Two-Step Cellulose Hydrolysis Using Modest Amount of Concentrated Sulfuric Acid or Autoclave and Fermentation of the Hydrolyzates into Ethanol Utilizing Recombinant Yeast

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Abstract: Tissue paper was hydrolyzed by the two-step acidic hydrolysis using 60-80 wt.% of concentrated sulfuric acid in the first step to produce hydrolyzates containing glucose as a main component. The hydrolyzate was fermented by means of ß-glucosidases-producing recombinant yeast to afford ethanol in the maximum yield of 7.7%. In the use of such modest amount of concentrated sulfuric acid, repetition of the two-step hydrolysis was effective. From wood powder, ethanol was obtained in the maximum yield of 6.9%. When autoclave heating at 190-200ºC was carried out as the first-step hydrolysis instead of concentrated sulfuric acid, the maximum ethanol yield was 2.7% and 1.0% from tissue paper and wood powder, respectively. These results indicate that mainly amorphous cellulose on the surface can be hydrolyzed by such amount of concentrated sulfuric acid and that thermomechanical operation might be necessary for hydrolyzing crystalline cellulose.

Introduction

Many researches to produce ethanol from cellulose contained in wood have not completely been successful [1,2]. The reason is that the glucoside bond in a crystalline region of celllulose was hardly hydrolyzed by acids or enzymes. On the other hand, since the glucoside hydrolysis in an amorphous region can take place with both acid and enzyme, it has been possible to produce glucose from the amorphous region of cellulose. Accordingly, it is assumed that the production of ethanol from the amorphous cellulose has been accomplished.

Previously we reported that when a two-step hydrolysis of cellulose was carried out with a large amount of concentrated sulfuric acid in the first step and with hot dilute sulfuric acid in the second step, the whole cellulose including the crystalline region was thoroughly hydrolyzed to produce a glucose and

cellooligosaccharide mixture [3]. The hydrolyzate was fermented by utilizing a ß-glucosidases-expressing recombinant yeast to produce ethanol in high conversion. So far, the concentrated sulfuric acid hydrolysis is the only successful method that the whole cellulose including crystallline-region cellulose can be completely hydrolyzed into glucose. However, defects in this method are the use of a large amount of concentrated sulfuric acid and difficulty in its recovery.

According to the crystal structure analysis, cellulose macromolecules are bonded by one intermolecular and two intramolecular hydrogen bonds per glucose residue [4]. An individually weak hydrogen bond of approximately 5 kcal/mol is multiplied by the number of glucose residues along the cellulose backbone, i.e., the degree of polymerization. Accordingly, the hydrogen bond between cellulose macromolecules becomes a few hundreds kilocalories per mole, forming a tightly-oriented crystalline region along the backbone. And, even protons can not penetrate into the cellulose microcrystal.

Microcrystalline cellulose has been industrially produced by the acidic hydrolysis of cellulose, which selectively decomposes the amorphous region possibly into glucose and its derivatives, remaining the crystalline region as widely-used microcrystalline cellulose [5, 6].

Recently, it was reported that an ethanol plant has been successfully operated in Japan in which glucose and xylose were produced from construction scrap wood by hydrolyzing with two different concentrations of dilute sulfuric acid at high temperatures in the autoclave, followed by individually fermenting into ethanol [7]. However, it has been considered that a considerable amount of cellulose, presumably crystalline region, remains after the hydrolysis.

Hardwood is composed approximately of 49% cellulose, 25% xylan, 20% lignin, and 5% others [8]. Crystallinity indexes of original and acid-treated eucalytpus wood chips were determined to be 0.30 and 0.48, respectively, indicating that the crystallinity of cellulose increased after the acidic treatment possibly by removing amorphous region [9]. When native cellulose was subjected to strong oxidation, surface-oxidized microfibrils were obtained, which contained crystalline structure inside [10, 11].

