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Pretreatment and enzymic saccharification of water hyacinth cellulose

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ABSTRACT

Water hyacinth was pretreated, under variable conditions, with NaOH, alkaline H_2O_2, peracetic acid and sodium chlorite. Combined pretreatments included sodium chlorite with each of NaOH, alkaline H_2O_2 and peracetic acid. Combined pretreatment with 0.1% $NaClO_2$ for 1 h at 100 °C and peracetic acid at 100 °C for 15 min afforded the most promising sample. The recovered lignin, cellulose and hemicellulose of this sample was 2.56%, 96.69%, and 81.38%, respectively. The same sample, by cellulase hydrolysis showed the highest cellulose conversion (80.8%) and 90% saccharification using 200 FPU/g substrate. Some ambient factors affecting saccharification of pretreated water hyacinth were investigated. Enzymic saccharification after 6 h was about 50% of that at 48 h, indicating a slow hydrolysis rate by time. Addition of 8% glucose at the beginning of the enzymic hydrolysis decreased the saccharification to about its half while addition of 8% ethanol brought about complete inhibition of the enzyme. Addition of cellobiase to the reaction mixture increased cellulose conversion and saccharification by 10%.

1. Introduction

Water hyacinth (*Eichhornia crassipes*) is a water plant, popularly called ward El Nile, is wide spread in the River Nile and covers vast areas of it surface. It thus represents an obstacle in River Nile navigation. Mechanical smearing of this water plant does not completely solve the problem since its grows again and the removed plant if kept on the ground will cause pollution.

Studies in our laboratory indicated the water hyacinth contains 60% cellulose, 8% hemicellulose and 17% lignin. Water hyacinth is thus a lignocellulosic biomass and for its better utilization it can be used as a good source for the production of reducing sugars, which can be further used for production of ethanol, xylitol, organic acids and other chemicals (Xia & Sheng, 2004; Chen, Zhao, & Xia, 2008). This can be achieved by enzymatic hydrolysis which produces better yields than acid-catalyzed hydrolysis (Pan et al., 2005). However, there are many problems hindering the effective enzymatic hydrolysis of lignocellulosic material. Of these is the lignin seal which prevents penetration by degrading enzymes (Taniguichi et al., 2005), crystallinity of cellulose and product inhibition by the accumulation of cellobiose on using cellulose preparations poor in cellobiase. Therefore, an ideal pretreatment is needed to reduce the lignin content and crystallinity of cellulose, which impedes enzymatic hydrolysis (Hendriks & Zeeman, 2009). A number of chemical pretreatments of lignocellulosics to enhance enzymic saccharifications of cellulose have been extensively investigated. These include pretreatment with sodium hydroxide (Carrillo, Lis, Colom, Lopez-Mesas, & Valldeperas, 2005; Chen et al., 2008; Goyal, Kalra, Sareen, & Soni, 2008; Hendriks & Zeeman, 2009; Okeke & Obi, 1995; Xiros, Topakas, Katapodis, & Christakopoulos, 2008), alkaline hydrogen peroxide (Adsul et al., 2005; Gould, 1984; Saha & Cotta, 2007; Wei & Cheng, 1985), Peracetic acid (Farid, Shaker, & El-Diwany, 1983; Gharpuray, Lee, & Fan, 1983), and sodium chlorite (Adsul et al., 2005; Saddler, Brownell, Clermont, & Levitin, 1982).

The present study aims at investigating chemical pretreatments of water hyacinth followed by enzymic saccharification. Emphasis was directed toward investigating the appropriate conditions leading to good delignification with the least loss of cellulose and hemicellulose contents. As far as we are aware, nothing has yet been reported on the enzymic saccharification of water hyacinth.

2. Methods

2.1. Water hyacinth

E. crassipes (water hayacinth) was collected from the River Nile, then freed from foreign substances. The whole plant was dried and milled.

2.2. Cellulase

It was a preparation from *Trichoderma viride* 100 (Abdel-Fattah, Ismail, & Abdel-Naby, 1995).

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2.3. Cellobiase

This a preparation from *Aspergillus niger* 1 (Ismail, Abdel-Naby, & Abdel-Fattah, 1995).

2.4. Determination and preparation of cellulose and hemicellulose

This was done by the method adopted by Whistler, Bachrach, and Bowman (1948).

2.5. Determination of lignin content

This was done according to the method of Bagby, Cunningham, and Maloney (1973).

2.6. Determination of reducing sugars

This was done according to Somogyi (1952).

