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ENZYMATIC SACHARIFICATION OF ALKALINE PRETREATED RICE STRAW BY CELLULASE FROM *CELLULOSIMICROBIUM* SP. MP1

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Abstract. The effects of different physical and technological parameter such as time, substrate to liquid ratio, enzyme concentration, temperature, and pH on enzymatic saccharification of alkaline pretreated straw cellulose were studied. For alkaline pretreatment, the straw was incubated with 10 % NaOH at ratio 1:20 (w/v) at 90 °C for 1 hour. After the alkaline pretreatment the cellulose content increased from 50.2 % (w/w) to 67.3 % (w/w). Enzyme used for saccharification of treated and untreated straw was produced from *Cellulosimicrobium sp* MP1 which was isolated from termite gut. Results from research showed that the highest percentage of saccharification of alkaline pretreated straw was 69.91 %, corresponding to 10.58 mg/mL of reducing sugar. The hydrolysis conditions for reaching this highest saccharification yield were: temperature of 55 ºC, substrate to liquid ratio of 2 g/100 mL, enzyme concentration of 37.5 U/g, pH of 5.5 and hydrolysis time of 48 hours.

Keywords: cellulase, cellulose, Termite gut, *Cellulosimicrobium*.

Classification numbers: 1.3.2, 1.4.3.

1. INTRODUCTION

As a staple food for much of the world, rice production is widespread. However, it also results in the generation of large quantities of non-food biomass, primarily in the form of straw and husks. Although they have been little utilized and much rice straw is still simply burned, these lignocellulosic material have considerable values for production of biofuel or feed [1].

The efficient bioconversion of lignocellulosic feedstock to cellulose biofuel via the sugar platform involves three key steps: feedstock pretreatment, enzymatic saccharification, and fermentation or catalytic conversion of sugars. Enzymatic saccharifications has been identified as one of the costliest step in cellulosic biofuel production [2].

A pretreatment step such as using dilute acid, ammonia, alkaline green, etc., is required to remove the nature resistance of lignocellulose cell wall for efficient enzymatic saccharification

[3]. The purpose of pretreatment is to remove lignin and hemicelluloses, reduce cellulose crystallinity, and increase the porosity of materials.

Cellulosimicrobium sp. MP1 was isolated from termites gut, showing ability to biosynthesize cellulase including endoglucanase, exoglucanase, β-glucosidase and xylanase and therefore is potential candidate for enzymatic saccharification of lignocellulosic material. The aim of this study is to investigate the ability of crude enzyme produced from *Cellulosimicrobium* sp. MP1 to hydrolyse the abundant lignocellulosic material in Viet Nam as straw. There are various factors, which effect the enzymatic hydrolysis rate such as substrate concentration, cellulase activity and reaction conditions (temperature, pH, enzyme concentration and etc.). The enzymatic hydrolysis rate can be improved by optimizing these conditions [4].

2. MATERIALS AND METHODS

2.1. Materials

Chemicals including 3,5 – Dinitrosalicylic acid (DNS) (Sigma, USA), CMC (Japan), avicel and pNP- β- glucopyranoside (pNPG) (Sigma, USA), birch wood xylan (Sigma, USA), xylose (Merck, Germany), glucose (Germany), NaOH (England), Na-K tartarate (England), Na metabisulfite (England), citric acid monohydrate (Germany) and the other chemicals of analytical grade were from China.

2.2. Method

2.2.1. Cellulase production

The *Cellulosimicrobium* sp. MP1 isolated from termite gut was used as a cellulase-producing strain. The cellulase production was performed at 37 ^{o}C and 150 rpm in production media composed of KH_2PO_4 0.5 g/L; $MgSO_4$ 0.25 g/L; Peptone 2 g/L, KCl 2.5 g/L, cellulose 5 - 10 g/L, pH adjuted to 6.8 - 7.2 (Gupta, 2011). Broth culture was incubated for 3 days, then centrifuged at 6000 rpm and 4 ^{o}C for 15 min. Supernatant was collected and was stored as crude enzyme preparation at 4 ^{o}C. The crude enzyme preparation possesses the endoglucanase activity of 3.05 U/ml, FPU of 0.1 U/mL, exoglucanase of 0.28 U/mL, β-glucosidase of 0.153 U/mL, xylanase of 0.55 U/mL.

