Enzymatic Hydrolysis of Bamboo Alkaline Sulfite Pulp for Glucose Production

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List of Abbreviations

Abbreviation	Description
AA	Active Alkali
AS-AQ	Alkaline Sulfite-Anthraquinone
Py-GC/MS	Pyrolysis-Gas Chromatography/Mass Spectrometry
NREL	National Renewable Energy Laboratory
FPU	Filter Paper Unit
ISO	International Organization for Standardization
IS	Internal Standard
ppm	parts per million
ppb	parts per billion
UV	Ultra Violet
IUPAC	International Union of Pure and Applied Chemistry
AcBr	Acetyl bromide

Chapter 1 Introduction

1.1 Glucose and ethanol production from lignocellulosic biomass

Lignocellulosic biomass refers to plant biomass that is composed of lignin, cellulose, and hemicelluloses, including forest products (softwood and hardwood), agricultural residues (wheat straw, corn stover, and sugarcane bagasse), and dedicated crops (switchgrass and salix).

Crude oil has been a major resource used to meet increasing energy demands. However, other energy sources are needed to reduce pollution and help satisfy the Kyoto protocol, established in 1997, by limiting the global net emission of carbon dioxide. Compared with fossil fuels, the advantages of biofuel sources are geographically distributed, generates low net green gas emissions, particularly climate change [1-3]. Cellulosic ethanol and ethanol produced from other biomass resources may have the potential to reduce greenhouse gas emissions by 86% [4].

1.2 Pretreatment of lignocellulosic biomass for glucose production

The pretreatment of lignocellulosic materials to remove lignin and hemicellulose can significantly enhance the enzymatic hydrolysis of cellulose in lignocellulosic biomass. Pretreatment procedures must meet the following requirements [1)]:

1. To improve the formation of monosaccharide by the pretreatment or by the subsequent enzymatic hydrolysis

2. To avoid the degradation of carbohydrate during the treatment

3. To avoid the formation of byproducts that inhibit the subsequent enzymatic hydrolysis and fermentation processes.

The cooking methods used in the papermaking industry can be divided into alkaline and acidic cooking methods, which include kraft and acid sulfite cooking, respectively. However, whether the wood cooking methods meet the three requirements is not known.

It has been reported that the delignification effect of alkaline cooking methods for pretreatment is better compared with acidic cooking methods, because there is less decomposition of carbohydrates and the glucose yield of subsequent enzymatic hydrolysis is high [5), 6)]. The alkaline wood cooking method is an established method in pulp and paper industry, therefore it is advantageous as pretreatment for bioethanol production

from lignocellulosic biomass. The Forestry and Forest Products Research Institute of Japan has studied the utilization of soda cooking pretreatment methods for the production of bioethanol from lignocellulosic biomass [7), 8)]. Takahashi *et al*. [9), 10)] reported the advantages of using acid sulfite cooking as a pretreatment for ethanol production, and Yamashita *et al*. [11), 12)] used sodium hydroxide for the pretreatment of Japanese cedar and alkaline peroxide for the pretreatment of bamboo.

Alkaline sulfite (AS) cooking is often used to produce pulp from non-wood materials. Hedjazi *et al*. [13)] used the AS-anthraquinone (AQ) method to cook wheat straw, which resulted in a higher pulp yield than by soda-AQ method. Latibari *et al*. [14)] clarified that the optimum cooking condition for corn stalks was an $NaOH/Na_2SO_3$ ratio of 50/50. Kamthai *et al*. [15)] used the AS-AQ method to cook bamboo and obtained a pulp yield of approximately 56%. However, few studies report the use of AS cooking as a pretreatment for ethanol production. Identifying the applicable materials and cooking conditions is useful when applying AS cooking as pretreatment.

1.3 Enzymatic hydrolysis treatment

Ethanol is mainly obtained from the fermentation of hexoses, such as glucose. Cellulosic hydrolysis by acid or enzyme for obtaining glucose is affected by lignin and hemicellulose content in biomass. Guo *et al.* [16] used sulfuric acid with different concentrations to pretreat herbaceous materials, such as bagasse, at 130 ℃ for 15 min. They demonstrated that enzymatic digestibility was significantly affected by lignin content of the pretreated lignocellulosic materials, and they suggested that lignin inhibits the cellulose accessibility of cellulase, leading to a reduction in enzymatic hydrolysis efficiency [17), 18)]. However, the mechanism of lignin action is not clear.

1.4 Estimation of residual lignin after enzymatic hydrolysis

Lignin inhibits enzymatic hydrolysis during saccharification for bioethanol production from woody biomass [19]. Lignin is a polymer that consists of phenylpropane units which can be divided into three groups: syrigyl, guaiacyl, and *p*-hydroxyphenyl types. It was suggested that these units cannot be degraded by hydrolysis enzymes such as cellulose; furthermore, they can absorb the enzyme, which can inhibit the accessibility of the enzyme to the cellulose [20].

The saccharification ratio of bamboo (*Phyllostachys pubescens*) stem alkaline sulfite pulp during enzymatic hydrolysis was much higher than that from larch (*Larix leptolepis*) pulp and cellulose filter paper [21]. In order to investigate the residual lignin and saccharification behavior of these pulps, it is necessary to precisely determine the lignin content after enzymatic hydrolysis.

Glucose is a raw material for bioethanol production. During enzymatic hydrolysis for glucose production, lignin and xylan remained in the saccharification residue. Further, even if the residue was thoroughly washed after enzymatic hydrolysis, there was still enzyme remaining in the residue. In order to determine the lignin content in the saccharification residue, it is necessary to find a rapid, accurate, and precise lignin determination method.

1.4.1 Lignin determination by using Acetyl bromide method

The acetyl bromide method and the Klason method are commonly used for the quantification of lignin. For the Klason method, 0.3–1.0 g of sample is required for the analysis. The comparative advantage of the acetyl bromide method is that only a small amount of sample (about 15 mg) is required. In 1961, Johnson *et al.* [22] first introduced the acetyl bromide method for the quantification of lignin. Since then, the original procedure has been modified to serve various purposes [23-28]. Iiyama and Wallis [23] incorporated perchloric acid to completely dissolve the plant cell wall and the cellulose fraction. Hatfield *et al.* [24] reported that acetyl bromide readily degrades xylan. In contrast, Marton [29] reported the successful use of this method to quantify lignin in softwood kraft and bisulfite pulps with kappa numbers in the 15–120 range. However, Marton [29] also reported that this method sometimes produces inconclusive results for hardwood pulps.

1.4.2 Lignin determination by using pyrolysis gas chromatography/mass spectrometry

Pyrolysis-gas chromatography/mass spectrometry (Py-GC/MS) is a sensitive and rapid method for characterizing the complex polymers in lignocellulosic biomass [30]. Only tens of micrograms are required for analysis using this method. Furthermore, it has the advantage of accurate identification of the pyrolysis products. Hasumi *et al.* [31] used the Py-GC/MS method to determine the lignin structure of eucalyptus pulp. In addition to eucalyptus [32, 33], Py-GC/MS has been used to determine the lignin content in softwood materials [34, 35] and non-wood fiber materials [36, 37].

1.5 Objectives of this study

The use of alkaline sulfite cooking as a pretreatment method for bioethanol production has not yet been reported. It will be useful to clarify the effect of this cooking method as compared to other cooking methods.

This study was structured as follows:

First, to evaluate the effect of the ratio of $NaOH/Na_2SO_3$ used in the pretreatment, in addition to cooking temperature and time, on pulp yield and delignification for both chips and meals. In addition to mentioned above, it is also to clarify the most effective cooking time, temperature, and the $NaOH/Na_2SO_3$ ratio.

Second, use filter paper to determine the cellulase activity of enzyme (GC220, provided by Genencor Kyowa Co. Ltd., Japan). The enzymatic digestibility of various materials with different lignin contents using different enzyme dosages and treatment times, to clarify the effect of lignin content on enzymatic hydrolysis. In this study, bamboo stem and kenaf bast pulps, other materials, such as LOKP, BCTMP, PPC, and cellobiose will be hydrolyzed in addition to larch heartwood pulp. The enzymatic digestibility of these materials will be evaluated by comparing them to filter paper.

Third, to estimate the lignin content in residual bamboo pulp after enzymatic hydrolysis, the possibility of using the acetyl bromide and Py-GC/MS methods was discussed. The difficulty of applying the acetyl bromide method to analyze the lignin in saccharification residue was clarified. The final aim of this study was to use the Py-GC/MS method to determine the lignin content and remaining enzyme in the residue.

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Chapter 2 Pretreatment of Lignocellulosic biomass

2.1 Introduction

There was little study report the use of AS cooking as a pretreatment for ethanol production. On applying the alkaline sulfite cooking as pretreatment, to clarify the applicable materials and cooking conditions is useful.

In this chapter, first, as a pretreatment, alkaline sulfite cooking was focused for the enzymatic hydrolysis of larch and bamboo, the cooking characteristics were examined of each material. Particularly, the effect of $NaOH/Na_2SO_3$ ratio in cooking liquor on pulp yield and delignification was clarified.

2.2 Materials and Methods

2.2.1 Materials

Wood from 30-year-old Japanese larch (*Larix leptolepis*) was obtained from the Agricultural and Forestry Research Center of University of Tsukuba, Yatsugatake Forest (Kawakami, Nagano, Japan). The larch wood was divided into heartwood and sapwood. Heartwood portions were cut with a chisel into wood chips (20 mm × 10 mm × 3 mm as longitudinal × radial × tangential directions) for cooking.

Bamboo (*Phyllostachys pubescens*) was obtained from Hitachiomiya in Ibaraki Prefecture. The bamboo stem was cut by a chisel into small blocks as the same size as wood chips for the cooking.

Filter paper (Advantec No. 1) was purchased from Toyo Roshi Co., Ltd.

LOKP was provided by the Niigata Mill of the Hokuetsu Kishu Paper Co., Ltd.

Canadian-made BCTMP was obtained from a Chinese papermaking company.

PPC was purchased from a local market.

2.2.2 Alkaline sulfite cooking and soda cooking

The cooking was performed in a 500-mL stainless steel autoclave (Taiatsu Techno Corporation) using 45 g (as oven-dried weight) of heartwood chips or bamboo stem blocks. The following conditions were applied in the cooking:

Active alkali charge (AA charge): 30%, calculated as Na_2O;

Heating time to maximum temperature: 45 min; and cooking time to maximum temperature: 60, 120 and 240 min.

Maximum temperature: 150 ℃ and 160 ℃; and anthraquinone (using SAQ, 1,4-dihydro-9,10-dihydroxyanthracene sodium salt provided by Kawasaki Chemicals Ltd.) charge: 0.1%

In addition, sodium hydroxide/sodium sulfite ($NaOH/Na_2SO_3$) ratios for the cooking were adjusted from 100/0 to 20/80, calculated as Na_2O. 100/0, is representative of soda-AQ cooking.

Alkaline cooking was performed in a 500-mL stainless steel autoclave (Taiatsu Techno Corporation) using 45 g (oven-dried weight) of wood chips.

On the other hand, wood meal cooking was performed in a 12-mL stainless steel autoclave using 1 g (oven-dried weight).

Detailed cooking conditions are shown from **Table 2-1** to **Table 2-3**.

Table 2- 1 The cooking conditions of larch heartwood chips

Samples	Liquor ratio (mL/g)	$NaOH/Na_2SO_3$ ratio	AA charge (%)*	AQ charge (%)	Temperature (℃)	Time (h)
L1	5.5	80/20	30	0.1	160	4
L2	5.5	70/30	30	0.1	160	4
L3	5.5	60/40	30	0.1	160	4
L4	5.5	60/40	30	0.1	160	2
L5	5.5	50/50	30	0.1	160	4
L6	5.5	100/0	30	0.1	160	4
L7	5.5	100/0	30	0.1	160	2

* As Na_2O

Table 2- 2 The cooking conditions of larch heartwood meals

Samples	Liquor ratio (mL/g)	$NaOH/Na_2SO_3$ ratio	AA charge (%)*	AQ charge (%)	Temperature (℃)	Time (h)
L8	10	80/20	30	0.1	160	4
L9	10	70/30	30	0.1	160	4
L10	10	60/40	30	0.1	160	4
L11	10	50/50	30	0.1	160	4
L12	10	40/60	30	0.1	160	4
L13	10	30/70	30	0.1	160	4
L14	10	20/80	30	0.1	160	4
L15	10	100/0	30	0.1	160	4

* As Na_2O

Table 2- 3 The cooking conditions of bamboo stem chips

Samples	Liquor ratio (mL/g)	$NaOH/Na_2SO_3$ ratio	AA charge	AQ charge (%)	Temperature (℃)	Time (h)
B1	5.5	80/20	0.3	0.1	150	4
B2	5.5	70/30	0.3	0.1	150	4
B3	5.5	60/40	0.3	0.1	150	4
B4	5.5	50/50	0.3	0.1	150	4
B5	5.5	100/0	0.3	0.1	150	4
B6	5.5	60/40	0.3	0.1	150	1
B7	5.5	60/40	0.3	0.1	150	2
B8	5.5	60/40	0.3	0.1	160	1
B9	5.5	60/40	0.3	0.1	160	2
B10	5.5	60/40	0.3	0.1	160	4

2.2.3 Determination of lignin content

The lignin content of the sample was determined using the method reported by Yoshihara *et al*. [1)] with minor modifications. Each sample (0.3 g, oven-dried weight) was initially hydrolyzed with a 72% sulfuric acid solution for 2.5 h and then hydrolyzed a second time with a 4% sulfuric acid solution at 121 ℃ for 1 h. The solution was then filtered with a glass filter (1GP16), and the residue weight was measured. The acid-soluble lignin (AL) was determined according to the TAPPI Test Method UM-250. The amount of lignin was a combination of acid-insoluble and acid-soluble lignin.

2.3 Results and Discussion

2.3.1 Relationship between $NaOH/Na_2SO_3$ ratio of cooking liquor and delignification

The alkaline cooking conditions and results of Japanese larch heartwood are listed in **Table 2-4**. The pulp yield was increased with the decrement of NaOH and the increment of Na_2SO_3 in the cooking liquor. The main reason is that the decomposition of hemicellulose is small. $NaOH/Na_2SO_3$ ratio of 80/20 (dosage 6%) produced the lowest pulp yield (L1) of 43.8%, as compared to other ratios. On the other hand, for larch, being cooked under 160 ℃ for 4 h, there was no correlation between the $NaOH/Na_2SO_3$ ratio and the lignin content of pulp, at a 60/40 $NaOH/Na_2SO_3$ ratio (L3), the pulp with lowest lignin content was obtained, it was 3.5%. It was found that Soda-AQ pulp (L6) has a lower pulp yield and higher lignin content compared with AS-AQ pulp (L3), even though under the same cooking condition.

Table 2- 4 Lignin content and pulp yield of larch pulps

	Pulp yield (%)	Lignin content (%) [*1]
Larch	-	32.0
L1	43.8	6.4
L2	46.9	6.9
L3	47.3	3.5
L4	52.5	14.3
L5	48.3	9.3
L6	44.5	4.4
L7	48.3	9.6
L8	47.7	9.3
L9	49.6	9.9
L10	49.7	9.7
L11	50.6	9.0
L12	52.2	5.8
L13	54.2	14.8
L14	55.5	13.9
L15	50.1	9.3

Note: [*1]: Larch wood is based on material, others based on pulp weights.

2.3.2 Estimation of cooking of larch heartwood in different scale

The relationship between $NaOH/Na_2SO_3$ ratio in cooking liquor and pulp yields of larch chip and meal was shown in **Fig. 2-1**.

A $NaOH/Na_2SO_3$ ratio of 80/20 produced the lowest pulp yield as compared to other ratios. When the sodium sulfite ratios were kept constant, the pulp yields of wood meal were higher than those of wood chips. At a 100/0 $NaOH/Na_2SO_3$ ratio, the pulp yield of wood meal was 5% higher than that of wood chips. As the $NaOH/Na_2SO_3$ ratio increased, the difference between the pulp yields from wood chips and wood meal decreased, and at a 50/50 $NaOH/Na_2SO_3$ ratio, this difference was only 3%.

Both wood chips and meals were cooked for 4 h under the same conditions except for the liquor ratio, which was varied. Lignin content and $NaOH/Na_2SO_3$ ratios are shown in **Fig. 2-2**.

When cooking with wood chips, the lignin content of pulp increased with an increase in the $NaOH/Na_2SO_3$ ratio to 70/30. However, at a 60/40 $NaOH/Na_2SO_3$ ratio, the lignin content decreased to 3.5%, which was the lowest value found. When the ratios of $NaOH/Na_2SO_3$ were constant, the lignin contents of meal pulps were generally higher than those of chip pulps, but at a 50/50 $NaOH/Na_2SO_3$ ratio, the chip pulp produced a higher

lignin content.

