Successive Saccharification and Fermentation of Cellulosic Agricultural Residues Using a Combination of Cellulase and Recombinant Yeast

Xuzhe Zhao^{*1}, Xianxiang Liang^{*1}, Shuqin Han^{*1}, Toshiyuki Uryu^{*1}, and Takashi Yoshida^{*1,*2,#}

^{*1}Department of Bio and Environmental Chemistry, Kitami Institute of Technology,
165 Koen-cho, Kitami 090-8507, Hokkaido, Japan

^{*2}Research Center for Environmentally Friendly Materials Engineering,
Muroran Institute of Technology, 27-1 Mizumoto-cho, Muroran 050-8585, Hokkaido, Japan

Abstract: Bioethanol was produced from agricultural residues in Hokkaido Prefecture, Japan, by dilute alkali pretreatment, and then successive saccharification with cellulase and fermentation with recombinant yeast pYBGA1 that is a recombinant laboratory yeast expressing β -glucosidase. The agricultural residues, beet pulp, beet leaf, corn stover, and weed were pretreated with 2% NaOH aqueous solution for 1 h at 121°C to remove lignin. The pretreated beet pulp (20 g/L), for example, was then saccharified for 24 h by Cellic CTec (10 wt% to beet pulp) as the cellulase at 50°C to give glucose (11.8 g/L) at relatively good rates. After the temperature decreased to 30°C, the resulting glucose was continuously fermented for 36 h by addition of 1×10^8 cells/mL of pYBGA1 yeast to give 5.54 g/L of bioethanol in 60% and 92% theoretical yields of cellulose in beet pulp and glucose saccharified, respectively. Other agricultural residues also afforded bioethanol by the successive saccharification and fermentation. It was found that the appropriate cellulase for saccharification differed with the kind of agricultural residue; beet pulp was effectively saccharified by Cellic CTec, beet leaf and corn stover by Sucrose C, and weed by Sumizyme C. These agricultural residues were found to be suitable for the production of bioethanol because Hokkaido Prefecture is the most abundant producer of beets and corn in Japan, and accordingly, agricultural residues are easily available. pYBGA1 was also found to be a good yeast for the fermentation of sugars saccharified from agricultural residues.

Keywords: Agricultural residue, Hokkaido Prefecture, Cellulases, pYBGA1 yeast, Successive saccharification and fermentation, Bioethanol

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1. Introduction

Bioethanol produced from renewable plant resources is expected to provide an environment-friendly and clean energy to replace fossil fuels. The raw materials currently used for the industrial process are mainly starches of grains and corn, which are foods. However, cellulosic bioethanol from non-food plants is becoming increasingly attractive because of the great abundance of cellulose produced by plants on earth [1]. Although both starch and cellulose are composed of glucose, unlike starchy biomasses, native cellulose is highly resistant to enzymatic saccharification because it has crystalline regions in the molecule that are present as a lignin-cellulose complex. Lignin inhibits enzymatic saccharification and fermentation. Therefore, pretreatment is needed to relax the highly crystalline

structure and to remove lignin. Although the crystalline region was completely hydrolyzed to glucose by concentrated sulfuric acid [2], a large amount of toxic sulfuric acid is required and it is difficult to recover. Therefore, industrial saccharification of cellulosic raw materials to glucose is accomplished by use of dilute sulfuric acid, followed by fermentation with yeast [3,4].

