Biotechnol. Bioprocess Eng. **2002**, 7: 268-274

Empirical Evaluation of Cellulase on Enzymatic Hydrolysis of Waste Office Paper

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Abstract Enzymatic hydrolysis of waste office paper was evaluated using three commercial cellulases, Acremonium cellulase, Meicelase, and Cellulosin T2. Varying the enzyme loading from 1 to 10% (w/w) conversion of waste office paper to reducing sugar was investigated. The conversion increased with the increase in the enzyme loading: in the case of enzyme loading of 10% (w/w), Acremonium cellulase yielded 79% conversion of waste office paper, which was 17% higher compared to Meicelase, 13% higher than that of Cellulosin T2. Empirical model for the conversion (%) of waste office paper to reducing sugar (x) was derived from experimental results as follow, $x = kE^m t^{(aE+b)}$ where k, m, a, and b denote empirical constants. E indicates initial enzyme concentration.

Keywords: cellulase, waste office paper, saccharification, recycle, reducing sugar

INTRODUCTION

Production of paper increased drastically this decade. Of sixty percent, among the flammable solid waste materials comes from waste papers. Recently, with the increase in understanding of recycling concept of resources, 50% of waste newspapers and 90% of cardboard were recycled in Japan. The rest of waste papers were sent for incineration or landfill. However, because of environmental restrictions, lack of suitable new sites, and green house effect of the earth, the incineration or disposal by landfill is probably impossible in the near future.

However, cellulose, a major building block of waste paper can be converted enzymatically to sugars including glucose, and subsequently fermented to ethanol [1, 2]. The potential for using cellulosic materials in bioconversion processes is well recognized, but the high cost associated with the hydrolysis of cellulose remains a major problems [3]. Concentrated acid has been used in wood-to-ethanol processes, but yield is limited and implementation may require the development of new, cost-effective methods for acid recovery and reuse [3]. Enzymatic hydrolysis provides an environmentally friendly means of depolymerizing cellulose and the potential for higher yields, but costs are also unfavorable. Moreover, the resulting reducing sugar concentration is not high enough to be used as carbon source in microorganism culture or chemical feedstock. It might be due to a low hydrolytic yield from waste paper.

Many researches have reported solutions to circumvent this problem and simultaneously to improve the hydrolytic yield. Supercritical carbon dioxide was used to increase the reactivity of cellulose and enhance the rate of cellulosic material hydrolysis as well as increases glucose yield by as much as 50% [4]. Ultrasound stimulated ethanol production in the simultaneous saccharification and fermentation of mixed waste paper [5]. Surfactants were reported to enhance the cellulose conversion; 2% addition of nonionic surfactants enhanced the cellulose conversion to 52% using pretreated newspaper [6].

However, with the viewpoint of recycling of waste paper, it is preferable to hydrolyze the waste paper without additional reagents, additional processes, or additional pretreatments. Moreover, with the progress of biotechnology several kinds of cellulase are available commercially for enzymatic hydrolysis of waste paper. Even though they are commercial products, there are few reports on investigation about which type of enzyme is suitable for the hydrolysis of waste paper.

In this work, we have tried to evaluate three kinds of cellulase, which are from different origin and are available commercially, on conversion of waste paper to reducing sugar during the enzymatic hydrolysis. Enzymatic hydrolysis was carried out at flask level, and an empirical model was applied to explain the conversion of waste paper. Using experimental results kinetic parameters were determined empirically and discussed.

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MATERIALS AND METHODS

Pretreatment of Waste Office paper

Office paper was used for this experiment. Waste papers were cut using shredder in rectangle with 0.6 cm in wide and 1.5 cm in length, and were used for enzymatic hydrolysis without any further pretreatments [7].

