Biodegradation of high molecular weight lignin under sulfate reducing conditions. : lignin degradability and degradation by-products

Jae-Jung Ko[1*], Yoshihisa Shimizu[2], Kazuhiro Ikeda[2], Seog-Ku Kim[1], Chul-Hwi Park [3], Saburo Matsui[4]

[1]Korea Institute of Construction Technology, 2311, Daehwa-Dong, Ilsanseo-Gu, Goyang-Si, Gyeonggi-Do, 411-712, Republic of Korea, E-mail: caryotes@hotmail.co.jp

[2]Research Center for Environmental Quality Management, Kyoto University, 1-2 Yumihama, Outsu, Shiga, 520-0811, Japan,

[3]Dept. of Environmental Engineering, University of Seoul, 90 Jeonnong-Dong, Dongdaemun-Gu, Seoul, 130-743, Republic of Korea

[4]Dept. of Technology and Ecology, Kyoto University, Yoshida Honmachi, Sakyoku, Kyoto, 606-8501, Japan

*Corresponding author: Tel: 82-31-910-0514, Fax: 82-31-910-0291, E-mail: caryotes@hotmail.co.jp

Abstract

This study is designed to investigate the biodegradation of high molecular weight (HMW) lignin under sulfate reducing conditions. With a continuously mesophilic operated reactor in the presence of co-substrates of cellulose, the changes in HMW lignin concentration and chemical structure were analyzed. The APPL (Acid Precipitable Polymeric Lignin) and lignin monomers, which are known as degradation by-products, were isolated and detected. The results showed that HMW lignin decreased and showed a maximum degradation capacity of 3.49 mg/l/day. APPL was confirmed as a polymeric degradation by-product and was accumulated in accordance with HMW lignin reduction. We also observed non-linear accumulation of aromatic lignin

monomers such as hydrocinnamic acid. Through our experimental results, it was observed that HMW lignin is biodegraded through APPL and aromatic monomers under anaerobic sulfate reducing conditions with a co-substrate of cellulose.

Keywords: Acid Precipitable Polymeric Lignin (APPL), Degradation products, HMW lignin, Sulfate reducing conditions

1.Introduction

Next to cellulose, lignin is the most abundant natural polymeric carbon source in theworld. Lignin is a three-dimensional network built up of dimethoxylated (syringyl, S),monomethoxylated (guaiacyl, G) and non-methoxylated (p-hydroxyphenyl, H)phenylpropanoid units, derived from corresponding p-hydroxycinnamyl alcohols, whichgive rise to a various subunits including different ether and C-C bonds (del Río et al.,2007; Martínez et al., 2005; Reale et al., 2004). The size, nonhydrolyzability,heterogeneity, and molecular complexity of lignin dictate that initial biodegradation beoxidative and nonspecific, and that it be mediated by an extracellular system (Pérez etal., 2002). Lignin is a barrier when cellulose is accessible to enzymes, such that thepresence of lignin in the cell wall impedes enzymatic hydrolysis of the carbohydrate (Mosier et al., 2005; Sun and Cheng, 2002).

Research on lignin biodegradation is important in the field of global carbon cyclingas well as in the bleaching and pulp industries. In addition, in the field of environmental engineering, it is well known that ligninolytic enzymes can biodegrade recalcitrant xenobiotics such as dioxins due to the broad substrate specificities of these enzymes (Manimekalai and Swaminathan, 2000; Ravinocvich et al., 2004). So far, most of these

studies have reported on the biodegradation of lignin under aerobic conditions, however, little is known about biodegradation of lignin in anaerobic environments. Anaerobic biodegradation of lignin is important as it is implicated in municipal solid waste (MSW) landfill stabilization, in waste treatment from the pulp industry, as an inhibitor in fermentation for bioethanol and in developing a clearer understanding of the global carbon cycle. Among the components of wood, it is reported that cellulose and hemicellulose are easily converted to methane or carbon dioxide anaerobically. However, the biodegradation of high molecular weight (HMW) lignin is prevented under anaerobic conditions and no studies to date have observed anaerobic degradation of lignin (de Boer et al., 2005; Micales and Skog, 1997; Tuomela et al., 2000). This isbecause the extracellular enzymes required for depolymerization of lignin need molecular oxygen, and their oxidative reactions would not be anticipated under anaerobic conditions (Hataka, 1994; Hataka, 2001; Kirk and Farrel, 1987; Zeikus, 1981).

However, Pareek et al. (2001) reported that one of the major inter-monomeric linkages in lignin, represented by a β-O-4 linked lignin model compound, was degraded under sulfate reducing conditions. Additionally, under the same anaerobic sulfate reducing conditions, lignin monomers of degradation by-products such as vanillic acid, were isolated after incubation with a sole carbon source such as newspaper, which contained 24% lignin. These results suggest that analysis of aromatic compounds is effective for assessing MSW landfill stabilization, as the most recalcitrant component of MSW is lignin (Barlaz, 2006).