2.7. Determination of glucose

The amount of glucose released in the reaction mixture was determined by using glucose oxide/peroxidase regent, which was supplied as enzyme kit from "Bio analytics corp".

2.8. Determination total carbohydrate

This was done according to the method of Dubois, Gilles, Hamilton, Rebers, and Smith (1956).

2.9. Filter paper activity

This was measured following the technique of Mandels and Sternberg (1976).

2.10. Cellobiase activity

This was assayed by the method reported by Berghem and Petterson (1974).

2.11. Xylanase activity

This was done according to the method reported by Warzywods, Chevron, Ferre, and Pourquie (1983).

2.12. Pretreatment of water hyacinth

All pretreatments were carried out using milled dry samples (200 mesh).

2.12.1. With NaOH

Suspensions of 4% (w/v) of water hyacinth in NaOH solutions (2–10%) were heated at 100 °C in boiling water both with stirring for 20–180 min. The pretreated samples were filtered off, washed with distilled water until the wash water was neutral and then solvent dried.

2.12.2. With alkaline H_2O_2

This was carried out by suspending the milled sample (4 g) in 100 ml 2–10% H_2O_2 after adjusting the pH to 10–13 with NaOH. The mixtures were incubated in a water bath at 30–100 °C for 1–5 h. Pretreated samples were filtered off, washed with water and solvent dried.

2.12.3. With peracetic acid

Peracetic acid was prepared just before pretreatment by mixing equal volumes of acetic anhydride and 30% H_2O_2. Pretreatment was carried out by suspending milled samples (4 g) in 100 ml of peracetic acid and the mixtures were incubated in a water bath at 60–100 °C for 20–120 min. The pretreated samples were filtered off, washed with distilled water until the wash water become neutral and then solvent dried.

2.12.4. With $NaClO_2$

This was done by suspending 4 g of milled water hyacinth in 100 ml sodium chlorite (0.1–0.4%, w/v). The mixtures were incubated in water bath at 60–100 °C for 20–120 min, then acidified with acetic acid (1 ml/g $NaClO_2$) and lasted for 60 min. The pretreated samples were then washed with water and solvent dried.

2.12.5. With $NaClO_2$–alkaline H_2O_2

Pretreatment of water hyacinth (4 g) with $NaClO_2$ was achieved as reported above with 0.1% $NaClO_2$ at 70 °C for 1 h after solvent drying, the sample was treated with alkaline H_2O_2 (8%) at pH 13 and 40 °C for 15–60 min. The final sample was solvent dried.

2.12.6. With $NaClO_2$–peracetic acid

Pretreatment of the milled water hyacinth (4 g) was performed with 0.1% $NaClO_2$ at 70 °C for 1 h as indicated above. The solvent dried sample was treated with peracetic acid, as described above at 100 °C for 15–60 min, filtered off, washed with water and solvent dried.

2.13. Solvent drying of pretreated samples

This was done as described by Lee and Fan (1982). The water retained in the pretreated sample was replaced by soaking successively in fresh methanol, and the latter was replaced by benzene and soaking three times. The benzene was removed from the sample in a vacuum oven. All samples were dried at 50 °C for longer than 48 h before use.

2.14. Enzymatic hydrolysis

The reaction mixture consisted of 10 ml of buffered enzyme solution (0.05 M citrate–phosphate buffer, pH 5.0). The enzyme solution obtained from *T. viride* 100 (Abdel-Fattah et al., 1995), contained in units: FP, 40; CMCase, 365; cellobiase, 11.2; xylanase, 222 and 200 mg of pretreated sample. Two drops of 1% sodium azide was then added and the reaction mixture was incubated in an incubator shaker (150 rpm) at 50 °C. Aliquots were withdrawn at different time intervals lasting for 48 h. After centrifugation of each aliquot sample, the supernatant was used for the determination of reducing sugars, total carbohydrate and glucose.

The saccharification value calculated as follows:

$$\text{Saccharification}\ (\%) = \frac{\text{Reducing sugars} \times 0.9}{\text{Carbohydrate content of sample}} \times 100$$

The value of cellulose conversion was calculated as follows:

$$\text{Cellulose conversion}\ (\%) = \frac{\text{Amount of relased glucose}}{\text{Cellulose content of untreated sample}} \times 100$$

3. Results and discussion

3.1. Effect of water hyacinth pretreatments on its composition, cellulose conversion and saccharification

In the present study, pretreatment of the water hyacinth plant was investigated with sodium hydroxide, peracetic acid, alkaline hydrogen peroxide and sodium chlorite. Combined pretreatments under optimum conditions with sodium chlorite and each of sodium hydroxide, peracetic acid and alkaline hydrogen peroxide were also studied. The contents of lignin, cellulose and hemicellulose were investigated and compared with the original untreated sample. The results indicated in Table 1 represent the composition of the pretreated water hyacinth under the optimum pretreating conditions, cellulose conversion and saccharification at enzymic reaction period of 48 h.