In the enzyme hydrolysis of cellulose, it was reported that the digestion scarcely occurred in the crystalline region, because the enzyme macromolecule can not penetrate into cellulose crystal [12]. Thus, in the field of cellulosic ethanol technology, major research subjects have been both enhancement in the enzyme activity and improvements in the pretreatment of cellulosic materials [13-19]. Recently, simultaneous saccharification and fermentation of switchgrass soaked in ammonium hydroxide solution gave a high ethanol yield [20].

In the two-step hydrolysis, a large amount of concentrated sulfuric acid was essential to hydrolyze the whole cellulose. Thus, it is considered that if the amount of sulfuric acid is decreased, the hydrolysis and fermentation of cellulose might be industrially possible.

In this paper, we report an acidic hydrolysis and fermentation of cellulosic materials such as tissue paper and wood powder in order to find out a minimum amount of concentrated sulfuric acid to cause the hydrolysis of cellulose. The hydrolyzate was fermented with a ß-glucosidases-expressing recombinant yeast to give ethanol. In addition, an autoclave heating up to 200ºC was examined if it can be substituted for the concentrated sulfuric acid hydrolysis.

Experimental Section

Materials. Commercial tissue paper was cut into small pieces, then it was immersed into acetone overnight, followed by filtering and drying. It was reported that in commercial hardwood bleached kraft pulp used for tissue paper, cellulose and water contents are 90% and 7%, respectively [21]. Wood powder (hardwood sawdust) was delignified by heating in 5 N NaOH aqueous solution at 70 - 90ºC for 24 h, then it was neutralized with dilute sulfuric acid. After filtration, the pretreated wood powder was dried in vacuo for 2 d. A recombinant yeast pYBGA1 was produced by encoding both bglA gene of A. kawachii IFO4308 and an uracil-encoding gene *Ura3* in a laboratory yeast *S. cerevisiae* YH499 (*MATa ura3 lys2 ade2 try1 leu2*) [22-24]. IFO4308 encoding both extracellular and cell wall-bound ß-glucosidases exhibited a function to degrade cellooligosaccharides into glucose [3].

Two-step hydrolysis using a modest amount of concentrated sulfuric acid in the first step. Using a modest amount, that is, 60-80 wt.% to tissue paper, of concentrated sulfuric acid, acetone-pretreated tissue paper was immersed into concentrated sulfuric

acid put in a beaker or concentrated sulfuric acid was sprayed over tissue paper. The obtained mass of paper was heated at 25-60ºC for 30 min. Then, the first-step mixture was diluted with water to prepare the first-step hydrolyzate suspension in 5% dilute sulfuric acid. The second-step hydrolysis was carried out by heating at 75-85ºC for a fixed time. After the hydrolysis solution was neutralized with Ca(OH) 2, the hydrolyzate was extracted with water, concentrated, and freeze-dried from water. In the case of wood powder, delignification was carried out by heating in 5% NaOH solution at 70-90ºC for 24 h, followed by neutralization.

Two-step hydrolysis using autoclave heating in the first step. 1.5 Grams of acetone-pretreated tissue paper or 3 g of delignified wood powder were heated with about 50 ml water at 190-200ºC for 0.2-1.5 h in a 100 ml TVS-1 type autoclave (Taiatsu Glass Ind., Tokyo). The resulting suspension was neutralized with aqueous Ca(OH) 2 suspension, then the whole material was freeze-dried to give the first-step hydrolyzate. In the second-step hydrolysis, it was hydrolyzed at 65-85ºC for 12-24 h in a 100 ml of 5% dilute sulfuric acid.

Fermentation. A recombinant yeast pYBGA1 kept at 4ºC was preincubated in a medium containing a minimum essential medium YNGw/oAA, uracil-deficient supplement, and glucose for 72 h. Fermentation was carried out at 28ºC in a 10 ml medium containing 0.5 g (5 w/v%) of a hydrolyzate, 0.2 g (2%) of peptone, 0.1 g (1%) of yeast extract by a 1 x 107 cells/ml concentration of the preincubated recombinant yeast.

