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Hydrolysis of Paper Mill Sludge Using an Improved Enzyme System

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Abstract The effects of water soluble materials in paper mill sludge on cellulase and β-glucosidase activities were studied while the optimization of enzyme system for hydrolysis of the paper mill sludge for production of glucose was made. Water soluble materials in the paper mill sludge showed stimulatory effect on carboxymethyl cellulose (CMC) activity, inhibitory effect on filter paper (FP) activity, and no effect on avicelase and β-glucosidase activities. CMC and β-glucosidase activities at 5 and 10, 5 or 10 and 10, and 10 and 10 U/ml were optimal for hydrolysis of 5, 10, and 20% of the paper mill sludge, respectively.

Key words: Paper mill sludge, hydrolysis, cellulase, β-glucosidase, glucose

Each year, about 10^6 tons of paper mill sludge are produced in Korea. The dried materials, comprising 40% of this sludge, are composed of 30 to 60% of cellulose, 5 to 10% of lignin, with the remaining composed of mainly ashes. Cellulose in paper mill sludge can be hydrolyzed chemically or enzymatically to produce glucose, which in turn can be used as a substrate to ferment for the production of fuel [14, 15], organic acids [16], and other useful products. Chemical hydrolysis of cellulose yields waste acid or alkaline, which will eventually lead to environmental pollution, while enzymatic hydrolysis will be free from this drawback. Many works have been carried out on enzymatic hydrolysis of biomass such as different types of wood [22] and rice straws [11]. These natural materials should be pretreated in order to be easily attacked by enzymes [11, 22, 25]. The commercial development has been hindered because the pretreatment process requires high amount of energy [4, 13] and the enzyme is of higher cost than acid or alkaline. On the other hand, paper mill sludge might need no pretreatment because it has already been treated for removing lignin and hemicellulose and is much more susceptibile towards enzymatic hydrolysis [6]. In the present study, the enzyme system was optimized to obtain high glucose yield with relatively less enzymes to reduce the cost for the enzymes.

Cellulose is degraded by the synergistic action of three types of enzymes in the cellulase complex, namely, endoglucanase, exoglucanase, and β-glucosidase. Endoglucanase acts randomly on the internal bond of amorphous cellulose to break the polymer. Exoglucanase cuts the cellulose polymers from their nonreducing terminals producing cellobiose unit. Exoglucanase is intensively inhibited by cellobiose. β-Glucosidase degrades cellobiose into glucose that relieves the feedback inhibition of cellobiose on exoglucanase. Glucose, the final product, inhibits various steps breaking cellulose molecule into glucose [8]. Therefore, it is necessary to employ a balanced enzyme system containing appropriate amounts of endo- and exo-type glucanases as well as β-glucosidase for achieving an efficient hydrolysis of cellulose. In this research, we investigated optimal conditions, especially the balance of the enzyme system for the hydrolysis of paper mill sludge.

MATERIALS AND METHODS

Enzymes and Reaction Condition

Raw cellulase powder [30] produced by *Trichoderma reesei* Rut C-30 (ATCC 56765) and commercial β-glucosidase solution (Novozym 188, Novo Nordisk, Denmark) mixed in various compositions were used in this research. Twenty ml citrate buffer (pH 4.8), and appropriate amounts of paper mill sludge and enzymes in 100 ml flasks were incubated at 50°C and 350 rpm in a shaking water bath (KMC1205KW1, KMC Vision Co., Korea). Oxymycin as an antibiotic was added to a final concentration of 25 mg/ml for preventing contamination. The flasks containing the mixture of paper mill sludge and buffer solution were sterilized at 121°C for 30 min and then cooled down to about 50°C before adding

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of the enzymes and antibiotics. Paper mill sludge (sludge for short is used in the following parts) was obtained from Samwha Paper Co., Korea.

Analytical Methods

CMC and FP degrading activities, and avicelase and β-glucosidase activities were determined according to the method of the International Union of Pure and Applied Chemistry (IUPAC). One unit of the enzyme activity was defined as the amount of enzyme releasing 1 μmol of glucose per minute. Glucose concentration in enzymatic hydrolysis experiments was measured using the glucose analyzer (YSI 2700, Yellow Springs Instrument Co., U.S.A.).