It was reported that sulfate-reducing bacteria (SRB) have a stronger electronic affinity than do methane-producing bacteria (MPB) for anaerobic biodegradation of lignocellulose (Kim et al., 1998; Pareek, 2000). The objective of this study is to

investigate the biodegradation of HMW lignin under sulfate reducing conditions. In particular, we isolated the degradation by-products of a soluble lignin polymer, because the production of depolymerized soluble lignin is known to be a rate limiting reaction in the biodegradation of HMW lignin (Crawford et al., 1983). Due to the structural characteristics of lignin, it cannot be used as a substrate directly by microorganisms, instead, lignin is degraded only during secondary metabolism where easily metabolizable nutrients such as sugars are used. Moreover, lignin biodegradation is not a hydrolytic reaction but an oxidation by radicals, which are also a byproduct of cellulose biodegradation (Evans et al., 1994; Couto and Rätto, 1998). Therefore, in this study, a cellulose co-substrate was included to investigate the degradation of HMW lignin under sulfate reducing conditions.

2. Materials and methods

2.1. Experimental apparatus and operational condition

The inoculum was obtained under anaerobic condition from a simulated landfill column reactor that had been designed for leachate circulation and continuously operated for over 3 years under sulfate reducing conditions using sawdust and newspapers as substrates (Kim et al., 1998). The carbon sources of Organosolv lignin and Avicel cellulose were obtained from Sigma-Aldrich, Japan, and other reagents were from Wako Chemicals, Japan. The reactor used was an anaerobic 10L glass rectangular semi-batch reactor, which was connected with a butyl rubber stopper, a temperature controller and a 5L gas bag. The reactor was operated at 37±1℃ with mixing, for 280

days under sulfate reducing conditions. Because HMW lignin is extremely recalcitrant, the operation was performed continuously over long periods of time, in 20, 20, 56, 59, and 99 day batch experiments. The variation in lignin content was monitored from day 40. A blank reactor containing 20 mM molybdate, an SRB inhibitor, was used to investigate the non-biological degradability alone.

2.2. General parameters and methods

The cellulose concentration and sulfate concentrations were measured as a general parameter for monitoring cellulose biodegradation. After homogenization, the variation in cellulose concentration was analyzed with phenol-sulfuric acid method using glucose as a standard (Dubois et al., 1956). Sulfate was analyzed using ion chromatography (DX 500, Dionex) on a liquid sample filtered through a 0.45µm membrane filter. In order to assess the lignin degradation, the acid-insoluble lignin content was measured using the Klason lignin method (Crawford et al., 1988; Lins and Dence, 1992). The lignin content value obtained was based on the averages of at least three replicates. Complete solubilization of cellulose in 72% H2SO4 was confirmed, and the filtrate was used for analysis of acid soluble lignin by UV spectrometry.

2.3. Degradation by-products

The isolation of acid precipitable polymeric lignin (APPL) was performed using the method suggested by Crawford et al. (1988). To a 50 ml sample, 50 ml of distilled water was added and autoclaved for 1 hr at 120oC, 3 ml of 30% HCl was added, then the

sample was filtered through a Whatman No.54 filter. The precipitate was allowed to settle overnight, then was collected by centrifugation and freeze dried. The APPL content was calculated from the weight of the freeze-dried matter, and was based on averages from at least 3 replicates. For the analysis of aromatic compounds, the acidified samples were extracted 3 times with 20 ml of anhydrous diethyl ether. The extracts were then derivatized with 50µl N,O-bis-trimethylsilyl trifluoroacetamide (BSTFA) at 70℃ for 20 min to form trimethylsilyl derivatives. One µl of derivatized sample was injected into a GC-MS (QP5000, Shimadzu). The detection and identification of the aromatic compounds were performed in the SCAN mode, and quantification was in SIM mode (Pareek et al., 2001).

2.4. py-GC/MS, Fourier-transform infrared spectroscopy (FT-IR) and gel permeation chromatography (GPC) analyses of APPL and HMW lignin

To investigate variations in lignin structure as a function of time, and to compare Klason lignin and APPL, FT-IR and GPC analyses were performed. Additionally, py-GC/MS analysis was carried out on APPL. Sample pyrolysis was performed in duplicate with a Double-shot pyrolyzer (Frontier Lab, Japan) coupled to a 6890N GC/5975i MS (Agilent, USA), using a 30 m × 0.32 mm i.d., 0.32 mm DB5-HT column. Approximately 200 mg of finely divided sample was deposited on a ferromagnetic wire, then inserted into the glass liner and immediately placed in the pyrolyzer. Pyrolysis was carried out at 600℃. The chromatograph was programmed from 100℃ (5 min) to 300℃ at a rate of 10℃/min, and the final temperature was held for 10 min. The injector

and the GC/MS interface were kept at 310℃. In the full-scan mode, electron ionization (EI) mass spectra in the range of 5-1050 (m/z) were recorded at electron energy of 70eV. The compounds were identified by comparing the mass spectra obtained with those obtained from NIST computer libraries. Quantification was based on peak areas (the total integral of identified compounds was equal to 100). Relative peak molar areas were calculated for the hydroxyphenyl, guaiacyl, and syringyl lignin pyrolysis products and the data from two replicate experiments were averaged.