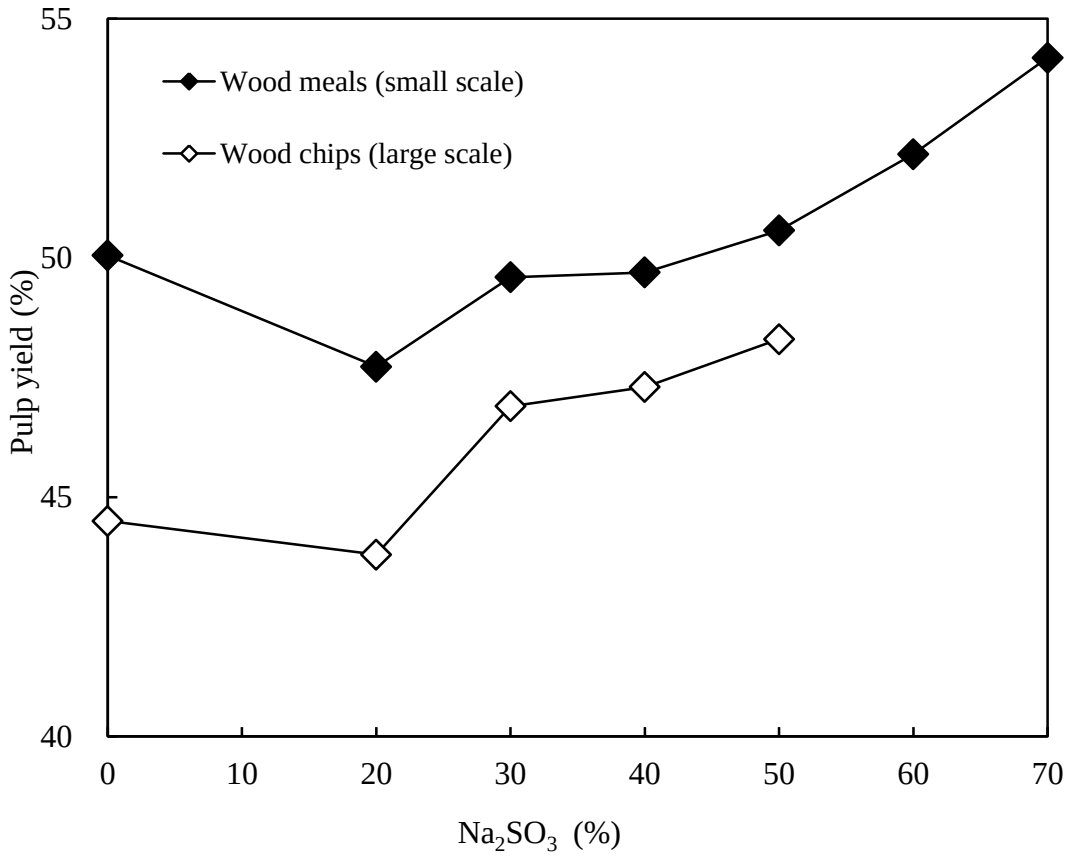

Figure 2- 1 Relationship between NaOH/Na_2SO_3 ratio and larch pulp yield

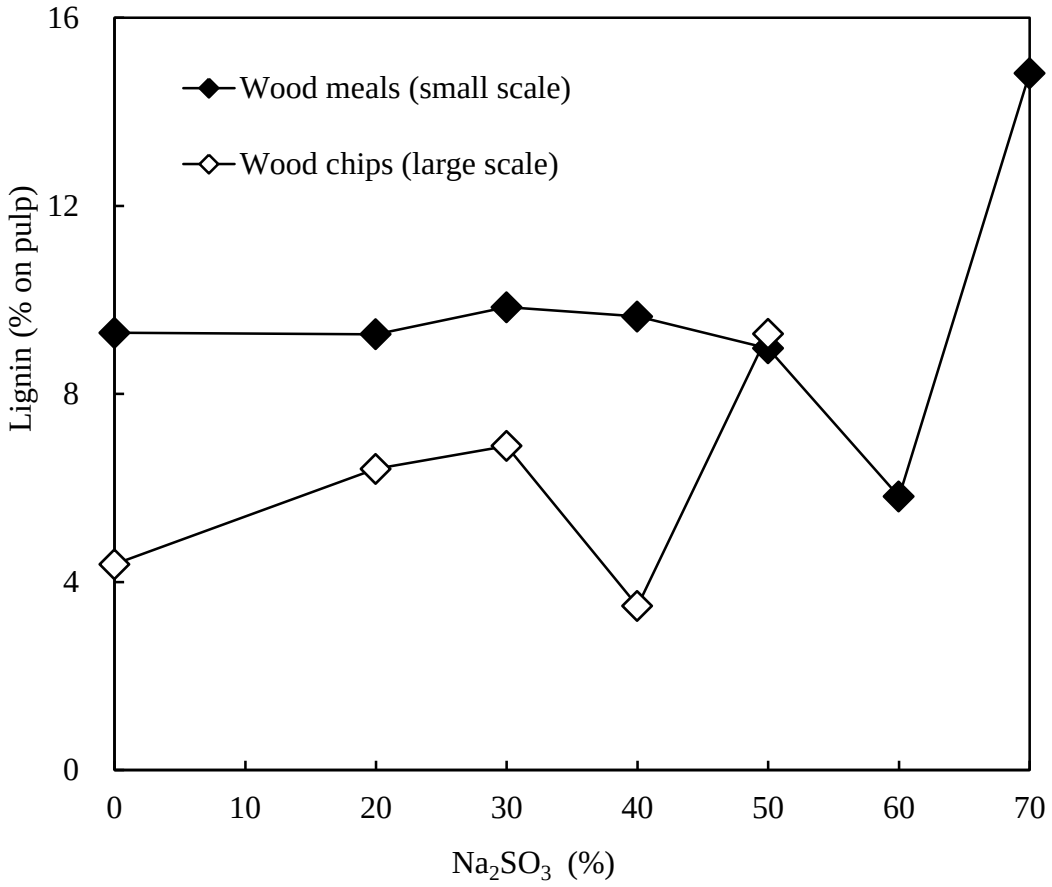

Figure 2- 2 Relationship between NaOH/Na_2SO_3 ratio and lignin content of pulp

2.3.3 AS-AQ cooking of bamboo

For bamboo stem, in order to confirm the optimum Na_2SO_3 ratio, the cooking was carried out with different Na_2SO_3 ratios. As shown in **Table 2-5**, with the increment of Na_2SO_3, the pulp yield increased, too. However, the tendency of lignin increment was also observed. Compared with larch material, the optimum cooing conditions could not been confirmed based on the viewpoints of pulp yield and lignin content.

Table 2- 5 Lignin content and pulp yield of bamboo pulps

	Pulp yield (%)	Lignin content (%) [*1]	Kappa number
Bamboo	-	28.6	-
B1	38.0	2.1	-
B2	40.0	2.2	-
B3	44.5	2.5	-
B4	47.2	2.7	-
B5	38.2	2.7	12.4
B6	42.6	9.1	44.6
B7	41.2	6.9	44.1
B8	42.9	4.2	23.9
B9	38.1	2.8	16.1
B10	39.8	1.7	10.1

Notes: [*1]: Bamboo sample is based on material, and others are based on pulp weights.

Hedjazi *et al*. [2, 3] confirmed the optimum NaOH/Na_2SO_3 ratio of 30/70 for bagasse, 50/50 for wheat straw by using AS-AQ cooking method.

The pulp yield with the highest Na_2SO_3 addition (B4, 50%) was approximate 7 percentage point higher than the lowest Na_2SO_3 addition (B1, 20%), thus the lignin content just only 0.6 percentage point higher. On the other hand, for Soda-AQ (B5), pulp yield was low, lignin content was high. It can be confirmed that compared with Soda-AQ method, AS-AQ method was more advantageous.

For the cooking of larch, the optimum NaOH/Na_2SO_3 ratio in the cooking liquor was 60/40 for the AS-AQ method. In the case of bamboo, even though the optimum cooking conditions could not be confirmed, it was found that the AS-AQ method produced the pulp with higher yield and lower lignin content compared to the soda-AQ method. Therefore, bamboo pulps with different lignin contents were prepared with a NaOH/Na_2SO_3 ratio of 60/40 using the AS-AQ method. **Table 2-5** shows the conditions and results of alkaline cooking of bamboo stem. It was found that the average pulp yield from bamboo stem was lower than that of larch heartwood; however, the delignification proceeded easily. Jin *et al*. [4] reported that the pulp yield of bamboo was lower than that of kenaf bast and softwood under the same kraft cooking conditions. The main reason was the substantial degradation of hemicellulose.

2.4 Conclusions

Cooking larch wood chips with a 60/40 $NaOH/Na_2SO_3$ ratio in the cooking liquor resulted in pulp with a lower lignin content and higher pulp yield. For bamboo stem cooking, even though the optimum ratio of Na_2SO_3 was not definitively known, the AS-AQ cooking method resulted in pulp with lower lignin content and higher pulp yield compared with the soda-AQ cooking method.

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Chapter 3 Enzymatic Hydrolysis Treatment

3.1 Introduction

In this chapter, the cellulase activity of GC220 enzyme was determined by the NREL method. The enzymatic saccharification of AS-AQ and Soda-AQ pulp with different lignin content were clarified. The effect of lignin in pulp on enzymatic hydrolysis was discussed. Furthermore, the most effective material and cooking method for glucose production was clarified.

3.2 Materials and Methods

Pulp was treated with an enzyme solution containing a cellulase (GC220, provided by Genencor Kyowa Co. Ltd., Japan). 50 mg (as oven-dried weight) of pulp was made into a strip (10 mm × 60 mm). The strip sample was rolled and put into a 13 mm × 100 mm (diameter × length) test tube. 1 mL of 0.05 M acetic acid buffer (pH 4.8) and 0.5 mL of the diluted enzyme solution was added into the test tube. The pulp strips should be totally saturated by the solution, and then were treated at 45 ℃ for 1, 2, 4, and 6 h, respectively.

After the enzymatic hydrolysis treatment, the suspension was heated in the test tube at 100 ℃ for deactivating the cellulase by using a block

heater. The amount of glucose reduced during the treatment in the filtrate (a resolution part of the pulp) was determined by using a Dionex ICS 3000 ion chromatograph (Dionex, Sunnyvale, CA, USA). This system consisted of a single pulp model (SP-1), an electrochemical detector (ED), an IonPac AS 7 column (Φ 4mm × 250 mm), an IonPac AS 7 guard column (Φ 4mm × 50 mm), and an auto smpler (AS).

The cellulase activity of the enzyme was measured according to the cellulase activity assay reported by the National Renewable Energy Laboratory (NREL) with minor modifications [1)]. A value of 2.0 mg of reducing sugar was calculated as glucose from 50 mg of filter paper for 60 min.

3.3 Results and Discussion

All materials and lignin contents used in this study for enzymatic hydrolysis are listed in **Table 3-1**.

Table 3- 1 Lignin content of various pulps for the enzymatic hydrolysis treatment

Sample	Lignin content (%) [*1]
L3	3.5
L4	14.3
L6	4.4
L7	9.6
B6	9.1
B7	6.8
B8	4.2
B9	2.8
B10	1.7
BCTMP ISO 70	22.4
BCTMP ISO 80	22.5
LOKP treated[*2]	0.7
LOKP untreated	0.5
Unbleached bamboo KP	3.0
Bleached bamboo KP	1.1
Unbleached kenaf KP	2.9
Bleached kenaf KP	0.4
PPC	8.2
Filter paper	0

Note: [*1]: Based on pulp weight; [*2]: 15% NaOH, 24 hours treatment.

KP: Kraft Pulp

3.3.1 Enzymatic hydrolysis behaviors of filter paper by GC220 enzyme

The filter paper cellulase unit (FPU) of GC220 solution was determined by the NREL method. It was calculated from the value of 2.0 mg of reducing sugar as glucose from 50 mg of filter paper in 1 h. The GC220 original solution was subjected to filter paper enzymatic treatment with 10-, 50-, 100-, 200-, and 250-fold dilution. The 200-fold dilution produced 1.92 mg of glucose after 1-h hydrolysis treatment, coming very close to 2.0 mg. The relationship between the released glucose amount after 1 h treatment and enzyme dilution inverse number was shown in **Fig. 3-1**. According to this calibration line, the inverse of the dilution ratio that can release 2.0 mg of glucose is 0.00583, which gives a dilution ratio of 171.5. This value indicates the cellulase activity of original GC220 solution is 63.5 FPU/mL.

Fig. 3-2 shows the results of enzymatic hydrolysis treatment of filter paper with different dosages (activity). With a cellulase dosage of 12.7 FPU per gram filter paper (FPU/g), glucose conversion ratio (the ratio of glucose conversion amount and filter paper weight) was only about 33%. With 250- or 200-fold dilution of original enzyme solution, the cellulase dosages such as 2.6 FPU/g and 3.2 FPU/g, this 1.25-fold increment in dosage produced a 1.54-fold increment in glucose yield. However, when

the cellulase dosage was increased from 12.7 FPU/g to 63.5 FPU/g (5 times), only 1.58 times of glucose amount as 12.7 FPU/g. It was suggested the possibility of the occurrence of “jamming effect” [2)], which because the limited amount of space on the surface of pulp, cellulase cannot be fully adsorbed onto the cellulose at high dosage of cellulase. It was decided that from the results of these, the cellulase dosage of 12.7 FPU/g was used during the enzymatic treatment of pulp.

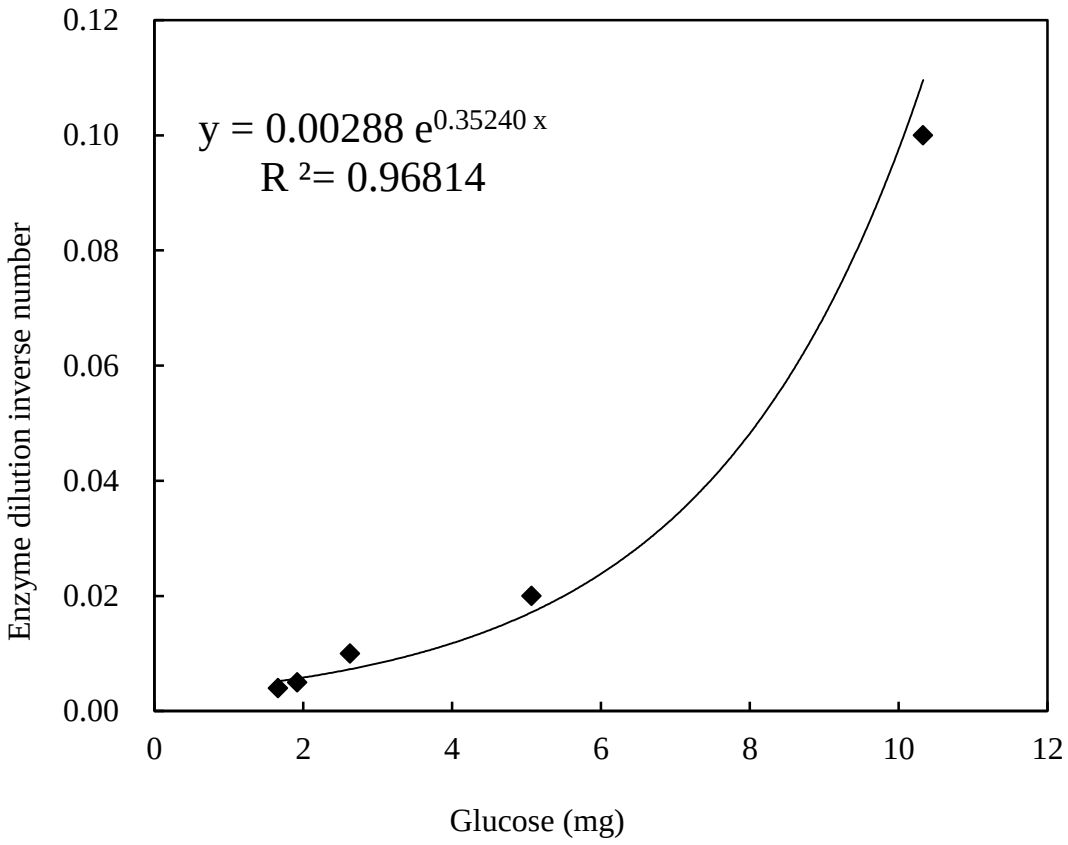

Figure 3- 1 Function of FPU calculation

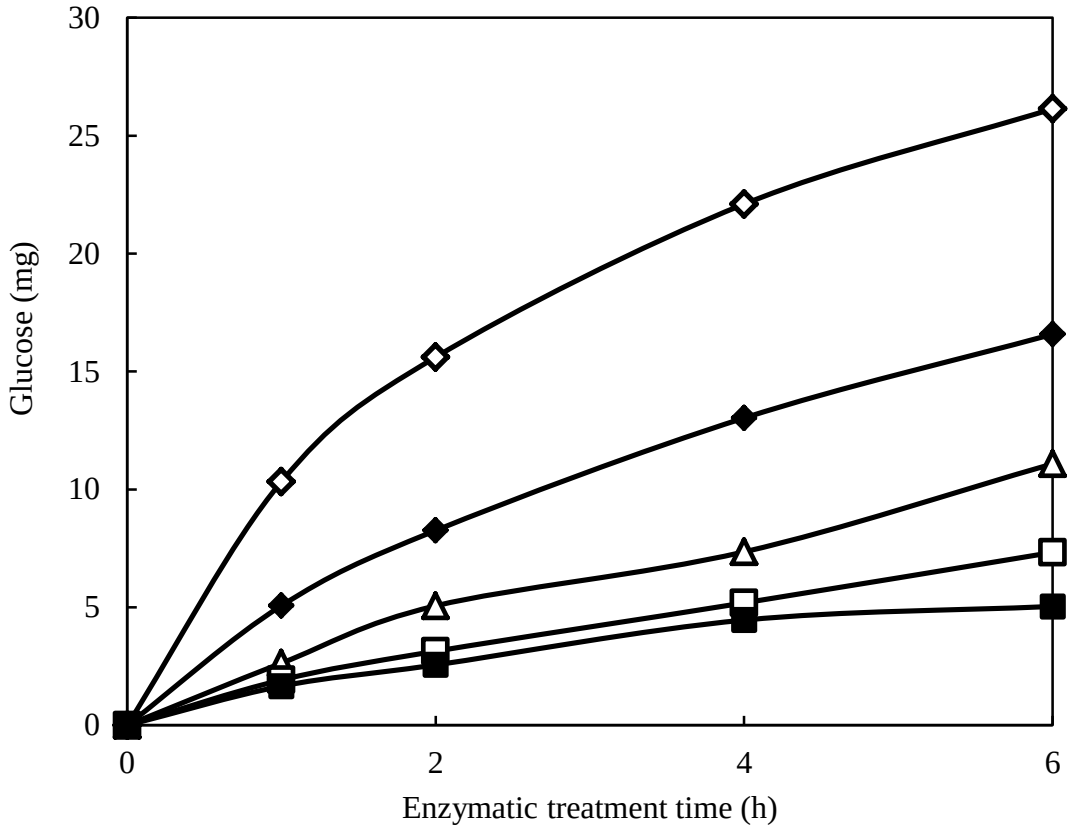

Figure 3- 2 Enzymatic hydrolysis of filter paper with different enzyme dilution factors

Legend: Dilution factor:◇10-fold dilution (63.5 FPU/g), ◆50-folddilution (12.7 FPU/g), ▵100-fold dilution (6.4 FPU/g), □200-fold dilution (3.2 FPU/g), ■250-fold dilution (2.6 FPU/g).

Notes: Filter paper: 50 mg; Solution of cellulase GC220, provided by Genencor Kyowa Co. Ltd., Japan: 0.5 mL.

3.3.2 Enzymatic sacharification of raw larch and bamboo

Fig. 3-3 shows that glucose was released quickly during the initial 1 h of enzymatic treatment using both larch heartwood and bamboo stem. Since these materials were not pretreated, the enzymatic digestibility was very low. After 6 h of treatment, only about 2.6% of larch and 1.4% of bamboo were converted into glucose. According to **Table 2-4** and **2-5**, the lignin content of larch raw material (32.0%) is higher than that of bamboo raw material (28.6%). The lignin content did not appear to have a negative effect on enzymatic hydrolysis.