RESULTS AND DISCUSSION

Effect of Water Soluble Materials in Sludge on Enzyme Activities

A sludge of 5% (dry weight/volume) was washed using distilled water by stirring at about 500 rpm for 2 h, and then centrifuged at 4°C, 8,000 rpm for 30 min to recover the sludge washing water. This water was then used for dilution of the cellulase powder and the β-glucosidase solution (described in Materials and Methods) for activity measurements. At the same time, the controls of cellulase and β-glucosidase diluted with distilled water were also measured for the enzyme activities. The results of cellulase and β-glucosidase activities are shown in Table 1. It shows that water extractable materials in the sludge indicated stimulating effect on CMC activities but inhibitory effect on FP activities, while almost no effect was shown on avicelase activities. Water extractable materials also have no effect on β-glucosidase activities.

Paper-mill industries use numerous chemicals during the extraction and purification of the cellulose which might have toxic effects on enzymes. Purification is performed by bleaching with chlorine, alkaline, and sometimes hypochlorite. Other additives are used in the preparation of pulp, such as kaoline, talc, titanium dioxide, calcium carbonate, zinc oxide and sulphate, diatomaceous earth, etc., whereas gelatine, vegetable glue, etc. are used as binders. About 50% of the dry sludge is constituted by organic matter, the mineral component is mainly calcium carbonate, talc and kaolin, and the metals are mostly aluminum and zinc. Although many chemicals which might be toxic to enzymes remained in the sludge, the above result showed that these chemicals do not affect the enzyme activities significantly. Therefore, no pretreatment to remove the toxins in the sludge was attempted prior to use for enzymatic hydrolysis.

Optimization of Enzyme System for Hydrolysis of 5% Sludge

Cellulase and β-glucosidase were mixed in various combinations. Six levels of CMC activities, 200, 100, 10, 5, 3, and 1 U/ml, respectively, were used. Four levels of β-glucosidase activities, 20, 10, 1, and 0.25 U/ml, respectively, were used. All combinations of CMC and β-glucosidase activities were tested for hydrolysis of 5% sludge in a batch mode. The time course of the hydrolysis showed that glucose concentration reached the maximum within 6 h of hydrolysis (Fig. 1). Glucose production was the highest in the case 5 with the CMC activities of 100 U/ml and β-glucosidase activities of 20 U/ml (Fig. 2). With the increase of CMC activity to 200 U/ml, which was the case 1 experiment, the glucose production decreased (Fig. 2). High glucose productions were obtained at high β-glucosidase activities (Fig. 2). Figure 3 showed that CMC activity at about 5 U/ml and β-glucosidase activity at about 10 U/ml was an optimal condition for hydrolysis of 5% sludge. Glucose yield was increased with high β-glucosidase activities. With CMC activity of 100 U/ml and β-glucosidase activity of 20 U/ml, glucose yield was almost one hundred percent (Fig. 4).

In general, the higher the enzyme activity, the more glucose was produced. However, with the same β-glucosidase activity of 20 U/ml, glucose production with CMC activity of 200 U/ml was lower than that with 100 U/ml. The

Table 1. Effect of water extractable materials on enzyme activities.

Dilution solvent	(1) Sludge washing water	(2) Distilled water	Activities in solvent (1)/ Activities in solvent (2)
Enzyme activity	(U/ml)	(U/ml)	
Cellulase (*T. reesei*)			
CMC activity	15.32	8.98	1.71
Avicelase	0.20	0.22	0.91
FP activity	0.64	3.51	0.18
CMC/FP activity	24.12	2.56	
β-glucosidase			
(NOVOZYM 188)	251	250	≈1

The measurements were made in duplicate.