2.2.2. Alkaline pretreatment of straw

Straw was throughly washed, chopped and dried until constant weight with following grinding for 10 min in a grinder and was used as the feedstock for pretreatment. The straw were alkaline pretreated according to method reported by Tsai *et al*. [5]. Briefly, the straw were incubated with 10 % NaOH at solid to liquid ratio of 1:20 (w/v) at 90 ^{o}C for 1 hour and the biomass (insoluble fraction) were collected and washed with tap water until the pH became neutral. After dried to remove water, the pretreated biomass was stored in sealed plastic bag at 4 ^{o}C. The pretreated biomass was dried in an oven at 65 ^{o}C for 24 hours.

2.2.3. Enzymatic saccharification

The enzymatic hydrolysis experiments were carried out in 250 mL flasks with a working volume of 100 mL. Alkaline treated straw biomass were hydrolyzed with crude enzyme

produced as described above. The flasks were incubated at 50 ºC. Sample aliquots were taken at different times, centrifuged and the supernatants were analyzed for reducing sugar. The percentage of saccharification was calculated based on the following formula:

$$\%\ \text{saccharification} = \frac{\text{reducing sugar} \times 0.9}{\text{cellulose+hemicellulose}} \times 100 \quad [6]$$

2.2.4. Analytical methods

The composition of the straw samples before and after pretreatment was determined according to a published method by NREL [7].

2.2.5. Enzyme assay

The enzyme activities of endoglucanase, exoglucanase, β-glucosidase were measured by spectrometric determination of reducing sugars at 540 nm by 3, 5-dinitrosalicylic acid (DNS) method as described by Ghose [8]. Endoglucanase, exoglucanase and β-glucosidase activities were determined using carboxymethyl cellulose, avicel and pNP- β- glucopyranoside (pNPG) as substrates, respectively. The xylanase activity was determined at the same condition as cellulase activity only using other substrate. Xylanase activity was determined by measuring the release of xylose from birch wood xylan. The amount of released reducing sugar was determined based on a standard curve which was constructed using the standard solutions of glucose and xylose. One unit of enzymatic activity was defined as the amount of enzyme that released 1 μmol of reducing sugar per minute.

2.2.5. Factors affecting enzyme saccharification of straw biomass

Various parameters such as hydrolysis time, temperature, substrate concentrations, cellulase concentration and pH were studied to find out the best hydrolyzing condition. The obtained optimized condition from previous experiment was used for the next optimization experiment unless otherwise stated. The optimization saccharification time was determined by withdrawing the sample at 6, 18, 24, 36, 48 and 72 h. To determine the optimum temperature of saccharification, the reaction mixture was incubated at different temperatures ranging from 40ºC to 70ºC. Three different solid loadings of 1, 2 and 3 (w/v) were investigated in the batch enzymatic hydrolysis. Concentration of enzyme varied from 7.5 U/g to 75 U/g.

3. RESULTS AND DISCUSSION

3.1. Pretreatment of straw

Rice straws contain a combination of cellulose, hemicellulose, lignin and ash. Prior to pretreatment, the straw composed of 38.4 % cellulose, 26.7 % hemicellulose, and 13.8 % lignin on a dried mass basis (Table 1).

Compared to the number reported by Goodman [1], our results showed the similarity. After the alkaline pretreatment the cellulose content in the biomass increased significantly from 38.4 % to 55.3 % due to the removal of hemicellulose and lignin. The alkaline treatment removed a significant amount of hemicellulose and some amount of lignin. The cellulose content became the major component of straw after treatment (55.3 %). Straws before and after alkaline pretreatment were used as substrate for enzyme saccharification. Enzymatic saccharificaion was

studied using the crude enzyme of *Cellulosimicrobium* sp. MP1. The saccharification was carried out at 50 ºC with substrate (g)/ liquid (mL) ratio of 1/100 (w/v), enzyme concentration of 150U Endoglucanase/g. Released reducing sugar after saccharification was determined after 6, 18 and 24 hours and result was shown in Table 2.

Table 1. Composition of straw before and after pretreatment (% db).