Several investigations of the production of bioethanol from cellulosic raw materials have been reported. Microorganisms appropriate for cellulase production and experimental conditions for saccharification were also reported. Among them, *Panicum virgatum*, commonly known as switchgrass, was pretreated by soaking in ammonium hydroxide (30%) at 15°C to 30°C in pilot scale bioreactors. Simultaneous saccharification and fermentation of the pretreated switchgrass with *Saccharomyces cerevisiae* gave 73% of the theoretical yield bioethanol in the 50 L reactor and 52-74% in the 350 L reactor [5]. An alkali pretreated rice

corresponding author

straw was saccharified by a microorganism to give glucose as a main component in the hydrolyzates, suggesting that *Tricoderma* ZM4-F3 is an effective and useful fungus for cellulase production [6]. Corn stover was used as a substrate for enzymatic saccharification by a cellulase produced from *Penicillium funiculosum*. The conversion of hemicellulose and cellulose in corn stover pretreated with dilute NaOH was more than 80% after 24 h of saccharification when using a 5 g/L initial concentration with 80 units/mL of the enzyme, respectively [7,8,9]. Extracellular enzymes produced by *Phanerochaete chrysosporium* degraded lignin in corn fiber and saccharified it, and the subsequent simultaneous saccharification and fermentation of the hydrolysates using *Saccharomyces cerevisiae* enhanced bioethanol production [10]. The sugar composition and enzymatic saccharification of fractionated beet pulp, which is a residue after extraction of sugar from beets, were carried out to investigate microorganisms having cellulase, and indicated that *Aspergillus niger* enzyme produced much higher saccharification of beet pulp [11].

Previously, we reported a two-step acid hydrolysis of tissue paper, cotton, and sawdust, with the result that whole celluloses including the crystalline region were hydrolyzed to obtain a mixture of glucose and cello-oligosaccharides; that is, these cellulosic materials were partially hydrolyzed by concentrated sulfuric acid (80 wt%) in the first step, and then the hydrolysis was continued in the second step with hot sulfuric acid that was diluted with water. The hydrolyzates from tissue paper, cotton, and sawdust were fermented with recombinant pYBGA1 yeast converting β -glucosidase into ethanol in good yields [12,13]. We also reported that ethanol was produced at good rates by direct saccharification and fermentation of cello-oligosaccharides with pYBGA1 yeast. For example, cellobiose in the concentration of 50 g/L was directly fermented for 60h with 1×10^8 cells/ml of pYBGA1 yeast at 30°C to give ethanol at an 80% theoretical conversion rate and a concentration of more than 20 g/L [14]. These results suggest that pYBGA1 yeast has some advantages as a simple procedure for effective fermentation of glucose and cello-oligosaccharides and for reducing time, cost, and energy consumptions [14].

Cellulosic agricultural residues are suitable raw materials for the production of bioethanol in local areas because they are wastes, not foods, and bioethanol, especially, is an ideal energy source for local production and local consumption. Hokkaido Prefecture is in the northern part of Japan, and agriculture is one of its

important industries [15]. Among the locally available agricultural products, beets and corn are the most abundant in Hokkaido Prefecture. The recent 5-year average of the productions was 3663 kt and 111 kt for beets and corn, respectively [16,17]. Therefore, these agricultural residues are promising raw materials for the production of bioethanol while protecting the environment, but effective application of agricultural residues has not been achieved yet.

In this paper, we report the saccharification of cellulosic agricultural residues by several cellulase enzymes, followed by fermentation of the resulting glucose by recombinant pYBGA1 yeast. A combination of cellulase and pYBGA1 yeast was also investigated with each agricultural residue.

2. Materials and Methods

2.1 Materials

Beet pulp was supplied by a local company, Hokkaido Togyo Co. Ltd., Hokkaido, Japan. Beet leaf and corn stover were provided by a local farm in Kitami City, Hokkaido. Weeds were obtained from the lawn of our university. These agricultural residues were washed several times with water and then dried in an oven at 40°C. Thereafter, the residues were shredded and ground to a powder by an absolute mill. The powdered raw materials were stored in sealed glass bottles at low temperature in a desiccator for further use. pYBGA1 yeast was kindly supplied by the National Research Institute of Brewing and was pre-incubated on agarose medium with cellobiose as a carbon source according to the procedure described in our previous paper [14]. The pYBGA1 yeast cells were counted using a cell counter. Cellulases used in this work, Cellic CTec (Novozymes Japan Ltd., 1000 EGU/g), Sucrase C (Mitsubishi-Kagaku Foods Corp., 3000 U/g), Sumizyme C (Shin Nihon Chemical Co.,Ltd., 1500 U/g), Meicelase (Meiji Seika Pharma Co.,Ltd., 400 FPU/g), Sumizyme AC (Shin Nihon Chemical Co.,Ltd., 2000 U/g), GODO-TCL (Oenon Holdings, Inc., 100 FPU/g), and “Onozuka”RS (Yakult Honsha Co.,Ltd., 20000 U/g) were obtained from the respective companies. Yeast extract and peptone were purchased from Merck Chemicals, Japan, and Kyokuto Pharmaceutical Industrial Co. Ltd., Japan, respectively.