Results and Discussion

Effects of the amount of concentrated sulfuric acid on hydrolysis of tissue paper. When the first-step hydrolysis was performed with 60-80 wt.% of

Table 1 Results of Two-Step Sulfuric Acid Hydrolysis, Sugar Composition of Hydrolyzates, and Fermentation for Tissue Paper and Wood Powder [a)]

No.	1st-Step hydrolysis[b)]			2nd-Step hydrolysis[c)]	Yield g (%)	Sugar composition, %						Fermentation		
	Sulfuric acid wt.%[k)]	Temp. °C	Time h	Temp., °C		Glu[d)]	C2[e)]	C3[f)]<	Man[g)]	Xyl[h)]	NS[i)]	Max. ethanol conc.[l)] %(w/v)	Ethanol yield[j)] %	Theoretical ethanol yield,[m)]%
Wood powder[t)]														
1[n)]	75	40	0.30	60	1.93 (48.3)	29.2	9.2	7.6	0	15.5	38.5	0.42	5.4	6.5
2[n)]	80	25-47	0.97	60	1.78 (44.4)	40.9	10.3	8.6	0	15.4	24.8	0.52	4.6	5.5
3[o)]	80	60	20.0	60	0.84 (28.1)	28.3	2.2	0	3.0	25.5	40.2	0.18	1.0	1.2
4	80	60	48.0	60	1.30 (43.5)	34.0	17.3	0	1.6	23.3	23.8	0.31	2.7	3.2
5[p)]	60	25[q)]	1.5[q)]	45[q)]	2.10 (42.0)	50.7	4.9	0	0	14.9	29.5	0.78	6.6	7.8
6[r)]	67	25-40[s)]	1.4[s)]	40[s)]	2.52 (50.3)	53.8	2.4	0	0	9.1	34.7	0.79	7.7	9.1
Tissue paper														
7[u)]	83	40	0.33	78	3.52 (48.6[v)])	29.1	6.0	3.6	0	4.0	57.3	0.61	4.3	———
8[w)]	78	40	0.33	75	3.83 (49.5[v)])	49.0	3.3	1.7	0	7.0	39.0	0.66	5.1	———
9[x)]	88	40	0.33	79	4.96 (71.7[v)])	51.1	6.8	6.8	0	5.6	29.7	0.70	6.9	———
10[y)]	91	40	0.33	75	4.21 (64.0[v)])	55.1	9.2	2.4	0	6.6	26.7	0.74	6.2	———

a) 3.0 g Tissue paper or 10.0 g wood powder (sawdust) was hydrolyzed in two-step hydrolysis. b) Concentration of sulfuric acid, 80%. c) Concentrated sulfuric acid was diluted to 5% concentration and heated for 24 h. d) Percent of glucose determined by HPLC. e) Cellobiose. f) Cellotriose and higher. g) Mannose. h) Xylose. i) Non-sugar components. j) Based on the starting tissue paper or untreated wood powder which is assumed to contain 100% cellulose. k) Weight percent of 80% sulfuric acid to tissue paper. l) Maximum ethanol concentration during fermentation utilyzing recombinant yeast. m) Based on the substantial cellulose content in tissue paper composed of 90% cellulose and 7% water [21]. n) Tissue paper, 4 g. o) 2nd-step hydrolysis time, 18 h. p) Tissue paper, 5.0 g. q) First- (totally 1.5 h) and second-step hydrolyses (totally 12 h) were repeated 3 times by dividing concentrated sulfuric acid. r) Tissue paper, 5.2 g. s) First- (totally 1.4 h) and second-step (totally 40 h) hydrolyses were repeated 4 times. t) 10.0 g Wood powder (sawdust) was pretreated by heating in 5 N NaOH aqueous solution at 90°C for 24 h, except for No. 8 (10. 4 g wood powder). u) Pretreated at 55°C; yield, 7.24 g. v) Based on the yield of pretreated powder. w) Pretreatment time, 4 h; yield, 7.73 g. x) Pretreatment yield, 6.92 g; second step sulfuric acid, 3%. y) Pretreatment yield, 6.61 g.

concentrated sulfuric acid to tissue paper, hydrolysis of tissue paper occurred to produce hydrolyzates in 28-50% yield. Results of the hydrolysis are summarized as well as the sugar composition in the hydrolyzates in Table 1.