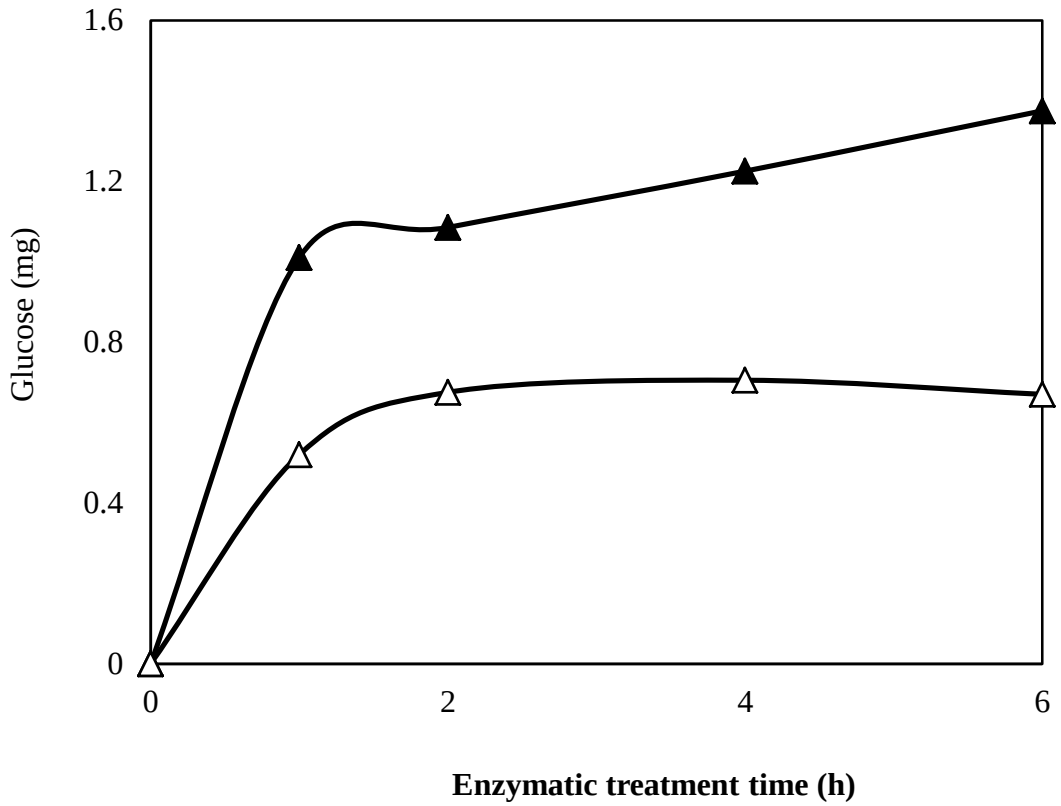

Figure 3- 3 Enzymatic hydrolysis of larch and bamboo raw material

Note: Enzyme dosage: 12.7 FPU/g

Legend: ▲Larch, △Bamboo

3.3.3 Enzymatic hydrolysis of larch AS-AQ and Soda-AQ pulp

Two types of pulps were prepared for this experiment: AS-AQ and soda-AQ pulps. These pulps were cooked for 2 h and 4 h.

Using a high cellulase loading (**Fig. 3-4**), i.e., 63.5 FPU/g, the conversion rates for the filter paper, AS-AQ 4h pulp (L3, lignin content: 3.5%) and AS-AQ 2h pulp (L4, 14.3%), were about 50%, 40%, and 20%, respectively.

Decreasing the cellulase loading to 12.7 FPU/g (**Fig. 3-5**) produced conversion rates of about 32%, 16%, and 13%. The lowest cellulase loading (6.4 FPU/g; **Fig. 3-6**), produced conversion rates of about 22%, 10% and 8%.

The Soda-AQ L7 (9.6%) and L6 (4.4%) were treated with the same enzyme dosages. The results of these treatments are shown in **Fig. 3-7**, **Fig. 3-8**, and **Fig. 3-9**. The shape of the glucose conversion ratio calibration curve of filter paper, Soda-AQ L7 and Soda-AQ L6 pulps were similar to the curve for the AS-AQ pulps, but the conversion rates were lower.

The enzymatic treatment results of larch AS-AQ and Soda-AQ pulp with the enzyme dosage of 12.7 FPU/g are shown in **Fig. 3-10**. For AS-AQ pulp, enzymatic saccharification ratio of L3 (lignin content: 3.5%)

was higher than L4 (14.3%). In addition, the enzymatic saccharification of all the AS-AQ and Soda-AQ pulps, were lower than that of filter paper. The amount of cellulase bound to the cellulose, affect the efficiency of enzymatic hydrolysis. Ooshima *et al.* [3] reported that cellulase not only cellulose, but also non-specifically adsorbed to lignin. The pulp with high lignin content, it is possible that the contributed active enzyme was reduced during the cellulosic enzymatic hydrolysis, rather than the lignin absorbed the enzyme. Being compared under same cooking method, it was found that enzymatic saccharification ratio of larch AS-AQ and Soda-AQ pulp have a tendency to the lignin content.

Despite the lignin content of AS-AQ pulp (L4, 14.3%) was higher than Soda-AQ pulp (L7, 9.6%), it shows the enzymatic saccharification ratio was higher than Soda-AQ.

Takahashi *et al.* [4] reported that the resolution ratio (equal to enzymatic saccharification ratio) of acid sulfite pulp was higher than Soda-AQ pulp. As one of the explanation, it was reported that the water retention value (WRV) of acid sulfite pulp is high. The WRV of pulp is a measurement of the degree of swelling. Mooney *et al.* [5] also reported that when the swelling of pulp is high, the amount of enzyme adsorbed to the pulp was also high. Therefore, the reason for larch AS-AQ pulp obtained a better enzymatic saccharification ratio than Soda-AQ, it was estimated that the swelling of AS-AQ pulp fiber is higher than Soda-AQ pulp, however,

compared the WRV of pulp, obvious difference was not obtained (**Table 3-2**).

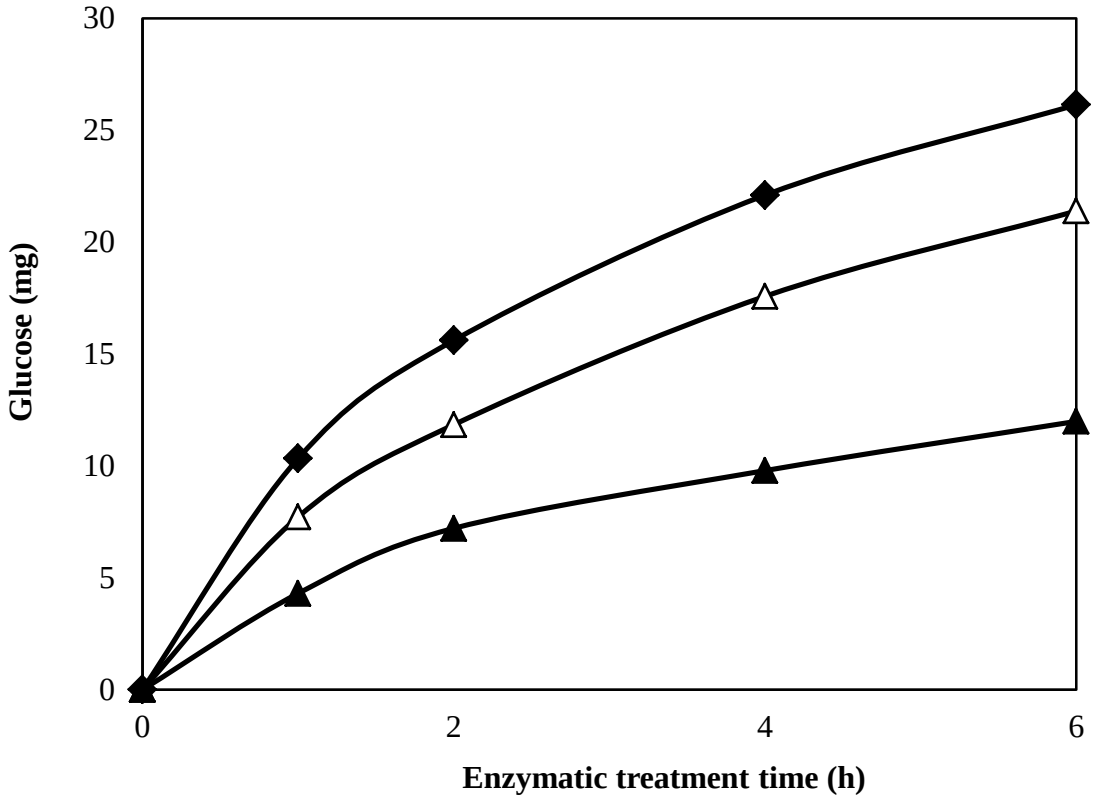

Figure 3- 4 Enzyamtic hydrolysis of AS-AQ larch pulp (high cellulase loading)

Note: Enzyme dosage: 63.5 FPU/g

Legend: ◆Filter paper, △AS-AQ L3 (3.5%), ▲AS-AQ L4 (14.3%)

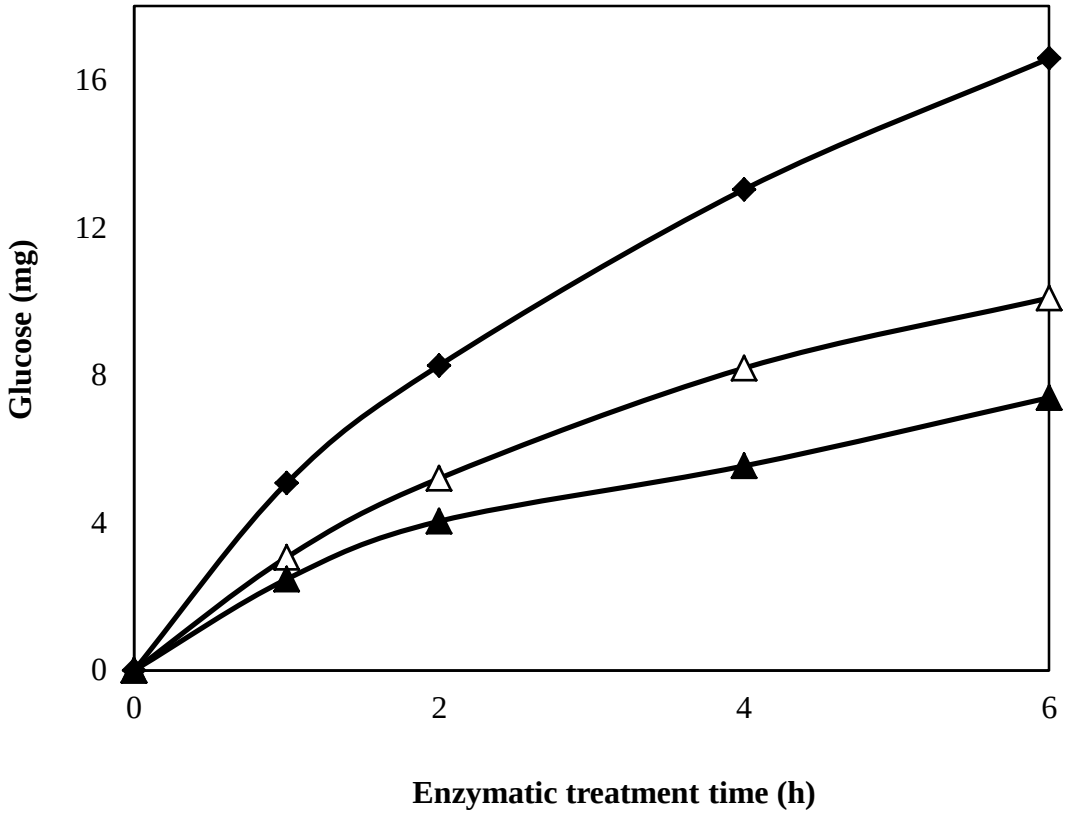

Figure 3- 5 Enzymatic hydrolysis of AS-AQ larch pulp (medium cellulase loading)

Note: Enzyme dosage: 12.7 FPU/g

Legend: ◆Filter paper, △AS-AQ L3 (3.5%), ▲AS-AQ L4 (14.3%)

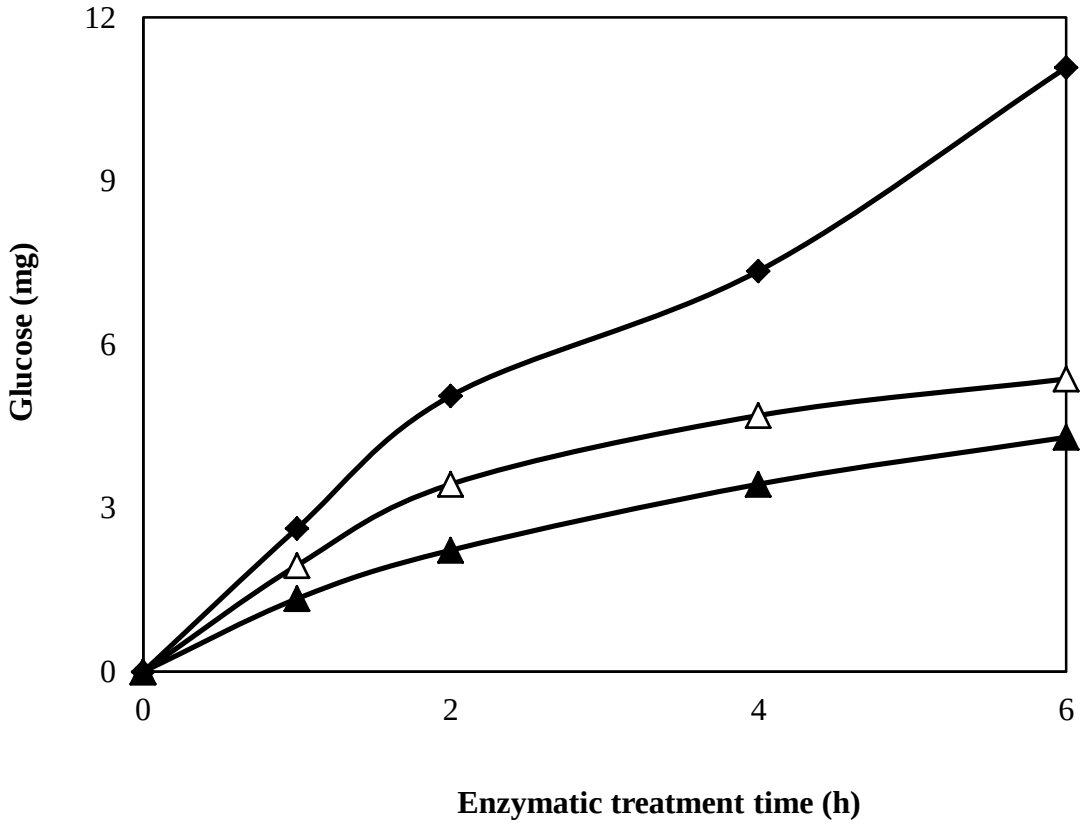

Figure 3- 6 Enzyamtic hydrolysis of AS-AQ larch pulp (low cellulase loading)

Note: Enzyme dosage: 6.4 FPU/g

Legend: ◆Filter paper, △AS-AQ L3 (3.5%), ▲AS-AQ L4 (14.3%)

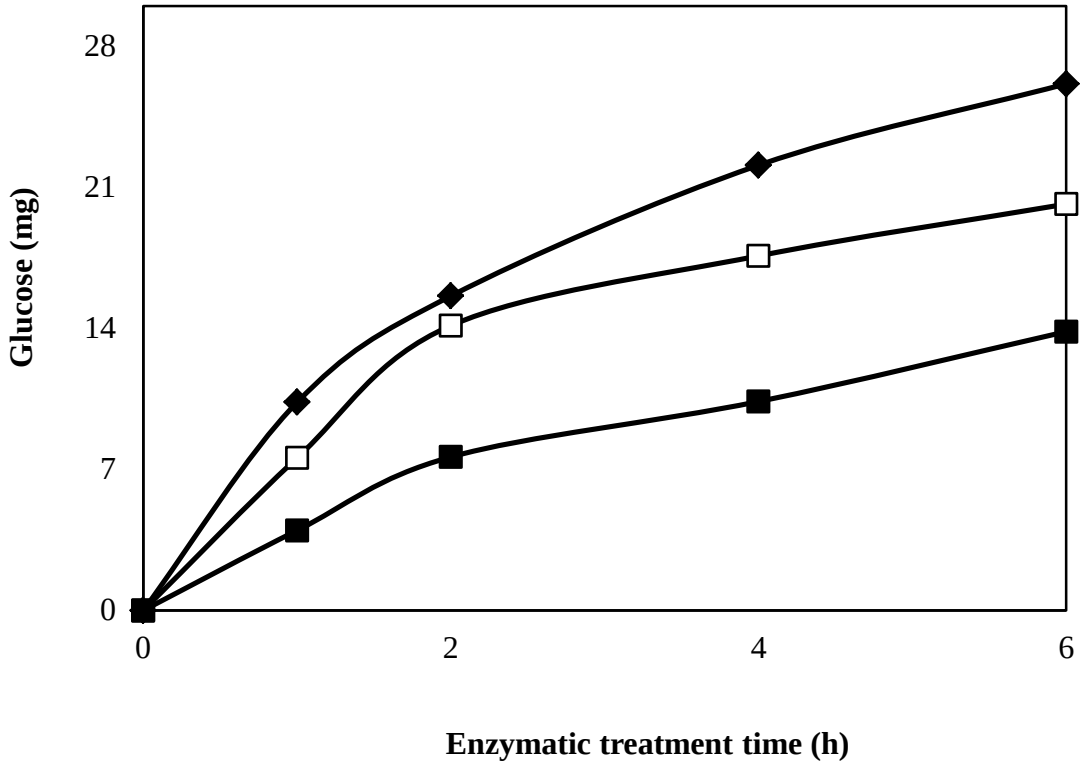

Figure 3- 7 Enzyamtic hydrolysis of soda-AQ larch pulp (high cellulase loading)

Note: Enzyme dosage: 63.5 FPU/g

Legend: ◆Filter paper, □Soda-AQ L6 (4.4%), ■Soda-AQ L7 (9.6%)