2.2 Measurement

Thermogravimetric analysis (TGA) was conducted with a Shimadzu DTG-60 thermoanalyzer during heating at 10°C/min under a nitrogen atmosphere. Saccharified sugars were measured quantitatively at 40°C by a reverse-

phase HPLC system with a Tosoh TSK-gel amide-80 column (5 μ m, 4.6 mm $\times$ 250 mm) eluted with a 1 : 1 acetonitrile and water solution at a flow rate of 0.5 mL/min with a Tosoh RI detector using glucose as a standard in the calibration curve. The concentration of ethanol produced was determined under a nitrogen atmosphere by a Shimadzu GC-8A gas chromatograph with a Shimadzu capillary column (SE-30, 3.2 mm $\times$ 30 m) and a hydrogen flame ionization detector. The column and injection temperatures were 60°C and 130°C, respectively.

2.3 Component analysis of agricultural residues

The content of water and ash in dried agricultural residues used here was performed by the TGA as shown in Figure 1. The concentrations of cellulose, hemicellulose, and lignin in the powdered agricultural residues before and after alkaline pretreatment were determined according to the modified method of the National Renewable Energy Laboratory (NREL), USA [18].

Fig. 1 Thermogravimetric analysis of dried beet pulp on the first heating run (10 °C/min) under N₂. The heating was kept for 10 min at 110 °C.

2.4 Dilute alkali pretreatment of agricultural residues

A typical procedure for the pretreatment of the agricultural residues with dilute alkali solution was as follows. The dried and powdered beet pulp (5.0 g) was treated with 2% aqueous NaOH (50 mL) for 1 h at 121°C in an autoclave. The pretreatment was carried out at 121°C in this work because the higher temperature gave by-products by an excessive degradation of cellulose and hemicellulose [19,20]. The residues were collected by centrifugation and then washed several times with water to neutral pH. The resulting beet pulp was dried in the oven at 50°C under vacuum to give 2.2 g of alkali treated beet pulp. Other agricultural residues (5.0 g each sample), beet leaf, corn stover, and weed, were also pretreated by the same procedure as above to give 2.1-2.3 g of pretreated residues, respectively.

2.5 Successive saccharification and fermentation of alkali-pretreated agricultural residues

A typical procedure for the successive saccharification and fermentation of alkali- pretreated agricultural residues is as follows. In a 300mL Erlenmeyer flask, 100 mL of deionized water was added to 2.0 g of the alkali-pretreated powdered beet pulp. The pH of the solution was adjusted to 4.5 by dilute aqueous HCl, and then Cellic CTec (10 wt% of beet pulp) was added. The flask was sealed with a sponge stopper and the mixture was incubated in an oven for 24 h at 45°C using gentle stirring by a rotary shaker. After lowering the temperature of the saccharified mixture to 30°C, 10 g/L of yeast extract and 20 g/L of peptone were added. The pH of the solution was adjusted to 5.0 with dilute aqueous NaOH and then pYBGA1 yeast (1×10^8 cells/mL) was added to the saccharified solution. The fermentation was continued at 30°C for further 36 h in an oven with gentle stirring using a rotary shaker. The saccharified glucose and fermented ethanol was sampled using a syringe with a long needle every 6 h to measure the concentration of the products.

