Degradation of lignin and lignin model compound under sulfate reducing condition

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Abstract The release of depolymerization products of lignin during the degradation of lignocellulsic material under sulfate reducing condition was investigated. In addition, we investigated the fate of the most common (β-O-4) link present in lignin under sulfate reducing condition, using a lignin model compound. The method of investigation was based on the selective inhibition of microbial uptake of released aromatic phenolic compounds, depolymerization product of lignin, by toluene. Eight different aromatic phenolic compounds were identified. Until day 17 only 3 phenolic compounds were regularly detected, thereafter 7 aromatic phenolic compounds could be regularly identified. The accumulation of identified phenolic acid was not linear with time. The lignin model compound was completely degraded within 13 days when either Avicel cellulose or newspaper was present as alternate source of carbon. On the other hand when lignin model compound was present as the sole source of carbon, it took more than 22 days for its complete degradation. But in either case complete degradation of lignin model compound was observed. Four degradation by-products of lignin model compound were identified, but the two most significant compounds identified were vanillic acid and 3-methoxy-4-hydroxy benzene propionic acid. The GC/MS analysis of the degradation by products of lignin model compound indicated that β-O-4 link was cleaved under sulfate reducing condition and the presence of additional carbon source enhanced this process.

Keywords Lignocellulose; lignin; lignin model compound; sulfate reducing condition

Introduction

Lignin is the second most abundant carbon source in the world, after cellulose. Lignin is a natural refractory material found in significant quantities in both domestic and agricultural wastes. It is a complex three-dimensional aromatic polymer consisting of basic phenyl-propane building blocks held together by irregular carbon-carbon and diaryl-ether linkages (Healy and Young, 1979). The most common linkage between the blocks is an arylglycerol-β-aryl ether or β-O-4 link (Adler, 1977).

The degradation of lignin has been thoroughly studied under aerobic condition. Lignin degradation process, best demonstrated by white rot basidiomycetes or fungi, *Phanerochate chrysoporium*, is thought to be an oxidative non specific process (Kirk and Farrell, 1987). In anaerobic condition lignin is generally thought to be refractory. Although methanogenic degradation of lignin derivatives has been reported by many investigators (Balba and Evans, 1977, Grbic-Galic and Young, 1985), to our knowledge only Benner *et al.* (1984) reported that lignin component of intact softwood and hardwood is partially degraded to gaseous end product under anaerobic condition. The result of one study, in which no degradation of synthetic lignin was observed in a variety of anoxic sediments and soils (Hachett *et al.*, 1977) has led to the general belief that the lignin component of lignocellulose is inert in an anaerobic environment (Zeikus, 1981).

In the past, many different lignin preparations such as kraft lignin, isotopically labelled lignin, milled wood lignin, synthetic lignin (DHP) and lignin model compounds have been used to study lignin biodegradation. Dimeric model compounds that represent the principal

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substructures of lignin have been used successfully to characterize lignin degradation. Dimeric models played a large role in the discovery of fungal lignin degradation enzymes which cleaves lignin between C_α and C_β and they also have been used to assess lignin degrading enzyme activity *in situ* in fungus-colonized wood (Hammel *et al.*, 1993).

Despite recent progress made in understanding the aerobic mechanism of lignocellulose catabolism, relatively little attention has been given to its microbial degradation under anaerobic conditions (Colberg, 1988), especially under sulfate reducing condition. Aromatic monomers (phenolic compounds), the basic building blocks of the lignin polymer, are released during its degradation in aerobic environments. In this study, we investigated the release of lignin depolymerization products (phenolic compounds) during the degradation of lignocellulosic material under sulfate reducing condition. The method of investigation was based on the selective inhibition of microbial uptake of released phenolic acids by toluene. The uptake of released phenolic acids depends on a concentration gradient over the semipermeable membrane, which collapses in the presence of toluene. The accumulation of depolymerization products, i.e., phenolic compounds were followed over time by gas chromatography (GC) and were identified by GC coupled with mass spectrometry (GC/MS). To investigate the potential for degradation of major inter monomeric linkage in lignin, i.e., β-O-4 link, a lignin model compound with β-O-4 link was synthesized and used in biodegradation study under sulfate reducing condition.