Substrate	Cellulose	Hemicellulose	Lignin
Straw untreated	38.4 ± 1.71	26.7± 1.3	13.8± 0.5
Straw pretreated	55.3 ± 1.43	12.8± 1.7	11.6± 1.3

Table 2. Effect of pretreatment on saccharification yield.

Duration time (hours)	Pretreated straw		Untreated straw	
	Reducing sugar concentration(mg/mL)	Saccharification (%)	Reducing sugar concentration(mg/mL)	Saccharification (%)
6	3.11 ± 0.26	41.10	1.59 ± 0.33	21.98
18	3.56 ± 0.25	47.04	2.07 ± 0.27	28.61
24	4.20 ± 0.32	55.50	2.19 ± 0.37	30.27

Table 2 showed that sacharification yield of alkaline pretreated straw was higher than untreated one. Furthermore prolonging time resulted on higher percentage of saccharification. Whithout pretreatment, the saccharification could achieve 30.27 % after 24 hours, which was about 1.8 times lower than that with preatreatment (55.50 %).

3.2. Effect of time on enzymatic hydrolysis

For the improvement of enzymatic hydrolysis, it is necessary to optimize the critical process parameters such as optimum cellulase loading, temperature, saccharification time and substrate to liquid ratio etc. The effect of temperature on saccharification yield was presented on Table 3.

Table 3. Effect of hydrolysis time on saccharification yield.

Duration time (hours)	Reducing sugar concentration (mg/mL)	Saccharification (%)
0	0.09 ± 0.02	1.19
6	3.13 ± 0.33	41.36
18	3.56 ± 0.25	47.05
24	4.20 ± 0.32	55.50
48	5.06 ± 0.31	66.87
72	5.09 ± 0.38	67.26

The enzymatic hydrolysis of pretreated straw biomass at biomass concentration of 1 % w/v proceeded rapidly till 48 hours reaching 66.87 % saccharification yield, corresponding to 5.06 mg/mL reducing sugars. Afterwards there was no significant increase in saccharification yield (Table 3). Therefore, hydrolysis time of 48 hours was chosen for the next experiment. The

similar result was also reported by Chiranjeevi *et al*. [9]. They also found that the reducing sugars released rapidly up to till 48 hours and only slightly increased afterwards. Since the pretreated straw composed of only 12.8 % hemicellulose, i.e only about 1.41 mg/mL of reducing sugar could be released when hemicellulose is hydrolysed completely, it could be concluded that cellulose was hydrolysed already at first 6 hours of hydrolysis. The very fast saccharification rate during the first 6 hours of hydrolysis could be explained by sufficient number of enzymatic accesible substare sites, which became limited during prolonged hydrolysis [10].

3.3. Effect of temperature on enzymatic hydrolysis

The enzymatic hydrolysic of pretreatment straw at biomass concentration of 1 % w/v was carried out at 40 ºC to 70 ºC for 48 hours. The results were presented on Table 4.

Table 4. Effect of hydrolysis temperature on saccharification yield.

Temperature (ºC)	Reducing sugar concentration (mg/mL)	Saccharification (%)
40	3.31 ± 0.35	43.74
50	5.06 ± 0.4	66.87
60	4.05 ± 0.35	53.52
70	2.54 ± 0.35	33.56

Table 4 showed that the maximum hydrolysis of pretreated substrates occurred at 50 ºC corresponding to degree of saccharification of 66.87 %. The temperature of 50 ^{o}C is also optimum temperature for endoglucanase from *Cellulosimicrobium* sp. MP1. The reducing sugar decreased almost 20 % at 60 ^{o}C (Table 4) in accordance with the reduction of 30 % of endoglucanase activity (data was not presented).

3.4. Effect of substrate concentration on the enzymatic hydrolysis of pretreated straw

Three different solid loadings of 1, 2 and 3 % (w/v) were investigated in the batch enzymatic hydrolysis. It was observed that the amount of reducing sugars released increased with the increase of the substrate concentration while the percent saccharification was reduced (Table 5). Indeed maximum sugars were released at the ratio of substrate to enzyme of 3 %, meanwhile the sacchrification yield was highest at the ratio of 1 %. However, the saccharification yield was only slightly decreased at the ratio of 2 %.

Table 5. Effect of solid to liquid ratio on saccharification yield.