3. Results and Discussion

3.1 Alkali-pretreatment of agricultural residues

Table 1 shows the results of pretreatment of dried and powdered agricultural residues used in this work with 2% aqueous NaOH solution for 1 h at 121°C in the autoclave. Dilute acid pretreatment was also reported, but, toxic furfural derivatives were produced as a by-product [2,3,13]. Therefore, we used a dilute alkali for the pretreatment to remove lignin, which inhibits enzymatic saccharification, followed by fermentation. As shown in Table 1, the concentrations of cellulose and hemicellulose of beet pulp increased and decreased, respectively, to 74.0 wt% and 10.9 wt% from 58.3 wt% and 16.7 wt%. The lignin concentration decreased to below one third.

Table 1 Component of agricultural residues in Hokkaido prefecture, Japan, before and after alkaline pretreatment^a.

	Beet pulp		Beet leaf		Corn stover		Weed	
	Before	After	Before	After	Before	After	Before	After
	wt%	wt%	wt%	wt%	wt%	wt%	wt%	wt%
Cellulose	58.3	74.0	36.6	61.5	46.2	66.4	44.5	53.6
Hemicellulose	16.7	10.9	23.3	13.2	28.0	19.5	30.8	28.3
Lignin	4.2	1.2	9.4	2.2	10.6	2.8	11.2	7.8
Water	5.6	6.5	10.1	11.3	5.4	5.8	5.2	6.3
Ash	2.5	2.2	18.0	11.5	6.5	4.8	6.2	3.7
Others	12.7	5.2	2.6	0.3	3.3	0.7	2.1	0.3

a) Alkaline pretreatment was carried out with 5 g of each agricultural residue in 2% NaOH aqueous solution for 1 h at 121 °C in an autoclave. Pretreated agricultural residues (2.1 ~ 2.3 g) were obtained.

The cellulose, hemicellulose, and lignin concentrations of other residues also showed the same tendency as beet pulp before and after pretreatment. The pretreatment caused a slight loss of polysaccharides but significantly reduced the lignin concentration of the agricultural raw materials, enhancing enzymatic saccharification.

3.2 Successive saccharification and fermentation of agricultural residues

We found recently that pYBGA1 yeast has higher fermentation ability than that of commonly used Sake yeast Kyokai no. 7 (K7) for brewing [21]. For example, after saccharification of cellulose (20 g/L) with 1 wt% (to cellulose) of Acremonium cellulase (Meiji Seika Pharma Co. Ltd.), ethanol was obtained in 2.6 g/L and 6.8 g/L, respectively, by fermentation with 1×10^8 cells/mL of K7 yeast and with 1×10^8 cells/mL of pYBGA1 yeast [22]. Therefore, we used pYBGA1 yeast in this work.

Figure 2 shows the successive saccharification and fermentation 20 g/L of alkaline-pretreated beet pulp with addition of 10 wt% of Cellic CTec as the cellulase to the feed and with 1×10^8 cells/mL of pYBGA1 yeast. The weight of the beet pulp decreased rapidly during the first 24 h at 45°C and pH 4.5 after starting saccharification, and then the weight was almost constant at 0.2-0.15 g until 168 h. As the saccharification proceeded, the concentration of glucose increased rapidly, and 11.8 g/L of glucose was produced after 24 h of saccharification. After 24 h saccharification, the temperature and pH of the saccharification solution had decreased, and we increased it to 30°C and 5.0 to obtain the optimal condition of fermentation. Then pYBGA1 yeast was added to the saccharified solution, and the fermentation was continued for 144 h further after addition of yeast extract and

Fig. 2 Time course of successive saccharification and fermentation of alkaline pre-treated beet pulp powder with Cellic CTec (10 wt% of beet pulp) as the cellulase and 1×10^8 cells/mL of pYBGA1 yeast. The saccharification was carried out for 24 h at 45 °C and pH 4.5, and fermentation for further 144 h at 30 °C and pH 5.0 after addition of yeast extract and peptone.