Materials and methods

Source of microorganism. The leachate from a simulated landfill column reactor was used as seeding inoculum. The reactor was operated under sulfate reducing condition for a period of over three years. The reactors were initially loaded with newspaper and sawdust, and anaerobic sludge was added to seed the reactor with anaerobic microorganisms. Sulfate was added as and when required to the reactor to maintain the sulfate concentration of 1 g/L in the leachate. The objective of the experiments with simulated landfill column reactor was to control methane emission and are described in greater detail elsewhere (Pareek *et al.*, 1998a).

Experimental condition. The serum bottle reactor (SBR) test outlined by Gutpa *et al.* (1996) was used with a few modifications to study the initial degradation of newspaper under sulfate reducing condition. A precisely weighed amount of dry sample was added to 100 mL serum vials along with 80 mL of nutrient and bacterial seed solution mixture. The mixture contained 90% by volume bacterial culture obtained as the leachate from a simulated landfill column reactor. All media preparation and organism transfer was conducted under oxygen free nitrogen gas. The nutrient medium used was modified Postgate medium C with the following composition (g/L) KH_2PO_4, 0.5; NH_4Cl, 10; $CaCl_2$, 0.06; $MgSO_4 \cdot 7H_2O$, 0.06; $FeSO_4 . 7H_2O$, 0.007 and trace elements B, Co, Cu, Mn and Zn 0.05 mg/L (each) (Reis *et al.*, 1992). 1.5 g of newspaper (dry weight) cut into strips 1×5 cm was added to each vial along with a mixture of bacterial inoculum and nutrient solution, while control vials contained newspaper and distilled water. The initial sulfate concentration in the vial was adjusted to 1 g/l (approximately), the sulfate concentration was monitored over the six-week incubation period and it was added as and when required to maintain a sulfate concentration of 500 mg/L (approximately). All the vials were sealed with butyl rubber stoppers and aluminium crimps and incubated anaerobically at 37±1°C in a reciprocating shaker at constant speed.

A new approach was used to measure the initial decomposition rates of lignin in newspaper under sulfate reducing condition. The method is based on the selective inhibition of microbial hydrolysis product uptake by toluene without effecting the extracellular hydrolysis of lignocellulosic materials (Boschker *et al.*, 1995). The vials were incubated

for a period of six weeks (approximately) and after a time interval of 7 to 10 days, toluene (3% v/v) was injected into vials. After the addition of toluene the vials were incubated for another 6 hours. The accumulation of hydrolysis products of lignin were followed over the 6 hours of incubation period after the addition of toluene.

Phenolic acid analysis. Liquid samples from the vials were first centrifuged at 20,000 × *g* for 15 min. The supernatants were acidified with HCl, after filtration through 0.45 µm membrane filters. The acidified samples were extracted by anhydrous diethyl ether, the extraction procedure was repeated 5 times. The samples were dried on a rotary evaporator and transferred to 1 mL vial using diethyl ether. The samples were dried and derivatized with 100 µL *N, O*-Bis-trimethylsilyl trifluoroacetamide (BSTFA) at 70°C for 20 min to allow complete formation of trimethyl silyl derivatives. One microlitre of the derivatized sample was injected into GC. The GC analysis was performed on a Shimadzu GC-15A equipped with a female ionization detector (FID) (Column: Shimadzu CBP1 fused silica capillary column coated with methylsilicon; column length: 25 m; column internal diameter 0.25 mm; carrier gas: He at 40 mL/min; column temp: Constant at 200°C; Injection temp: 210°C; Detection temp. 230°C; Split mode: 1:53).