The FT-IR spectra of Klason lignin and APPL were analyzed using the KBr technique with a FTIR-8400S (Shimadzu). The molecular size distribution of HMW lignin and APPL was determined using GPC with a HPLC (Waters 616LC) system equipped with an Asahi pak GF-310HQ column. The lignin samples were dissolved in dimethylformamide at a concentration of 0.1% (w/v) and centrifuged. Subsequently, the supernatants were eluted at a flow rate of 0.5ml/min at room temperature. Calibration was performed with a polystyrene standard and the column effluent was monitored using a UV detector at 280 nm.

3. Results and discussion

3.1. Biodegradation of cellulose

Fig. 1 shows the variation in sulfate concentration as a function of time. Arrows indicate the time when sulfate, lignin and cellulose were added. From the first phase of the operation, sulfate decreased gradually without any lag phase. During this time, the sulfate concentration in the molybdate-containing reactor was not reduced. It is thought

that sulfate was used as an electron acceptor during the metabolism of cellulose,indicating the activity of SRB. Fig. 2 describes the hydrolysis of cellulose. Although the phenomenon of cellulose degradation was not observed by the 2nd operational phase, cellulose degradation occurred from the 40th day. Avicel cellulose used in this study was not linked to lignin chemically, and it is therefore considered that there was no inhibitory effect due to lignin during cellulose hydrolysis. Lignocellulosic carbon sources, except hemicellulose in this study, are made up of only insoluble organics. Therefore, the variation of total solid weight is a very effective parameter in investigating the biodegradability of lignocellulose. Fig. 3 illustrates the variation in total solid concentration as a function of time, which indicates decomposition of lignocellulose. From day 40 to day 96, the maximum reduction rate was 30%, from 12.7 g/l to 8.9 g/l.

3.2. Characteristics of HMW lignin degradation

Klason lignin is acid-insoluble lignin, and can be described as insoluble HMW lignin. Therefore, a variation in Klason lignin content indicates not total lignin content but the depolymerization rate of HMW lignin. Fig. 4 describes the change of Klason lignin content. In this HMW lignin biodegradation test, Klason lignin decreased slightly, 11.6 % of 3.49 mg/l/day, from the 97th day to the 156th day (3rd operational period), and 12.0 % of 3.03 mg/l/day, from the 181th day to the 280th day (4th operational period). From these results, although the amount of depolymerized lignin was small, HMW lignin was shown to be degraded with a co-substrate of cellulose under sulfate reducing conditions. Zeikus et al. (1982) reported that HMW lignin (>Mw 850) was not

degraded in 30 days; in contrast, Kraft lignin, which is a chemically modified and partially soluble lignin, was significantly decomposed under anaerobic conditions. They also suggested that lignin must be chemically modified or depolymerized before significant decomposition occurs in anoxic sediments. The results of the UV spectrum on acid soluble lignin exhibited the typical UV spectrum of lignin. The absorption maximum at λ 280 nm originates from nonconjugated phenolic groups (aromatic rings) and the absorption band is broad due to the structural complexity of lignin. As a function of time, the value of E280 nm/E260 nm decreased from day 40 to day 96, from 1.07 to 0.93, and from day 97 to day 156, from 1.10 to 0.95. The decrease in absorbance at 280 nm in the spectrum of lignin, due to microbial attack, can only be attributed to cleavage of the aromatic ring structures within the polymer (Janshekar et al., 1981).

Crawford et al. (1983) proposed that, when HMW lignin is degraded, the degradation by-product APPL, a soluble polymeric LMW lignin, is produced (Reale et al., 2004). Fig. 5 shows the variation in APPL concentration as a function of time. APPL content increased from day 40 to day 96 and from day 181 to day 261. This might be explained by accumulation of APPL as a result of solubilization and depolymerization of HMW lignin. In the meantime, from the 97th to the 156th day, APPL content decreased after its gradual growth. This is likely evidence that this polymeric degradation by-product was further degraded to lignin monomer aromatic compounds that, in comparison to polymeric lignin, are easily degradable. The decline in APPL content was likely attributable to the fact that the amount of APPL produced was smaller than that further degraded to aromatic compounds.

Py-GC/MS has several great advantages as a technique for enabling rapid analysis of small samples, for avoiding problematic isolation of partially biodegraded lignin and for

yielding structural information that can contribute to a better understanding of the transformation of lignin macromolecules (Telysheva et al., 2007). Table 1 lists the detected aromatic compounds produced by pyrolysis from the day 97 and day 156 APPL samples. The symbols H, G and S indicate the pyrolyzates derived from hydroxyphenyl-, guaiacyl- and syringyl propane units, respectively, and 27 peaks were assigned to 4 S-type and 7 G-type compounds with corresponding side-chain structures. The distribution of each pyrolyzate showed a similar tendency. Total phenolic acid in APPL was 44% on the 97th day and 46% on the 156th day, respectively. The high phenolic content found in the APPL was probably due to degradation of aryl-ether linkages, which are known major inter-unit linkages in lignin polymerization (Camarero et al., 1999). In terms of total APPL phenolic content, the total H: G: S ratio was 18:16:10 on the 97th day and 16:16:6 on the 156th day. The high content of H in the ratio, compared to generally known HMW lignin ratios, might be the result of depolymerization due to biodegradation under sulfate reducing conditions. The S/G ratio has proven useful for the investigation of the process of wood and lignin biodegradation (Vinciguerra et al., 2007; Telysheva et al., 2007; Nonier et al., 2006). The S/G ratio decreased as a function of incubation time from 0.6 to 0.37, indicating changes in the chemical structure of APPL, which would include reactions such as demethylation of the methoxyl groups.