Since in the first step, low temperature and short time (No. 1, 2) gave higher yields and higher proportions of fermentable glucose and cellobiose than high temperature and long time (No. 3, 4), it was revealed that optimum hydrolysis conditions are approximately 40ºC and 1 h.

Next, by dividing the quantity of concentrated sulfuric acid into 3 or 4 portions, the first- and second-step hydrolysis were repeated 3 or 4 times under mild conditions (No. 5, 6). While the yield slightly increased to approximately 50%, the glucose content considerably increased to 51-54%. This phenomenon indicates that the first-step hydrolysis occurred at the surface of cellulosic material and the fresh surface formed after the second step was in turn hydrolyzed by the repeated operation.

High proportions of non-sugar components were mostly furfural derivatives formed by decomposition of xylose and glucose [25].

It has been found that (1) in this study utilizing a modest amount (60-80 wt.%) of concentrated sulfuric acid, the yield was less than 50%, (2) tissue paper was not at all hydrolyzed with 5% dilute sulfuric acid (corresponding to the second-step hydrolysis), and (3) the use of a large amount (200 wt.%) of concentrated sulfuric acid caused the hydrolysis of amorphous cellulose, xylan, and glucomannan, but not crystalline cellulose.

Taking into account that a clear solution was obtained after hydrolysis with a large amount of concentrated sulfuric acid [3], it was revealed that concentrated sulfuric acid also plays a solvent role for the low-molecular-weight cellooligosaccharides.

Effects of the amount of concentrated sulfuric acid on the hydrolysis of wood powder. To remove lignin from wood powder, 10 g of wood powder was pretreated by immersing in 5N NaOH aqueous solution. Then, in the first-step hydrolysis, the whole pretreated wood powder (6.6-7.7 g) was hydrolyzed with 6 g of concentrated sulfuric acid which corresponded to 78-91 wt.% to the wood powder. The second-step hydrolysis was performed with 3 or 5% sulfuric acid. The result and sugar composition in hydrolyzates are also shown in Table 1.

Comparing the sample No. 8 pretreated at 90ºC with No. 7 at 55ºC, the glucose proportion of the former was higher than the latter. Furthermore, No. 9 pretreated for 24 h showed much higher hydrolyzate yield than No. 8 pretreated for 4 h. Thus, an alkali pretreatment at 90ºC for 24 h might have satisfactorily removed lignin to produce glucose in high yield.

All hydrolyzates contained a considerable amount of non-sugar components. Unlike the tissue paper hydrolyzate, the wood power hydrolyzate showed low xylose proportion, suggesting that a considerable amount of xylose had been removed during the alkali pretreatment.

Consequently, hydrolysis behaviors of the pretreated wood powder were almost equivalent to those of tissue paper.

Two-step hydrolysis of tissue paper and wood powder using autoclave heating as the first step. In order to investigate if the temperature much higher than 100ºC helps to cause hydrolysis of the crystalline cellulose, autoclave heating was used as the first step. The reason is that the hydrogen bond preventing the crystalline cellulose from hydrolyzing becomes weak at high temperatures.

When tissue paper and wood powder were hydrolyzed by autoclave heating at 190-200ºC in the first step, followed by the second-step hydrolysis with 5% sulfuric acid, hydrolyzates were obtained in 18-79% yields. The result is summarized in Table 2.

Glucose proportions in tissue paper hydrolyzates were in the range of 14-38%, revealing that the autoclave heating hydrolyzed cellulose. In addition, taking into account of high xylose proportions, except for No. 2, the hydrolysis of xylan might have mainly taken place in these conditions. In No. 2 containing only 3% xylose, it is suggested that the acid pretreatment at 91ºC followed by the autoclave heating decomposed once-formed xylose into non-sugar components.