Identification of phenolic acids. The derivatized sample used for the phenolic acid analysis was diluted 10 times and then 1 µL of the diluted derivatized sample was injected in GC for the identification of phenolic acid. The identification of phenolic acid was performed on a GC coupled with mass spectrometry GC/MS Shimadzu QP-2000 (Column: Shimadzu CBP1 fused silica capillary column coated with methylsilicon; column length: 25 m; column internal diameter 0.25 mm; carrier gas: He at 40 mL/min; column temp.: 120 to 210°C at 2°C/min; Injection temp.: 210°C; detection temp.: 230°C).

Sulfate analysis. Sulfate was analyzed using ion chromatography Dionex QIC Analyzer (Column: Dionex Ionpac, AS4A 4 mm; carrier gas: Nitrogen 5 kg/cm^2; Eluent: 0.25 NH_2SO_4, 1.8 mM Na_2CO_3, 1.8 mM $NaHCO_3$).

Lignin model compound. The lignin dimeric model was synthesized according to the method described by Hosoya *et al.* (1980) and is shown in Figure 1. The purity of the synthesized lignin model compound was checked by C^{13} NMR and was found to be satisfactory as compared to the published results. Vial batch experiments as described previously were used to study the degradation of the lignin model compound. The effect of relatively easily biodegradable substrate (Avicel cellulose and newspaper) on the degradation of lignin model compound was also investigated.

The lignin model compound concentration during the biodegradation study was measured by high performance liquid chromatography by using standard calibration curve of the standard lignin model compound. Samples (5 µL) were injected into a Jasco system equipped with a C18 column, and elution condition as described by Caramelo *et al.* (1999) were used. The biodegradation by-products of lignin model compound were identified by GC/MS Shimadzu QP-2000 (Column: Shimadzu DB5 fused silica capillary column coated with methylsilicon; column length: 25 m; column internal diameter 0.25 mm; carrier gas: He at 40 mL/min; column temp.: 140 to 210°C at 20°C/min; injection temp.: 210°C; detection temp: 230°C). Before the GC/MS analysis, liquid samples (1 mL) were first centrifuged at 20,000 × *g* for 15 min. The supernatants were acidified with HCl, after filtration through 0.45 µm membrane filters. The acidified samples were extracted by anhydrous diethyl ether, the extraction procedure was repeated 5 times. The samples were dried and derivatized with 20 µl BSTFA at 70°C for 20 min.

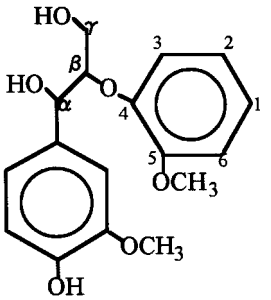

Figure 1 Lignin model compound with β-O-4 link

Results and discussion

Accumulation of aromatic phenolic compounds during the degradation of lignocellulose under sulfate reducing conditions

Lignin is a phenyl propanoid structural polymer, thus phenolic aromatic compounds are the most probable depolymerization products. Based on this hypothesis we analyzed the vial culture for aromatic phenolic compound accumulation after the injection of 3% toluene (v/v). The unit used to express the accumulation of phenolic acids was micro mole phenolic acid accumulation per gram dry weight of the newspaper used. The gas chromatograph obtained had many large and small peaks making identification of the peaks difficult on the basis of retention time. The samples were further analyzed by a GC/MS and a library search was conducted.