The major peaks known as the specific band for lignin were observed at 1,213 Cm^{-1}, 1,460 Cm^{-1}, 1,511 Cm^{-1}, 1,650 Cm^{-1} and 3,400 Cm^{-1} for both Klason lignin and APPL samples, by FT-IR analysis. That indicates that APPL is a polymeric lignin in chemical structure. However, there were differences in the peaks, illustrating the existence of structural distinctions between Klason lignin and APPL. The peak at 1,113 Cm^{-1},

attributed to syringyl C-H in lignin, was detected only in APPL, while the peaks at 850 Cm^{-1} and 1,068 Cm^{-1}, which originated from C-H out of plane and C-O in secondary alcohols, were observed only in Klason lignin. The peak strength at 1,288 Cm^{-1}, which is known to be a typical guaiacyl ring, was larger in APPL than in Klason lignin, indicating that the ratio of guaiacyl rings was greater in APPL than in Klason lignin. In terms of structural changes in the same kind of lignin as a function of time, little change appeared in Klason lignin. However, the band at 1,213 Cm^{-1}(C-O of syringyl ring) for APPL became larger with increased incubation time.

The results of GPC analysis indicated that both HMW lignin and APPL have two regions of molecular distribution, one around Mw 20,000 and the other around Mw5,000. Compared to HMW lignin, the profile of APPL was shifted to a smaller region and the height around Mw was lower, indicating that the Mw of APPL is smaller than that of HMW lignin and APPL is a more depolymerized lignin than is HMW lignin. At the same time, little change in Mw distribution was observed as a function of time for either HMW lignin or APPL.

The aromatic compounds detected as degradation products are summarized in Table 2 and the variation of total aromatic compounds is shown in Fig. 6. The concentration of almost all of the aromatics did not begin to increase until the 96th day. The reason for this can be explained by the complex steps involved in HMW lignin degradation. By the 96th day, although depolymerization to APPL (thought to be the rate limiting step) had occurred, the further steps involved in the degradation to aromatics had not yet begun. However, from the 97th day, it appeared that APPL was gradually being degraded to lignin monomers of aromatic compounds following its accumulation. The nonlinearity of the accumulation of aromatic compounds may be attributed to the fact that lignin

consists of many different kinds of phenolic units, unsymmetrically polymerized by complicated heterogeneous links (Pareek, 2000). Another reason is that lignin monomers of aromatics are easily biodegraded, compared to polymeric lignin itself. Phelps and Lovely (1997) reported that ferulic and syringic acids, typical lignin monomers, were well metabolized without lag phases under sulfate reducing conditions. In view of the chemical structure, phenolic acids that should be the primary compounds detected among the aromatics. The concentration of aromatics having a phenylpropane unit was higher than that of aromatics having a carboxyl substitute. Among the detected compounds originating from synapyl alcohol, vanillic acid and syringic acid were the most prevalent, but their concentration was very low compared to other compounds originating from p-coumaryl alcohol or coniferyl alcohol.

3.3. Discussion on the chemical pathway of HMW lignin biodegradation under sulfate reducing conditions

Because lignin has a complex chemical structure, high insolubility and high molecular weight, lignin model compounds consisting of lignin dimers are typically used in biodegradation tests. It was reported that the β-O-4 link lignin model compound, which is a soluble LMW lignin dimer, was degraded under anaerobic conditions, in the complete absence of molecular oxygen (Pareek et al., 2001; Kajikawa et al., 2000). However, it is doubtful that HMW lignin was degraded under anaerobic conditions by the mechanism proposed in that research. Anaerobic degradation of labeled lignocelluloses, as well as synthetic lignin, Kraft lignin, and beechwood has also been studied with the result that, after several weeks or months, very little HMW lignin was