peptone. After 36 h of fermentation, pYBGA1 produced the highest concentration of ethanol at 5.54 g/L in 60% and 92% theoretical yields based on cellulose content in beet pulp and glucose saccharified, respectively. After fermentation for 60 h, the ethanol concentration decreased gradually, suggesting that ethanol produced was being consumed by pYBGA1 yeast as a carbon source. Glucose had almost disappeared after 48 h of fermentation. Both glucose production and ethanol consumption with the cellulases used in this work and with pYBGA1 yeast for beet leaf, corn stover, and weed had almost the same tendency as those of the combination of Cellic CTec and pYBGA1 yeast shown in Figure 2. These results suggest that saccharification occurred for 24 h, and then the fermentation was continuously performed for a further 48 h.

Table 2 represents the results of successive saccharification and fermentation of dilute alkali-pretreated beet pulp with several cellulases with saccharification for 24 h and then saccharified glucose fermented with pYBGA1 yeast for further 48 h. Cellulase played an important part in the saccharification of plant raw materials. The saccharification was carried out at the optimal pH and temperature for each cellulase. With Cellic CTec as a cellulase, glucose (11.8 g/L) was obtained in 72% conversion based on the concentration of cellulose. Sucrase C as a cellulase gave glucose (9.6 g/L) at 58%. When Meicelase was used, glucose was produced at 8.0 g/L at 49%. Glucose was also obtained from other cellulases, Sumizyme AC, GODO-TCL, and "Onozuka" RS, but the conversion rates were somewhat lower. Therefore, for the 24 h saccharification of beet pulp, Cellic CTec was found to be an effective cellulase.

For the fermentation, yeast extract and peptone were successively added to the saccharified solution, the temperature was decreased to 30°C, and the pH was adjusted at 5.0 for the optimal fermentation condition of pYBGA1 yeast. After adding pYBGA1, the fermentation was continued for a further 144 h. Saccharified glucose

Table 2 Successive saccharification and fermentation of beet pulp with cellulases^a.

Cellulase	Saccharification					Fermentation ^b			
	wt%	Temp °C	pH	Sugar ^c g/L	Conversion %	pYBGA1 x10 ⁸ cell/ml	Ethanol ^c g/L	% ^d	% ^e
Cellic CTec	10	45	4.5	11.8	72	1	5.54	60	92
Sucrase C	10	45	5.0	9.6	58	1	4.56	59	93
Sumizyme C	15	55	4.5	8.9	54	1	4.04	43	89
Meicelase	10	45	4.5	8.0	49	1	3.68	39	90
Sumizyme AC	15	55	4.0	7.0	43	1	3.14	34	88
GODO-TCL	10	55	4.5	6.5	39				
"Onozuka" RS	10	45	4.5	6.0	37				

a) Dry beet pulp (2 g) in water (100 ml) was used.

b) The saccharification was performed for 24 h and then fermentation was started by using pYBGA1 yeast for further 48 h at 30°C and pH 5.0 after addition of yeast extract and peptone.

c) The concentration of glucose and ethanol was determined by HPLC and GC, respectively.

d) Based on cellulose content of alkaline-pretreated agricultural residues.

e) Based on glucose saccharified.

was converted to ethanol at the rates of around 90%. The pYBGA1 yeast also has a high ability to directly ferment cello-oligosaccharides to ethanol [12,14]. pYBGA1 itself produced β -glucosidase on the cell wall, and cello-oligosaccharides are hydrolyzed into glucose, followed by fermentation into ethanol. Therefore, ethanol was directly obtained from cello-oligosaccharides that were saccharified from cellulose by exo-type cellulases. Among the cellulase complexes used in this work, cellulases, including the exo-type cellulases Sumizyme C and Meicelase, should saccharify beet pulp to glucose and cello-oligosaccharides, which were fermented with pYBGA1 to give ethanol. On the other hand, because Cellic CTec is also a complex type cellulase, glucose is the only sugar saccharified with β -glucosidase. Therefore, pYBGA1 fermented the glucose to give ethanol.

