0273-2289/1559-0291 (Online)/06/135/0015/$30.00

Chemo-Enzymatic Production of Fuel Ethanol From Cellulosic Materials Utilizing Yeast Expressing β-Glucosidases

Toshiyuki Uryu,[1,*] Masashi Sugie,[1] Satoshi Ishida,[1] Shinya Konoma,[1] Hironori Kato,[1] Kaname Katsuraya,[2] Kohsaku Okuyama,[3] Gereltu Borjihan,[4] Kazuhiro Iwashita,[5] and Haruyuki Iefuji[5]

[1]Department of Environmental and Material Engineering, Teikyo University of Science and Technology, 2525 Yatsusawa, Uenohara, Yamanashi 409-0193, Japan, E-mail: uryu@ntu.ac.jp; [2]Wayo Women's University, 2-3-1 Konodai, Chiba 272-8533, Japan; [3]Institute of Industrial Science, University of Tokyo, Meguro-ku, Tokyo 153-8505, Japan; [4]Institute of Macromolecular Chemistry and Mongolian Medicine, Inner Mongolia University, Hohhot 010021, P. R. China; and [5]National Research Institute of Brewing, 7-3-1 Kagamiyama, Higashihiroshima, Hiroshima 739-0046, Japan

Received September 30, 2005; Revised February 28, 2006; Accepted February 28, 2006

Abstract

Ethanol was produced in a considerably high yield by fermenting hydrolyzates from cellulosic materials by means of a recombinant laboratory yeast expressing β-glucosidases. Tissue paper, cotton, and sawdust were hydrolyzed by two-step sulfuric acid hydrolysis to give mixtures containing glucose, cellobiose, and higher cello-oligosaccharides. After the cellulosic material was partially hydrolyzed with 80% sulfuric acid, the hydrolysis was continued with 5% sulfuric acid. Except for non-carbohydrate components, all constituents in the hydrolyzates were fermented by the yeast that was preincubated in the medium that the plasmid encoded by the β-glucosidases gene was kept in the multiplicated yeast. A solution containing 4% hydrolyzates from paper was fermented to give as high as 1.9% maximum ethanol concentration and 70% ethanol conversion. Cotton also gave a similar result.

*Author to whom all correspondence and reprint requests should be addressed.

Sawdust was converted into ethanol in approx 22% conversion. Accordingly, it was revealed that the β-glucosidases-expressing yeast can ferment the cello-oligosaccharides obtained by hydrolysis of cellulosic materials into ethanol. In addition, a hydrolyzate containing a high glucose proportion gave a high ethanol concentration in a short time.

Index Entries: Recombinant yeast; fuel ethanol fermentation; β-glucosidase; cellulose; two-step hydrolysis; paper; wood.

Introduction

Production of fuel ethanol by fermentation was started in Brazil in 1975, followed by United States in 1978, and by Canada in late 1990s *(1)*. In Brazil, ethanol was first produced by fermentation of sugar cane juice. Recently, ethanol has also been produced from maize and corn as starting material *(2)*. These raw materials are composed of mainly α-1,4-linked oligo- and polyglucoses. When the polyglucoses have high-molecular weight, they are degraded by enzymes secreted from microorganisms into glucose and low molecular weight malto-oligosaccharides, which the yeast *Saccharomyces cerevisiae* can take into the body and ferment to ethanol, as in production of the liquor alcohol.

Production of glucose and ethanol from cellulosic materials existing as the most abundant natural organic materials has been investigated for more than a few decades. Because cellulose is a 1,4-β-linked polyglucose included in such structural materials as woods and grasses, its degrading enzymes are not so abundant in nature.

Ingram and coworkers used a recombinant bacteria *Klebsiella oxytoca* for fermenting mixed waste office papers into ethanol in high yield, after the cellulosic material was hydrolyzed by enzymes into cellobiose and cellotriose *(3)*. Pretreatment was carried out by heating the waste paper with 1% sulfuric acid at 140°C for 30 min to produce pulp. Although a recombinant *K. oxytoca* expressing a cellobiose-degradating enzyme, a considerable concentration of a cellulose-degradating enzyme cellulase was added in case of saccharification and fermentation. Recently, it was reported that cellulose was pyrolyzed into levoglucosan, which was then hydrolyzed into glucose, and that the obtained glucose was fermented by yeasts *S. cerevisiae* and *P. sp. YZ-1* to produce ethanol in high yield *(4)*.

Recombinant *Escherichia coli* were produced so that the genes encoding the NADH-oxidizing system of *Zymomonas mobilis (5)* or a yeast exo-β-1,3-glucanase *(6)* were inserted. It was found that during anaerobic growth, the *E. coli* expressing the genes from *Z. mobilis* converted 10% (v/v) of glucose into as high as 4.5% of ethanol *(5)*.

Concerning the production of glucose from cellulose, in 1948 a pilot plant was constructed in Japan, in which two-step sulfuric acid hydrolysis of ground wood was used *(7)*. After the ground wood was first treated with 9% sulfuric acid at 140°C and then with 1.2 to 1.5% sulfuric acid, concentrated sulfuric acid was added to the pressed-out wood powder to increase

the acid concentration to 80%. With the concentrated sulfuric acid hydrolysis, the yield of glucose was as high as 96%. However, this process was not industrialized, probably because the aim of the plant was to produce glucose.