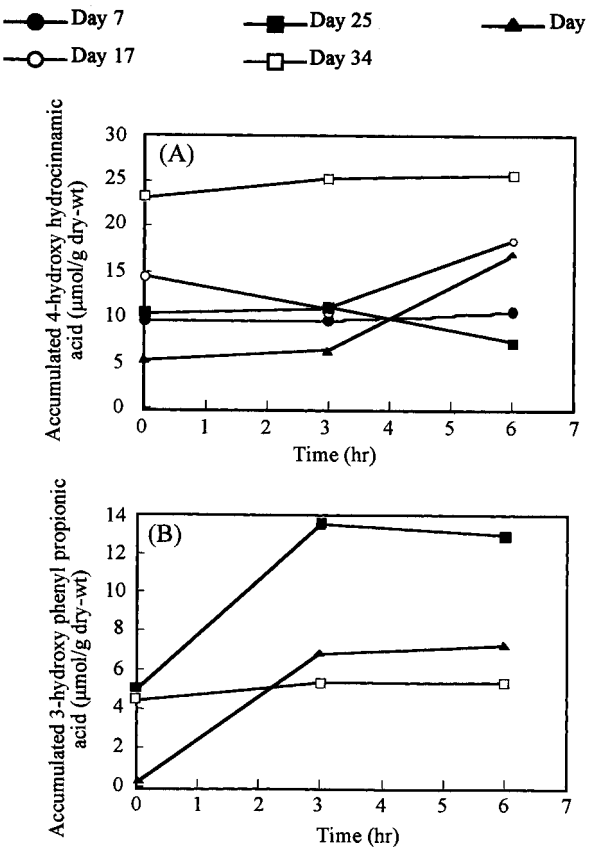

Figure 2 4-Hydroxy hydrocinnamic acid (A) and 3-Hydroxy phenyl propionic acid (B) accumulation

Only those peaks with high reliance (%) were considered for further investigation. The corresponding standards of the phenolic aromatic compounds with high reliance were bought and analyzed on GC and GC/MS. Eight phenolic aromatic compounds were identified: 4-hydroxy benzoic acid, 4-hydroxy phenyl acetic acid, 4-hydroxy hydrocinnamic acid, 3-hydroxy benzoic acid, 2-hydroxy phenyl acetic acid, 3-hydroxy phenyl propionic acid, 3-methoxy phenyl acetic acid and vanillic acid. The compounds were identified on the basis of retention time and the fragmentation pattern of the purchased standards. After the addition of toluene an accumulation of the aromatic phenolic compounds was observed but the accumulation was not linear with time (Figure 2). In a previous study, a linear accumulation of sugars, hydrolysis products of cellulose and hemicellulose, were observed during the degradation of lignocellulose (Pareek *et al.*, 1998b). The accumulation of phenolic compounds, like the accumulation of sugars, was expected to be linear with time, but that was not the case. To investigate further as to why the accumulation was not linear with time, we studied the recovery of two aromatic phenolic compounds in presence of 3% toluene. Para hydroxy benzaldehyde and 4-hydroxy phenol acetic acid (80 mg each) was dissolved in distilled water and introduced to vials (80 mL). The vials were sealed and 3% toluene (v/v) was injected into the vials. The aromatic phenolic compounds were extracted and analyzed after 0 hr, 3 hr and 6 hr of incubation with toleuene (Figure 3). As can be seen from Figure 3, the amount of the two aromatic phenolic compounds recovered after incubating with 3% toluene decreased with time. The non linear accumulation of the aromatic phenolic compounds may be due to the reason explained above, i.e., probably the release of the aromatic phenolic compounds was linear with time but reduced recovery of aromatic phenolic compound in the presence of toluene lead to non linear accumulation of depolymerization product of lignin.

In Table 1 the maximum concentrations of aromatic compounds is observed, after the addition of toluene is presented. Vanillic acid was only identified and detected on day 7. Until day 17 only three aromatic compounds could be regularly identified, after which, i.e., day 25 onwards, seven different aromatic compounds were observed to accumulate as depolymerization products. The increase in the number of identifiable aromatic phenolic compounds' accumulation as depolymerization product is indicative of the fact that the lignin degradation/depolymerization increased with time.