The pretreated sample with sodium hydroxide (10%, w/v) for 1 h at 100 °C effected 86% delignification and the removal of about 14% cellulose and 87.5% hemicellulose (Table 1). This pretreated water hyacinth showed 60.35% cellulose conversion and 84% saccharification. The drawback of sodium hydroxide pretreatments is its known solubilization of hemicellulose. In general, these results are in agreement with those reported by many authors (Goyal et al., 2008; Hendriks & Zeeman, 2009; Lin, Ladisch, Voloch, Petterson, & Noller, 1985; Rao, Desphande, Seeta, Srinivassan, & Mishra, 1985; Xiros et al., 2008).

Peracetic acid was more effective than sodium hydroxide in delignifying water hyacinth. This pretreatment with peracetic acid at 100 °C for 120 min afforded a sample which, by enzymic hydrolysis 67.7% cellulose conversion and 91.68% saccharification were achieved for a reaction of 48 h. In that sample peracetic acid effected 94.14% delignification and the removal of 19.15% cellulose and 79.46 hemicellulose. Comparatively the adverse effect of peracetic acid on hemicellulose is less than that of sodium hydroxide. The high cellulose conversion and saccharification of the peracetic acid pretreated water hyacinth sample may be related to the effective delignification occurred. Similar results were reported for other substrates with peracetic acid (Farid et al., 1983; Gharpuray et al., 1983; Taniguichi, Tanaka, Matsuno, & Kamikubo, 1982).

Delignification and accessibility of the water plant material by pretreatment with alkaline hydrogen peroxide depended on the concentration of hydrogen peroxide, pH value of pretreating solution, temperature, and duration of the process (unrecorded data). Pretreatment with 8% hydrogen peroxide at pH 13 at 40 °C for 1 h afforded a sample which, by enzymatic attack, showed 68% cellulose conversion and 79.3% saccharification (Table 1). In this sample solubilization of lignin, cellulose and hemicellulose reached 87.7%, 9.5% and 50%, respectively. Under the aforementioned conditions, extending the pretreatment process to 2.5 h delignification reached about 92%, but the loss of cellulose and hemicellulose reached 18.5% and 64.7%, respectively (data not shown). Accordingly, saccharification of the latter sample increased to 85% but the increase of released glucose was not compensated by the loss of cellulose and hence the value of cellulose conversion decreased to 65.9% (data not shown). Wei and Cheng (1985) reported that pretreatment of rice straw at 60 °C for 5 h in a solution with 1%; (r/w) H_2O_2 and NaOH resulted in 60% delignification and the extent of enzymatic hydrolysis of this sample reached 53.2%. On the other hand, Saha and Cotta (2007) found an increase of total sugars by enzymic saccharification of rice hulls pretreated with alkaline H_2O_2.

Pretreatment of the water hyacinth plant material with 0.1% (w/v) sodium chlorite for 1 h at 70 °C afforded an ideal substrate. This pretreatment effected 83.7% delignification without loss in cellulose or hemicellulose (Table 1). After incubation of this pretreated sample with *T. viride* NRC100 cellulase, 64% of the original cellulose was converted to glucose while the extent of sample saccharification reached 71% (Table 1).

Pretreatments at 70 °C for 1 h with higher sodium chlorite concentrations up to 0.4% (w/v) resulted in more delignification up to 94.8% but this was associated by loss in cellulose and hemicellulose up to 13% and 23.8%, respectively (data not shown). Pretreatment of rice straw, sugarcane bagasse and aspen wood with sodium chlorite was also reported (Adsul et al., 2005; Saddler et al., 1982).

Aiming at increasing the cellulose conversion and saccharification of the sodium chlorite pretreated sample (0.1% chlorite for 1 h at 70 °C) by effecting more delignification it was thought to employ combined pretreatments with sodium chlorite followed by NaOH, alkaline H_2O_2, or peracetic acid separately. Thus, treatment of the $NaClO_2$ sample with 10% NaOH at 100 °C for 15–60 min effected more delignification (96–98%) but solubilized 10–20% of cellulose and 39.5–100% hemicellulose (data not shown). Of the $NaClO_2$ samples that treated with 10% NaOH for 15 min showed the highest cellulose conversion (75.5%) and good saccharification (89%) (Table 1).