Enzymatic Hydrolysis

To investigate the effect of cellulase on the hydrolysis of waste office paper, the 50 mL of acetate buffer (pH 4.4) containing 1.5 g of dried waste office paper was pre-incubated at 45°C for 10 min. Hydrolysis of waste paper began by addition of cellulase. Amounts of enzyme used were 1%, 3%, 5%, and 10% (w/w) to the dried waste office paper as substrate. Three kinds of cellulase were used: Cellulosin T2 (Hankyu Bioindustry Co. Ltd., Osaka), Meicelase CEP 1070 and Acremonium cellulase AUS0301 (Meiji Seika Co., Tokyo). Their characteristics are listed in Table 1. When 1% enzyme to waste paper is used in the hydrolytic reaction, the activity of Acremonium cellulase, Meicelase, and Cellulosin T2 is corresponded to 0.34, 0.24, and 0.16 filter paper units per mL (units/mL), respectively. The hydrolytic reaction was carried out in 500-mL Erlenmeyer flask using a reciprocal shaker with agitation rate of 110 strokes per min for 15 h at a constant temperature of 45°C.

Furthermore, cardboard, comic magazine, catalog paper, and newspaper were tested to evaluate enzymatic hydrolysis with the same kind of enzyme.

Empirical Model

To quantitatively evaluate various enzymes a model describing the hydrolytic reaction of waste paper is required. Many models have been developed with postulated mechanisms for hydrolysis, product inhibition, enzyme adsorption and deactivation, *etc.* [8,9]. Because the complex waste paper was used in this study, and an empirical model proposed by Walseth [10] is chosen as following:

$$x = K t^{n} \tag{1}$$

x is conversion of waste paper to reducing sugar (%), t is time (h), and K and n are empirical constants, respectively. K has been proposed to be a function of the initial enzyme concentration, E (units/mL), in the form

$$K = kE^{m} \tag{2}$$

where k and m are also empirical constants.

Analytical Methods

After hydrolysis, the sample contained in strictly capped vials was boiled to deactivate the hydrolytic enzyme in boiling water for 10 min, the slurry was centrifuged and the supernatant was kept for sugar assay. Glucose concentration was measured by glucose analyzer (2700, YSI, Co. Inc. Yellow Spring, Ohio, USA), and reducing sugar was measured by HPLC. To analyze the composition of reducing sugars an HPLC (PU-980, JASCO, Co. Ltd., Tokyo) was used: Shim Pack column (CLC-NH_2, Shimadzu, Kyoto) and 75% acetonitorile solution as eluent with flow rate of 0.8 mL/min were used. Column temperature was controlled at 25°C and the reducing sugars were detected by RI detector (RI-930, JASCO, Co. Ltd., Tokyo). Standard glucose, xylose, and cellobiose for HPLC analysis were purchased from Wako Pure Chem. Ind. (Tokyo).

Filter paper assay [11] was performed to measure cellulase activity of different kinds of enzymes using Whatman No. 1 filter paper strip, 1 × 6 cm, and the activity was expressed as filter paper cellulase unit (units /mL). The conversion (%) of waste paper to reducing sugar (x) in reactor was calculated as follow,

$$x = \frac{\text{Reducing sugar concentration (g/L)} \cdot \text{Working volume (L)}}{\text{Waste paper weight (g)} \cdot \text{Cellulose content in waste paper (\%)}} \times 100 \tag{3}$$

where cellulose content in office paper was 99.1%, which was cited from reference [12].

Table 1. Optimal temperature and pH for the enzymatic hydrolysis with various enzymes

Cellulase[a]	Origin	Substrate	Temperature (°C)	pH	Specific activity (units/g)[b]
Acremonium cellulase [c]	*Acremonium cellulolyticus*	Watman No. 1 filter paper strip (1 × 6 cm)	50-70	4.0-5.0	1,127.0
Meicelase [c]	*Trichoderma viride*		50-70	4.0-5.0	787.7
Cellulosin T2 [d]	*Trichoderma viride*		50	4.5	677.7

[a] Trade name provided by maker.
[b] Activity was measured as filter paper cellulase units per gram of used enzyme.
[c] Acremonium cellulase and Meicelase from Meijiseika Co. Ltd., Japan.
[d] Cellulosin T2 from Hankyu Bioind. Co. Ltd., Osaka.