Since in Nos. 4 and 5 the third-step hydrolysis caused an increase in the yield, an absolute amount of glucose increased. Heating at 200ºC for 0.5 h gave low yield (No. 7).

When wood powder was heated at 200ºC for 0.2-1.5 h, both yield and glucose proportion were low. It is noted that a considerably high proportion of mannose

was contained. Long-time heating resulted in increases in the yield and glucose proportion, while the mannose and xylose proportions decreased (No. 10).

As a result, it was revealed that the autoclave heating as the first-step hydrolysis caused the hydrolysis of amorphous cellulose and xylan in addition to glucomannan, but not possibly of crystalline cellulose.

Ethanol fermentation of the hydrolyzate obtained with a modest amount of concentrated sulfuric acid. Tissue paper hydrolyzates obtained by the concentrated sulfuric acid hydrolysis were fermented with ß-glucosidases-expressing yeast to produce ethanol. Ethanol concentration was plotted against incubation time (Figure 1). Ethanol yield was calculated from maximum ethanol concentration.

As shown in Figure 1, the fermentation curve exhibited two peaks appearing in 24-48 h and 70-96 h. Previously, it was revealed that the first and second peaks correspond mainly to fermentation of glucose and cellooligo- saccharides as a carbon source, respectively [3]. Almost all first peaks exhibited the maximum ethanol concentration. The maximum ethanol concentration and ethanol yield are given in Table 1.

The ethanol yield is defined as the weight percent of ethanol to the starting cellulosic material. Since we defined the ethanol conversion (%) in the previous paper [3], the relation between the ethanol conversion and the ethanol yield in this report is shown in Equation 1.

$$\underset{162\ g/mol}{(C_6H_{10}O_5)_n} \longrightarrow \underset{2 \times 46\ g/mol}{2C_2H_5OH + 2CO_2}$$

$$\text{ethanol yield (\%)} = \text{ethanol conversion (\%)} \times \frac{92}{162} \quad (1)$$

For tissue paper, the maximum ethanol concentration in the incubation medium and the ethanol yield were 0.18-0.79% and 1.0-7.7%, respectively. Assuming that the tissue paper contained 7% water, the theoretical ethanol yield was 1.2-9.1%.

The highest ethanol yield (7.7%) was obtained in the case of the repeated hydrolysis (No. 6). The repeated

Table 2 Results on First-Step Autoclave Heating Followed by Second-Step Acid Hydrolysis, Sugar Constitution of Hydrolyzates, and Fermentation for Tissue Paper and Wood Powder [a]

No.	Autoclave heating		2nd Step	Yield	Sugar constitution, %						Fermentation	
	Temp. °C	Time h	hydrolysis[b] temp., °C	g (%)	Glu[c]	C2[d]	C3[e]	Man[f]	Xyl[g]	NS[h]	Max. ethanol conc.[j] %(w/v)	Ethanol yield[i] %
Tissue paper												
1[k]	190	1.5	65	1.20 (28.6)	27.9	12.2	0	3.0	24.4	32.5	0.48	2.7
2[l]	190	1.5	65	1.30 (35.1)	37.7	7.5	0	0	3.0	51.8	0.30	2.1
3[m]	190	1.5	70	0.55 (18.3)	16.9	3.5	0	8.1	45.3	26.2	0.20	0.7
4[n]	190	0.5	70	1.03 (68.7)	22.3	5.8	0	3.3	51.2	17.4	0.14	1.9
5[n]	190	1.5	70	1.19 (79.3)	14.3	6.5	0	4.1	30.0	45.1	nd[o]	nd
6	190	0.2	85	1.12 (74.7)	27.2	0	0	4.7	40.2	27.9	0.14	1.1
7[p]	200	0.5	85	0.63 (42.0)	34.0	0	0	8.5	37.8	19.7	0.19	1.6
Wood powder[q]												
8	200	0.2	85	0.69 (23.0)	11.3	0	0	7.5	42.1	39.1	0.20	0.9
9[r]	200	0.5	85	0.59 (19.6)	21.4	7.0	0	10.8	24.4	36.4	0.21	0.8
10[s]	200	1.5	85	1.02 (34.0)	22.3	3.7	0	4.4	11.1	58.5	0.15	1.0