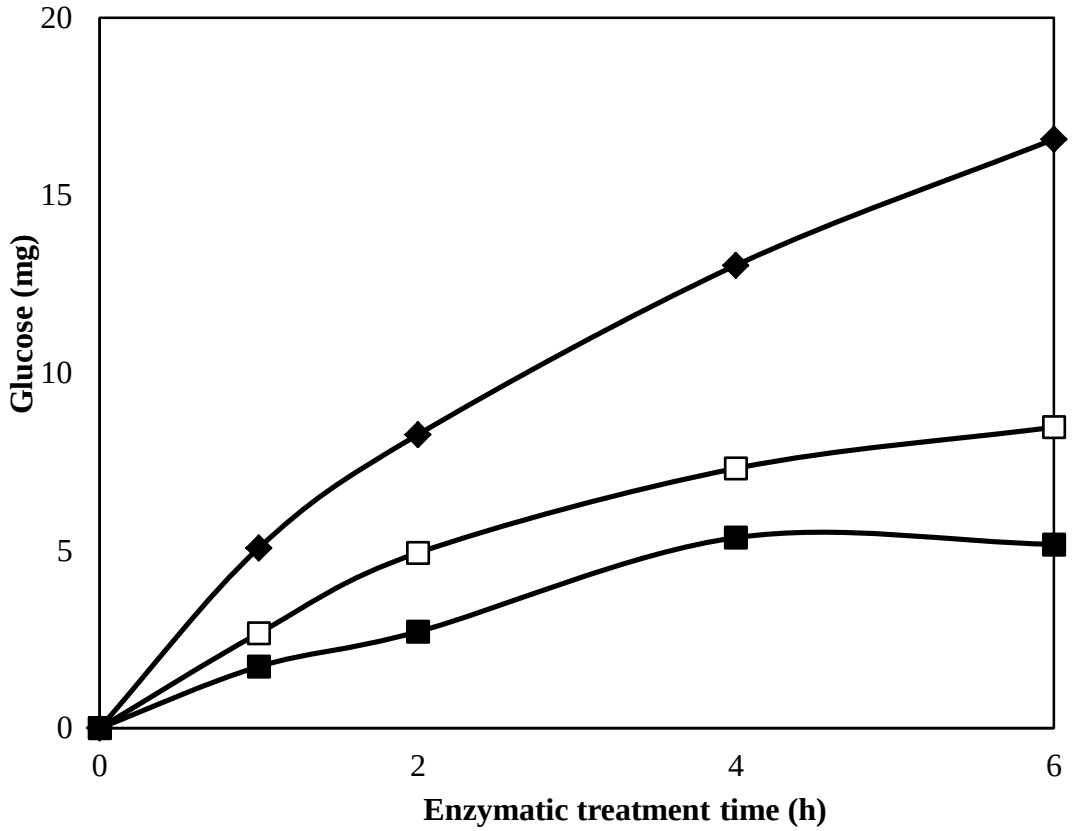

Figure 3- 8 Enzymatic hydrolysis of soda-AQ larch pulp (medium cellulase loading)

Note: Enzyme dosage: 12.7 FPU/g

Legend: ◆Filter paper, □Soda-AQ L6 (4.4%), ■Soda-AQ L7 (9.6%)

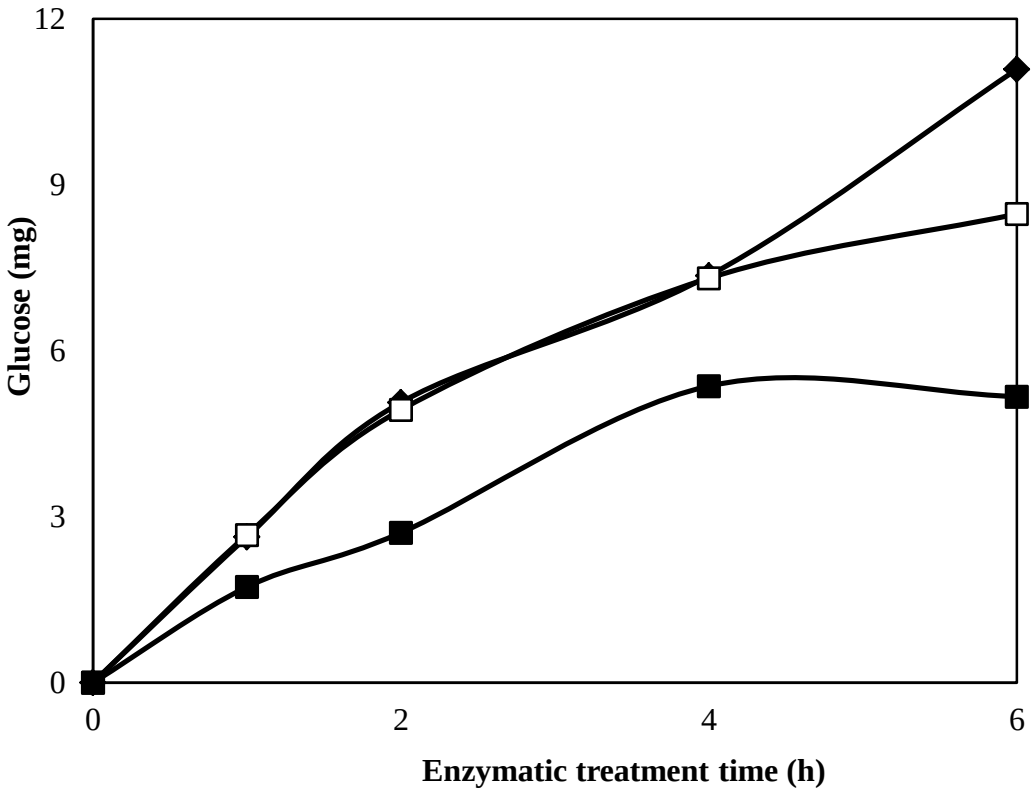

Figure 3- 9 Enzyamtic hydrolysis of soda-AQ larch pulp (low cellulase loading)

Note: Enzyme dosage: 6.4 FPU/g

Legend: ◆Filter paper, □Soda-AQ L6 (4.4%), ■Soda-AQ L7 (9.6%)

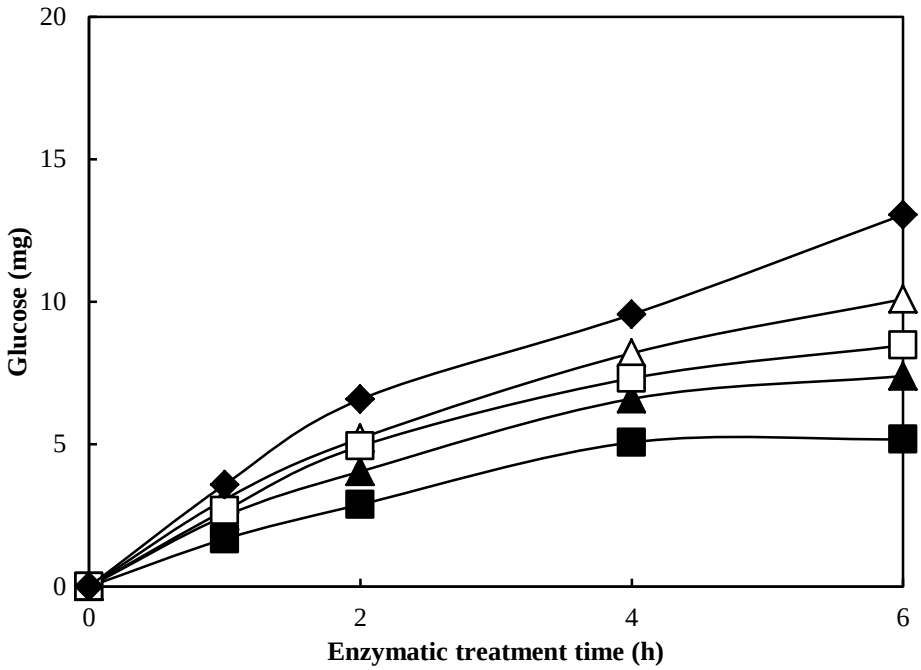

Figure 3- 10 Comparison of AS-AQ and soda-AQ larch pulps

Note: Enzyme dosage: 12.7 FPU/g

Legend: ◆Filter paper, △AS-AQ L3 (3.5%), ▲AS-AQ L4 (14.3%), □Soda-AQ L6 (4.4%), ■Soda-AQ L7 (9.6%).

3.3.4 Enzymatic hydrolysis of bamboo AS-AQ pulps

Bamboo pulps cooked for 1 and 2 h (at 150 ℃) with lignin contents of 9.1% (B6) and 6.8% (B7), respectively, are shown in **Fig. 3-11**. The lignin content of the B6 pulp was higher than that of B7, but the enzymatic digestibility was lower than that of the B7 pulp. The enzymatic digestibilities of these two pulps were higher than that of the filter paper, which does not contain lignin.

Bamboo was also cooked at 160 ℃ for 1, 2, and 4 h. The lignin contents of these pulps were 1.7% (B8), 2.8% (B9), and 1.7% (B10), respectively. These results are shown in **Fig. 3-12**. The enzymatic digestibility of the B9 and B10 pulps were higher than that of the B8 pulp. At the initial 2 h of enzymatic treatment, the enzymatic digestibility of B9 was lower than that of B10 pulp, but from 4 h to 6 h of treatment, the B9 had a higher enzymatic digestibility than the B10. The enzymatic enzymatic digestibilities of these pulps were also higher than those of the filter paper.

The pulp-to-glucose conversion ratios were more than 12% for all bamboo AS-AQ pulps.

On the other hand, the amount of released xylose was almost the same in all treatment (**Fig. 3-13** and **Fig. 3-14**), regardless of differing lignin contents. According to the figures, the amount of xylose released from the pulp corresponds with treatment time, not the pulp’s lignin content.

The saccharification ratio of B9 and B10 was higher than that of B8. For bamboo stem AS-AQ pulp, when lignin content was lower than 4.2%, no matter the presence of lignin, the saccharification ratio was higher than filter paper, it also can obtain a nearly saccharification ratio to filter paper, with the lignin content of 6.9%.

As one of the reason, it was considered whether the swelling of bamboo stem pulp is higher than filter paper or not, the WRV of pulp was compared (**Table 3-2**). The WRV of bamboo stem AS-AQ pulp was 122%, higher than filter paper (85%). However, the WRV of larch AS-AQ and Soda-AQ pulp was slightly higher than filter paper, on the other hand, saccharification ratio was low, it cannot explain the difference of saccharification ratio between AS-AQ pulp and Soda-AQ pulp from the difference of WRV.

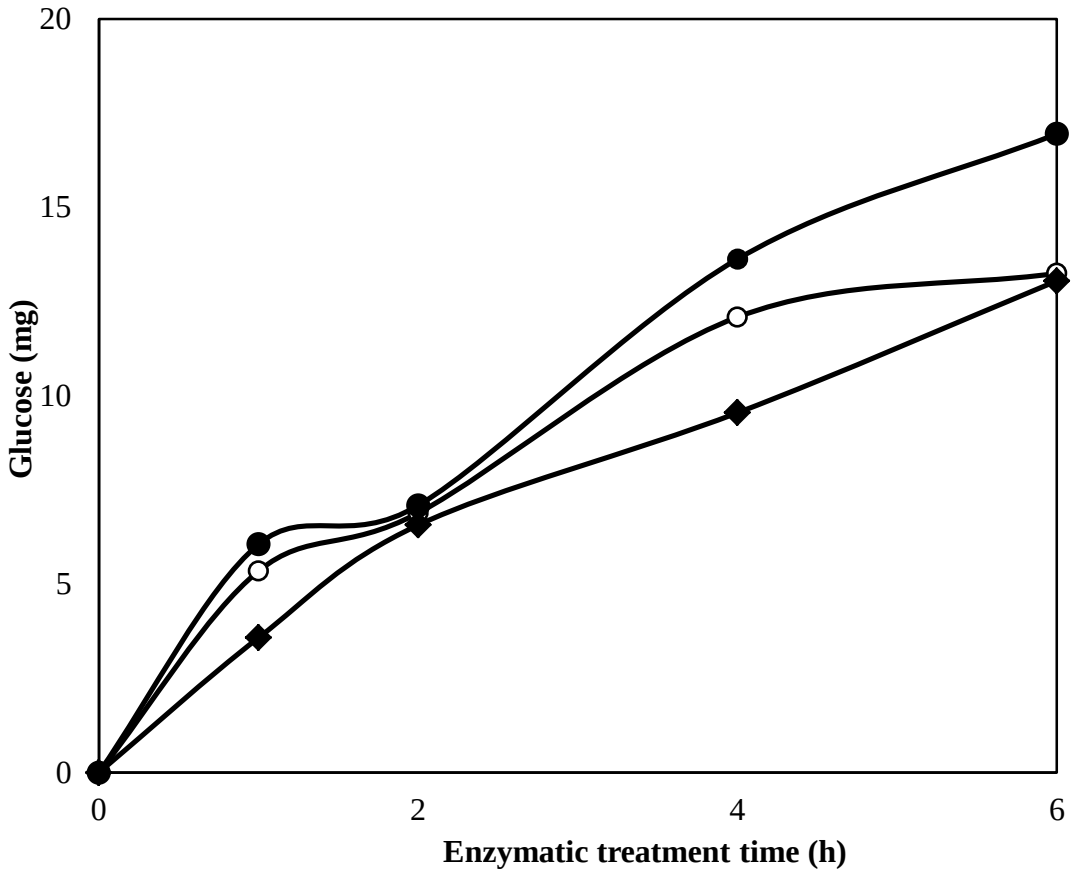

Figure 3- 11 Enzymatic hydrolysis of AS-AQ bamboo pulp (150℃ cooking)

Note: Enzyme dosage: 12.7 FPU/g

Legend: ◆Filter paper, ○B6 (9.1%), ●B7 (6.8%)

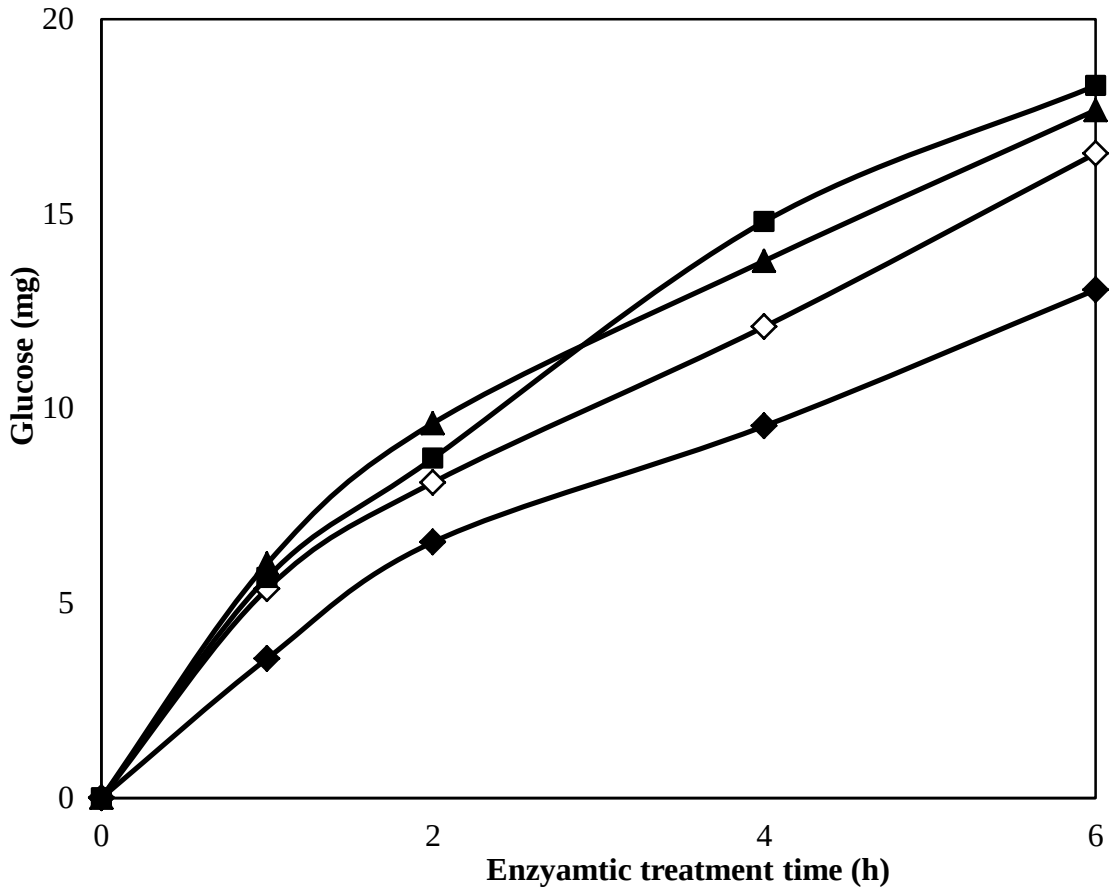

Figure 3- 12 Enzymatic hydrolysis of AS-AQ bamboo pulp (160℃ cooking)

Note: Enzyme dosage: 12.7 FPU/g

Legend: ◆Filter paper, ◇B8 (4.2%), ■B9 (2.8%), ▲B10 (1.7%)

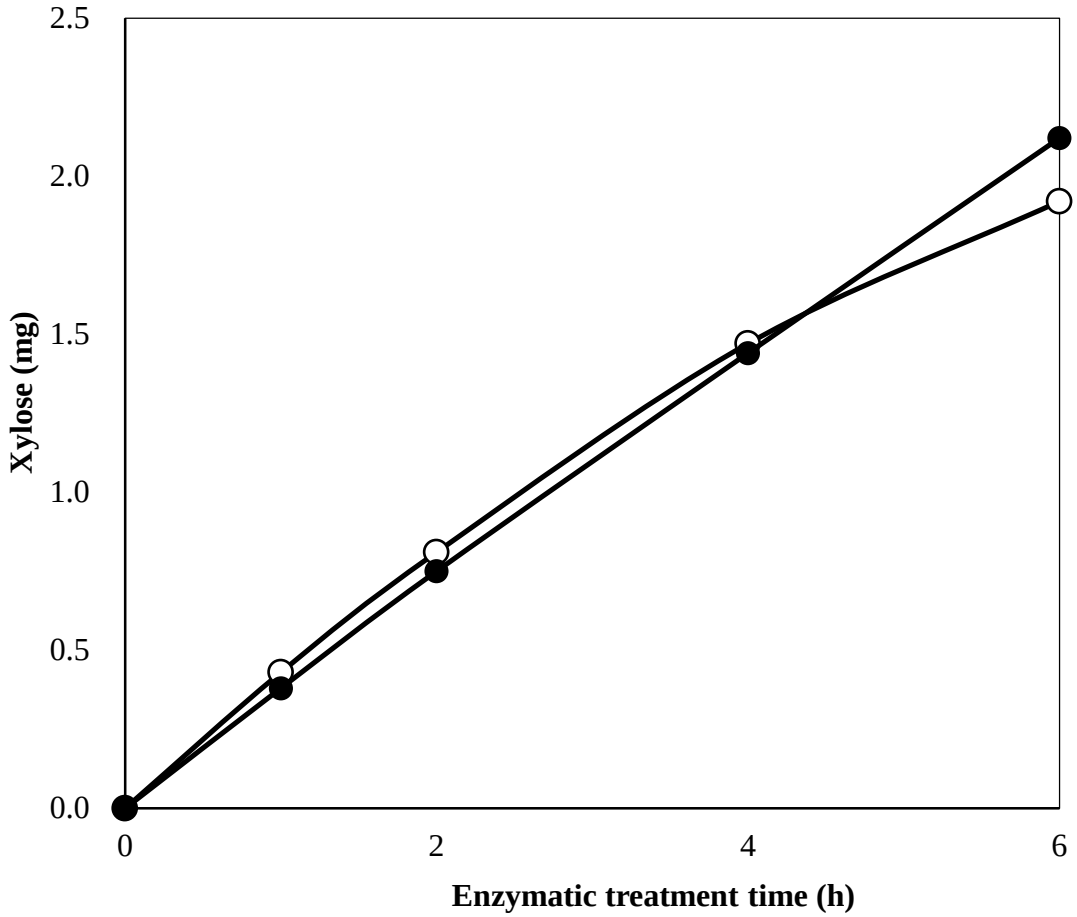

Figure 3- 13 Xylose content of AS-AQ bamboo pulp (150℃ cooking) after the enzymatic hydrolysis treatment