RESULTS

Effect of Different Kinds of Cellulase and Its Amount on Reducing Sugar Composition in Saccharification of Waste Office Paper

When Acremonium cellulase was used the glucose concentration increased with the increase in the enzyme loading, and so also xylose percentage increased to 13% with 10% enzyme loading. In the case of Meicelase, when the enzyme concentration higher than 5%

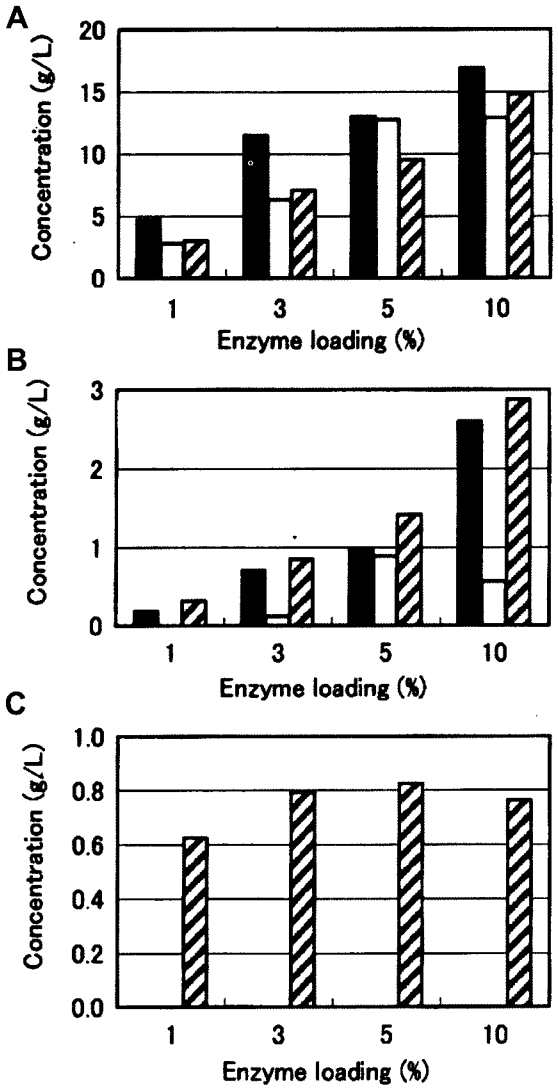

Fig. 1. Effect of different kinds of enzymes and its amount on reducing sugar composition in the enzymatic hydrolysis of waste office paper. A, B, and C denote Acremonium cellulase, Meicelase, and Cellulosin T2, respectively. Cellulase loading was represented as weight percent of enzyme to dried waste office paper. Black, white, and slashed bars indicate the concentration of glucose, xylose, and cellobiose, respectively.

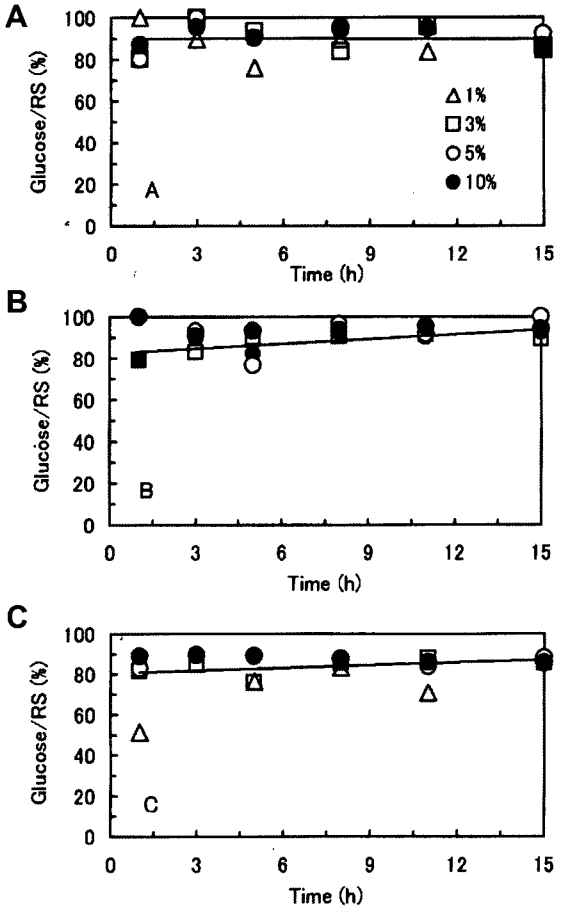

Fig. 2. Time course of the concentration ratio of glucose to reducing sugar during enzymatic hydrolysis with various types of enzyme and their concentrations. A, B, and C denote Acremonium cellulase, Meicelase, and Cellulosin T2, respectively. Used enzyme concentrations were 1% (triangle), 3% (square), 5% (open circle), and 10% (closed circle) to waste office paper.

was used glucose concentration was constant, and xylose concentration was lower than 1 g/L. Reducing sugar concentration was not proportional to Meicelase concentration, which was different from the other two enzymes. Cellulosin T2 produced reducing sugar in a similar amount to Acremonium cellulase, but the amount of xylose and cellobiose content reached 20%, which was the highest among the three kinds of cellulase.