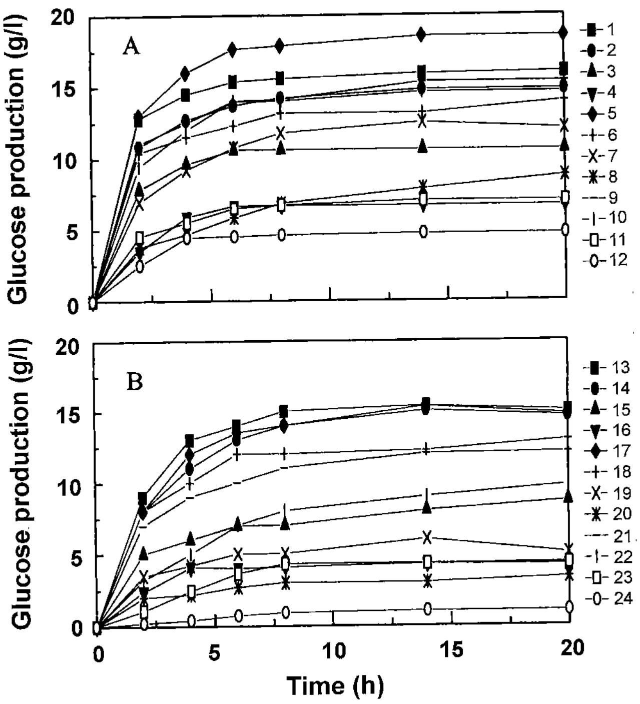

Fig. 1. Time course of 5% sludge hydrolysis.
A: Cases 1 to 12 stand for CMC and β-glucosidase activities of 200/20, 200/10, 200/1, 200/0.25, 100/20, 100/10, 100/1, 100/0.25, 10/20, 10/10, 5/1, 10/0.25 U/ml, respectively. B: Cases 13 to 24 stand for CMC and β-glucosidase activities of 5/20, 5/10, 5/1, 5/0.25, 3/20, 3/10, 3/1, 3/0.25, 1/20, 1/10, 1/1, 1/0.25 U/ml, respectively.

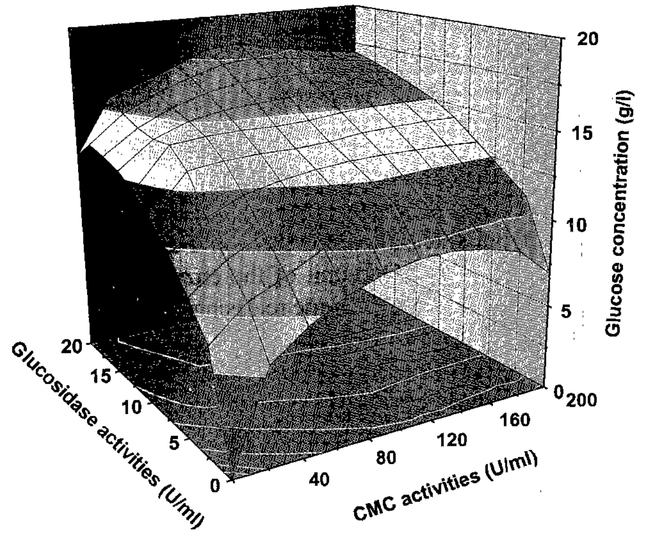

Fig. 3. Relation of glucose production to CMC and β-glucosidase activities.

former simulation result showed that with high activities of cellulase, not only glucose, but also cellobiose concentration reached a great level in the early stage of the hydrolysis (data not shown). Cellobiose accumulation is known to inhibit the enzymatic activity of both the endo- and exo-types of glucanase components of the fungal cellulase complex [10]. This should be the reason why too high CMC or cellulase activities can lead to the decrease of final glucose concentration.

Optimization of Enzyme System for Hydrolysis of 10% Sludge

Four levels of CMC activities, 200, 100, 10, and 5 U/ml, respectively, and two levels of β-glucosidase activities, 20 and 10 U/ml, respectively, were tested. All combinations of the levels of CMC and β-glucosidase activities were tried in hydrolysis experiments with 10% sludge in the batch mode. Glucose concentration was fairly high after one hour and almost reached the maximum level after 10 h of hydrolysis (Fig. 5). The highest glucose production