degraded anaerobically (Eriksson et al., 1990). Zeikus et al. (1982) further investigated the recalcitrance of lignin to biological decomposition under anaerobic conditions. LMW (Mw <1,000) [14C]-DHPs (Dehydrogenative Polymerizates) were significantly decomposed (>5% conversion to $^{14}CO_2$ and $^{14}CH_4$ in 30 days), but HMW lignin (Mw >1,000) was not degraded. They suggested that O_2 is required for the biologically mediated depolymerization of lignin, but not for the microbial decomposition of model lignins that contain ether linkages or soluble aromatic compounds. Additionally, Kuhnigk et al. (1994) reported that depolymerization of HMW lignin, which is known to be the rate limiting step in HMW lignin biodegradation, was not confirmed; although the reaction of side chain oxidation and demethylation could be carried out by termites under anaerobic conditions (Ohkuma, 2003). Lignin degradation is not a hydrolysis reaction but a radical reaction, due to the characteristics of its chemical structure. On the basis of research about aerobic white-rot fungi, it was advocated that lignin depolymerization in oxidative reactions is attributable to production of aryl and phenoxy radicals by lignin peroxidase and manganese peroxidase (Breznak and Brune, 1994). Theoretically, the biodegradation of HMW lignin can be limited due to the fact that extracellular enzymes, which are responsible for production of radicals, produce H2O2 using molecular oxygen as a substrate (Breznak and Brune, 1994; Gómez-Toribio et al., 2001; Reale et al., 2004). Hydrogen peroxide appears to be involved in cellulose degradation by white-rot fungi and brown-rot fungi, which are known as the most ubiquitous microorganisms involved in lignin decomposition (Evans et al., 1994). In the present study, cellulose degradation might bring about HMW lignin decomposition under sulfate reducing conditions, as the hydrolysis of the cellulose co-substrate would produce hydrogen peroxide, which could then act as a substrate for

extracellular enzymes.

As proposed by Filley et al. (2001), HMW lignin degradation can be considered to have occurred by three main reactions, namely depolymerization, side chain oxidation and demethylation. The most important aspects of HMW lignin biodegradation are depolymerization and solubilization, which result from oxidative reactions following β-ether linkage cleavage by extracellular enzymes. In this study, it was confirmed that the APPL of soluble depolymerized lignin, which is a degradation by-product of HMW lignin, that was isolated and quantified not only had the chemical structure of the specific lignin but also had different characteristics from those of acid-insoluble HMW lignin fractions, as indicated by spectrometric analysis. The accumulation of APPL appeared during the process of HMW lignin degradation; however, when the concentration of APPL became high, as shown in Fig. 5, the degradation of APPL into aromatic compounds occurred, as shown in Fig. 6. The highest rate of increase in aromatics was seen in the period in which reduction of APPL concentration was also occurred. This suggests that HMW lignin is depolymerized to APPL, then APPL is degraded into aromatic compounds, randomly by side chain reactions and demethylation, and finally, lignin monomer aromatic compounds are decomposed into inorganic carbon.

4. Conclusion

This study confirmed that HMW lignin, analyzed as acid insoluble lignin in this study, was degraded under sulfate reducing conditions. The degradation rate was not high, with a maximum of 3.49 mg/l/day as measured by Klason lignin analysis. However,

polymeric soluble lignin, APPL, was isolated and appeared to have a chemical structure that originated from the starting lignin and this accumulated during the period that Klason lignin quantification indicated that HMW lignin was declining. Other evidence of lignin degradation, in the form of lignin degradation by-products, was the detection of aromatic compounds. The concentration of aromatics increased as APPL content decreased, indicating that APPL might be converted to lower molecular weight lignin monomers. From these results, it can be concluded that the analysis of APPL and aromatic compounds is an effective parameter for determining the degradation of HMW lignin under sulfate reducing conditions.

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Table 1 Principal pyrolysis products of APPL and area ratio

name	structure	m/z	RT	97th day area (%)	156th day area (%)
Toluene		92	1.7	15.41	15.25
3-Methylpyrrole		81	1.78	3.36	4.39
Styrene		104	1.9	1.99	1.84
Phenol	H	94	2.12	5.34	5.99
Benzene, 2-propenyl-		118	2.39		0.88
Phenol, 2-methyl-	H	108	2.49	0.93	1.18
Phenol, 4-methyl-	H	108	2.6	8.66	9.31
Phenol, 2-methoxy-	G	124	2.76	2.76	2.05
Benzeneacetonitrile		117	2.09	0.94	1.21
3-Ethylphenol	H	122	3.46	2.17	2.35
Phenol, 2-methoxy-4-methyl-	G	138	3.86	1.79	1.33
p-vinyl phenol	H	120	4.21	1.44	1.54
Benzofuran, 2,3-dihydro-		120	4.24		
1,2-Benzenediol, 3-methoxy-	G	140	5.09	3.71	1.8
Phenol, 4-ethyl-2-methoxy-	G	152	5.46	0.77	0.47
Indole		117	5.74	3.12	3.74
2-Methoxy-4-vinylphenol	G	150	6.18	1.62	1.1
Phenol, 2,6-dimethoxy-	S	154	6.91	6.25	4.16
1H-Indole, 6-methyl-		131	7.53	1.64	1.92
3-Hydroxy-4-methoxybenzoic acid	G	168	8.64	4.91	3.31
4-Hydroxyphenylacetic acid nitrile	H	133	9.14	0.65	0.78
2,3,5-trimethoxytoluene	S	182	9.89	1.03	0.7
1,4-Dihydrophenanthrene	H	180	10.44		1.4
Cinnamic acid, 4-hydroxy-3-methoxy-	G	194	10.97	0.54	
Phenol, 2,6-dimethoxy-4-(2-propenyl)-	S	194	12.19	1.68	1.04
Ethanone, 1-(4-hydroxy-3,5-dimethoxyphenyl)-	S	196	12.55	0.78	
9H-Pyrido[3,4-b]indole		168	15.09	1.36	