Treatment of the $NaClO_2$ sample with 8% H_2O_2 at pH 13 and 40 °C for 15–60 min was more efficient than with NaOH in delignifying the residual lignin (97.5–100% delignification). The drawback of alkaline H_2O_2 is its effective solubilization of hemicellulose. Maximal cellulose conversion (76%) was found with the $NaClO_2$ sample treated with alkaline H_2O_2 for 30 min at 40 °C (Table 1). The action of alkaline H_2O_2 seems not to be limited to delignification. The significant saccharification values reached point out to its amending effect on crystalline cellulose and thus transferring it to accessible cellulose (Eveleigh, Mandels, Andreotti, & Roche, 2009). Almost complete saccharification (97.3%) was obtained with chlorite sample treated with alkaline H_2O_2 at 40 °C and pH 13 for 60 min. The loss in cellulose and hemicellulose components by this treatment, however, lowers the economical value of the pretreatment process.

Treatment of the chlorite sample with peracetic acid at 100 °C for different periods (15–60 min) was the most efficient. Treatment for 45 min was sufficient to bring about complete delignification of the sample. However, the chlorite sample treated for 15 min with peracetic acid at 100 °C showed the highest cellulose conversion (80.8%) by *Trichoderma viside* NRC 100 cellulase. This sample showed also good saccharification (90%) (Table 1). It contained 0.63 hemicellulose. This chlorite–peracetic acid sample fulfilled all the necessary requirements in containing negligible amount of lignin and comparatively the highest amounts of cellulose and hemicellulose all together with the highest cellulose conversion and high saccharification. That sample was therefore used in studying some ambient factors affecting the enzymic saccharification process.

3.2. Ambient factors affecting saccharification of pretreated water hyacinth

3.2.1. Hydrolysis course

Following the hydrolysis course (0.25–48 h) of each pretreated sample, it can be shown that the enzymic saccharification after 6 h was about 50% of that at 48 h. This indicates a show hydrolysis rate by time. The reduction in the hydrolysis rate, in spite of the presence of residual substrate, might presumably be due to partial enzyme inactivation, product inhibition of cellobiose and glucose and recrystallization of amorphous cellulose. In an unrecorded experiment, the cellulose of *T. viride* NRC 100, in absence of substrate, lost 49% of its activity after 48 h incubation at 50 °C and pH 5.0. Although, in presence of substrate, the enzyme would be more stable than in its absence, some loss of enzyme activity is expected during extended periods of hydrolysis. On the other hand, Lee and Fan (1983) indicated that cellobiose is a stronger inhibitor

Table 1
Effect of water hyacinth pretreatment on its composition, cellulose conversion and saccharification.

Pretreating agent	Recovered component (%)			Cellulose conversion (%)	Saccharification (%)
	Lignin	Cellulose	Hemicellulose		
NaOH	14.03	86.1	12.5	60.35	84.0
Peracetic acid	5.86	80.85	20.54	67.7	91.68
H_2O_2	12.3	90.5	50.0	68.0	79.3
$NaClO_2$	16.3	100	100	64.0	71.0
$NaClO_2$ + NaOH	3.96	89.55	60.44	75.5	88.92
$NaClO_2$ + H_2O_2	1.98	92.24	46.82	76.2	90.0
$NaClO_2$ + peracetic acid	2.56	96.69	81.38	80.8	90.11

Composition of untreated water hyacinth (%): lignin, 17; cellulose, 60.0; hemicellulose, 8.0.

Table 2
Effect of enzyme/substrate ratio on the hydrolysis of $NaClO_2$–peracetic acid pretreated water hyacinth with *T. viride* 100 cellulase.

Substrate concentration (mg/ml)	Enzyme/substrate ratio (FPU/g)	Reducing sugars released (mg/ml)	Glucose released (mg/ml)	Saccharification (%)	Cellulose conversion (%)
20	200	18.56	15.58	90.11	80.92
50	80	43.69	35.22	84.2	73.18
100	40	77.0	63.10	74.2	65.54
150	26.6	112.6	85.42	72.2	59.16
200	20	130.72	98.07	62.98	50.94

for cellulose than glucose and suggested that the inhibitory effect of cellobiose is exerted mainly on the initial hydrolysis rate. Following the degree of polymerization of the hydrolysis products during the reaction course with any pretreated sample, it was found that it gradually decreased with time (data not shown), indicating the accumulation of cellobiose in the first few hours which would thereafter partially inhibit cellulose and hence the reaction rate decreases.