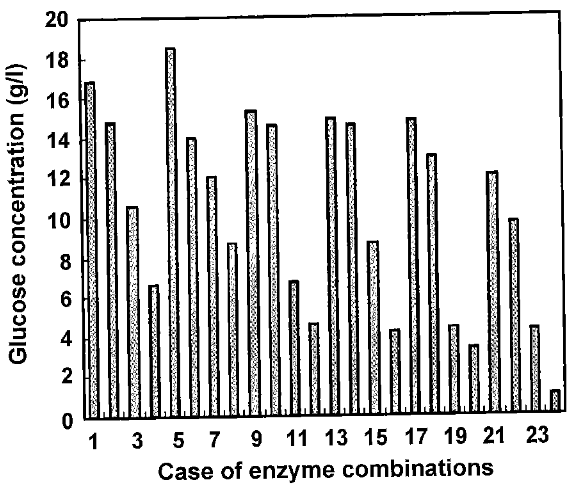

Fig. 2. Hydrolysis of 5% sludge using various enzyme systems.
Cases from 1 to 24 are the same as the cases in Fig. 1.

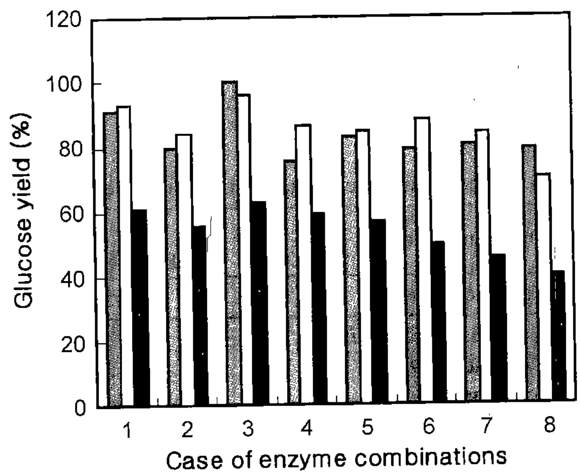

Fig. 4. Glucose yields of sludge hydrolysis.
▩: 5% sludge; □: 10% sludge; ■: 20% sludge. CMC and β-glucosidase activities are 200/20, 100/20, 200/10, 100/10, 10/20, 10/10, 5/20, and 5/10 U ml for cases from 1 to 8, respectively.

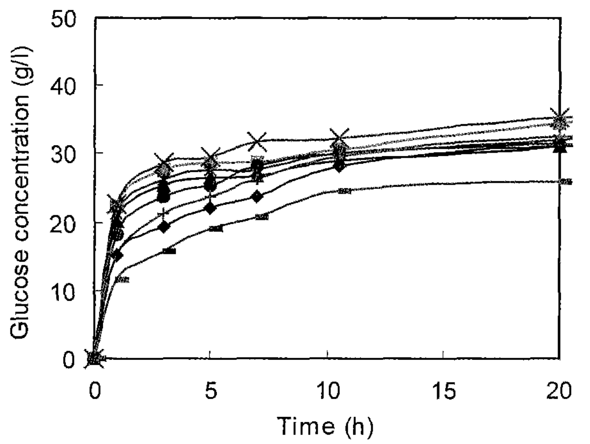

Fig. 5. Time course of 10% sludge hydrolysis.
CMC and β-glucosidase activities are ■: 20/20; ▲: 200/10; ✕: 100/200; ✳: 100/10; ●: 10/20; +: 10/10; ◆: 5/20; ▬: 5/10 in U/ml.

was obtained with CMC and β-glucosidase activities of 100 and 20 U/ml, respectively. At the same β-glucosidase activity of 20 U/ml, glucose production with CMC activity of 200 U/ml was lower than that with 100 U/ml, which was the same pattern as that in hydrolysis of 5% sludge (Fig. 6). Glucose production was not varied too much in all eight cases of enzyme combinations (Fig. 6). Glucose yields were also high enough and had similar values as in hydrolysis of 5% sludge (Fig. 4). Glucose production almost doubled without any indications of an apparent product inhibition effect that occurred with sludge of 10% compared to 5%. The CMC activity of 5 or 10 U/ml and β-glucosidase activity of 10 U/ml was found to be an optimal enzyme combination for hydrolysis of 10% sludge.