a) 1.5 g Tissue paper or 3.0 g pre treated wood powder (sawdust) was used as starting material. b) 5% Sulfuric acid was used. Hydrolysis time, 20 h. c) Percent of glucose determined by HPLC. d) Cellobiose. e) Cellotriose. f) Mannose. g) Xylose. h) Non-sugar constituents. i) Based on the starting tissue paper or untreated wood powder. j) Maximum ethanol concentration during fermentation utilyzing recombinant yeast. k) 4.2 g Tissue paper was pretreated by heating in 5% sulfuric acid at 28°C for 25 h. Hydrolysis time, 24 h. l) 3.7 g Tissue paper was pretreated by heating in 5% sulfuric acid at 91°C for 48 h. Hydrolysis time, 24 h. m) 3.0 g Tissue paper was used. n) Third-step hydrolysis was carried out with 5% sulfuric acid at 70°C for 20 h. o) Not determined. p) For fermentation, beer yeast was used instead of recombinant yeast. q) Wood powder was pretreated in 5 N NaOH aqueous solution. r) Hydrolysis time, 12 h. s) Third-step hydrolysis was carried out with 5% sulfuric acid at 85°C for 20 h.

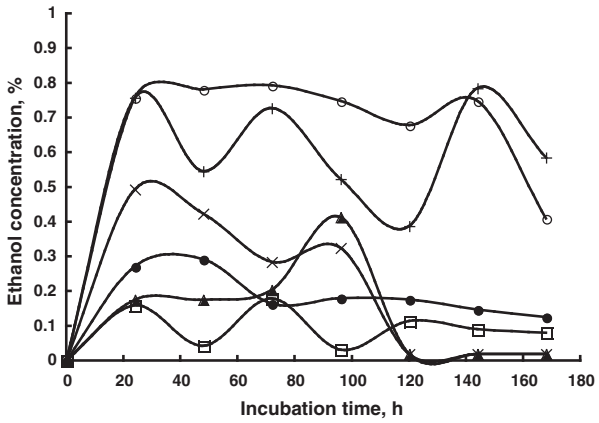

Fig. 1 Ethanol concentration in fermentation of 5% (w/v) hydrolyzates from tissue paper. ▲ : No. 1; × : No. 2; □ : No. 3; ● : No. 4; + : No. 5; ○ : No. 6. The number corresponds to that in Table 1.

operation might have promoted the hydrolysis of newly-formed cellulose surface, because a small amount of concentrated sulfuric acid could not penetrate into the inside.

Previously, we reported that the hydrolysis with 200 wt.% sulfuric acid afforded as high as 70.3% ethanol conversion which corresponds to 40% ethanol yield [3].

For wood powder, the ethanol fermentation curve and results are shown in Figure 2 and Table 1, respectively. The maximum ethanol concentration and ethanol yield are 0.61-0.74% and 4.3-6.9%, respectively, being similar to those for tissue paper.

The highest ethanol yield (6.9%) was obtained when the pretreatment with NaOH solution was carried out at 90ºC for 24 h (No. 9).

Ethanol fermentation of the hydrolyzate obtained by use of high temperature autoclave heating. Ethanol was produced from all hydrolyzates by autoclave heating. Three representative fermentation curves are shown in Figure 3. The ethanol concentration reached a maximum in 24-48 h, then it kept almost constant.

Results are summarized in Table 2. For tissue paper, when the autoclave heating was carried out at 190ºC, the maximum ethanol concentration and ethanol yield were in the range of 0.14-0.48% and 0.7-2.7%, respectively, being less than half of the sulfuric acid hydrolysis.

When wood powder was hydrolyzed in the autoclave at 200ºC, the maximum ethanol concentration and ethanol yield were 0.15-0.21% and 0.8-1.0%, respectively, lower than those of tissue paper.

As a consequence, it was revealed that the autoclave heating was not effective as the first-step hydrolysis.