Note: Enzyme dosage: 12.7 FPU/g

Legend:○B6 (9.1%), ●B7 (6.8%)

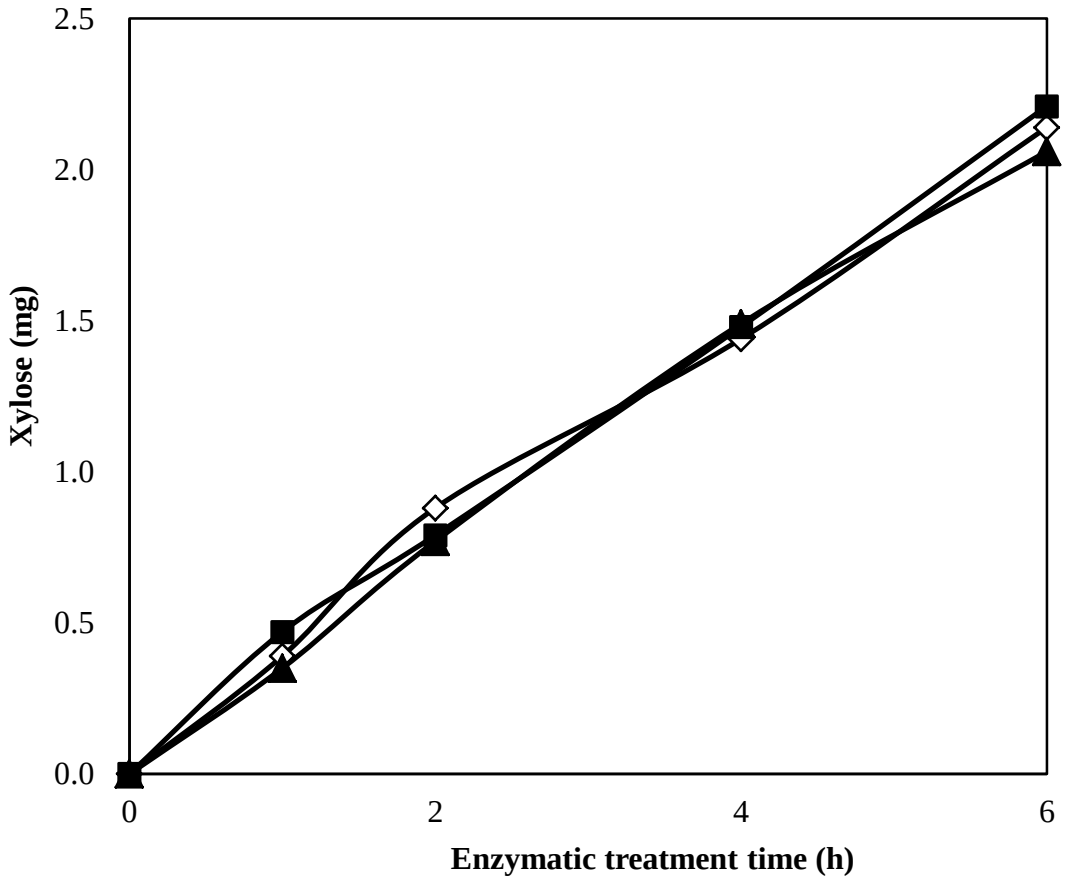

Figure 3- 14 Xylose content of AS-AQ bamboo pulp (160 ℃ cooking) after the enzymatic hydrolysis treatment

Note: Enzyme dosage: 12.7 FPU/g

Legend:◇B8 (4.2%), ■B9 (2.8%), ▲B10 (1.7%)

Table 3- 2 Water retention value of AS-AQ and Soda-AQ pulps

Sample	Pulp yield (%)	Lignin content (%)	Water retention value (%)
B3 AS-AQ bamboo	44.5	2.5	122
L1 AS-AQ larch	43.8	6.4	104
L6 Soda-AQ larch	44.5	4.4	109
Filter paper	-	-	85

3.3.5 Enzymatic hydrolysis of kraft pulps

Bleached and unbleached kraft bamboo and kenaf pulps were obtained from Jin. The chemical characteristics and bleaching conditions are described in the articles [6, 7]. According to **Fig. 3-15**, the saccharification rate of bleached bamboo kraft pulp was greater than those of unbleached pulp and filter paper. Unbleached pulp also showed a higher saccharification rate than that of filter paper.

Fig. 3-16 shows that the unbleached kenaf pulp produced the best saccharification rate as compared to bleached kenaf pulp and filter paper.

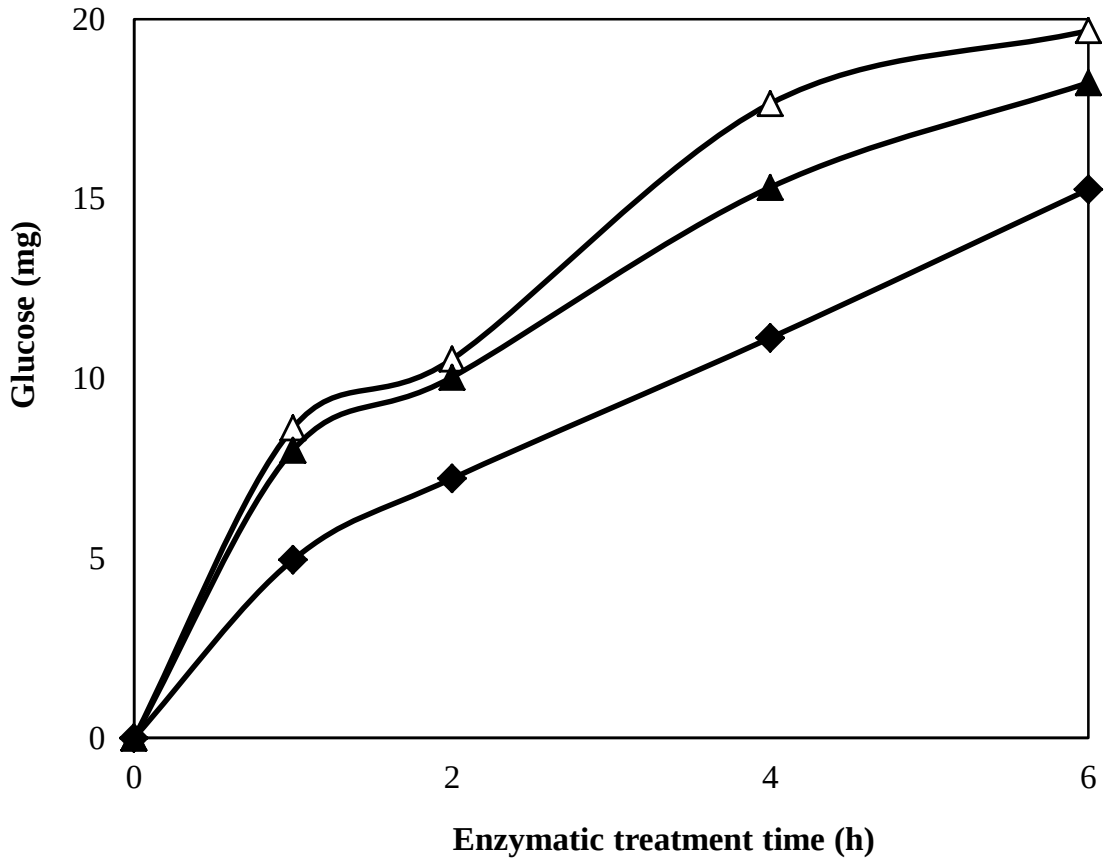

Figure 3- 15 Enzymatic hydrolysis of bleached and unbleached bamboo kraft pulp

Note: Enzyme dosage: 12.7 FPU/g

Legend: ◆Filter paper, △Bamboo KP bleached (1.1%), ▲Bamboo KP unbleached (3.0%)

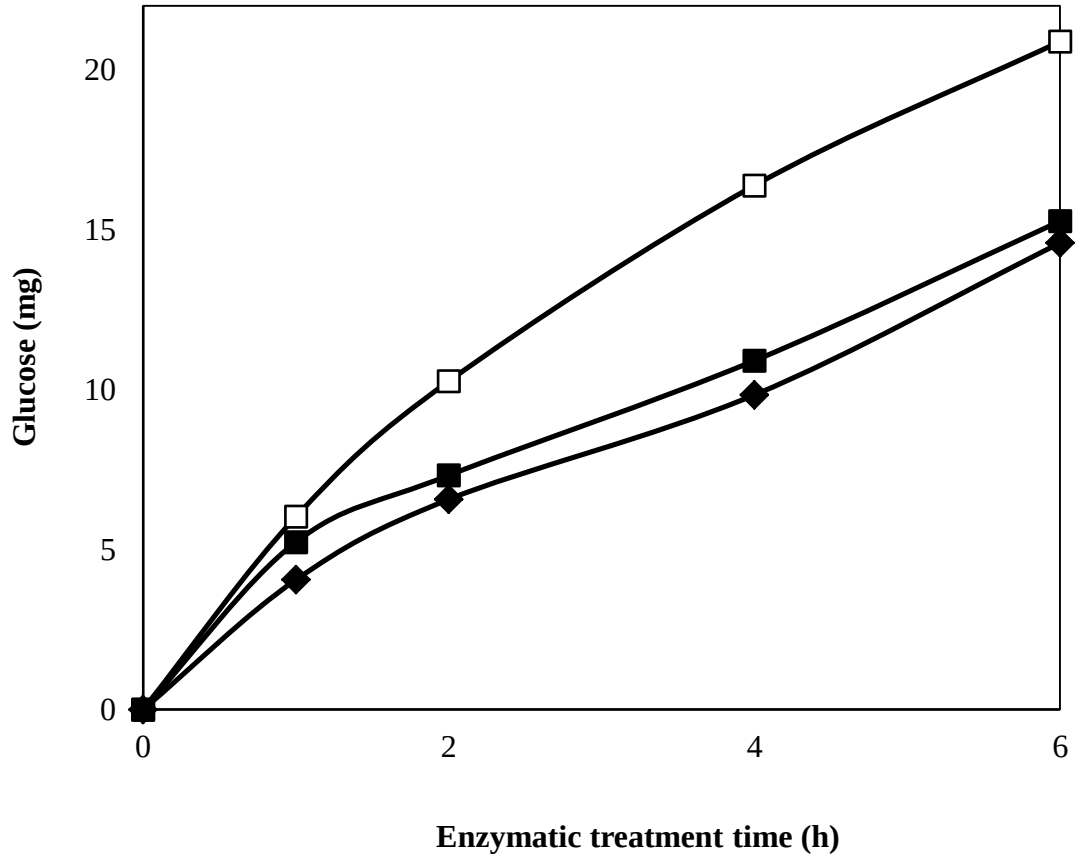

Figure 3- 16 Enzymatic hydrolysis of bleached and unbleached kenaf kraft pulp
Note: Enzyme dose: 12.7 FPU/g
Legend: ◆Filter paper, ■Kenaf KP bleached (0.4%), □Bamboo KP unbleached (2.9%)

3.3.6 Enzymatic hydrolysis of chemi-mechanical pulp

PPC (Plain Paper Copier), is made from waste paper and is used widely across Japan. The lignin content of PPC is 8.2%. Black ink was used to print on the PPC, covering both 50% and 100% portions of PPC samples, and compared the enzymatic digestibility of PPC with those of filter paper (**Fig. 3-17**). The enzymatic digestibilities of these samples were slightly lower than that of filter paper. After 6 h of enzymatic treatment, the paper with no laser printing showed a 35% rate of sugar conversion.

BCTMP with ISO 70 and ISO 80 were used in this experiment (**Fig. 3-18**). The glucose released from ISO 80 pulp was slightly higher than that from ISO 70. However, both released glucose at rates much lower than that of filter paper, with an average sugar conversion rate of approximately 4% after 6 h of treatment.

LOKP was treated by alkali for extracting its xylan. I compared the enzymatic digestibility of the treated and untreated pulps with that of filter paper, and the results are shown in **Fig. 3-19**. Both treated and untreated LOKP pulps produced higher enzymatic digestibility than that of filter paper. Nearly 40% of the LOKP pulp was converted into glucose after 6 h treatment.

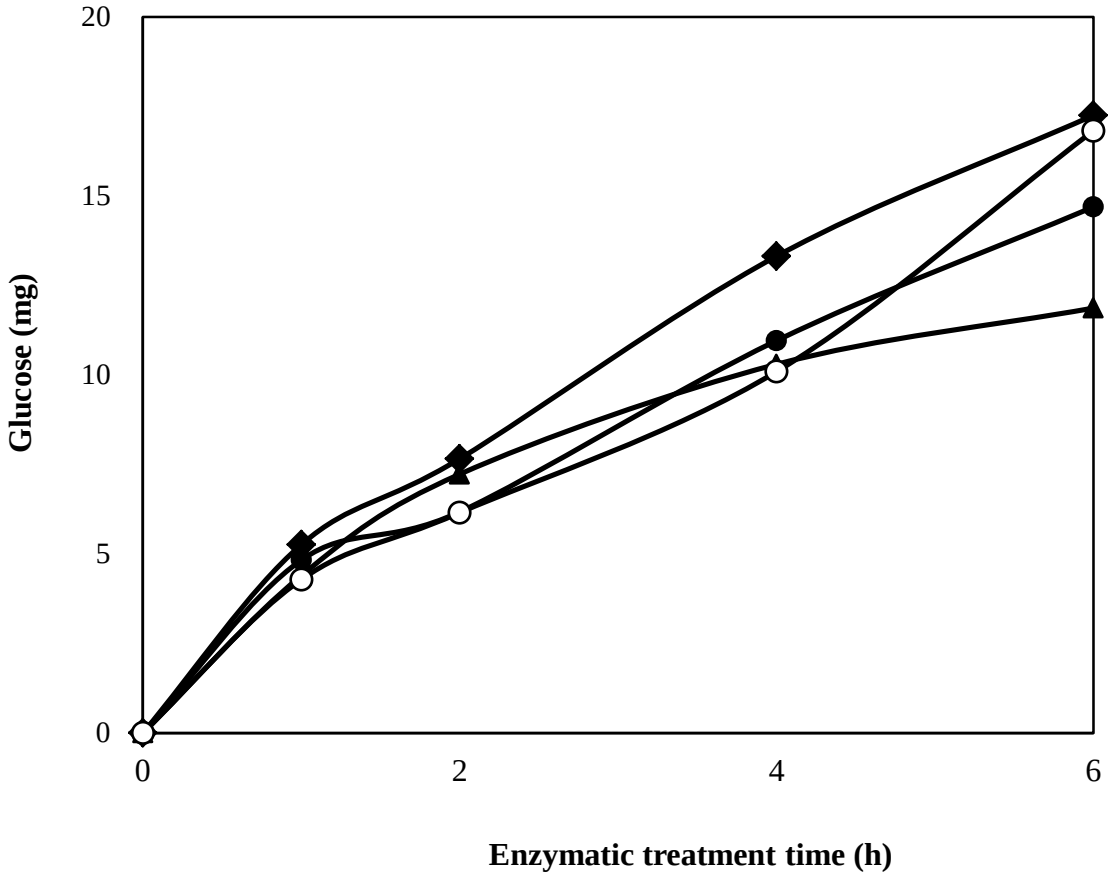

Figure 3- 17 Enzymatic hydrolysis of PPC

Note: Enzyme dosage: 12.7 FPU/g

Legend: ◆Filter paper, ●Black 100%, ▲Black 50%, ○Black 0.

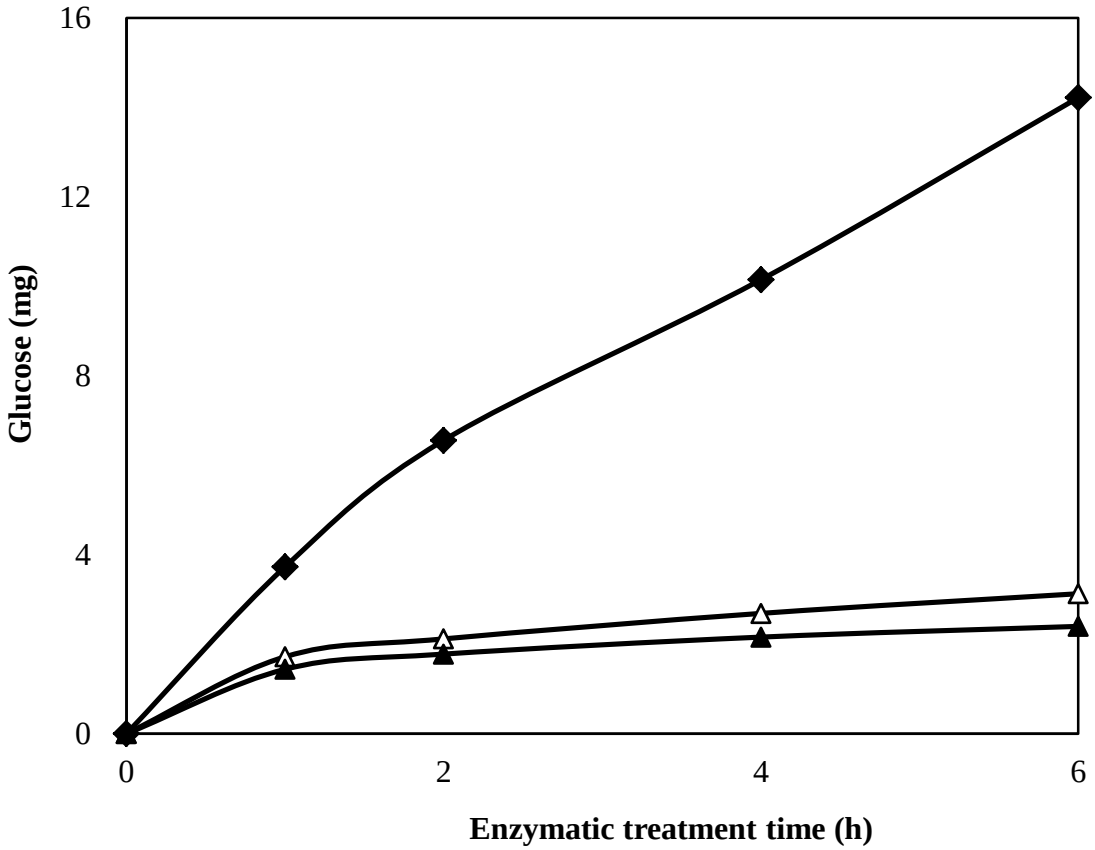

Figure 3- 18 Enzymatic hydrolysis of BCTMP with different brightness

Note: Enzyme dosage: 12.7 FPU/g

Legend: ◆Filter paper, △BCTMP ISO 80 (22.5%), ▲BCTMP ISO 70 (22.4%).