Industrial production of fuel ethanol has been reported that wood cellulose is hydrolyzed by dilute sulfuric acid into glucose, which is then fermented with a normal yeast *(8,9)*. Because a 1,4-β-linked disaccharide cellobiose formed during hydrolysis is not fermented by the yeast, it must be hydrolyzed into glucose *(10)*.

We have investigated synthesis of an azidothymidine-releasing drug as an AIDS drug, in which the azidothymidine bound to polysaccharide is designed to be severed by an enzyme from the bound polysaccharide *(11)*. It was recognized that the enzymatic severance is difficult and slow for high-molecular weight polymers such as polysaccharides. Therefore, a proper combination of the chemical hydrolysis of cellulosic materials and the enzymatic fermentation of the obtained 1,4-β-linked oligosaccharides might efficiently produce fuel ethanol from the cellulosic material.

Iwashita and coworkers reported that β-glucosidase genes in *Aspergillus kawachii* were expressed in both extracellular and cell wall-bound forms in the recombinant yeast *S. cerevisiae (12)*.

In this article, we report that cellulosic materials, such as tissue paper, adsorbent cotton, and sawdust, are hydrolyzed by a two-step sulfuric acid hydrolysis to give a mixture of glucose and cello-oligosaccharides. In addition, the hydrolyzates are efficiently fermented by the recombinant β-glucosidases-expressing yeast to produce ethanol in high yields up to 70%.

Materials and Methods

A recombinant laboratory yeast pYBGA1 was produced by encoding both *bgl*A gene of *A. kawachii* IFO4308 and an uracil-encoding gene *Ura3* in *S. cerevisiae* YPH499 (*MATa ura3 lys2 ade2 trp1 his3 leu2*) *(12)*. IFO4308 encodes both extracellular and cell wall-bound β-glucosidases *(13,14)*.

Two-Step Hydrolysis of Cellulosic Materials

In the first-step hydrolysis, tissue paper consisting of almost pure cellulose was cut into small pieces, then it was put into 80% (w/w) sulfuric acid and kept at a fixed temperature and time. The tissue paper readily dissolved in concentrated sulfuric acid to form a cello-oligosaccharides solution. In the second step, the solution was diluted with water to yield a 5–20% (w/w) sulfuric acid solution, and heated under stirring at 75–85°C for 1–15 h. After cooling, the hydrolyzate solution was neutralized with calcium hydroxide to form calcium sulfate. The calcium sulfate precipitate was removed by filtration, and then the filtrate was concentrated by evaporation. The concentrated solution was freeze-dried, followed by drying *in vacuo* to afford hydrolyzates. The oligosaccharide composition of the hydrolyzate was determined by liquid chromatography using a Toso HPLC 8020 equipped with Toso Amido 80 packed columns. Ash tree

sawdust was treated with sodium hydroxide solution to remove lignin, followed by filtering and neutralizing with dilute sulfuric acid. The dry treated sawdust was used as cellulose raw material for the two-step hydrolysis.

Preincubation of Recombinant Yeast

Yeasts kept at 4°C were incubated in four kinds of preincubation media at pH 6.0. Compositions of the preincubation medium are summarized in Table 1. Yeast peptone dextrose (YPD) and yeast peptone cellobiose (YPC) media were composed of yeast extract and peptone as well as glucose or cellobiose as carbon source, respectively. Synthetic dextrose or cellobiose minus uracil medium (SD-*Ura* or SC-*Ura*) was the medium composed of the minimum essential medium, a supplement containing nucleosides other than uracil, and glucose or cellobiose, respectively. After preparing the medium, it was sterilized in an autoclave. For YPC medium, preincubation was performed for 11 d, while for other media it was 7 d to produce individual yeasts.

Fermentation

Fermentation of cellobiose was carried out at 26°C in a medium containing 4.0% (w/v) cellobiose, 1.0% yeast extract, 2.0% peptone with the preincubated yeast in the concentration of 1×10^8 cells/mL for 2 wk. The initial pH was adjusted to 6.0. For fermentation of cellulose hydrolyzates, 4.0– 10.8% (w/v) hydrolyzates were fermented in a similar condition as that of cellobiose in the yeast concentration of 1×10^7 cells/mL. During 2-wk fermentation, the ethanol concentration produced in the liquid medium was measured at fixed intervals by use of a Shimazu gas chromatograph GC-14B, followed by calculating the ethanol conversion from the starting cellulosic materials.

Results and Discussion

Effects of Preincubation Medium on Fermentation of Cellobiose

Because a laboratory yeast pYBGA1 has the β-glucosidase gene in the plasmid instead of the nucleus, it was examined using the fermentation of a 1,4-β linked disaccharide cellobiose if the β-glucosidase is expressed from the preincubated pYBGA1. The result of fermentations is shown in Fig. 1.

Of the five kinds of media examined, yeasts preincubated in YPD could not ferment cellobiose. On the other hand, other four media such as YPC, SD, SD-*Ura*, and SC-*Ura* gave yeasts fermenting cellobiose. Especially, yeasts preincubated in SC-*Ura* and SD-*Ura* fermented cellobiose to produce ethanol in a maximum concentration of 1.7% in a short time. The decrease in ethanol concentration with time might be as a result of proliferation of yeasts by reversely using the produced ethanol as a carbon source in the later stage.