On the other hand, amorphous cellulose is known to be more accessible to enzymatic attack than crystalline cellulose. Lee and Fan (1983) and Bertran and Dale (1985) suggested that recrystallization of amorphous cellulose probably occurs during enzymatic hydrolysis of cellulose. Such recrystallization of amorphous cellulose, if accurs, may integrate in showing the enzymatic hydrolysis rate of cellulose (Liu & Ming, 2011).

3.2.2. Effect of enzyme/substrate ration

In this and the succeeding studies the sodium chlorite–peracetic acid sample was used as well as *T. viride* NRC 100 cellulose. In the foregoing saccharification processes a reaction mixture was used containing 40 FP units and 200 mg substrate, i.e., the enzyme/substrate ratio was 200 FPU/g. The effect of this ratio on the extent of saccharification and cellulose conversion was investigated by increasing the substrate (pretreated sample) concentration in 10 ml reaction mixture from 200 mg (2% substrate) up to 2 g (20% substrate) with constant amount of 40 FPU (from 200 to 20 FPU/g). The results (Table 2) indicated that by decreasing the enzyme/substrate concentration from 200 to 20 FPU/g substrate led to the decrease in the calculated saccharification from 90.11% to 62.98% and cellulose conversion from 80.92% to 50.94% after a 48 h reaction. Due to the high substrate concentration, the yield of either reducing sugars or glucose by the 20 FPU/h was about 7-fold that by the 200 FPU/g. The decrease in the hydrolysis rate of the 20 FPU/g sample may be related to the high viscosity of the reaction mixture which has an adverse effect on the mobility of the reactants and the release of products. On the other hand, the concentrations of the released glucose in the reaction mixtures of 200 FPU/g and 20 FPU/g were about 1.56% and 9.8%, respectively. Such high concentration of glucose may thus lead to the decrease in reaction rate by partially inhibiting the cellulose system of *T. viride* NRC 100. Similar observations were previously reported (Chen et al., 2008; Zheng, Pan, Zhang, & Wang, 2009).

3.2.3. Effect of added sugars and ethanol

The effect of added glucose, mannose, fructose, xylose, arabinose, maltose, sucrose and ethanol on the hydrolysis of the chlorite–peracetic acid pretreated sample was investigated. All showed inhibitory effects which depended on the concentration of the added substance (2–8%) (data not shown). Glucose, fructose, and maltose showed the highest inhibitory effects. Addition of 8% glucose at the beginning of the hydrolysis decreased the saccharification to about its half. On the other hand, the inhibitory effect of ethanol was more power full and 8% ethanol brought about complete inactivation of enzyme action. These results are in accord with those reported by other authors (Ferchak & Pye, 1983; Oshima, Ishitani, & Harano, 1985; Vallander & Eiriksson, 1985).

Table 3
Effect of exogenous *A. niger* 1 cellobiase on the hydrolysis of $NaClO_2$–peracetic acid pretreated water hyacinth with *T. viride* 100 cellulase.

Ratio of FPU/cellobiase U		Hydrolysis time (h)				
		1	2	6	24	48
1:0.35	Cellulose conversion (%)	1.47	3.43	9.2	38.2	50.94
	Saccharification (%)	4.26	7.17	18.93	48.2	62.98
1:0.5	Cellulose conversion (%)	1.77	3.68	1091	40.4	52.24
	Saccharification (%)	4.41	7.89	20.22	55.3	67.32
1:1	Cellulose conversion (%)	1.88	4.27	11.34	44.7	59.0
	Saccharification (%)	4.60	9.67	22.4	56.72	70.33
1:2	Cellulose conversion (%)	2.0	4.89	12.22	47.2	60.3
	Saccharification (%)	5.77	11.3	24.5	60.4	74.5

3.2.4. Effect of addition of exogenous cellobiase

The effect of addition of exogenous cellobiase from *A. niger* 1, on the enzymic saccharification of the chlorite–peracetic acid sample was also investigated. The results (Table 3) showed that increasing the ratio of FP–cellulose to cellobiase from 1:0.35 to 1:2 in the reaction mixture led to increase of saccharification from 63%, to 74.5% and cellulose conversion from 51% to 61% after 48 h hydrolysis. It is expected that the removal of cellobiose by exogenous cellobiase reduces the inhibition of cellulose and hence promotion of saccharification occurs. On the other hand, this limited increase in saccharification might be related to the inhibitory effect of the increasing glucose concentration as the hydrolysis proceeded. Addition of cellobiase to enhance the enzymatic hydrolysis of cellulose has also been reported by some authors (Chem et al., 2008; Zheng et al., 2009).

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