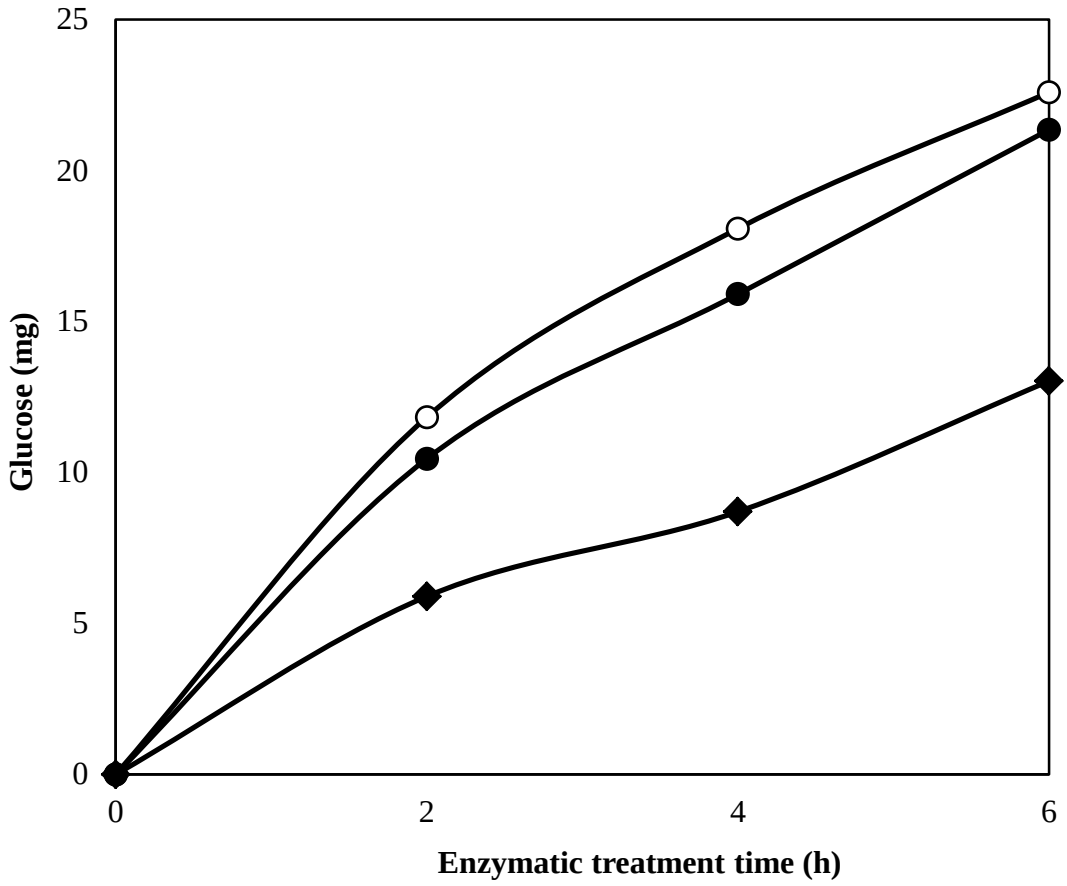

Figure 3- 19 Enzymatic hydrolysis of LOKP pulp

Note: Enzyme dosage: 12.7 FPU/g; LOKP treated: 15% NaOH, 24 hours treatment

Legend: ◆Filter paper, ○LOKP untreated (0.5%), ●LOKP treated (0.7%).

3.3.7 Enzymatic hydrolysis of cellobiose

Cellobiose is a disaccharide. The molecule is derived from the condensation of 2 glucose molecules. It can be hydrolyzed using enzymes to produce glucose. The results are shown in **Fig. 3-20**.

A comparison of sugar conversion rates after 6 h reveals that nearly 80% of cellobiose is converted into glucose using an enzyme dosage of 12.7 FPU/g. The enzyme dosages of 6.4, 3.2, and 1.6 FPU/g produced enzymatic digestibility of 50%, 30%, and 18%, respectively.

Bleached and unbleached kraft pulps of bamboo and kenaf were obtained from Dr. Jin. The chemical characteristics and bleaching conditions are described in his articles [6), 7)]. The enzymatic digestibility of bamboo bleached pulp was greater than those of unbleached pulp and filter paper. Unbleached pulp also showed a higher enzymatic digestibility than that of filter paper.

The unbleached kenaf pulp produced the best enzymatic digestibility as compared to bleached kenaf pulp and filter paper.

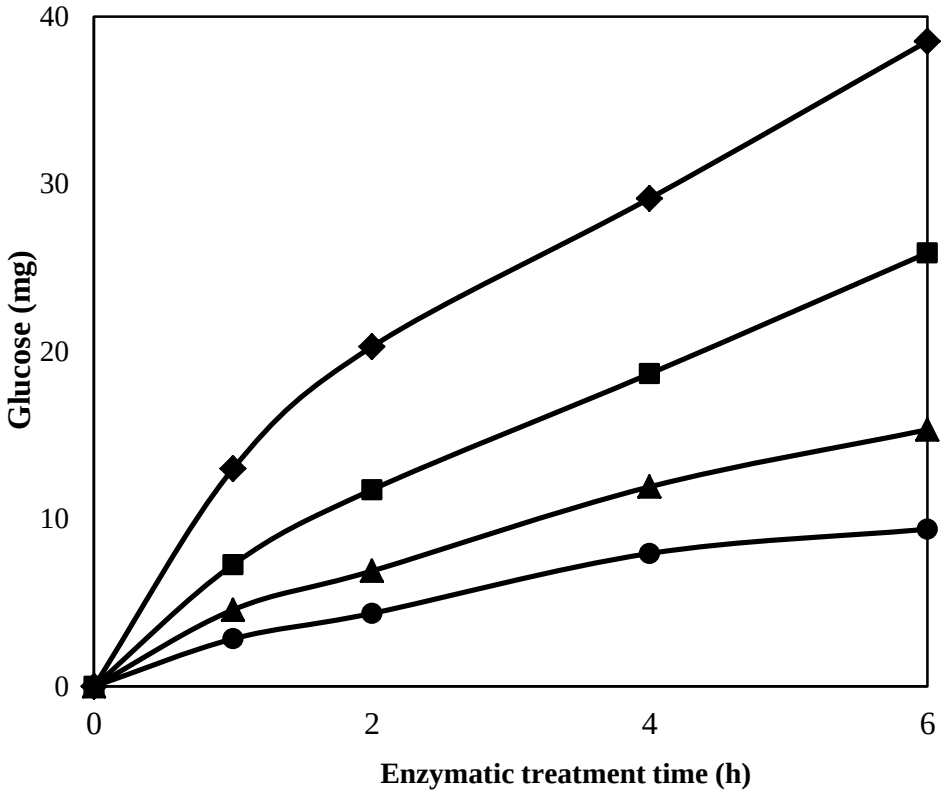

Figure 3- 20 Enzymatic hydrolysis treatment of cellobiose at different enzyme dosages

Legend: ◆12.7 FPU/g, ■6.4 FPU/g, ▲3.2 FPU/g, ●1.6 FPU/g.

According to **Fig. 3-21**, although the lignin content of the AS-AQ bamboo pulp was higher than that of the bleached and unbleached bamboo kraft pulps, the AS-AQ pulp had nearly the same effect on enzymatic hydrolysis as the bleached kraft pulp.

Fig. 3-22 shows that under the same cooking temperatures, the saccharification rate of bamboo pulp was higher than that of larch pulp and filter paper, and larch pulp had a lower saccharification rate than that of filter paper.

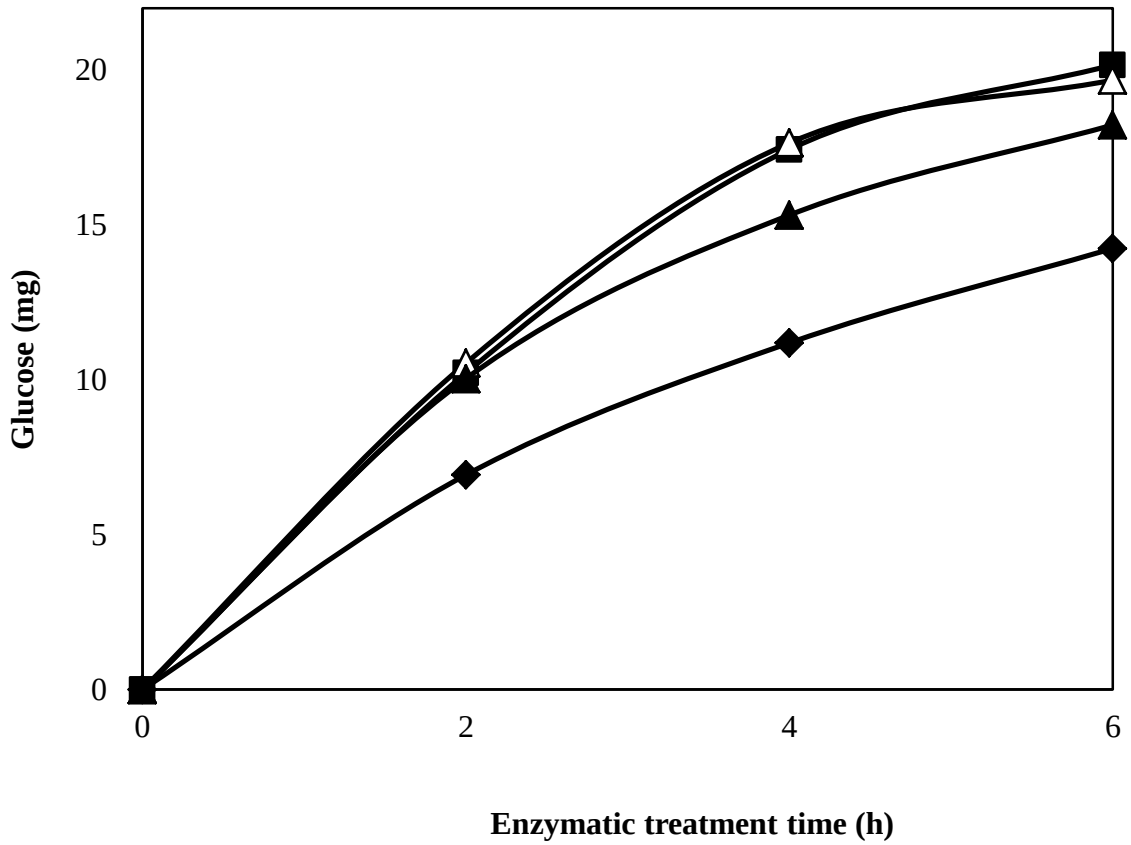

Figure 3- 21 Enzymatic hydrolysis of bamboo bleached and unbleached pulps compared with AS-AQ bamboo pulp and filter paper

Note: Enzyme dosage: 12.7 FPU/g

Legend: ■AS-AQ B10 (1.7%), △Bleached KP (1.1%), ▲Unbleached KP (3.0%) ◆Filter paper.

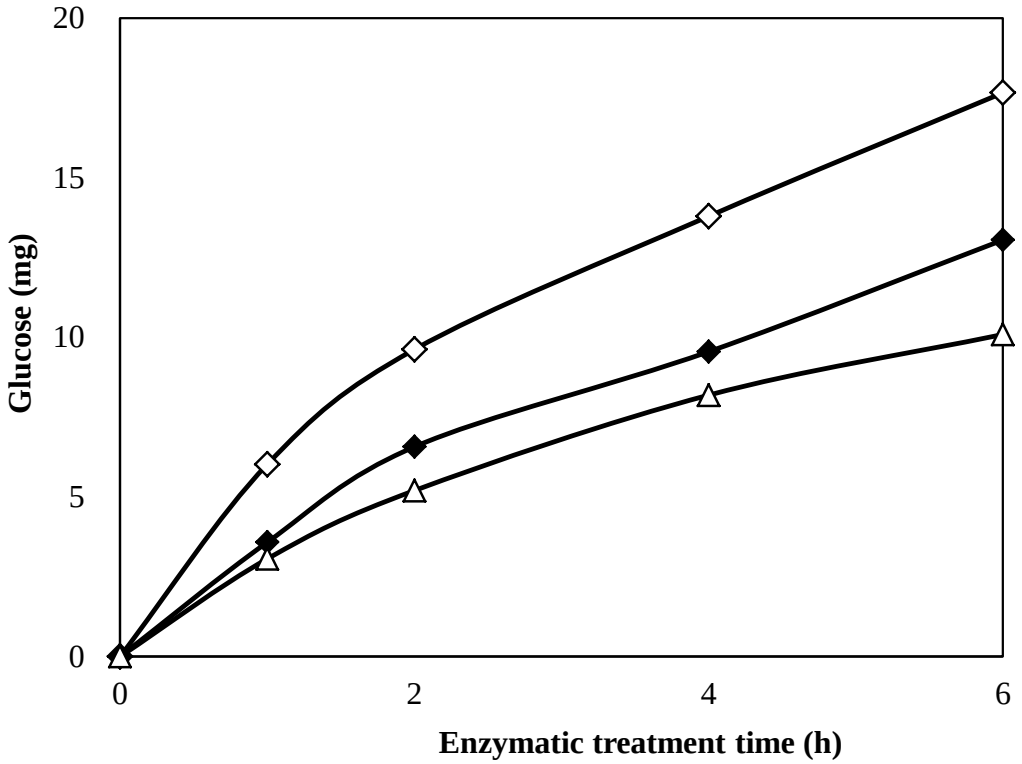

Figure 3- 22 Comparison of AS-AQ bamboo and larch pulps to filter paper

Note: Enzyme dosage: 12.7 FPU/g

Legend: ◇AS-AQ B8 (4.2%), ◆Filter paper, △AS-AQ L3 (3.5%).

3.4 Conclusions

According to the test method of filter paper cellulase activity, when AS-AQ pulp and Soda-AQ pulp was treatment by cellulase, it shown that the enzymatic saccharification was inhibited in the high lignin content pulp.

It was found that larch AS-AQ pulp have higher enzymatic saccharification ratio than Soda-AQ pulp.

Furthermore, it was also found that bamboo stem AS-AQ pulp with the lignin content of more than 9%, have higher enzymatic sacchrification ratio than filter paper.

It was clarified that AS-AQ is a promising cooking method as the pretreatment for bio-ethanol production by using bamboo as a raw material. Utilization of bamboo stem, glucose can be produced efficient by using AS-AQ cooking as pretreatment.

3.5 Reference

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Chapter 4 Estimation on Residual Lignin of Bamboo Alkaline Sulfite Pulp after Enzymatic Hydrolysis

4.1 Introduction

After enzymatic hydrolysis of pulp for production of monosaccharides and bio-ethanol, there was still lignin and xylan remaining in the residue. Even though the enzymatic residue was washed adequately after the enzymatic hydrolysis, it was considered that a slight amount of enzyme still remains in the residue. In order to determine the lignin content in enzymatic residue, it is necessary to estimate the effect of residual xylan and enzyme and find a speedy quantitative method for precise lignin content determination.

In this chapter, in order to estimate the lignin content in residual bamboo pulp after enzymatic hydrolysis, the possibility of using the acetyl bromide and Py-GC/MS methods was discussed. The difficulty of applying the acetyl bromide method to analyze the lignin in saccharification residue was clarified. The final aim of this study was to use the Py-GC/MS method to determine the lignin content and remaining enzyme in the residue.

4.2 Materials and Methods

4.2.1 Materials

Bamboo (*Phyllostachys pubescens*) stem was obtained from Hitachiomiya in Ibaraki Prefecture. The bamboo stem was cut into chips (20 × 10 × 3 mm as longitudinal × radial × tangential directions) prior to cooking. 1,4-Dihydro-9,10-dihydroxyanthracene sodium salt (SAQ), obtained from Kawasaki Kasei Chemicals, was used as an additive during cooking. Xylose and furfural were purchased from Wako Pure Chemical Industries, and xylan was purchased from SERVA Electrophoresis GmbH. Filter paper (ADVANTEC No. 1) was purchased from Toyo Roshi, Ltd.

4.2.2 Acetyl Bromide Treatment of Pulp

Pulps (15 mg) were dried at 70℃ for 5 minutes in a test tube. The solution of 25% (w/w) acetyl bromide in acetic acid (5 mL) was added into it. The tubes were sealed with screw caps and placed in a dry block heater at 70℃ for 30 min. After treatment, the mixture was cooled and transferred to a 100 mL volumetric flask containing 10 mL of 2 M sodium hydroxide and 20 mL of acetic acid. The solution was diluted to 100 mL with acetic acid, and its UV absorption spectrum was measured at 280 nm.

Lignin content was calculated from the formula [1] as shown below.

Lignin %= $100(\boldsymbol{A_s}-\boldsymbol{A_b})\boldsymbol{V} \div \boldsymbol{aW}-\boldsymbol{B}$

$\boldsymbol{A_s}$ and $\boldsymbol{A_b}$: absorbance of sample and blank;

$\boldsymbol{V}$: solution volume, L

$\boldsymbol{a}$: absorptivity of lignin, L $g^{-1}cm^{-1}$, using 20.09 based on the method improved by Iiyama and Wallis [2].

$\boldsymbol{W}$: sample weight, g

$\boldsymbol{B}$: Marton [3] factor, kraft pulp 1.70 (softwood pulp)

4.2.3 Determination of furfural

Filter paper (15 mg), xylan (5 mg), xylose (5 mg) and xylose 0.5 mL of 10mg/mL solution were placed in a test tube with a solution of 25% (w/w) acetyl bromide in acetic acid (5 mL). The tubes were sealed with screw caps and placed in a dry block heater at 70℃ for 30 min. After treatment, the mixture was cooled and transferred to a 100 mL volumetric flask containing 10 mL of 2 M sodium hydroxide and 20 mL of acetic acid. The solution was diluted to 100 mL with acetic acid or distilled water, and its UV absorption spectrum was measured at 280nm. Furfral was determined by HPLC (column: ZORBAX COLUMN : ODS, 25cm X4.6mm, flow rate: 0.5 mL/min, column temp.: 40℃, eluting solution: formic acid aqueous solution and acetonitrile (4:1 v/v, pH2.5).