Table 1
Liquid Medium Used for Preincubation of a Yeast pYBGA1[a]

No.	Medium name	Carbon source		Yeast extract % (w/v)	Peptone % (w/v)	YNBw/oAA[b] % (w/v)	*-Ura* supplement[c] % (w/v)	*-Trp* supplement[d]
		Glucose % (w/v)	Cellobiose % (w/v)					
1	YPD[e]	2.0		1.0	2.0			
2	YPC[f]		2.0	1.0	2.0			
3	SD[g]	2.0				0.67	0.077	0.074
4	SD-*Ura*[g]	2.0				0.67	0.077	
5	SC-*Ura*[h]		2.0			0.67	0.077	

[a]pH, 6.0.
[b]Minimum essential medium.
[c]Supplement containing nucleoside bases except for uracil.
[d]Supplement containing amino acids except for tryptophan.
[e]YPD designates a medium composed of yeast extract, peptone, and dextrose (= glucose).
[f]YPC designates a medium composed of yeast extract, peptone, and cellobiose.
[g]SD or SD-*Ura* designates minimum essential medium containing dextrose with or without uracil.
[h]SC-*Ura* designates minimum essential medium containing cellobiose without uracil.

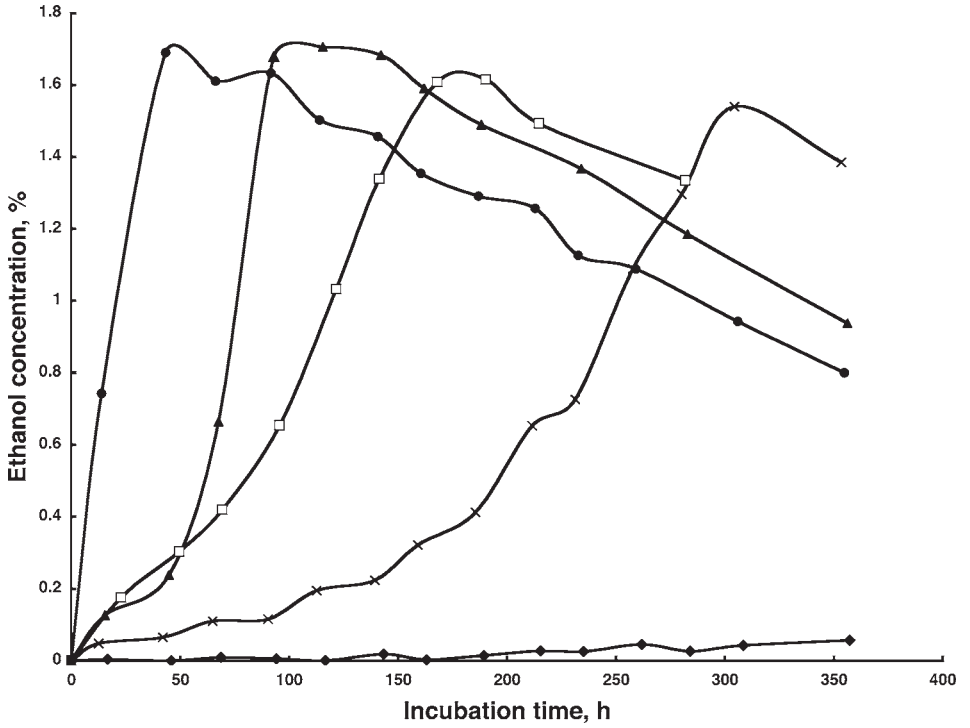


Fig. 1. Effects of preincubation medium on ethanol concentration in fermentation of 4% (w/v) cellobiose. ◆: YPD; □:YPC; ×: SD; ▲: SD-*Ura*; ●: SC-*Ura*. YPD or YPC designates yeast (Y), peptone (P), and dextrose (D)(=glucose) or cellobiose (C), respectively. SD and SD-*Ura* designate minimum essential medium containing dextrose with and without uracil, respectively. SC-*Ura* designates minimum essential medium containing cellobiose without uracil (in Table 1).

For YPC and SD, the maximum ethanol concentration was high in 1.6 and 1.5%, respectively. However, the rate of ethanol production was low. Especially, for SD, the time until the maximum concentration was 310 h.

As a result, it was revealed that the yeast preincubated in the YPD medium lacked in a β-glucosidase. The reason is assumed to be that the yeast not containing the plasmid encoded by the β-glucosidase gene was exclusively proliferated or that the plasmid encoded by the β-glucosidase gene was removed during cell division.

On the other hand, the yeasts preincubated in SC-*Ura* and SD-*Ura* had the plasmid containing the β-glucosidase gene, the selection of which occurred by the –(minus) *Ura* supplement taking either cellobiose or glucose as a carbon source. Accordingly, a recombinant yeast containing both β-glucosidase gene and uracil-producing gene was selected by the preincubation medium.

Based on this result, the yeast preincubated in the SD-*Ura* medium was chosen as the yeast to ferment cellulose hydrolyzates in the next step.

Two-Step Sulfuric Acid Hydrolysis of Tissue Paper and Its Composition

In the first place, in order to examine enzymatic properties of the yeast expressing β-glucosidases, a hydrolysis method producing as much and many as possible kinds of cello-oligosaccharides was sought. Although tissue paper consisting of high-purity cellulose was hydrolyzed with 5 or 10% dilute sulfuric acid at 80°C for a couple of days, no soluble hydrolyzates were obtained. In addition, when tissue paper was immersed in 80% concentrated sulfuric acid at room temperature, soluble hydrolyzates were produced. However, after treatment, the hydrolyzate cannot be fermented by the yeast preincubated in SD-*Ura*, probably because the terminal group of the cellulose hydrolyzate was a nonglucosic compound, which was not severed by the β-glucosidase into glucose.