From the viewpoint of microbial cultivation, both glucose concentration and glucose percentage contained in reducing sugar should be as high as possible, because reducing sugars such as xylose, cellobiose or cellotriose are hardly consumed by microorganisms. When cellulose was hydrolyzed enzymatically, glucose, cellobiose, and cellotriose was produced as reducing sugars, sometimes lot of xylose might be produced. In the case of hydrolytic reaction using Cellulosin T2, xylose percentage rose to 16%. Xylose might originate from hemicellulose consisting of xylans, polymers of xylose in β (1-4) linkage with side chains of other sugars. Since Acremonium cellulase, Meicelase, and Cellulosin T2 have xylanase activity, xylan that contained in waste paper during paper manufacturing process might be hydrolyzed, which might be one possibility.

Glucose Percentage to Total Reducing Sugar During Enzymatic Hydrolysis using Various Kinds of Cellulase

Increase of the yield of glucose concentration among the total reducing sugar is a very important factor to

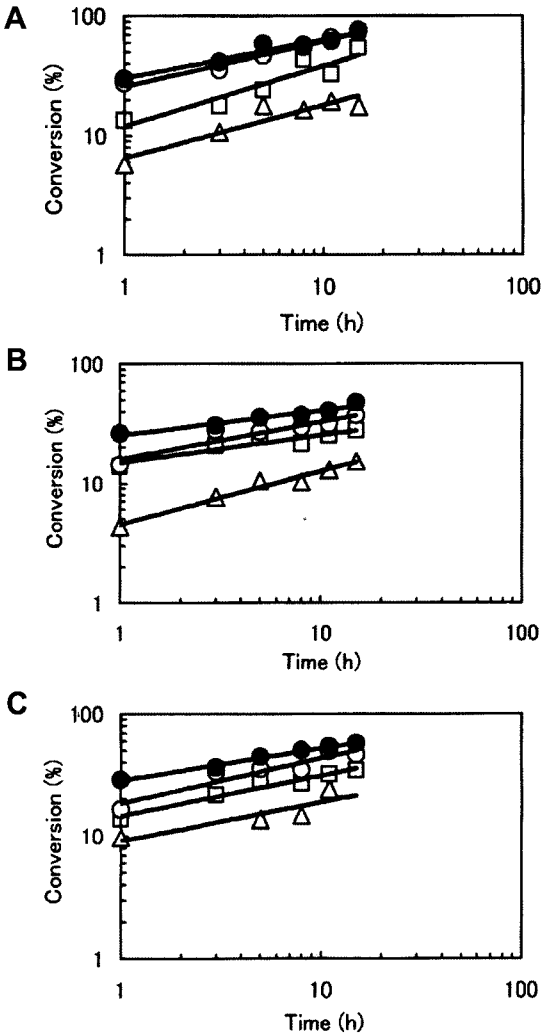

Fig. 3. Conversion kinetics of three cellulases from waste office paper to reducing sugar at different enzyme concentration. A, B, and C denote Acremonium cellulase, Meicelase, and Cellulosin T2, respectively. Symbols are the same as those in Fig. 2.

evaluate cellulase. The concentration ratio of glucose and total reducing sugar was investigated under various conditions like varying amounts of enzyme loadings and their reaction times, as shown in Fig. 2. In the case of Acremonium cellulase, the glucose percentage did not depended upon the amount of enzyme loaded and reaction time, and maintained at 90%. Meicelase and Cellulosin T2 tended to increase from 80% to 90% with the increase in the reaction time. There is no difference in glucose percentage in reducing sugar among the used kinds of cellulase.