Table 3 summarizes the successive saccharification and fermentation of agricultural residues under the optimal conditions. Agricultural residues at 20 g/L and cellulase at 10 wt% or 15 wt% of the feed were used. As shown in Table 2, several cellulases were tested to determine the optimal saccharification condition. For beet leaf and corn stover, Sucrase C was the most effective cellulase for saccharification and produced glucose at 9.4 and 7.1 g/L, respectively, at 60% and 43% conversions based on the cellulose concentration for 24 h saccharification. Weed was saccharified with Sumizyme C to give 6.1 g/L of glucose at 51% conversion. Thus, the most appropriate cellulase for the saccharification depended on the kind of agricultural residue. As shown in Table 1, although the cellulose concentration in weed was lower than that of other agricultural residues, it was considered a convenient raw material because of the abundance of plant waste in general. The fermentation shown in Table 3 was also carried out successively with pYBGA1 yeast for 48 h at 30°C and pH 5.0. Ethanol was obtained at good conversion rates based on sugars in each saccharified agricultural residue.

4. Conclusion

As described above, cellulose in agricultural residues was mainly converted to ethanol; that is, the successive saccharification and fermentation of agricultural residues were carried out by a combination of cellulase for saccharification and pYBGA1 yeast for fermentation under the optimal conditions to give ethanol at good conversion rates. We tested several cellulases for saccharification of the agricultural residues. Among them, Cellic CTec was the most effective for beet pulp, Sucrase C for beet leaf and corn stover, and Sumizyme C for weed, respectively, for the 24 h saccharification. When beet pulp was saccharified with Cellic CTec for 24 h at pH 4.5 and 45°C, 11.8 g/L of glucose was obtained in 72% conversion based on the cellulose concentration of the alkali-pretreated beet pulp. The fermentation was successively performed by adding pYBGA1 yeast for further 36 h at 30°C and pH 5.0 to produce 5.54 g/L of ethanol at 60% and 92% conversions of cellulose content in beet pulp and of the glucose saccharified, respectively. Other agricultural residues, beet leaf, corn stover, and weed, gave ethanol at 91 and 96% conversions, respectively, in each optimal condition. However, based on the cellulose concentration, the conversion to ethanol decreased slightly, probably because of incomplete saccharification of cellulose having crystalline moiety. In this work, we mainly investigated successive saccharification and fermentation of glucose saccharified from cellulose in the agricultural residues in Hokkaido Prefecture. Bioethanol production fermented by cello-oligosaccharides and pentoses from cellulose and hemicellulose in agricultural residues will be further investigated to find the best combination of cellulase, hemicellulase, and yeast for good conversion rates.

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Table 3 Successive saccharification and fermentation of agricultural residues under optimal condition^a.

Sample	Saccharification ^b				Fermentation ^b					
	Cellulase	wt%	Temp °C	pH	Glucose ^c g/L	Conversion %	pYBGA1 x10 ⁹ cell/ml	Ethanol ^c g/L	% ^d	% ^e
Beet pulp	Cellic CTec	10	45	4.5	11.8	72	1	5.54	66	92
Beet leaf	Sucrase C	10	45	5.0	9.4	69	1	4.39	63	91
Corn stover	Sucrase C	10	45	5.0	7.1	48	1	3.32	44	91
Weed	Sumizyme C	15	55	4.5	6.1	51	1	3.00	49	96

a) Dry beet pulp (2 g) in water (100 ml) was used.

b) The saccharification was performed for 24 h and then fermentation was started by using pYBGA1 yeast for further 48 h at 30°C and pH 5.0 after addition of yeast extract and peptone.

c) The concentration of glucose and ethanol was determined by HPLC and GC, respectively.

d) Based on cellulose content of alkaline-pretreated agricultural residues.

e) Based on glucose saccharified.

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