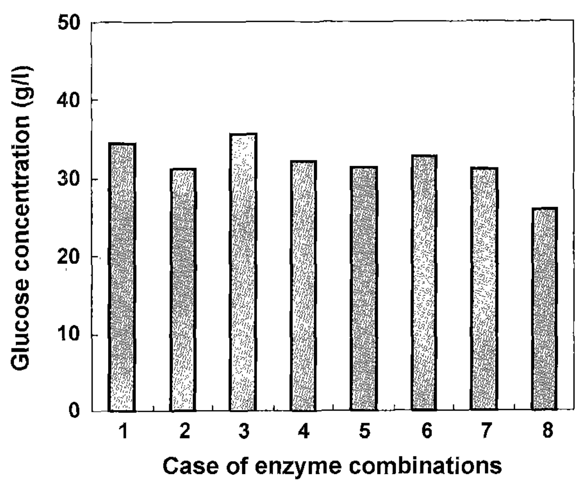

Fig. 6. Results of 20 h hydrolysis of 10% sludge with various enzyme systems.
Cases 1 to 8 stand for CMC/β-glucosidase activities of 200/20, 200/10, 100/20, 100/10, 10/20, 10/10, 5/20, and 5/10 U/ml, respectively.

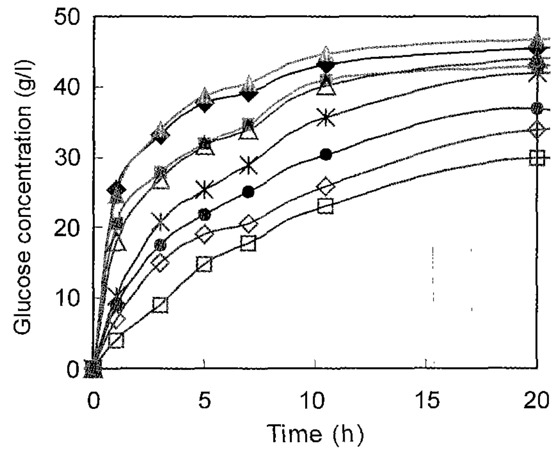

Fig. 7. Time course of 20% sludge hydrolysis.
◆: 200/20; ■: 200/10; ▲: 100/20; △: 100/10; ✕: 10/20; ●: 10/10; ◇: 5/20; ⊞: 5/10.

Optimization of Enzyme System for Hydrolysis of 20% Sludge

Same enzyme combinations as hydrolysis of 10% sludge were used in the hydrolysis of 20% sludge in the batch mode. All combinations of four levels of CMC activities, 200, 100, 10, and 5 U/ml, respectively, and two levels of β-glucosidase activities, 20 and 10 U/ml, respectively, were used. In the cases of CMC activities of 200 or 100 U/ml, glucose concentration almost reached the maximum level after 10 h of hydrolysis (Fig. 7). However, in cases of lower CMC activities, such as 10 or 5 U/ml, glucose concentration reached the maximum level after 20 h of hydrolysis (Fig. 7). The highest glucose production was also obtained with CMC activity of 100 U/ml and β-glucosidase activity of 20 U/ml, which was the same optimal condition as hydrolysis of sludge of 5 or 10% (Fig. 8). Except in a case of CMC activity of 200 U/ml, glucose

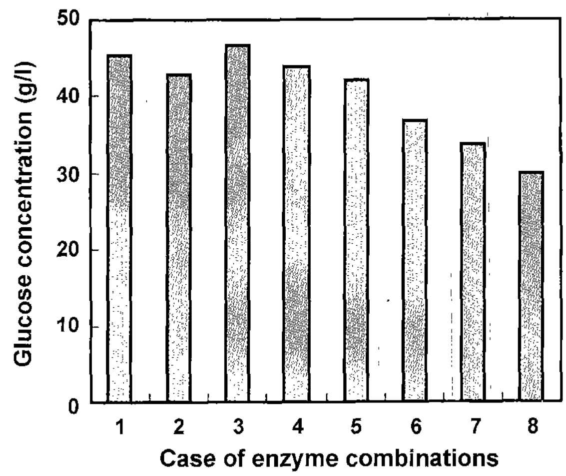

Fig. 8. Results of 20 h hydrolysis of 20% sludge with various enzyme systems.
Cases 1 to 8 are the same as that of Fig. 6.