Empirical Kinetic Model of Enzymatic Hydrolysis of Waste Office Paper to Reducing Sugar

The conversion kinetics of cellulose from waste paper to reducing sugar was fitted logarithmically as shown

Table 2. Kinetic parameters, a and b in the empirical model proposed by Eq. (4)

Cellulase	a (mL/units)	b (–)	Correlation coefficient
Acremonium cellulase	-0.059	0.51	0.93
Meicelase	-0.100	0.41	0.72
Cellulosin T2	-0.054	0.37	0.97

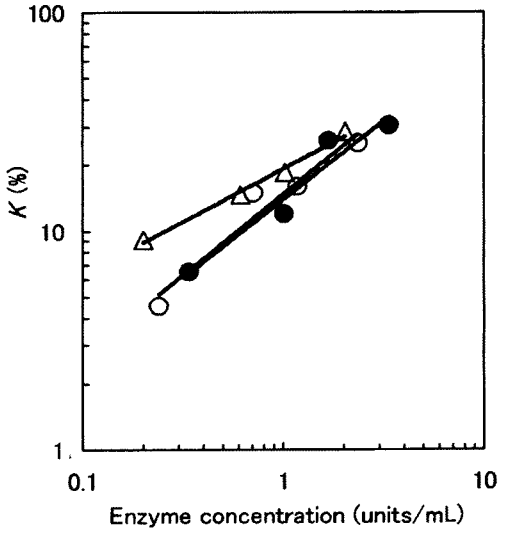

Fig. 4. Correlation between K determined from Fig. 3 and enzyme concentration. Closed circle, open circle, and triangle, denote Acremonium cellulase, Meicelase, and Cellulosin T2, respectively.

in Fig. 3. The K values in Eq. (1) were depended on both used type of enzyme and its amount. The parameter, n assumes to be depended only on the type of enzyme used and the enzyme concentration, because the slopes in Fig. 3 were varied on the enzyme concentration used. Since the n value was affected by the initial enzyme concentration, which can be expressed as follow:

$$n = aE + b \tag{4}$$

where, a and b denote empirical constants that indicate the enzyme deactivation that may be dependent upon inhibition by reducing sugar, the enzyme concentration and the type of enzyme. The determined constants are shown in Table 2. The a value of both Acremonium cellulase and Cellulosin T2 showed similar value, but that of Meicelase was two-fold as high as other enzymes. This indicates that the Meicelase might be easy to be deactivated under reaction conditions. The correlation coefficients of Acremonium cellulase and Cellulosin T2 were higher than 0.93, but Meicelase was 0.72 that might be due to instability in hydrolytic reaction of waste paper. The constant b seems to be dependent

Table 3. Kinetic parameters involved in the enzymatic hydrolysis with various enzymes

Cellulase	k^a	m (–)	Correlation coefficient
Acremonium cellulase	13.93	0.71	0.94
Meicelase	14.72	0.74	0.94
Cellulosin T2	19.23	0.48	0.99

[a] Unit is [% (units/mL)$^{-m}$].