Thus, tissue paper was hydrolyzed by a two-step hydrolysis with concentrated and dilute sulfuric acids in the first and second steps, respectively. Results of the hydrolysis of paper, as well as those of cotton and wood powder, are summarized in Table 2. Oligosaccharide composition of the hydrolyzates, which was determined by high-performance liquid chromatography (HPLC), is shown in Table 3.

In nos. 1 and 2 of Table 2, in the first step, tissue paper was hydrolyzed with 80% sulfuric acid at low temperature of 30 and 45°C for the short time of 0.17 and 0.25 h, respectively. Then, the obtained light-brown solution was diluted with water to form 3 and 5% sulfuric acid solutions, respectively. The dilute sulfuric acid solution was heated at high temperatures of 75 and 50°C for 20 and 10 h in no. 1 and 2, respectively. Hydrolyzates were obtained in more than 100% yields, which contained impurities (Table 2). As shown in Table 3, the hydrolyzate of no. 1 is composed of 16.7% glucose, 14.3% cellobiose, 3.2% C3, 36.3% over C5, and 29.5% other (indicating noncarbohydrates). For no. 2, the oligosaccharide composition was similar, except for a higher proportion of noncarbohydrates.

It was revealed that the second-step temperature up to 75°C produced high proportions of non-carbohydrates (= other) (nos. 3–6). Because the change in condition of the first-step hydrolysis did not affect much on the oligosaccharide composition, the second-step temperature was raised to 80–85°C, fixing the first step temperature at 25°C (nos. 7– 11).

Under these conditions, the proportion of non-carbohydrates decreased to 17.5–31.9%. Except for no. 7, which had a short second-step time, the proportion of glucose to a large extent increased to 56.8–70.5%, indicating that the second-step hydrolysis at a high temperature proceeded to produce mainly glucose. Especially, the hydrolysis at 85°C for 10 h yielded as high as 70.5% glucose (no. 11). In addition, the proportion of noncarbohydrates was low in 17.7%.

Table 2
Conditions and Yield of Two-Step Hydrolysis of Cellulosic Materials[a]

			Hydrolysis						
			First-step			Second-step			
	Material		Sulfuric	Temperature,	Time,	Concentration	Temperature,	Time,	Yield
No.	Kind	g	acid[b], g	°C	h	of sulfuric acid[c], %	°C	h	g (%)
1	TP[d]	10.0	20	30	0.17	3	75	20	11.0 (110)[e]
2	TP	5.0	10	45	0.25	5	50	10	6.0 (120)[e]
3	TP	5.0	10	45	0.25	5	50	15	6.0 (120)[e]
4	TP	10.0	20	25	0.17	10	75	20	9.0 (90)
5	TP	5.0	10	50	0.25	20	75	1	5.7 (114)[e]
6	TP	5.0	10	50	0.25	20	75	3	5.4 (108)[e]
7	TP	7.9	20	25	0.25	5	80	4	6.3 (80)
8	TP	7.9	20	25	0.25	5	80	15	4.5 (58)
9	TP	5.0	10	25	0.33	5	85	5	4.1 (82)
10	TP	5.0	10	25	0.33	5	85	8	4.2 (84)
11	TP	5.0	10	25	0.33	5	85	10	4.2 (84)
12	Cotton[f]	10.0	20	30	0.17	10	75	20	9.4 (94)
13	WP[g]	9.3	40	40	0.33	5	75	24	4.7 (51)
14	WP[g]	9.3	20	40	0.33	10	85	6	4.5 (49)

[a]Cellulosic materials were dissolved in concentrated sulfuric acid, then the solution was diluted with water to 5 to 20 wt% dilute sulfuric acid.
[b]80% Sulfuric acid.
[c]Dilute sulfuric acid (5–20 wt%).
[d]Tissue paper.
[e]Contained impurities.
[f]Adsorbent cotton.
[g]Wood powder (ash tree sawdust).

Table 3
Oligosaccharide Composition of Hydrolyzates Obtained by Two-Step Hydrolysis

No. (Same as in Table 1)	Cellulosic material	Oligosaccharide[a] (mole %)					
		Glucose	C2	C3	C4	C5<	Other
1	TP[b]	16.7	14.3	3.2	0	36.3	29.5
2	TP	17.2	12.5	8.8	3.2	20.7	37.6
3	TP	43.3	5.9	5.7	3.6	0	41.5
4	TP	50.0	9.6	6.3	3.6	1.3	29.2
5	TP	49.1	4.6	1.7	1.8	0	42.8
6	TP	51.8	13.9	10.4	6.2	2.1	15.6
7	TP	29.2	18.1	8.2	4.4	8.2	31.9
8	TP	60.8	9.2	5.8	1.8	0	22.4
9	TP	56.8	12.6	7.4	3.0	0	20.2
10	TP	62.0	8.3	5.5	3.2	0	21.0
11	TP	70.5	7.1	4.7	0	0	17.7
12	Cotton	63.0	10.9	5.8	0	0	17.5
13	WP[c]	77.4	3.4	0	0	0	19.2
14	WP	63.6	6.0	2.9	0	0	27.5

[a]C2: cellobiose; C3: cellotriose; C4: cellotetraose; C5<: cellopentaose and larger oligosaccharides; Other: low-molecular-weight non-saccharide materials.