Steam explosion technique which explodes cellulose suspension at high temperature has been reported to cause increases in enzyme-digestable polysaccharides [26-28], in pentose yield [29], and in the activity of cellulases [30,31]. In this technique up to 250ºC, it is assumed that the removal of lignin from wood and corn stover occurred, leaving cellulose bundle and xylan.

When microcrystalline cellulose was thermomechanically hydrolyzed by use of flowing high-pressured hot water, water-soluble saccharides were produced in high yields [31]. On the other hand, no reaction occurred in case of its hydrolysis in high pressured water without stirring [32]. Therefore, it is suggested that not only

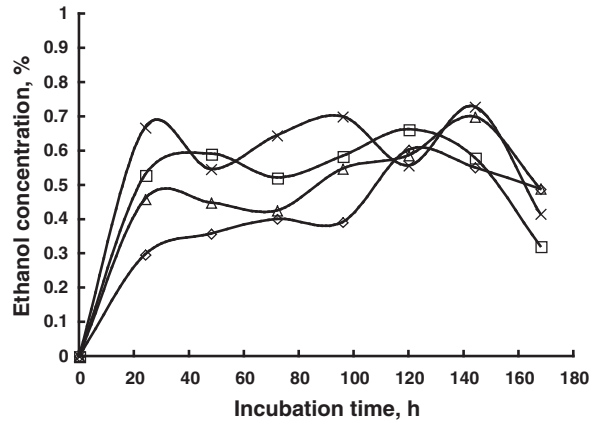

Fig. 2 Ethanol concentration in fermentation of 5% (w/v) hydrolyzates from wood powder. ◇ : No. 7; □ : No. 8; △ : No. 9; × : No. 10. The number corresponds to that in Table 1.

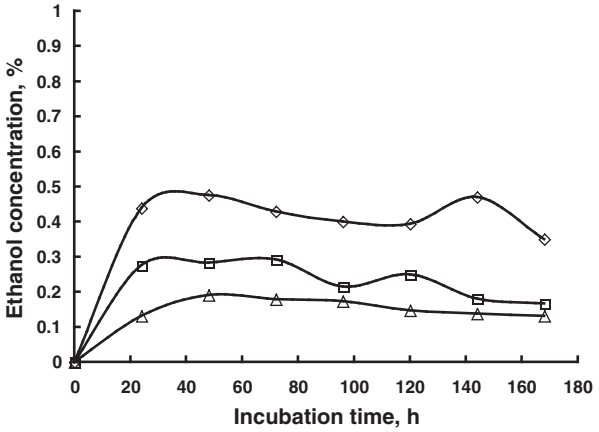

Fig. 3 Ethanol concentration in fermentation of 5% (w/v) hydrolyzates from tissue paper and wood powder. □ : No. 1; ◇ : No. 2; △ : No. 8. The number corresponds to that in Table 2.

high temperature but also flowing water must be necessary for hydrolyzing microcrystalline cellulose.

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中程度量の濃硫酸又はオートクレーブを使用する二段階加水分解と組換え酵母を利用する加水分解物のエタノール発酵

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要旨：ティッシュペーパーを第一段階で 60-80 wt.% 量の濃硫酸を使用する二段階酸加水分解で加水分解し、グルコースを主成分とする加水分解物を生成させた。加水分解物は、*β*- グルコシダーゼ産生組換え酵母によって発酵され、最高収率 7.7% でエタノールを与えた。この中程度量の濃硫酸を使用する時、二段階加水分解の繰返し操作が有効であった。木粉からは最高収率 6.9% でエタノールが生成した。190-200ºC におけるオートクレーブ加熱を濃硫酸の代りに第一段階加水分解に使用すると、最高エタノール収率は、ティッシュペーパーと木粉からそれぞれ 2.7% および 1.0% であった。これらの結果は、表面に存在する主にアモルファスセルロースがそのような量の濃硫酸によって加水分解されること、および高温における機械的操作が結晶セルロースの加水分解には必須であることを示している。