H: hydroxyphenyl type, G: guaiacyl type, S: syringyl type

Table 2 The detected aromatic compounds and chemical structure

	name	m/z	RT	major ion	target ion	structure
1	benzoic acid	179	7.8	77,105,135,179,194	179,105	
2	2,3-dimethlyl phenol	105	8.0	75,91,105,149,163,179,194	105,179	H
3	methlyl mandelate	79	8.8	79,89,107,166	79,107	
4	phenylacetate	73	9.1	73,91,105,164,193,196	73,193	
5	catechol	73	9.6	73,91,151,207,254	73, 254	H
6	p-hydroxybenzaldehyde	179	10.9	75,95,151,179,194	179,194	H
7	hydrocinnamic acid	104	12.0	75,91,104,207,222	104,207	
8	phenoxyacetic acid	73	12.0	73,91,135,165,181,224	73,224	
9	5-methyl resocinol	253	12.9	73,105,119,133, 253,268	253,268	H
10	veratraldehyde	166	I.S	77,95,151,166	166,95	I.S
11	2-methoxyphenylacetic acid	73	13.9	73,91,179,223,237	73,179	G
12	salycylic acid	73	14.5	73,91,135,149,209,267	73,267	H
13	vanillin	194	15.0	73,89,194,209,224	194,224	G
14	trans-cinnamic acid	205	15.2	77,103,131,145,161	205,220	
15	m-hydroxybenzoic acid	267	15.8	73,91,193,223,267,282	267,282	H
16	m-hydroxyphenylacetic acid	73	17.2	73,147,164,281,296	73,296	H
17	p-hydroxybenzoic acid	267	17.2	73,126,193,223,267	267,223	H
18	p-hydroxyphenylacetic acid	73	17.5	73,164,179,252,281	73,296	H
19	3-(2-hydroxyphenyl)propionic acid	147	18.6	73,147,177,192,253	147,192	H
20	phthalate	147	18.9	73,247,221,295	147,295	
21	4-methoxymandelic acid	209	19.0	73,135,147,209,283	147,209	G
22	3,5-dimethoxyphenylacetic acid	73	19.6	73,89,209,253,268	73,268	S
23	p-hydroxyhydrocinnamic acid	179	20.3	73,179,192,310,379	179,192	H
24	vanillic acid	312	20.5	73,89,126,223,253,267,282,297,312	297,312	G
25	protocatechuic acid	193	21.8	73,193,311,355,370	193,370	H
26	syringic acid	73	23.4	73,89,141,253,297,312,327,342	73,327	S
27	p-coumaric acid	73	24.1	73,219,249,293,308	73,293	H
28	caffeic acid	219	28.1	73,191,219,396	219,396	H

H: hydroxyphenyl type, G: guaiacyl type, S: syringyl type

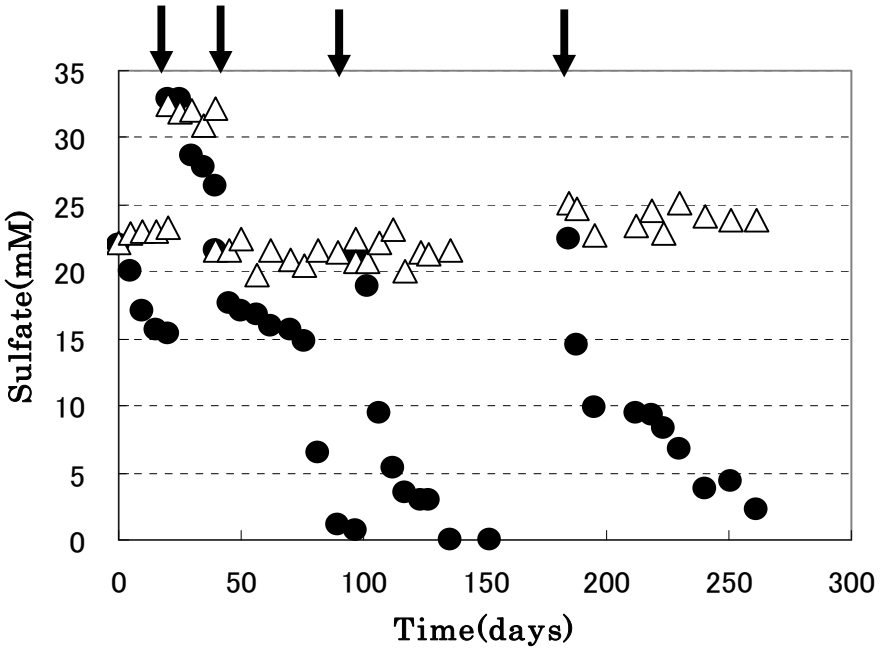

Fig.1. Variation of sulfate concentration as a function of experimental time.
(● : Sulfate reducing condition △ : Molybdate inhibition)