Two-Step Hydrolysis of Cotton and Wood Powder and Their Compositions

In the first step, cotton was hydrolyzed at 30°C for 0.17 h, then in the second step, the hydrolysis was continued at 75°C for 20 h (no. 12 in Tables 2 and 3). The hydrolyzate obtained in 94% yield was composed of 63% glucose, 10.9% C2 (cellobiose), 5.8% C3 (cellotriose), and 17.5% other, which was similar to that obtained from tissue paper under similar conditions (no. 11).

After wood powder was hydrolyzed at 40°C for 0.33 h in the first step, it was heated at 75°C for 24 h (no. 13) or at 85°C for 6 h (no. 14) in the second step. In the former, the glucose proportion was high in 77.4%, whereas in the latter the proportion of the useless others increased to 27.5%, indicating that the second-step temperature of 85°C was too high. This result also suggests that the wood powder was hydrolyzed more easily than the tissue paper. Because the wood powder was only subjected to alkali treatment, its cellulose structure might have consisted of high proportion of an amorphous region, which is known to be easily hydrolyzed. However, it is a defect that a large amount of sulfuric acid (344%) was used for the hydrolysis.

Ethanol Fermentation of Cellulose Hydrolyzates as the Carbon Source

Hydrolyzates were fermented with a recombinant yeast. Effects of fermentation time on ethanol concentration are shown in Fig. 2.

Hydrolyzates nos. 1 and 2 from tissue paper containing high proportions of oligosaccharides were fermented in a liquid medium containing 6% of hydrolyzates. For nos. 1 and 2, ethanol was produced in low concentrations of 0.14 and 0.17% in 48 h, and of 0.15 and 0.21% in 118 h, respectively. One of the reasons that the ethanol concentration was so low is assumed to be the result of high proportions of noncarbohydrates designated as other, which might be decomposed compounds from glucose because they appeared before glucose in HPLC. It has been reported that furfural and its derivatives toxic to glucolytic enzymes are formed during dilute-acid hydrolysis at high temperature *(9)*. In addition, over C5 portions might have a terminal group other than the glucose unit, which cannot be fermented by the yeast.

Hydrolyzates no. 3 to 6, which contain 43–51% glucose as well as considerably large amounts (8–30%) of oligosaccharides, were fermented. In a short time, glucose and no. 4 (9% concentration) gave a maximum ethanol concentration of 2.4 and 2.3%, respectively. Thereafter, the ethanol concentration gradually decreased. In nos. 3, 5, and 6, the ethanol concentration reached to 1–1.6% in a short time, followed by a gradual decrease.

Taking into account a relationship between the composition and the ethanol concentration, the two-step hydrolysis might proceed in the mechanism depicted in Scheme 1.

In the first step with concentrated sulfuric acid, it is assumed that oligosaccharides having the non-glucosic terminal were produced.