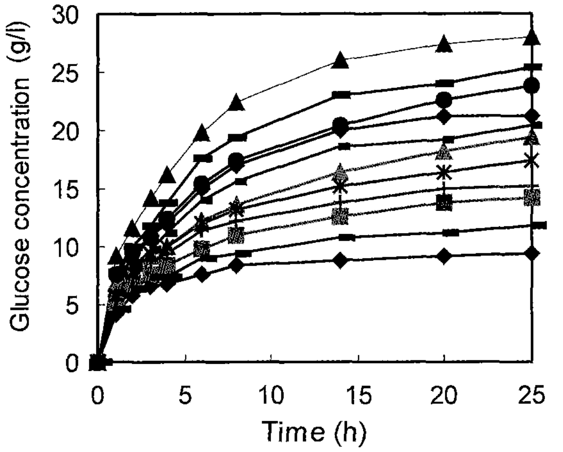

Fig. 9. Time course of hydrolysis with gradual feeds of sludge. At 0, 1, 2, 3 h of hydrolysis, 5% sterilized sludge was added to reach the final sludge concentration of 20%. CMC and β-glucosidase activities are -◆-: 5/2.5; -■-: 5/5; -▲-: 5/10; -▬-: 10/2.5; -✳-: 10/5; -●-: 10/10; -+-: 30/2.5; -▬-: 30/5; -▬-: 30/10; -◆-: 50/2.5; -▲-: 50/10 in U/ml.

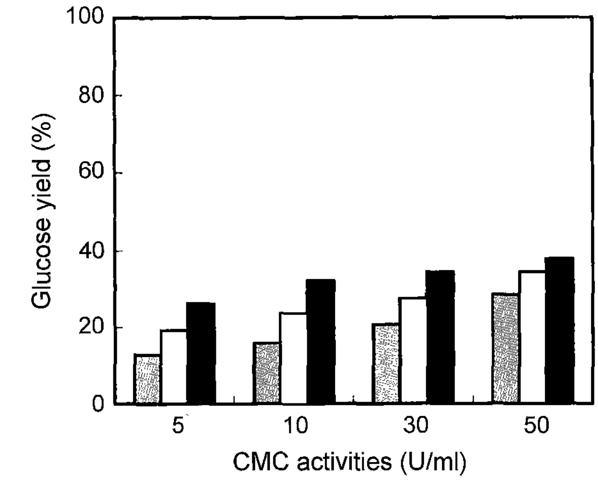

Fig. 11. Glucose yields of sludge hydrolysis in fed-batch mode. β-glucosidase activities are ▩: 2.5; □: 5; ■: 10 in U/ml.

production decreased more obviously with the decrease of enzyme activities for both CMC and β-glucosidase in hydrolysis of sludge of 20% compared to that of 5 or 10% (Figs. 2, 6, and 8). Glucose yields in hydrolysis of sludge of 20% were apparently lower than that of 5 or 10% (Fig. 4). This was because the product inhibition effect caused by cellobiose and glucose was stronger in the case of 20% than that of 5 or 10%. Besides the product inhibition problem, mass transfer resistance also occurred in the case 20%, especially with lower enzyme activities. Gradual addition of sludge could not increase either glucose production or glucose yield even though it could overcome the mass transfer problem (Figs. 9, 10, and 11). The reason was that, in the case of product inhibition, high initial concentration of substrate favored the reaction. CMC activity of 10 U/ml