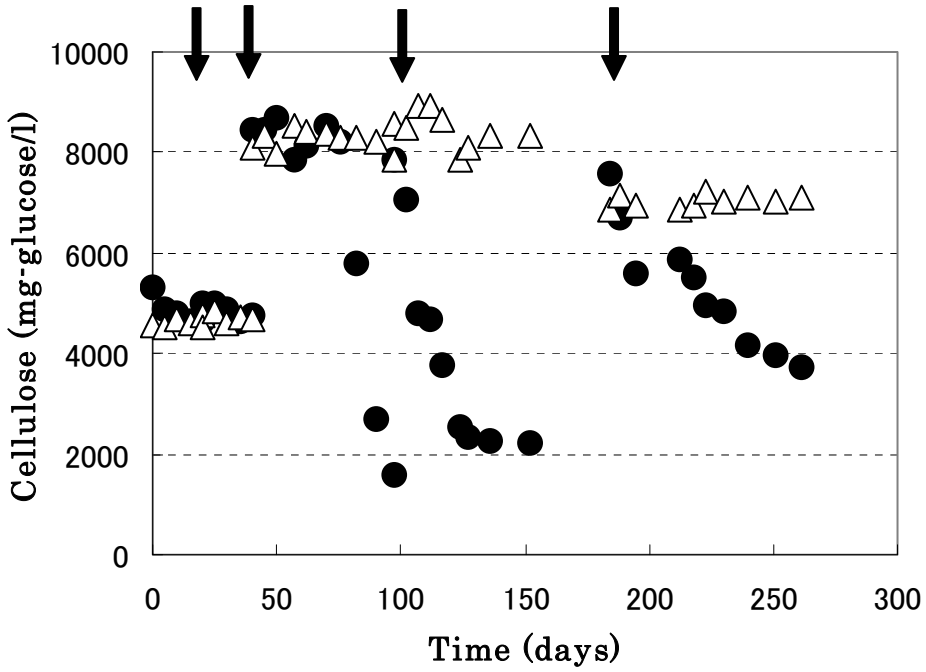

Fig.2. Variation of cellulose concentration as a function of experimental time.
(●：Sulfate reducing conditions △：Molybdate inhibition)

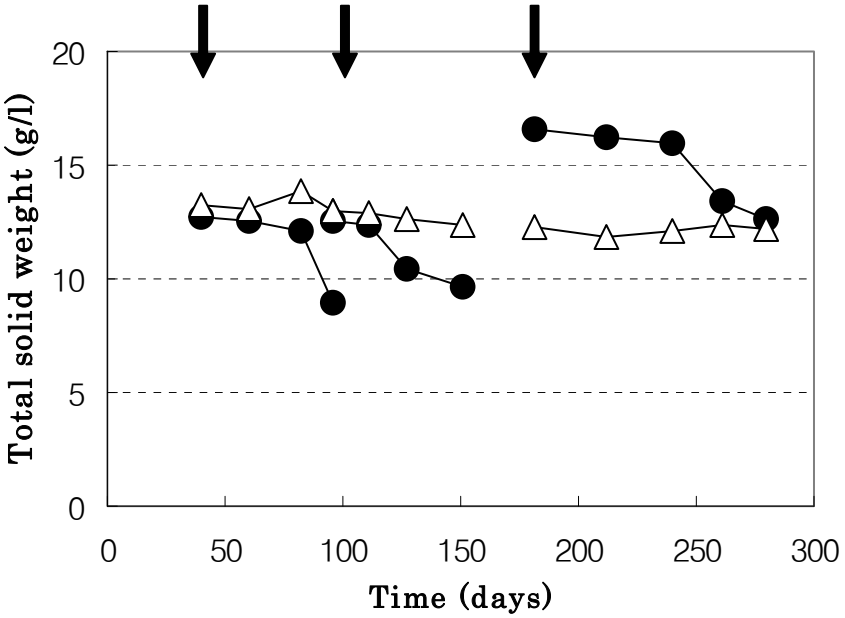

Fig.3. Variation of total solid concentration as a function of experimental time.
(● : Sulfate reducing condition △ : Molybdate inhibition)