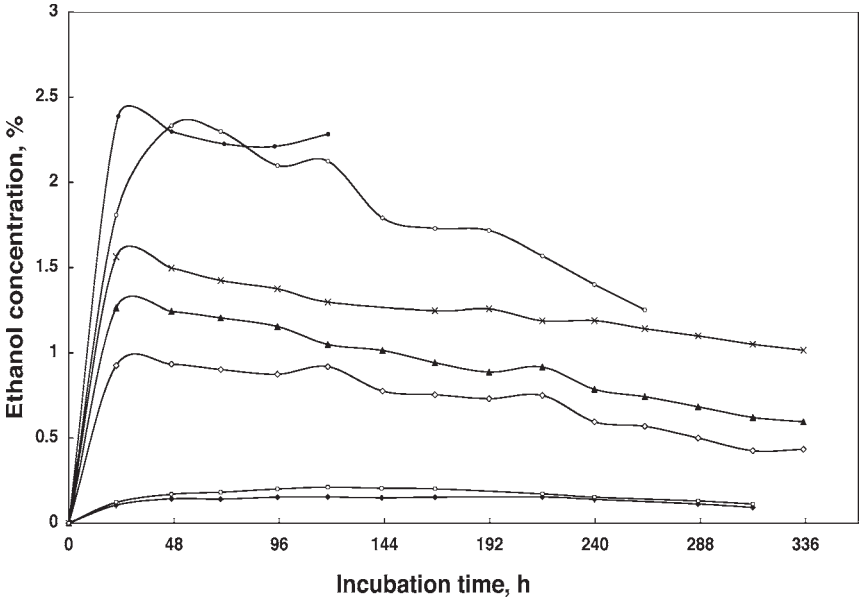


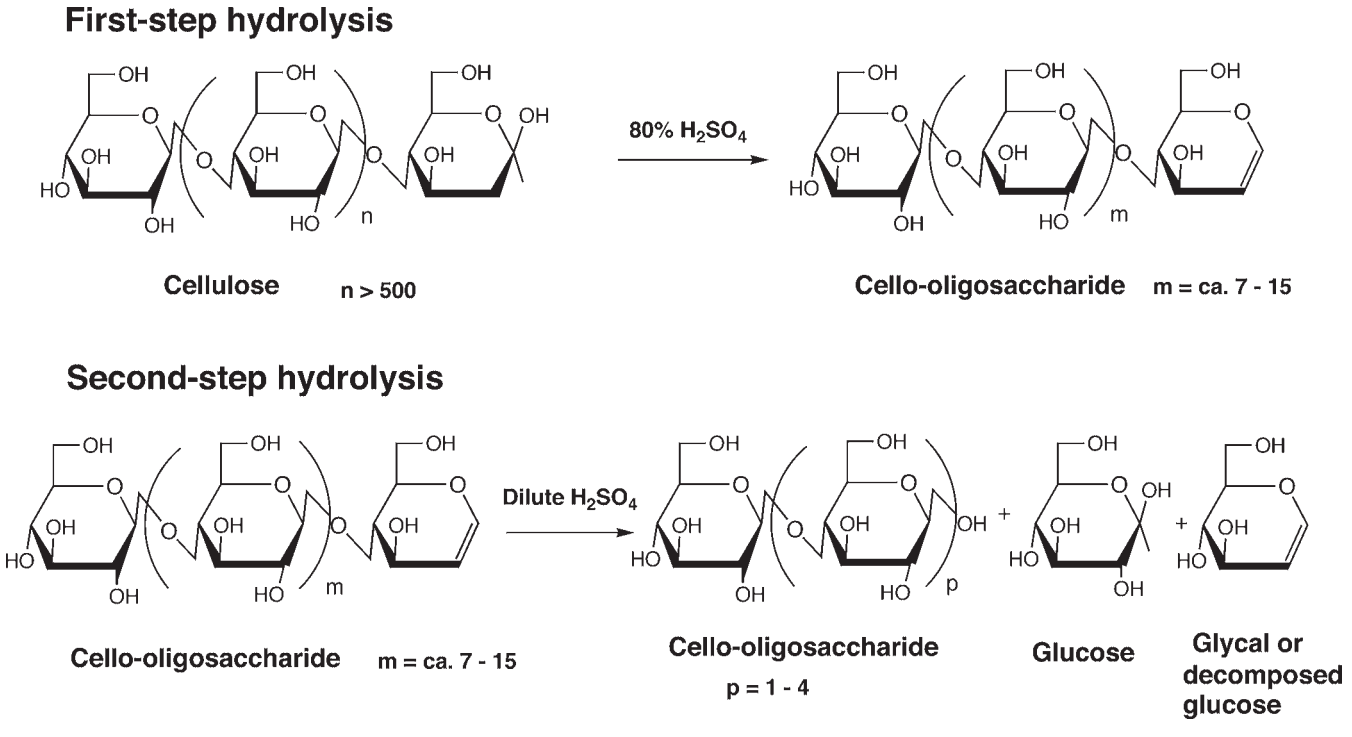
Fig. 2. Ethanol concentration in fermentation of 5% (w/v) paper hydrolyzates containing large proportions of cello-oligosaccharides. ◆: No. 1; □: No. 2; ◇: No. 3; ○: No. 4; ▲: No. 5; ×: No. 6; ●: Glucose. The number corresponds to that in Tables 2 and 3.

Then, in the second step with dilute sulfuric acid, the main chain of the oligosaccharides was severed into glucose, cellobiose, larger cello-oligosaccharides, and other including glycal, which is not confirmed. The last component (other) cannot be used for fermentation.

In Fig. 3 it is shown that a relationship exists between ethanol concentration and incubation time for hydrolyzates containing high proportions of glucose and considerable amounts of oligosaccharides. Except for nos. 7 and 9, all produced high concentrations of ethanol in a short time. Number 11, which had a high glucose concentration (70%) and produced 1.9% ethanol in 48 h, was especially high. Because nos. 7 and 9 included high proportions of oligosaccharides, which have low rate of fermentation, the ethanol concentration gradually increased with time.

For cotton and sawdust hydrolyzates in the 10% concentration, the fermentation curve is shown in Fig. 4. All were fermented in a short time to produce as high as 2.4–3.1% concentration of ethanol. The concentration decreased with time, probably because the ethanol was reversely consumed by the yeast as carbon source.

In Table 4, the maximum ethanol concentration and the ethanol conversion, which is based on both the weight of the starting material and the yield of the hydrolyzate shown in Table 3, are summarized. For nos. 1 and 2, the ethanol conversion was low as 4.5–6.3%. On the other hand, nos. 3 and 5 afforded the maximum ethanol concentration of 1–1.3%, which corre-



Scheme 1. Proposed mechanism for two-step sulfuric acid hydrolysis.

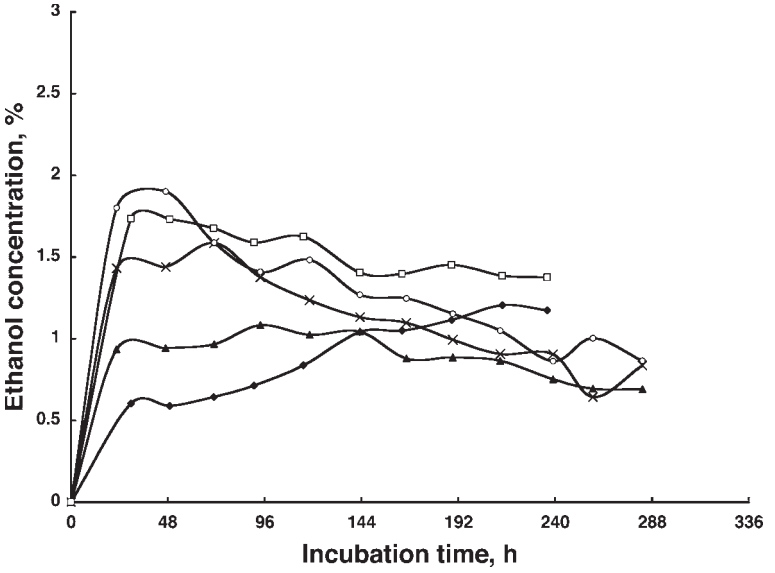


Fig. 3. Ethanol concentration in fermentation of 4% (w/v) paper hydrolyzates containing large proportions of glucose and small proportions of cello-oligosaccharides. ◆: No. 7 (5% hydrolyzate); □: No. 8 (5% hydrolyzate); ▲: No. 9; ×: No. 10; ○: No. 11. The number corresponds to that in Tables 2 and 3.