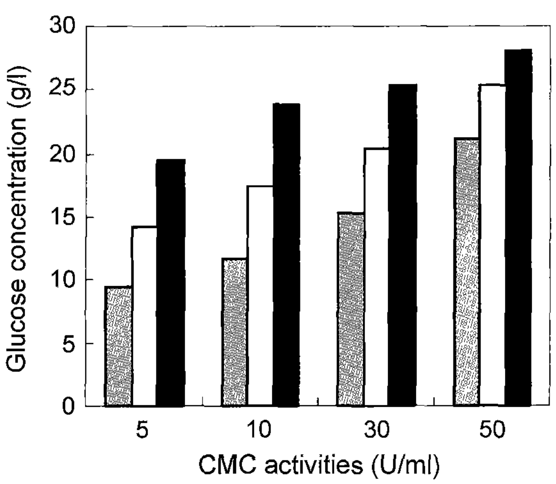

Fig. 10. Glucose concentrations at 25 h of hydrolysis with gradual feeds of sludge.
β-glucosidase activities are ▩: 2.5; □: 5; ■: 10 in U/ml.

and β-glucosidase activity of 20 U/ml was an optimal enzyme combination for hydrolysis of sludge of 20% in batch mode.

In order to develop a process of practical importance, crude cellulase of fixed endo- and exo-type glucanase composition was used. Therefore, no optimization on the composition of endo- and exo-type glucanase was made in this research.

High glucose yield can be obtained by enzymatic hydrolysis especially at sludge concentrations lower than 20%. This result shows that the sludge needs no pretreatment before enzymatic hydrolysis in order to obtain high glucose yield. Besides the chemicals that are added in the paper mill, the properties of the cellulosic materials such as particle size, degree of polymerization (DP), and crystallinity index (CrI) also affect the enzymatic hydrolysis of paper mill sludge. Cellulose is usually present in a bundle of fibrillar units with a supramolecular structure consisting of crystalline and amorphous regions [28]. The amorphous regions of the fiber are supposed to occur at similar intervals within the fiber. The initial action of the enzyme is to attack the less ordered regions of the fiber, resulting in a rapid reduction in particle size. Rapid decrease in viscosity of the sludge during the hydrolysis process was observed, but the influences of DP and crystallinity in restricting the ease of enzymatic hydrolysis are unclear. The susceptibility of sludge to complete enzymatic hydrolysis can not be easily predicted from the differences in the initial DP and crystallinity [12, 17]. Therefore, DP and crystallinity were not measured in this research.

Enzyme related factors that are influential in the hydrolysis process include end product inhibition of the enzyme, thermal and mechanical inactivation, and irreversible adsorption of the enzymes [3, 7, 24]. In this research, glucose production and the glucose yield were increased by the optimization of the enzyme system through the addition of β-glucosidase, which hydrolyzes cellobiose to glucose, thereby, preventing inhibition of cellulase by cellobiose [2]. Optimization of the enzyme system can also be made using mixed culture of *T. reesei* and *Aspergillus niger* [5, 9, 21], which are the efficient organisms to produce cellulase and β-glucosidase, respectively. The mixed culture is being done in our laboratory in order to produce the optimized enzyme system more efficiently. Thermal and mechanical inactivation should not be severe at the temperature not higher than 50°C and the agitation speed lower than 100 rpm during the reaction period within two days, the condition which is going to be used in our later research. Lignin is reported to be responsible for irreversible adsorption of cellulases [18-20, 27], but there is no lignin in the sludge that we use.

The glucose concentration produced by hydrolysis of 10% sludge, which is about 30 g/l, is high enough for a fermentation process operated in a continuous or repeated fed-batch mode. In order to make the hydrolysis process more economical, we are using sludge as the material to produce the enzymes in low cost after confirming that *T. reesei* and *A. niger* can grow and produce enzymes normally on sludge. Simultaneous saccharification and fermentation (SSF) processes can improve the hydrolysis efficiency by prevention of the accumulation of glucose and cellobiose to reduce the severe feedback inhibition effect on hydrolytic enzymes [1, 23, 26]. It has been reported that lactic acid, acetic acid, citric acid, itaconic acid, α-ketoglutaric acid, and succinic acid scarcely inhibit cellulase [1]. As a result, SSF has the advantage in producing the organic acids using cellulosic materials. We have confirmed that conditions for L-lactic acid fermentation using *Lactobacillus* and for cellulase and β-glucosidase reactions are compatible, which make it possible to produce L-lactic acid using paper mill sludge and the SSF process. This process is hopefully to reduce the cost of L-lactic acid, low enough for making degradable plastic [29].

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