Colberg and Young (1985) have reported the accumulation of aromatic intermediates when the methanogenic culture was inhibited by BESA. Although the accumulated aromatic intermediates observed in this study and that of Colberg and Young were similar,

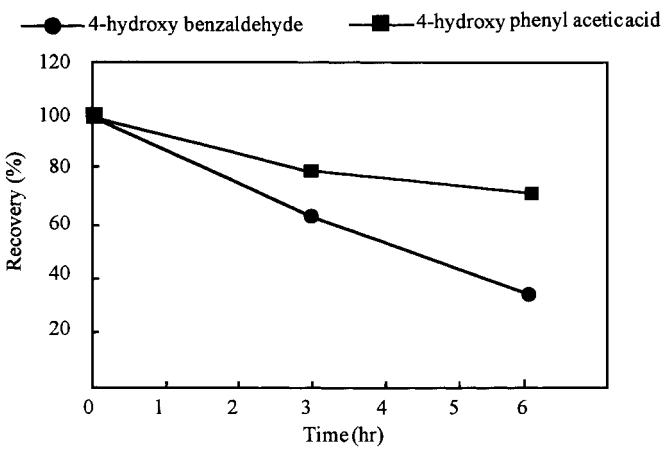

Figure 3 Effect of toluene on the recovery of phenolic compounds

Table 1 Maximum concentration of aromatic compounds observed during the degradation of lignocellulose under sulfate reducing condition

Aromatic phenolic compound	Day 7	Day 17	Day 25	Day 34	Day 43
4-Hydroxy hydrocinnamic acid	10.63	18.43	11.09	25.23	16.91
2-Hydroxy phenyl acetic acid	9.20	9.71	13.22	22.44	16.72
4-Hydroxy benzoic acid	7.72	17.89	50.67	73.38	ND
3-Methoxy phenyl acetic acid	4.31	ND	ND	5.51	2.96
4-Hydroxy phenyl acetic acid	ND	ND	57.06	73.34	46.44
3-Hydroxy phenyl acetic acid	ND	ND	13.53	5.34	7.25
3-Hydroxy benzoic acid	ND	ND	13.85	68.02	6.63
Vanillic acid	1.15	ND	ND	ND	ND

Unit: mg/g dry-wt of lignocellulosic material
ND: not detected

the lignocellulosic substrate used as the carbon source in the two studies was different. In this study newspaper was used as lignocellulosic substrate. Newspaper lignin is considered to retain its original structural and chemical properties. Colberg and Young used lignin oligolignol mixtures (average molecular weight 600) as the sole source of carbon.

Degradation of lignin model compound under sulfate reducing condition

We investigated the degradation of lignin model compound with β-O-4 link under sulfate reducing condition. In addition, the fate of lignin model compound in the presence of additional carbon source was also investigated. Three different vial groups with either lignin model compound as the sole carbon source or lignin model compound and Avicel cellulose or lignin model compound and newspaper as carbon source were investigated and the result is presented in Figure 4.

Lignin model compound was completely degraded in both the vial groups in 13 days, with either Avicel cellulose or newspaper as additional carbon source. Although complete degradation of lignin model compound was observed in vial group with lignin model compound as the sole source of carbon, it took more than 22 days for its complete degradation. The presence of an additional carbon source might have increased the microbial growth/activity, enhancing the degradation of lignin model compound.

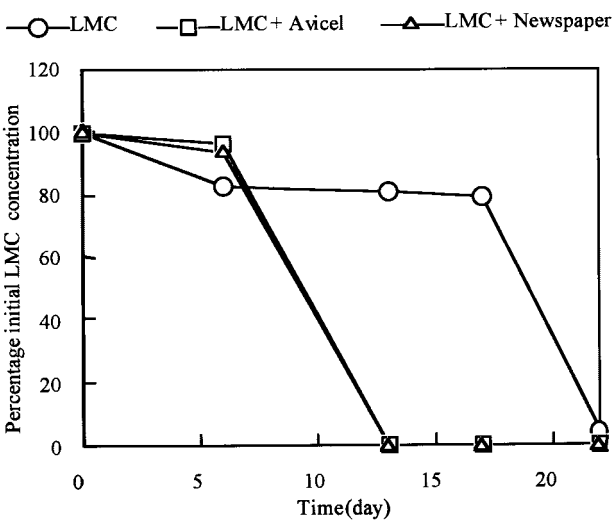

Figure 4 Degradation of lignin model compound (LMC) with (□ & △) and without (O) additional source of carbon