Solid to liquid ratio (g/mL)	Reducing sugar concentration (mg/mL)	Saccharification (%)
1:100	5.06±023	66.87
2:100	10.1±0.35	66.74
3:100	11.34±0.24	49.95

Similar phenomenom have also been reported [9], which could be explained by lower enzyme to substrate ratio. Beside high solid load can lead to retarding of enzyme difussion into the substrate so longer incubation time might be required. In addition, the low saccharification

could be attributed to the inaccessible insoluble cellulose. The solid load of 2 % was used for the next experiment.

3.5. Effect of cellulase concentration on the enzymatic hydrolysis of pretreated straw

When the amount of enzyme was increased from 7.5 to 75 U/g substrate, the reducing sugar concentration increased significantly from 2.96 to 10.1 mg/mL, which corresponded to the increase of percent saccharification from 19.56 % to 66.74 % after 48 hours of incubation (Table 6). Further increase of enzyme concentration from 37.5 U/g to 75 U/g resulted in no increase of reducing sugar. The enzyme concentration generally had a great effect on reducing hydrolysis time. In this case, at enzyme concentration of 37.5 U/g, 48 hours seemed long enough for obtaining maximal saccharification yield. For that reason, the enzyme concentration of 37.5 U/g was chosen for the next experiment. Compared to the results from Table 3, the "site limiting theory" is true again in this case when the decrease on saccharification yield could be negligible (66.74 % compared to 67.24 %) despite much lower enzyme concentration (37.5 U/g compared to 150 U/g) and shorter hydrolysis time (48 hours compared to 72 hours).

Table 6. Effect of enzyme concentration on saccharification yield.

Enzyme/Substrate (U/g)	Reducing sugar concentration (mg/mL)	Saccharification (%)
7.5	2.96 ± 023	19.56
15	5.67 ± 0.35	37.46
30	8.87 ± 0.30	58.61
37.5	10.10 ± 0.24	66.74
75	10.12 ± 0.25	66.87

3.6. Effect of pH on the enzymatic saccharification of pretreated biomass

pH is a very important parameter since pH affects the enzyme activity and in consequence the saccharification yield. In this experiment, enzymatic saccharification was carried out at different pH values from pH 4.0 to pH 7.0.

Table 7. Effect of pH on the Saccharification yield.

pH	Reducing sugar concentration (mg/mL)	Saccharification (%)
4.0	2.56 ± 0.35	16.91
4.5	8.85 ± 0.45	58.48
5.0	10.35 ± 0.25	68.39
5.5	10.58 ± 0.37	69.91
6.0	10.55 ± 0.37	69.71.
6.5	10.15 ± 0.39	67.07
7.0	10.1 ± 0.25	66.74

Table 7 showed that high reducing sugar was achieved at pH range from pH 5.0 to pH 7 with optimum at pH 5.5 - 6. The reducing sugar concentration was only slightly reduced at pH 6.5 and 7. The results are in accordance with the characteristics of endoglucanase from

Cellulosimicrobium sp. MP1, which possesses similar high activity and is stable in wide range of pH from 5-7 (data is not presented).

Thus the highest saccharification yield of 69.91 %, corresponding to reducing sugar concentration of 10.58 g/L or 529 mg/g substrate was achieved with alkaline pretreated straw. The reducing sugar concentration of 600 mg/g was achieved with cellulase from *Trichoderma harzianium* SNRS3 [11] but only 237.8 mg/g reducing sugar was released by crude cellulase from *Aspergilus niger* BK01 [12]. The results suggested that cellulase from *Cellulosimicrobium* sp. MP1 could be used for hydrolysis of lignocellulosic material.

4. CONCLUSIONS

The present study was aimed to investigate the enzymatic hydrolysis of straw biomass using crude cellulase produced by *Cellulosimicrobium* MP1 isolated from gut of termite. The alkaline pretreated biomass reached maximum saccharification yield of 69.91 % at 50 ºC, hydrolysis time of 48 hours, substrate to liquid ratio of 2 g/100 mL, cellulase concentration of 37.5 U/g and pH of 5.5. Compared to repoted publications, this saccharification yield in this study was rather good. Further study is needed to determine the composition of reducing sugars in the hydrolysates.

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