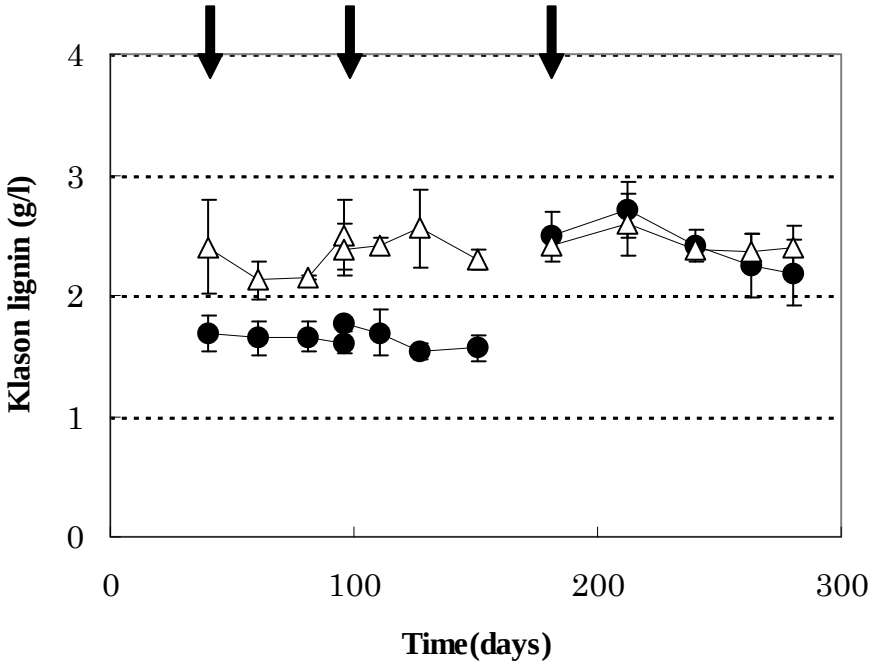

Fig.4. Variation of Klason lignin as a function of incubation time.
(● : Sulfate reducing condition △ : Molybdate inhibition)

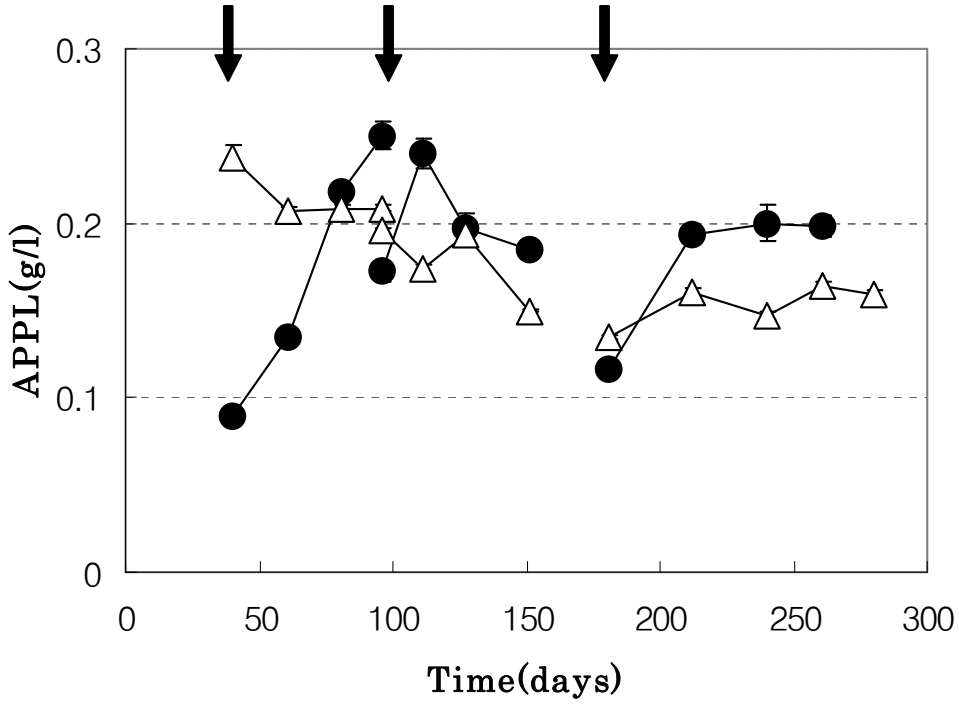

Fig. 5. Variation of APPL concentration as a function of incubation time.
(● : Sulfate reducing condition △ : Molybdate inhibition)

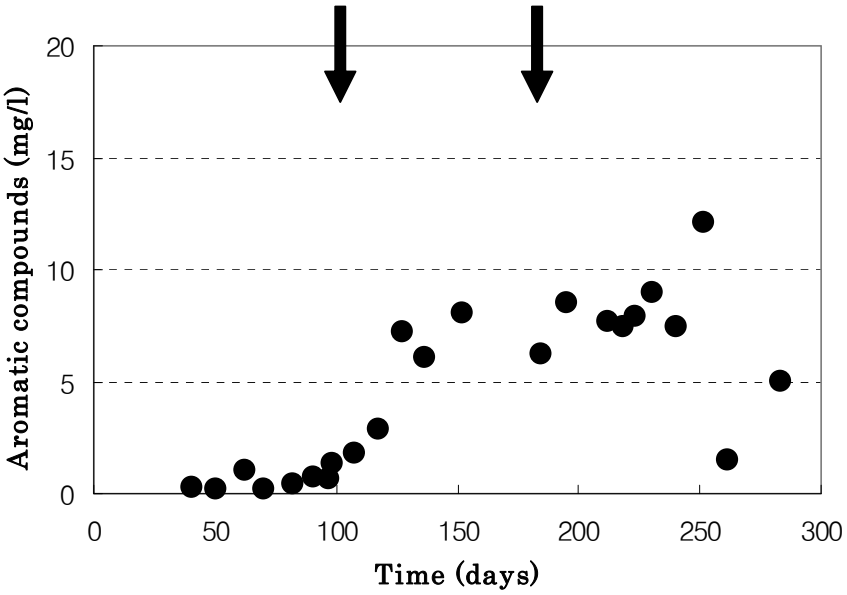

Fig. 6. Variation of total aromatic compounds concentration as a function of time.