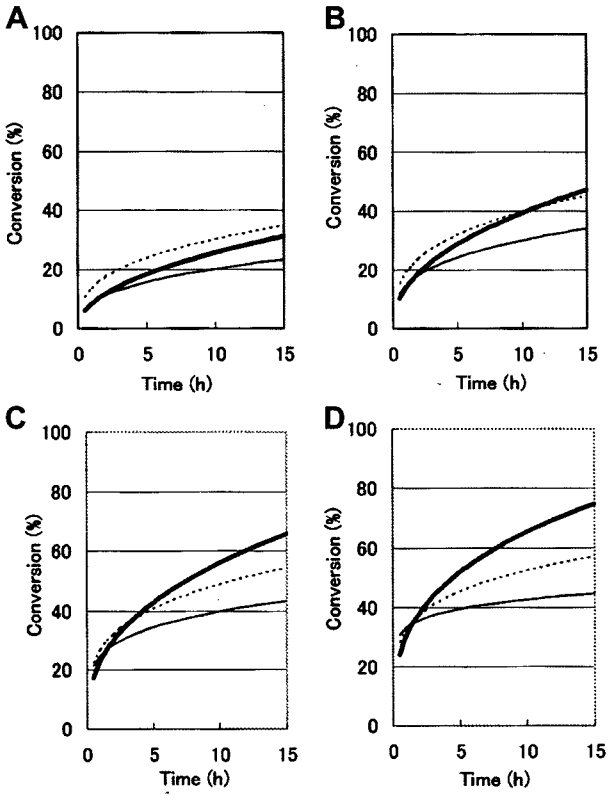

Fig. 5. Simulated results of the conversion of waste office paper to reducing sugar. A, B, C, and D denote results of enzyme concentration of 0.5, 1, 2, and 3 units ml^{-1}, respectively. Bold, plain, and dotted lines denote Acremonium cellulase, Meicelase, and Cellulosin T2, respectively.

upon the specific enzyme activity: Acremonium cellulase has 1.3-fold as high as those of other kinds of cellulase.

The K value has logarithmical linear relationship with the enzyme concentration as shown in Fig. 4, which demonstrated that the suggested empirical model could explain the conversion of waste paper to reducing sugar. Using the kinetics shown in the Eq. (2), parameters, k and m were determined and summarized in Table 3. The k value was similar except for Cellulosin T2, which means that initial enzyme reaction rate of three kinds of cellulase may not make much difference. The kinetic data were extremely precise, for each correlation

Table 4. Evaluation of empirical parameters of various waste papers

	Comic paper	Card board	News paper	Catalog	OA paper
Empirical parameters					
K	12.54	11.89	10.42	1.32	15.54
n	0.35	0.37	0.33	-0.06	0.42
Correlation coefficient	0.95	0.99	0.91	0.29	0.99
Saccharification of waste paper at 15 h reaction					
Glucose concentration (g/L)	7.8	8.4	6.8	0.4	12.8
Glucose percentage (%)	92	93	92	97	93
Conversion (%)	28.3	30.1	24.6	1.4	45.9

lation coefficient it was higher than 0.94. Therefore, the conversion kinetics of waste paper to reducing sugar is expressed empirically as followed,

$$x = kE^m t^{(aE+b)} \quad (5)$$

where k, m, a, and b denote empirical constants, and E indicates enzyme concentration.

Simulation of Enzymatic Hydrolysis of Waste Office Paper

Eq. (5) expresses enzymatic hydrolysis of waste office paper using various kinds of cellulase. With the range of enzyme concentration from 0.5 units/mL to 3 units/mL each type of enzyme the conversion of waste paper to reducing sugar was simulated as shown in Fig. 5. Upon fixed enzyme concentration, Acremonium cellulase showed the highest conversion. At 1 unit/mL, which corresponds to 2.9% enzyme of substrate, 45% of waste office paper was converted to reducing sugar, which explained well the results of Fig. 3.

In the case of Cellulosin T2, when the enzyme concentration was lower than 1 units/mL the conversion was compatible to that of Acremonium cellulase, but at higher than 1 units/mL the increase in the conversion was lower than that of Acremonium cellulase. This means that Cellulosin T2 might be inhibited by some byproducts. In the case of 2 units/mL of cellulase activity the conversion of waste paper to reducing sugar was 66% for Acremonium cellulase, 43% for Meicelase, 55% for Cellulosin T2. The results in Fig. 5 reveal that it would be possible to achieve 75% conversion of waste office paper to reducing sugar if 3 units/mL of cellulase was used. The 3 units/mL corresponds to the amount of 8.7% to waste office paper in Acremonium cellulase, 12.6% in Meicelase, 15.0% in Cellulosin T2.

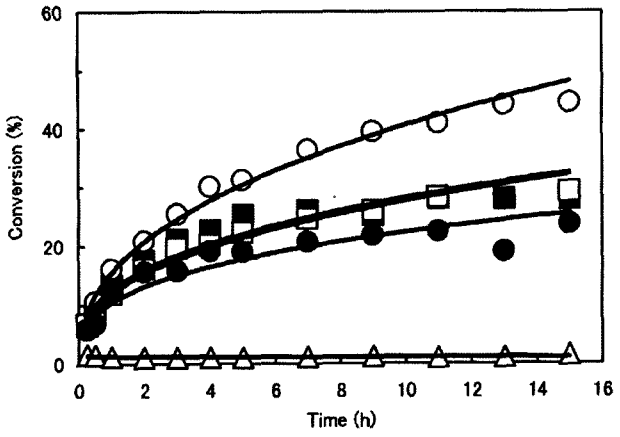

Fig. 6. Effect of various kinds of waste paper on the conversion of cellolosic material from waste paper. Office paper (open circle), cardboard (open square), comic magazine (closed square), newspaper (closed circle), and catalog paper (triangle) were used. Five percentage of Acremonium cellulase were used to hydrolysis of waste paper.

