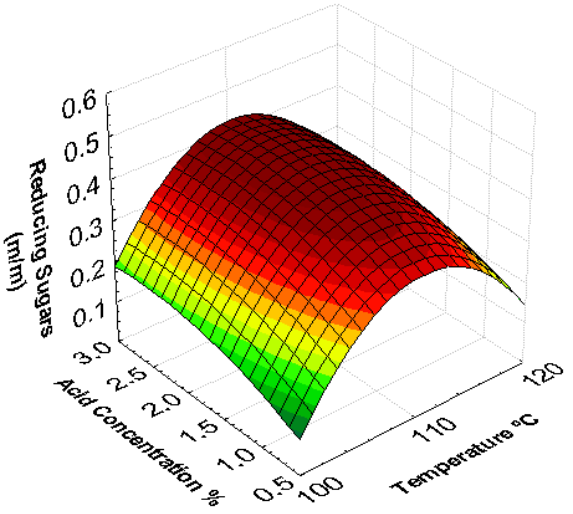

Fig. 3. 3D surface plot showing the influence of acid concentration and temperature on reducing sugars yield during acid pretreatment of *E. crassipes*

Three percent sulfuric acid could be considered as a high acid concentration for pretreatment because it can convert reducing sugars into furfurals and decrease fermenting sugars yield significantly.

The highest yield of reducing sugars using a pretreatment was obtained with 2% (v/v) sulfuric acid at 110 °C for 90 min. However, the statistical analysis indicated that the concentration of sulfuric acid had no statistically significant effect on yield (p=0.03).

Enzymatic Hydrolysis

The solid residues from pretreatment were used for enzymatic hydrolysis. The experimental matrix (Table 3) based on the Plackett-Burman design was used to investigate the influence of selected variables with two levels of repetition, -1 for the lower level and +1 for the upper level. This design allows all possible combinations of variables to be evaluated using a relatively small number of observations (Singh *et al*. 2011). Table 3 and Fig. 4 illustrate that combination 14 (Celluclast®1.5 L at 25 FPU/g, 0.001 % [w/v] Polyoxyethylene-20-sorbitan monooleate, pH 4.6, 50 °C, 24 h) provided the highest yield of reducing sugars (0.54 g/g).

Table 3. Plackett-Burman Design Matrix Used to Perform the Experiments for the Seven Independent Variables Analyzed during Enzymatic Hydrolysis of *E. crassipes*

Run	Time (h)	pH	Temperature (°C)	Substrate (g)	Surfactant (%)	Enzyme (FPU/g)	Enzyme type	Reducing sugars (g/g)
14	24	4.6	50	0.5	0.03	25	Celluclast	0.54^{a}±0.002
1	12	4.6	46	1	0.03	30	Celluclast	0.2^{d}±0.009
9	12	4.6	46	1	0.03	30	Celluclast	0.19^{d}±0.008
22	24	4.6	50	0.5	0.03	25	Celluclast	0.51^{c}±0.002
21	12	4.6	50	1	0.01	25	Powercell	$0.14^{d,e}$±0.006
15	12	5.0	50	0.5	0.01	30	Celluclast	0.20^{d}±0.001
12	24	5.0	46	1	0.01	25	Celluclast	$0.52^{b,c}$±0.002
4	24	5.0	46	1	0.01	25	Celluclast	$0.53^{b,c}$±0.002
6	24	4.6	50	0.5	0.03	25	Celluclast	0.54^{a}±0.002
11	12	5.0	46	0.5	0.03	25	Powercell	$0.03^{h,i}$±0.002
18	24	4.6	46	0.5	0.01	30	Powercell	$0.06^{h,i}$±0.003
19	12	5.0	46	0.5	0.03	25	Powercell	$0.04^{g,h}$±0.002
23	12	5.0	50	0.5	0.01	30	Celluclast	$0.19^{d,e}$±0.008
20	24	5.0	46	1	0.01	25	Celluclast	0.53^{b}±0.002
16	24	5.0	50	1	0.03	30	Powercell	$0.07^{g,h,i}$±0.003
7	12	5.0	50	0.5	0.01	30	Celluclast	$0.18^{d,e}$±0.007
13	12	4.6	50	1	0.01	25	Powercell	$0.06^{g,h}$±0.003
8	24	5.0	50	1	0.03	30	Powercell	0.09^{g}±0.004
2	24	4.6	46	0.5	0.01	30	Powercell	$0.08^{f,g}$±0.003
3	12	5.0	46	0.5	0.03	25	Powercell	$0.04^{h,i}$±0.002
17	12	4.6	46	1	0.03	30	Celluclast	$0.07^{g,h}$±0.003
5	12	4.6	50	1	0.01	25	Powercell	$0.04^{g,h,i}$±0.002
10	24	4.6	46	0.5	0.01	30	Powercell	$0.05^{h,i}$±0.002
24	24	5.0	50	1	0.03	30	Powercell	$0.08^{g,h}$±0.003