4.2.4 Analysis of lignin using Pyrolysis gas chromatography/ mass spectrometry (Py-GC/MS)

Samples of the pulp (150–250 μg) were weighed out and wrapped with pyrofoil. The pyrofoil was placed in a quartz tube, which was placed in the holder of a pyrolyzer and applied to Py-GC/MS. The products of pyrolysis were identified and quantified using a GC/MS. Pyrolyzer: JHP-5 (Japan Analytical Industry), GC/MS: QP-5050A (Shimadzu), pyrolysis conditions: 500 ℃ for 4 s, internal standard (I.S.): *n*-eicosane; column: HP 1-MS (30 m × 0.25 mm); film thickness: 0.25 μm; column temperature: 50 ℃ for 1 min, raised to 280 ℃ at 5 ℃/min, then maintained at 280 ℃ for 13 min; carrier gas: helium; injection temperature: 280 ℃; interface temperature: 280 ℃; ion source temperature: 210 ℃; ionization energy: 70 eV.

4.3 Results and Discussion

4.3.1 Application of the acetyl bromide method to bamboo alkaline sulfite pulp

Iiyama and Wallis [2)] reported that the lignin contents of pine kraft and bisulfite pulps determined by acetyl bromide method agreed reasonably well while the lignin contents of eucalypt kraft pulps were 10-20% higher than those obtained by the Klason method.

The lignin contents of bamboo AS-AQ pulp samples prepared under various cooking conditions with kappa numbers ranging from 10.1 to 44.6 were determined using the acetyl bromide and Klason methods. **Fig. 4-1** shows the correlation of the lignin contents determined by the Klason and acetyl bromide methods. The Klason method results are shown on the x–axis; acetyl bromide method results are shown on the y-axis; the slope of the linear approximation is 1.16, which means that the value determined by the acetyl bromide method was higher than that by the Klason method. This is the same tendency observed by Iiyama and Wallis [2)].

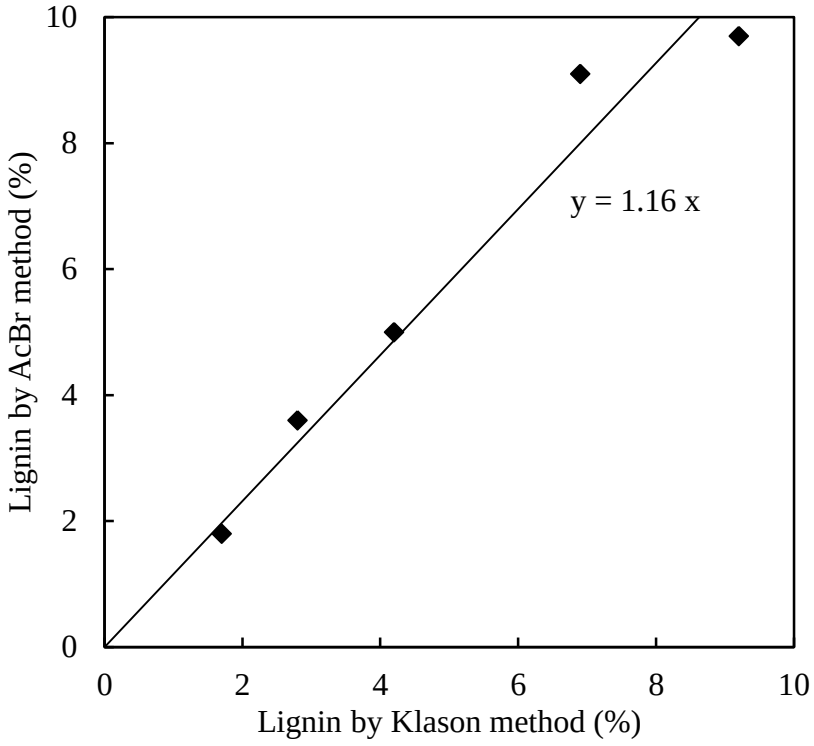

Figure 4- 1 Correlation between the lignin contents of bamboo AS-AQ pulps as determined by the Klason and acetyl bromide methods

As shown in **Fig. 4-2**, the difference between the value of lignin content determined by the acetyl bromide and Klason methods was correlated to the xylan content in the bamboo AS-AQ pulp. It was considered that the xylan in the bamboo pulp may affect the estimation of lignin by the acetyl bromide method due to UV absorption by the reaction products.

Bamboo AS-AQ pulp was used in this study, but the correction factor of 1.70 for softwood kraft pulp, reported by Marton, was used in this study [3)]. This correction factor means that the lignin content determined by the acetyl bromide method is calculated to be 1.70% when the kappa number of the pulp is assumed to be zero, and this value should be subtracted.

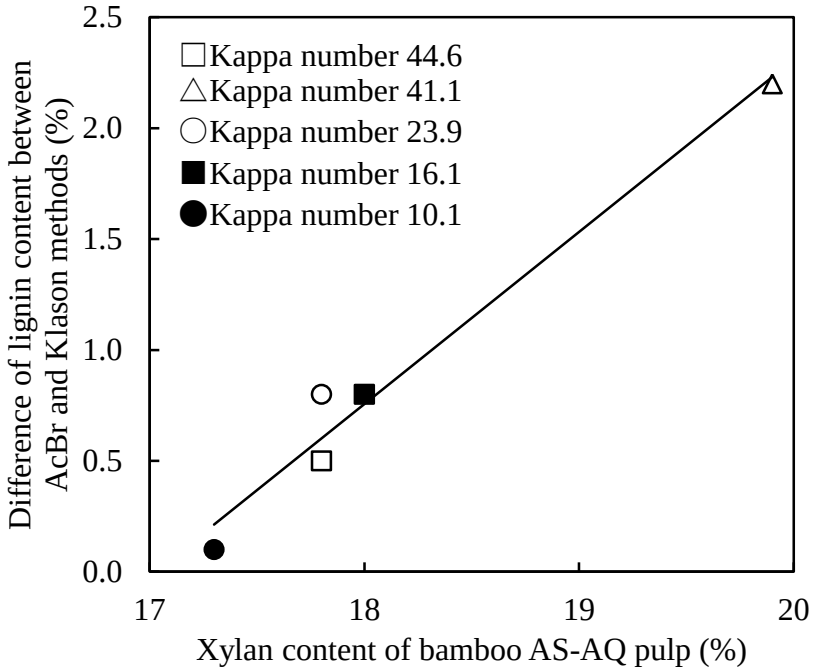

Figure 4- 2 Effect of the xylan content in bamboo AS-AQ pulp on the difference of the lignin contents determined by the acetyl bromide and Klason methods

In order to clarify whether the xylan and cellulose in the pulp affect the UV absorbance after being treated by acetyl bromide, xylose, xylan, glucose, and cellulose filter paper were tested. As shown in **Table 4-1**, at 280 nm, the absorbances of glucose and cellulose were very low; in contrast, the absorbances of xylose and xylan were very high. The existence of glucose and cellulose has little effect on the absorbance; it was considered that glucose and cellulose do not affect the quantification of lignin by the acetyl bromide method. However, during the acetyl bromide treatment, it was difficult to estimate the lignin content because of the UV absorption at 280 nm by the reaction products from xylose and xylan. This result showed that the acetyl bromide method gives higher values than the Klason method because of the existence of xylan in the pulp.

Table 4- 1 UV absorbances at 280 nm in the analysis of carbohydrates by the acetyl bromide method

Sample	UV absorbance at 280 nm per 1 mg
Xylose	0.0266
Xylan	0.0200
Glucose	0.0016
Cellulose (Filter paper)	0.0005

4.3.2 Behavior of xylose during acetyl bromide treatment

As shown in **Table 4-1**, comparing the UV absorbance of derivative of 1 mg of glucose, xylose, and their polymers, the UV absorbances of the xylose derivative and the xylan were higher than those of the glucose and the cellulose. The xylose absorbed about 20 times more than the glucose, and the xylan about 35 times more than the cellulose. It was considered that during the acetyl bromide treatment, some reaction products were produced from xylose or xylan, which affects the UV absorbance. It is known that furfural or hydroxymethylfurfural are formed from hemicellulose during the acid treatment [4)]. It was reported that under various acidic conditions, xylose can convert into furfural [5)]. It was suggested that the formation of furfural affects the UV absorbance during the acetyl bromide treatment.

Therefore, 1 mL (1000 mg) of xylose aqueous solutions with different concentrations were prepared and diluted with acetyl bromide and acetic acid. Then, the UV absorbance was measured after the acetyl bromide treatment. Furfural was analyzed by HPLC. The results of the UV absorbance and furfural quantification are shown in **Table 4-2**. When the amount of water is 1000 mg, the amount of furfural increased as the xylose content increased, and the UV absorbance also increased. During the acetyl bromide treatment, the formation of furfural was confirmed

with water contents of 1000 or 500 mg. Along with the effect of the xylose amount, the UV absorbance was also affected by the amount of water amount. With 1 mg xylose, the UV absorbance increased as the amount of water was increased. Particularly, in the case of 50 or 100 mg of water, even though the amount of furfural was lower than the limit of quantification, the increase in the UV absorbance was still observed.

Table 4- 2 UV absorbances and the formation of furfural in the analysis of xylose with water by the acetyl bromide method

Xylose (mg)	H_2O (mg)	UV absorbance at 280 nm	Furfural (mg)
1	50	0.025	0
1	100	0.027	0
1	500	0.494	0.193
1	1000	0.679	0.363
2	1000	1.356	0.747
5	1000	-	1.700

It was clarified that lignin and xylan affect the UV absorbance when using the acetyl bromide method. After the enzymatic hydrolysis treatment, the lignin content of the residue increases, and xylan is also present. Thus, it is necessary to know the xylan content to accurately analyze the residue using the acetyl bromide method. If the xylan content in the pulp or enzymatic saccharification residue is known, it is necessary to calculate the corrected absorbance ($\boldsymbol{A_n}$) by subtracting the UV absorbance caused by xylan. It was assumed that no water existed in the analysis system, and the corrected absorbance was calculated using the following formula.

$$\boldsymbol{A_n} = \boldsymbol{A_s} - \boldsymbol{A_b} - \boldsymbol{A_x} = \boldsymbol{A_s} - \boldsymbol{A_b} - 0.020 \times \boldsymbol{W_{xylan}}$$

$\boldsymbol{A_n}$: absorbance after correction for xylan absorption

$\boldsymbol{A_s}$ and $\boldsymbol{A_b}$: absorbances of the sample and blank

$\boldsymbol{A_x}$: absorbance of xylan (according to **Table 4-1**, 0.020 per one mg of xylan)

$\boldsymbol{W_{xylan}}$: xylan content in the sample, mg

The lignin content in the sample was calculated by using the following formula, and the result is shown in **Fig. 4-3**.

Lignin % = $100\boldsymbol{A_n V \div aW}$

V: volume of solution, L

a: lignin absorptivity, 20.09

W: sample weight, g

(Marton's [3] correction was not used)

Even though the pulp was washed thoroughly after enzymatic hydrolysis, some enzyme remained would adsorb to the residue. In order to investigate the effect of the enzyme amount on the UV absorbance, enzyme was added to the sample and treated by acetyl bromide without enzymatic hydrolysis. The results of AS-AQ pulp with kappa number 23.9 and Soda-AQ pulp with kappa number 12.4 are shown in Fig. **4-4**. For one gram of pulp, 12.7 FPU enzyme solution (GC220) was added, and the sample was dried prior to the acetyl bromide treatment. The result showed that the value was 0.5% higher than that without the addition of enzyme. Compared with the influence of xylan, the effect of a low amount of enzyme on the lignin quantification is small.

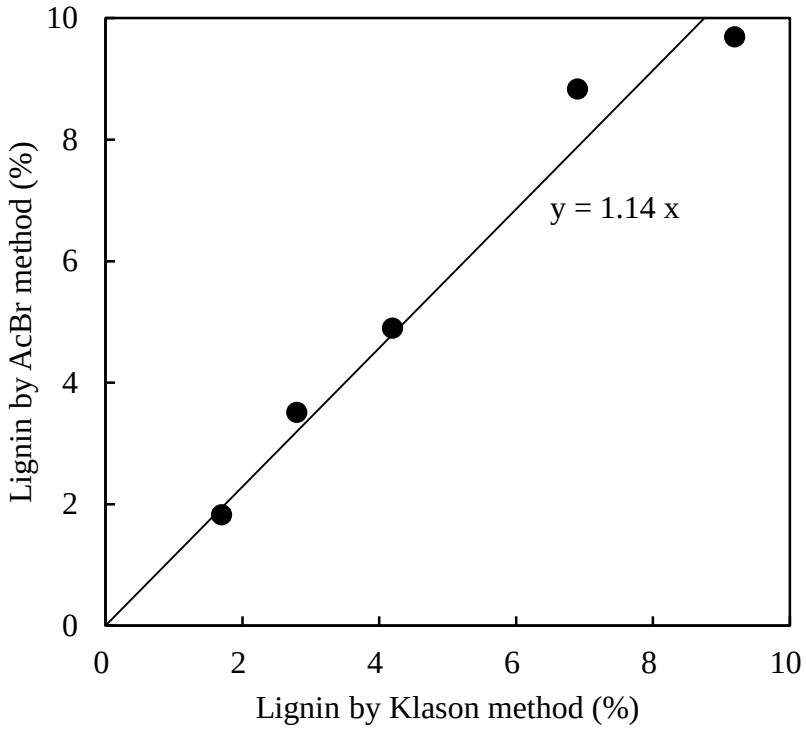

Figure 4- 3 Determination of the lignin content using the acetyl bromide method adjusted for the xylan content in bamboo AS-AQ pulp

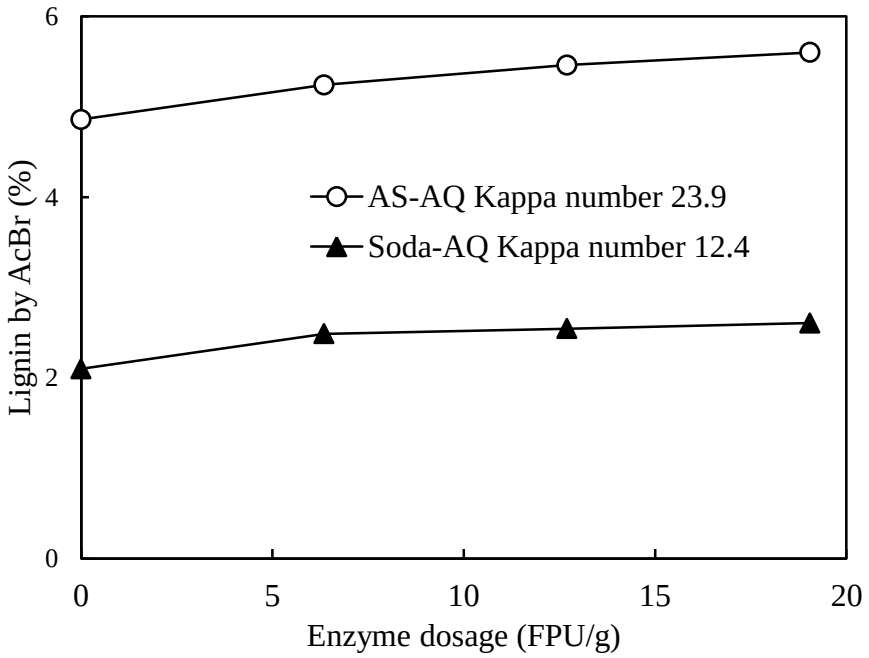

Figure 4- 4 Lignin content of bamboo AS-AQ and Soda-AQ pulps with various enzyme dosage determined by the acetyl bromide method

4.3.3 Identification of lignin pyrolysis products from pulp using Py-GC/MS

The quantification of residual lignin in the pulp after enzymatic hydrolysis treatment using Py-GC/MS was discussed. **Fig. 4-5** shows the total ion chromatogram (TIC) of the pyrolysis products from bamboo AS-AQ pulp. The pyrolysis products from the residual lignin in pulp were identified by 15 lignin pyrolysis products. The peak numbers, pyrolysis products, molecular ions, and main fragment ions are listed in **Table 4-3**. The lignin content in the residue was calculated from the sum of the TIC peak areas ($\boldsymbol{T_{15}}$) of these 15 pyrolysis products.

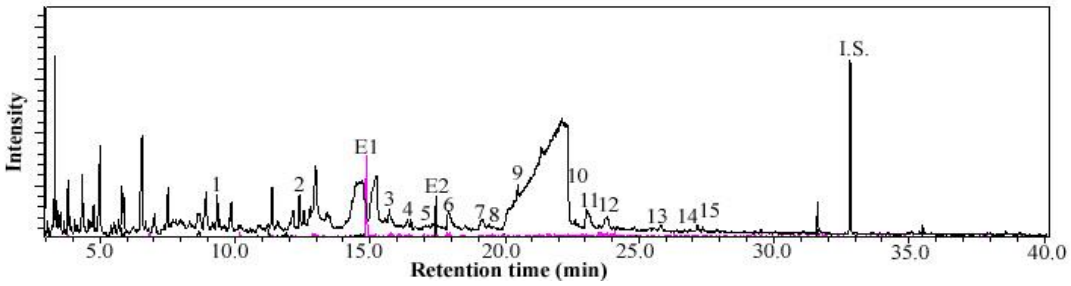

Figure 4- 5 Analysis of the residual lignin in AS-AQ bamboo pulp by Py-GC/MS

Note: Pyrolysis products from enzyme GC220: E1: indole and other pyrolysis products; E2: methylindole.

Peaks 1–15 of the pyrolysis products are listed in Table 4-3.

Table 4- 3 Peak data of the lignin pyrolysis products

Peak number	Pyrolysis product	Main ion peaks selected	
		Molecular ion	Fragment ion
1	Guaiacol	124	109, 81
2	4-Methylguaiacol	138	123, 95
3	4-Vinylguaiacol	150	135, 107
4	Syringol	154	139, 111
5	Eugenol	164	149, 77
6	Vanillin	152	151, 123
7	4-Methylsyringol	168	153, 125
8	*trans*-Isoeugenol	164	149, 131
9	Acetoguaiacone	166	151, 123
10	4-Vinylsyringol	180	165, 137
11	4-Allylsyringol	194	179, 163
12	Syringaldehyde	182	181, 167
13	*trans*-4-Propenylsyringol	194	179, 152
14	Acetosyringone	181	196, 153
15	*trans*-Coniferaldehyde	178	147, 135

First, five types of bamboo AS-AQ pulps with kappa numbers ranging from 10.1 to 44.6 were analyzed. The Klason lignin content (acid-soluble lignin + acid-insoluble lignin) of these pulps were 1.7, 2.8, 4.2, 6.9, and 9.2%. The weight of the Klason lignin content ($\boldsymbol{K}_{\mathbf{w}}$, μg) in the sample for pyrolysis analysis, the weight of the internal standard ($\boldsymbol{S}_{\mathbf{w}}$), and the ratio of these ($\boldsymbol{K}_{\mathbf{wr}} = \boldsymbol{K}_{\mathbf{w}} \div \boldsymbol{S}_{\mathbf{w}}$) were calculated. Next, the ratio of the sum of the TIC peak areas of the 15 lignin pyrolysis products ($\boldsymbol{T}_{\mathbf{15}}$) to the TIC peak area of the internal standard ($\boldsymbol{S}_{\mathbf{a}}$) was calculated as $\boldsymbol{T}_{\mathbf{ar}} = \boldsymbol{T}_{\mathbf{15}} \div \boldsymbol{S}_{\mathbf{a}}$. A calibration curve was created (**Fig. 4-6**) using the values of $\boldsymbol{K}_{\mathbf{wr}}$ and $\boldsymbol{T}_{\mathbf{ar}}$ obtained from the five types of pulp mentioned above. The calibration line is described as follows.