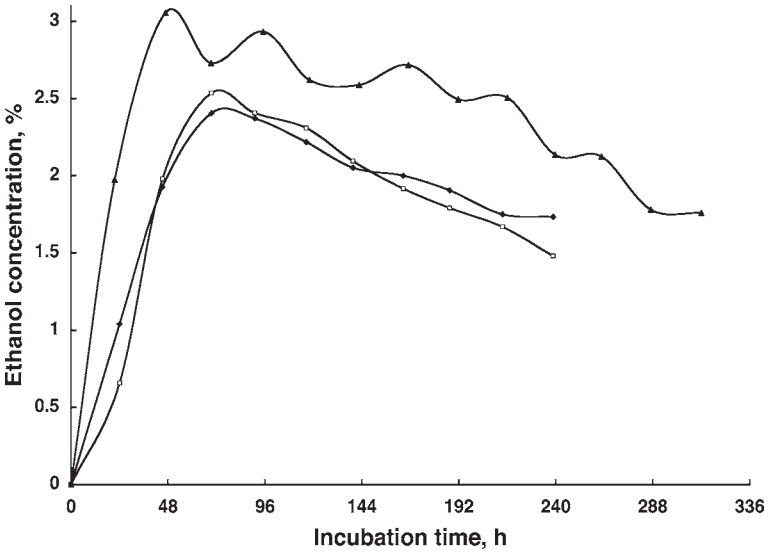


Fig. 4. Ethanol concentration in fermentation of 10% (w/v) cotton and sawdust hydrolyzates containing a large proportion of glucose. ▲: No. 12 (cotton); ◆: No. 13 (sawdust); □: No. 14 (sawdust). The number corresponds to that in Tables 2 and 3.

Table 4
Maximum Ethanol Concentration and Ethanol Conversion Obtained by Fermentation of Hydrolyzates

No.[a]	Concentration of hydrolyzate % (w/v)	Maximum ethanol concentration % (w/v)	Incubation time h	Ethanol conversion[b] %
1[c]	6.0	0.15	118.2	4.5
2[c]	6.0	0.21	118.2	6.3
3[c]	5.7	0.93	46.8	28.7
4[c]	9.0	2.33	46.8	41.0
5[c]	5.4	1.26	21.7	41.1
6[c]	10.8	1.56	21.7	25.4
7[c]	5.0	1.20	213.0	33.8
8[c]	5.0	1.73	29.0	35.3
9[c]	4.0	1.08	93.0	39.0
10[c]	4.0	1.59	70.0	58.8
11[c]	4.0	1.90	46.0	70.3
12[d]	10.0	3.06	46.8	50.6
13[e]	10.0	2.40	69.0	21.7
14[e]	10.0	2.53	69.0	21.8
Glucose	10.0	4.27	69.0	73.6

[a]Same as in Tables 1 and 2.
[b]Based on the weight of the starting material.
[c]Tissue paper.
[d]Cotton.
[e]Sawdust.

sponds to the ethanol conversion of 29–41% based on the theoretical ethanol concentration of 2.82%. In nos. 4 and 6, where 9.0–10.8% hydrolyzate concentration was used, the ethanol concentration and ethanol conversion were 1.6–2.3 and 25–41%, respectively. Similarly, nos. 7–11 showed the maximum ethanol concentration of 1.1–1.9% and the ethanol conversion of 34–70%.

Because 10% cotton and sawdust hydrolyzates were fermented, they gave high ethanol concentrations. The former afforded a high 51% conversion. As the sawdust must have contained approx 40–45% lignin and xylan, the cellulosic portion must have converted into ethanol in high yield.

Figures 2–4 and Table 3 indicate that in the fermentation of hydrolyzates with high glucose proportion, a high concentration of ethanol was produced in a short time. Thus, an ethanol concentration at 48-h incubation obtained from Fig. 2–4 and the glucose proportion exhibited in Table 3 were converted on the basis of 5% hydrolyzate concentration to give the converted ethanol concentration at 48 h and the calculated glucose concentration, respectively, as summarized in Table 5 and plotted in Fig. 5.

As seen in Fig. 5, the converted ethanol concentration at 48 h is closely related to the calculated glucose concentration, exhibiting a linear relationship as follows.

Table 5
Relationship Between the Converted Ethanol Concentration at 48-h Incubation and the Calculated Glucose Concentration in the Tissue Paper Hydrolyzates Used for Incubation

No.[a]	Concentration of hydrolyzate[b] % (w/v)	Ethanol concentration at 48 h incubation[c] % (w/v)	Converted ethanol concentration at 48 h[d] % (w/v)	Glucose proportion[e] wt %	Calculated glucose concentration[f] wt %
1	6.0	0.14	0.12	16.7	0.84
2	6.0	0.17	0.14	17.2	0.86
3	5.7	0.80	0.70	43.3	2.17
4	9.0	2.34	1.30	50.0	2.50
5	5.4	1.24	1.15	49.1	2.46
6	10.8	1.49	0.69	51.8	2.59
7	5.0	0.60	0.60	29.2	1.46
8	5.0	1.75	1.75	60.8	3.04
9	4.0	0.94	1.18	56.8	2.84
10	4.0	1.44	1.80	62.0	3.10
11	4.0	1.90	2.38	70.5	3.53

[a]Same as the number in Tables 2–4.
[b]The initial concentration of tissue paper hydrolyzates used for incubation.
[c]The ethanol concentration at 48-h incubation.
[d]Converted ethanol concentration at 48 h was calculated from the ethanol concentration at 48-h incubation assuming that the 5% hydrolyzate was used for incubation.
[e]Glucose proportion in the hydrolyzates shown in Table 3.
[f]Glucose concentration in the medium in the case of 5% hydrolyzate.