Comparison of Conversion from Five Kinds of Waste Papers

Recently there are several kinds of waste papers, and those are ready for recycle, because they are segregated disposal. The amount is rising year be year. Waste comic paper, card board, news paper, catalog paper and office paper are chosen for the investigation of cellulose conversion with the same reaction conditions. Five percent of Acremonium cellulase to waste paper was used, which corresponded to 1.69 units/mL of cellulase activity. Fig. 6 shows the results. After 15 h hydrolysis office paper showed the highest conversion 46%, while the conversion of comic paper and card board were similar with conversion of 28%, news paper with 25% conversion. Glucose percentage was higher than 92% in all cases. Conversion of catalog papers was only 1.4%. The surface of catalog paper was coated with unknown materials, which might be a hindrance of hydrolytic reaction. Therefore, in the case of catalog paper it needs an extra process to separate the coated materials in the waste paper.

Fig. 6 provided empirical parameters to evaluate the hydrolysis of waste papers according to Eq. (1). Since the same amount of enzyme is used, K and n represent the hydrolytic efficiency of used waste papers. Table 4 showed the results. Comic paper and cardboard have almost the same K value, 11.9-12.5; newspaper showed slightly low 10.4. In the case of office paper K value was the highest, 15.5. The n value is also one parameter to evaluate waste paper. Comic paper and cardboard had similar value; newspaper had slightly low. But office paper showed 1.2-fold higher value.

DISCUSSION

From the viewpoint of mass hydrolysis using cellulase, there are several constraints like reduction of amount of cellulase, production of high percentage of glucose with high conversion rate of waste paper to reducing sugar, and low product inhibition. In this research, we evaluated commercially produced cellulase using empirical kinetics on enzymatic hydrolysis of waste paper. The empirical kinetic model is convenient to predict the enzymatic conversion of waste paper. The empirical model described precisely the experimental results. Acremonium cellulase obtained the highest conversion of hydrolysis of waste paper. From simulation it is possible to achieve 75% conversion of waste paper using only 3 units/mL of Acremonium cellulase within 15 h hydrolysis.

Tested three kinds of cellulase showed the glucose percentage higher than 90%. Xylose, cellobiose, and cellotriose including glucose were detected as reducing sugar. However cellobiose and cellotriose were negligible in Acremonium cellulase and Meicelase, but amounted to 1 g/L for the Cellulosin T2. Ten percentage of reducing sugar was xylose, which might be caused by the decomposition of xylans that were commingled during the pulp producing process. The xylose is hardly to use carbon source of microorganisms. Therefore, it is very important to reduce the xylose percentage for cultivation of microorganism using reducing sugar contained in enzymatically-hydrolyzed solution.

When five kinds of waste papers were used, the conversion was different from the kinds of waste papers used. The difference in the empirical parameters might be due to the lignin content or inhibitory materials containing in paper. The presence of inhibitory materials has not been known and the amounts are also not exactly uncovered. The lignin contents of office paper, comic paper, cardboard, and newspaper are 0.9, 14.4, 11.3, and 18.8%, respectively [12]. The cellulose conversion and n in Eq. (1) were inversely proportional to the lignin contents with correlation coefficient of 0.99, while K in Eq. (1) was also inversely proportional to the lignin content with correlation coefficient of 0.92 (data not shown). This indicates that the cellulose conversion and parameter n is correlated closely with the lignin content containing in paper.

Through our experiments, Acremonium cellulase showed the highest conversion as far as three kinds of commercially available kinds of cellulase were tested. However, research to achieve higher conversion yield with effective cost are still being pursued for the mass hydrolysis of waste paper and for recycle the waste paper as renewal resources.

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[Received March 10, 2002; accepted July 10, 2002]