$\boldsymbol{K}_{\mathbf{wr}} = 131 \times \boldsymbol{T}_{\mathbf{ar}}$ $(R^2=0.917)$

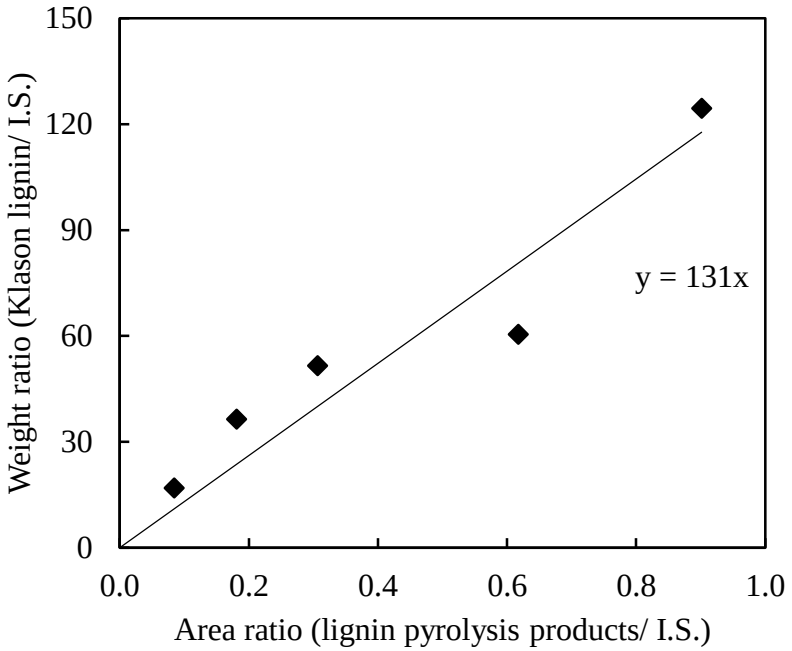

Figure 4- 6 Calibration line of the Klason lignin weight in pulp by Py-GC/MS
Legend: I.S.: internal standard

4.3.4 Behavior of lignin content in pulp during enzymatic hydrolysis treatment

It was shown that the saccharification ratio of bamboo AS-AQ pulp was higher than those of larch AS-AQ pulp and cellulose filter paper, and that the ratio for larch AS-AQ pulp was higher than that for Soda-AQ pulp [6].

In this study, for bamboo pulps, it was shown that the saccharification ratios of both AS-AQ pulp and Soda-AQ pulp were higher than that of cellulose filter paper. **Fig. 4-7(A)** shows the amount of glucose produced from AS-AQ pulp with a kappa number of 23.9 and Soda-AQ pulp with a kappa number of 12.4, and **Fig. 4-7(B)** shows the residue ratio of the pulp.

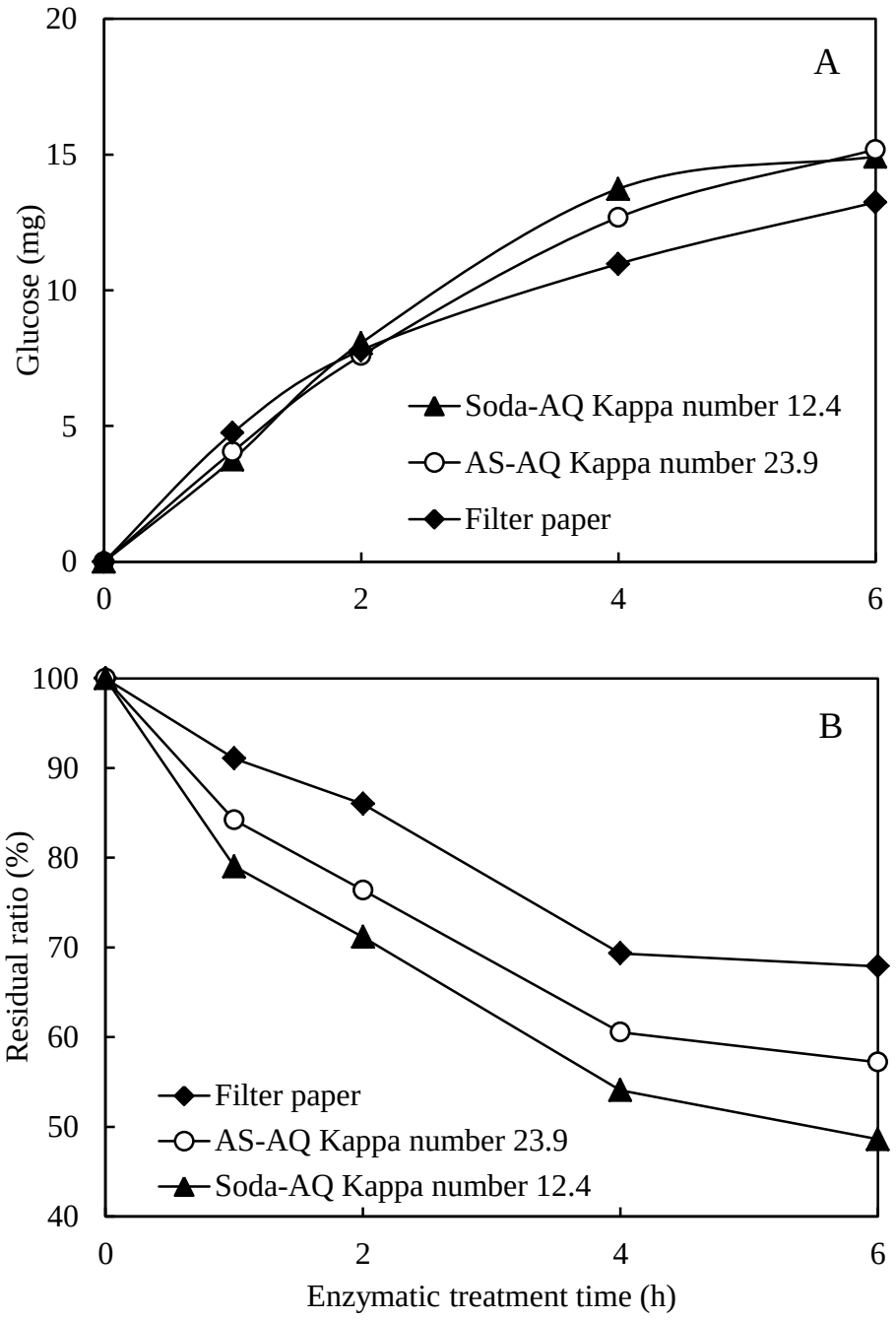

Figure 4- 7 Enzymatic hydrolysis of bamboo AS-AQ and Soda-AQ pulps (A: Glucose production; B: Residue ratio)

Note: Enzyme dosage: 12.7 FPU/g; Initial substrate weight: 50 mg.

Fig. 4-8 shows the lignin contents in the enzymatic saccharification residue as determined by the acetyl bromide method. The values of the lignin contents were corrected by xylan contents and calculated based on the % of untreated pulp weight. The lignin content of untreated AS-AQ pulp was 5.5%, and after a 6 h saccharification, it decreased to 4.1%. For Soda-AQ pulp, the lignin content of the untreated pulp was 2.5%, and during the 1–6 h enzymatic saccharification, the lignin content was almost 1.7%.

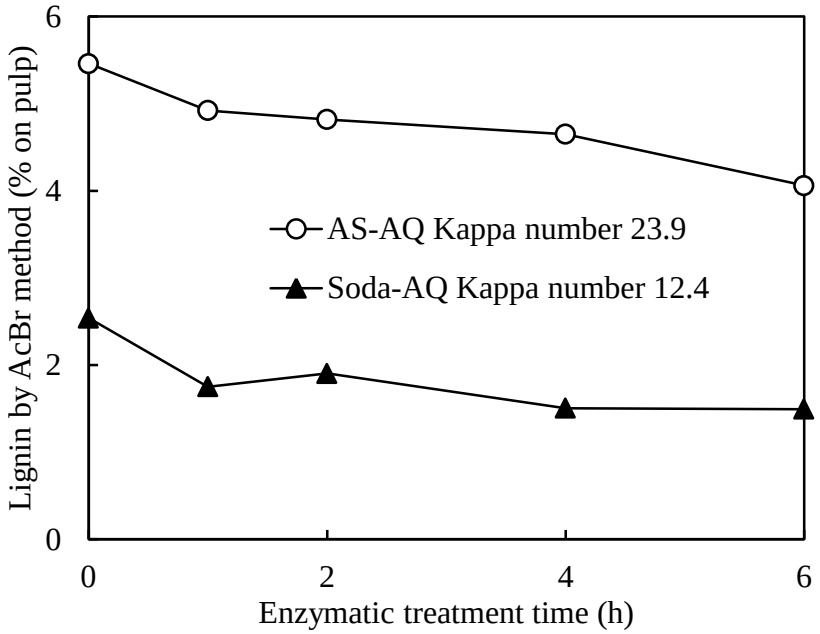

Figure 4- 8 Residual lignin content in bamboo pulps determined by the acetyl bromide method after enzymatic hydrolysis

Fig. 4-9 shows the lignin contents (calculated based on the % of untreated pulp) in the enzymatic saccharification residue as determined by the Py-GC/MS method. For AS-AQ pulp, the lignin content decreased from 4.4% to 3.2%. In Soda-AQ pulp, the lignin content of the untreated pulp was 2.1%, and during the 1–6 h treatment, the lignin content was almost 1.7%.

From the results shown above, it was clarified that part of the lignin was dissolved during the enzymatic hydrolysis treatment, especially for the AS-AQ pulp. Considering the results of Fig. **4-7(A)** as well, it was suggested that the lignin dissolved from the bamboo pulp enhanced the efficiency of the enzymatic saccharification.

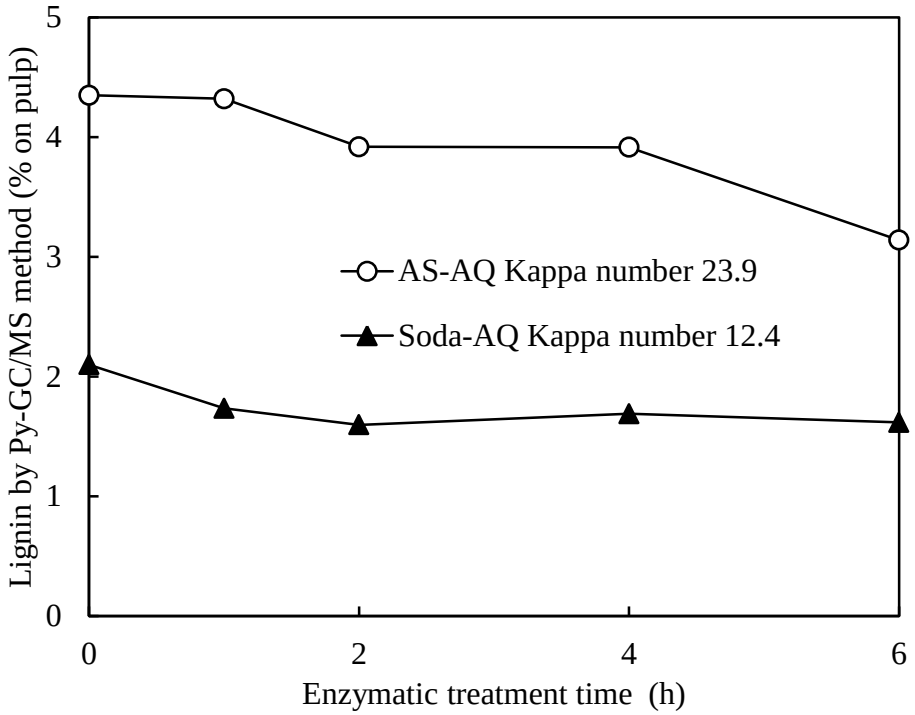

Figure 4- 9 Residual lignin content in bamboo pulps as determined by the Py-GC/MS method after enzymatic hydrolysis

Compared with the acetyl bromide method, which determines the lignin content based on UV absorption measurements, the Py-GC/MS method can easily identify and quantify the components present using the mass spectrum, even though there were many enzymes and polysaccharides in the sample, such as cellulose and xylan; non-lignin components have no effect on the lignin quantification. For the lab-scale enzymatic saccharification experiment, it is convenient to use the Py-GC/MS method, which only requires 0.1–0.3 mg for analysis, because there are many samples with small amounts of analysis.

4.4 Conclusions

It was shown that the saccharification ratios of bamboo AS-AQ and Soda-AQ pulps were higher than that of cellulose filter paper during enzymatic hydrolysis. To investigate the behavior of the enzymatic hydrolysis and the residual lignin of these pulps, methods that can precisely determine the lignin content after enzymatic hydrolysis were discussed. One of the widely used methods for determining lignin content is the acetyl bromide method, and by using this method, it was shown that the xylan in bamboo pulp affected the determination of the residual lignin due to UV absorption by the reaction products. Furthermore, the amount of water in reaction system also affected the UV absorbance. The application of the acetyl bromide method to bamboo alkaline pulp is difficult. On the other hand, it was shown that by using the Py-GC/MS method, the lignin content of pulp after enzymatic hydrolysis can be precisely estimated. It was suggested that the lignin dissolved from bamboo pulp enhanced the efficiency of enzymatic saccharification.

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Chapter 5 Summary

In this study, the application of alkaline sulfite-anthraquinone (AS-AQ) cooking was investigated as a pretreatment for enzymatic saccharification and bioethanol production from Japanese larch (*Larix leptolepis*) and bamboo (*Phyllostachys pubescens*) stem.

In order to investigate the residual lignin and saccharification behavior of pulp during enzymatic hydrolysis, it is necessary to precisely determine the lignin content of the pulp after enzymatic hydrolysis. The behavior during enzymatic hydrolysis and the residual lignin of these pulps, methods that can precisely determine the lignin content after enzymatic hydrolysis were considered. One of the widely used methods for determining lignin content is the acetyl bromide method, the application of the acetyl bromide method to bamboo alkaline pulp is difficult. On the other hand, using a pyrolysis-gas chromatography/mass spectrometry method, the lignin content of the pulp after enzymatic hydrolysis can be precisely estimated.

In Chapter 2, the ratio of $NaOH/Na_2SO_3$ was investigated. Besides AS-AQ cooking, Soda-AQ cooking was also performed. The raw materials which selected for the cooking were Japanese larch heartwood and Moso bamboo stem. As the results:

In the chips cooking, the lignin content of pulp also increased with the increase of $NaOH/Na_2SO_3$ ratio by 70/30 $NaOH/Na_2SO_3$ ratio.

At 60/40 of the $NaOH/Na_2SO_3$ ratio, the lowest lignin content of chip-based pulp was obtained. With the same ratios of Na_2SO_3, the lignin contents of meal-pulps were generally higher than chip-pulps.

Even though the raw material was the same, the variation of pulp yields between chips and meals cooking was almost similar to each other.

The optimum cooking condition of AS-AQ was a 60/40 ratio of $NaOH/Na_2SO_3$ which produced the lowest lignin content when cooking was compared to other ratios. The AS-AQ method is more effective than Soda-AQ method as pretreatment.

In Chapter 3, the enzymatic hydrolysis of larch amount of pulps were subjected, with different enzyme dosage, and different enzymatic treatment time.

At a cellulase dosage of 12.7 FPU/g, can made about 33% of the filter paper converted into glucose after 6 h of treatment. When cellulase dosage was increased to 63.5 FPU/g (5 times of 12.7 FPU/g), the glucose produced was only 1.58 times that produced using 12.7 FPU/g.

When comparing the low cellulase dosages of 2.6 FPU/g and 3.2 FPU/g, an increase in cellulase dosage by 1.25-fold produced a 1.54-fold increase in converted glucose.

When larch and bamboo raw materials were not pretreated, the enzymatic digestibility of the larch was higher than that of the bamboo.

After 6 h of enzymatic hydorlysis treatment of larch pulps, the glucose from the filter paper was about twice that of the AS-AQ 4h pulp (lignin content, 3.5%), and 2.5 times that of the AS-AQ 2h pulp (lignin content, 14.3%), though the lignin content of the 4h pulp was about 1/5 that of the 2h pulp.

The enzymatic digestibility of the soda-AQ 2h pulp (lignin content, 9.6%) was lower than that of the soda-AQ 4h pulp (lignin content, 4.4%).

After the enzymatic hydrolysis treatment of bamboo pulps, the enzymatic digestibility of 2h and 4h pulps were higher than that of the 1h pulp. After the initial 2 h of enzyme treatment, the enzymatic digestibility

of the 2h pulp was lower than that of the 4h pulp, but from 4 h to 6 h, the 2h pulp showed a higher enzymatic digestibility than that of the 4h pulp. The enzymatic digestibilities of these pulps were also higher than that of filter paper.

The amount of xylose released from bamboo pulps was not related to the lignin content of pulps but only related to the time of enzymatic hydrolysis treatment.

After pretreatment, the enzymatic digestibility of bamboo pulps were higher than those of larch pulps, based on a comparison with filter paper. The AS-AQ method is more effective than soda-AQ method as a pretreatment for bioethanol production. In addition, bamboo was more effective than larch as a raw material for bioethanol production.

In Chapter 4, acetyl bromide method and pyrolysis-gas chromatography/ mass spectrometry (Py-GC/MS) was subjected to determining the lignin content and adsorbed enzyme in the residual after enzymatic hydrolysis treatment. The results as:

It was shown that the saccharification ratios of bamboo AS-AQ and Soda-AQ pulps were higher than that of cellulose filter paper during enzymatic hydrolysis.

One of the widely used methods for determining lignin content is the acetyl bromide method, and by using this method, it was shown that the xylan in bamboo pulp affected the determination of the residual lignin due to UV absorption by the reaction products.

Furthermore, the amount of water in reaction system also affected the UV absorbance.

The application of the acetyl bromide method to bamboo alkaline pulp is difficult.

On the other hand, it was shown that by using the Py-GC/MS method, the lignin content of pulp after enzymatic hydrolysis can be precisely estimated.

Lignin dissolved from bamboo pulp enhanced the efficiency of enzymatic saccharification.

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Publications

1) Yi ZHANG, Hiroshi OHI, Shiho TAKAHASHI, Guangfan JIN, Keiichi NAKAMATA: Enzymatic Hydrolysis of Bamboo and Larch Alkaline Sulfite Pulps for Glucose Production. *JAPAN TAPPI* in printing (2014)
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