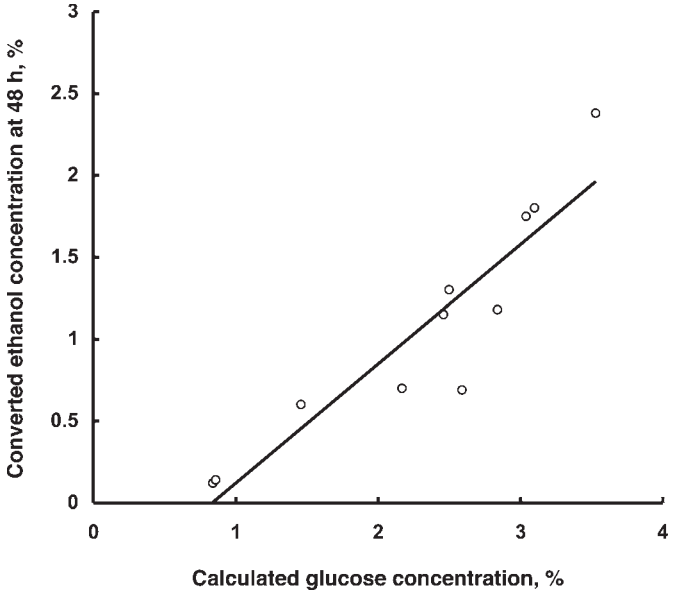


Fig. 5. Effects of the calculated glucose concentration (%) in the medium containing 5% hydrolyzate on the converted ethanol concentration at 48-h incubation (%).

$$\text{Converted ethanol concentration at 48 h, \% (w/v)} = 0.74 \times (\text{calculated glucose concentration, \% [w/v]}) - 0.63$$

As a result, it was revealed that the β-glucosidase-expressing yeast can ferment 1,4-β-linked cello-oligosaccharides to produce ethanol. However, as the rate of degradation by the β-glucosidase was low, the fermentation of cello-oligosaccharides took a long time.

In the present experiment, hydrolyzates containing a high proportion of glucose gave a high ethanol concentration as well as a high ethanol conversion. Accordingly, the use of yeast expressing β-glucosidases with high degradation rate might give a high ethanol concentration. Alternately, if a microorganism that can degrade solid-state cellulose directly into glucose is discovered, an efficient ethanol production from cellulose can be achieved by normal yeast. A search for conditions to decrease the quantity of sulfuric acid is in progress.

References

1. Wheals, A. E., Basso, L. C., Alves, D. M. G., and Amorim, H. V. (1999), *Trends Biotechnol*. **17,** 482–487.
2. Ring, K.-L., Barron, C., Jansheker, H., and Sakuma, H. (1995), *Ethyl Alcohol*, CEH Marketing Research Report, SRI International/Chemical Economics Handbook.
3. Brooks, T. A. and Ingram, L. O. (1995), *Biotechnol. Prog*. **11,** 619–625.
4. Zhang, H. X. and Yu, Z. S. (2003), *Macro Review* **16,** 53–57.
5. Ingram, L. O., Conway, T., Clark, D. P., Sewell, G. W., and Preston, J. F. (1987), *Appl. Environ. Microbiol*. **53,** 2420–2425.

6. Suzuki, K., Yabe, T., Maruyama, Y., Abe, K., and Nakajima, T. (2001), *Biosci. Biotechnol. Biochem*. **65,** 1310–1314.
7. Wenzl, H. F. J. (1970), *The Chemical Technology of Wood*, Academic Press, New York, NY.
8. Taherzadeh, M. J., Eklund, R., Gustafsson, L., Niklasson, C., and Liden, G. (1997), *Ind. Eng. Chem. Res*. **36,** 4659–4665.
9. Brandberg, T., Franzen, C. J., and Gustafsson, L. (2004), *J. Biosci. Bioeng*. **98,** 122–125.
10. Nagle, N. J. and Elander R. T. (2002), *Biotechnol. Prog*. **18,** 734–738.
11. Gao, Y., Katsuraya, K., Kaneko, Y., Nakashima, H., and Uryu, T. (1999), *Macromolecules* **32,** 8319–8324.
12. Iwashita, K., Nagahara, T., Kimura, H., Takano, M., Shimoi, H., and Ito, K. (1999), *Appl. Environ. Microbiol*. **65,** 5546–5553.
13. Ohta, T., Ohmori, T., Shimojo, H., Hashimoto, K., Samuta, T., and Ohba, T. (1991), *Biotechnol. Biochem*. **55,** 1811–1816.
14. Iwashita, K., Todoroki, K., Kimura, H., Shimoi, H., and Ito, K. (1998), *Biosci. Biotechnol. Biochem*. **62,** 1938–1946.