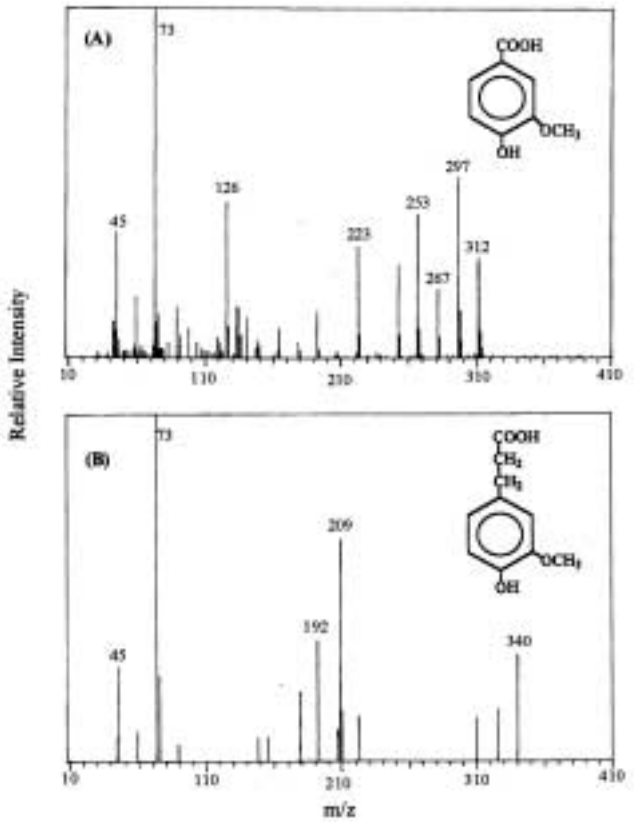

Figure 5 Degradation by-products of lignin model compound under sulfate reducing condition (A) vanillic acid (B) 3-methoxy-4-hydroxy benzene propionic acid

The formation of degradation by-products of lignin model compound was investigated by GC/MS analysis.

Four aromatic compounds were identified namely: vanillic acid, 3-methoxy-4-hydroxy benzene propionic acid, protocathechic acid and 3,4-hydroxy hydrocinnamic acid. The mass spectra and structural formulae of vanillic acid and 3-methoxy, 4-hydroxy benzene propionic acid are shown in Figure 5. Detection of aromatic intermediates particularly vanillic acid and 3-methoxy-4-hydroxy benzene propionic acid is suggestive of β-O-4 link cleavage.

Conclusion

Toluene (3%) was used to inhibit the microbial uptake of depolymerized products of lignin during the degradation of lignocellulosic material, under sulfate reducing condition. Eight aromatic phenolic compounds were identified as depolymerization products of lignin. Until day 17 only three aromatic phenolic compounds could be detected; thereafter 7 aromatic phenolic compounds were regularly detected; this may be indicative of the fact that lignin depolymerization increased with time. Non-linear accumulation of the released phenolic compounds could be attributed to the reduced recovery of phenolic acid in the presence of toluene. Lignin model compound with β-O-4 link was degraded under sulfate reducing condition and the degradation process was enhanced in the presence of an additional source of carbon. The results presented here provide evidence that lignin degradation occurs in anaerobic sulfate reducing condition. Clear evidence of β-O-4 link (the most common link present in lignin) cleavage could be inferred from the realties provided here.

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