The optimal temperature for the enzymatic hydrolysis of cellulose is 50 ºC. Alkasrawi *et al*. (2003) reported the best enzymatic activity of cellulase using 0.0025%

(w/v) polyoxyethylene-20-sorbitan monooleate. Enzymatic hydrolysis is enhanced by the addition of polyoxyethylene-20-sorbitan monooleate because the nonionic surfactant reduces the interaction between the enzyme and lignin (Ericksson *et al*. 2003).

The Pareto chart in Fig. 4 shows the standardized effect for the seven variables evaluated during enzymatic hydrolysis. Enzyme type, hydrolysis time, and enzyme concentration had a significant influence on the yield of reducing sugars. A lower value for enzyme type was achieved, which means Celluclast® was better for reducing sugars production, with lower enzyme concentration (25 FPU/g). Meanwhile, a higher hydrolysis time (24 h) was required to raise the reducing sugars yield.

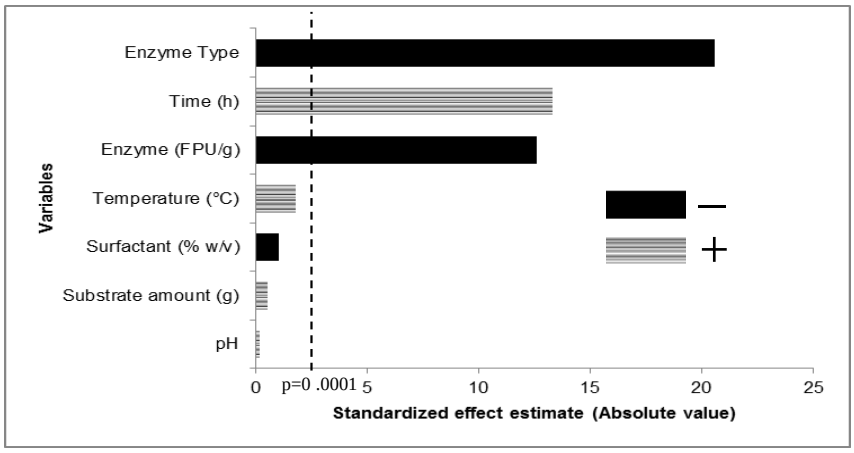

Fig. 4. Standardized effects for the seven variables analyzed. Significance level (p=0.0001) is shown. Lower value and higher value significances are shown with (-) and (+), respectively.

Water hyacinth is considered a suitable raw material because it can be found in large quantities, does not compete with agricultural foods for cultivation area, and its cost is relatively low (Awasthi *et al*. 2013). Considering its very high growth rate of 140 tons dry material of water hyacinth per ha per year, this plant seems to be an excellent raw material for several uses, including bioethanol from reducing sugars via fermentation (Girisuta *et al*. 2008).

In summary, the optimum conditions for the acid hydrolysis pretreatment are 2% (v/v) sulfuric acid at 110 °C for 90 min. The optimum conditions for the enzymatic hydrolysis use the commercial enzyme concentrate Celluclast® 1.5 L (25 FPU/g) to hydrolyze 0.5 g of substrate in the presence of 0.001 % (w/v) polyoxyethylene-20-sorbitan monooleate as a surfactant at 50 °C for 24 h.

CONCLUSIONS

1. Dilute acid pretreatment can be performed by pretreatment with 2% (v/v) sulfuric acid at 110 °C for 90 min to raise the reducing sugars yield.
2. During enzymatic hydrolysis, Celluclast from Novozymes was the most effective enzyme to hydrolyze cellulose than Powercell from Prozyn.
3. The highest reducing sugar yield during enzymatic hydrolysis was obtained using 0.5 g of substrate and 0.0001% polyoxyethylene-20-sorbitan monooleate as a surfactant at 50 ºC for 24 h.

4. Due to its high content of hemicellulose, water hyacinth provides a suitable raw material for products such as bioethanol.

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