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메탄생성균에 대한 Wood resin 구성성분의 독성에 관한 연구

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The Methanogenic Toxicity of Wood Resin Constituents

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Abstract

펄프폐수 내에 존재하는 대표적인 수지 구성성분의 일종인 wood resin 화합물이 메탄생성균에 미치는 독성을 평가하기 위한 회분식 독성실험을 수행하였다. 수지는 극성용매로 추출 가능한 몇몇 wood 구성성분의 혼합물로서 수지의 주요 구성성분은 긴 사슬 휘발성유기산, terpenes, resin acids, 리그난과 극성 페놀류들이다. 메탄생성균의 독성실험은 30℃에서 표준회분식 실험방법을 채택하였고 식종물질로서는 입상슬러지를 사용하였다. 극성페놀의 한 종류인 4-hydroxystilbene가 가장 높은 독성을 나타내었으며, 50%저해를 일으키는 농도는 20mg/ℓ이었다. Resin acid와 휘발성 terpene 역시 메탄생성균에 독성을 나타내었으며, 50%독성을 일으키는 농도는 43 ~ 330mg/ℓ이었다. 반면에 triterpenes은 1,000 to 1,300 mg/ℓ의 상대적으로 높은 농도에서도 메탄생성균에 독성을 일으키지 않았다. 따라서 wood resin의 구성성분이 몇몇 펄프폐수의 혐기성 처리에 있어서 독성을 일으키는 주요물질이었다.

I. INTRODUCTION

Resin is by definition the mixture of wood components extractable with apolar solvents. Major resin constituents are long chain fatty acid(LCFA), terpenes, resin acids, lignans, and apolar phenols. The averaged composition of the wood resin from softwood and hardwood is shown in Table 1.

The resin content in wood usually ranges from 3 to 5% of the wood dry weight, but it can be much higher in certain tropical and subtropical woods. Generally, softwoods are richer in resin than hardwoods. Softwoods extractives also differ in composition with respect to those of hardwoods. The resin of coniferous trees contains all classes of terpenes from monoterpenes to tri- and tetraterpenes, whereas in deciduous wood mainly higher terpenes are present[1].

The pulp and paper industry is a major source contributing to the discharge of wood extractives in the environment. Examples of other waste streams containing wood extractives or related compounds include citrus processing effluents and chemical manufacture wastewaters. In paper mills wood is subjected to pulping and bleaching operations in order to purify the cellulose. As a result of these treatments, hemicellulose, lignin and extractives are separated to different extents from the cellulose and extracted into the process waters. The resin content of various forest industry wastewaters reported in the literature is shown in Table 2[1,2].

Table 1. Literature averaged composition of wood and bark resin

Components	Hardwood	Softwood	
	Wood(% of total resin)	Wood(% of total resin)	Bark(% of total resin)
Resin acids	T	30	7
Volatile terpenes	NM	2	NM
Triterpenols	36	T	3
LCFA	48	33	13
Apolar phenols	NM	25	NM
Lignins	NM	10	NM

T : trace, NM : not measured, LCFA : long chain fatty acids

Pulp and paper mill wastewaters often exert inhibitory effects on microorganisms that can disturb biological treatment systems. They are derived either from extracted wood components, from chemicals added or products formed in the industrial processes. The methanogenic toxicity of inorganic wastewater components, such as sulfate, sulfite, hydrogen peroxide and other chemicals added during pulping and bleaching processes has been extensively investigated[2].

Table 2. Resin content in paper mill wastewaters

Wastewaters	Extractives (mg/ℓ)	LCFA [a](mg/ℓ)	Resin acids(mg/ℓ)
Debarking	51	2	7
Ground pulping	105	NR [d]	NR
Building board	69-154	NR	NR
Corrugated medium	228	NR	NR
TMP [b]	90	NR	NR
TMP	394	NR	NR
TMP	NR	43	380
TMP	NR	46	245
TMP	NR	37-53	63-143
TMP	NR	NR	48-370
TMP	NR	NR	15-62
CTMP [c]	1,000	NR	NR
Acid bleaching of KP [e]	46	8	0.2
Alkaline bleaching of KP	185	9	0.4
H_2O_2 bleaching of ground pulping	100	NR	NR

[a] *Long chain fatty acid*
[b] *Thermomechanical pulping*
[c] *Chemi-thermomechanical pulping*
[d] *Not reported*
[e] *Kraft pulping*

In contrast, very limited information describing the fate of natural organic wood components in anaerobic systems is available.

The microbial toxicity of wood resin components should be suspected because these components increase the decay resistance in trees. Timber durability has been related to the antifungal activity of resin components[3]. Additionally, the toxicity of resin constituents to aquatic organisms has been extensively investigated and a substantial amount of data is available to indicate that resin containing paper mill wastewaters display acute toxicity to fish and other

Table 3. Literature data on toxicity of wood resin components and related compounds

Compounds	Substrate [b]	50%IC (mg/ℓ)	Reference No.
Long chain fatty acids			
Lauric acid	C_2	525	(15)
Lauric acid	C_2	869	(9)
Caproic acid	C_2	1,027	(9)
Myristic acid	C_2	1,104	(9)
Oleric acid	C_2	1,235	(9)
Linolic acid	H_2/CO_2	897	(19)
Linolic acid	H_2/CO_2	501	(19)
Linolic acid	Pyruvate	278	(20)
Mixture of LCFA	C_4	250 [c]	(17)
Mixture of LCFA	H_2	250 [c]	(17)
Alcohols			
Octyl alcohol	Glucose	500(99%)[d]	(21)
Resin acids			
Abietic acid:oleric acid [a]	C_2	1,178(99%)[d]	(8)
Abietic acid	C_2, C_3, C_4	114	(22)
Volatile terpenes			
P-cymene	C_2	500[c]	(5)
Limonene	C_2	250[c]	(5)
Limonene	C_2	122(79%)[d]	(10)
α-pinene	C_2	122(0%)[d]	(10)
Resin related aromatics			
Eugenol	C_2	250	(5)

[a] *Abietate : oleate ratio on a dry weight basis was 52:48.*
[b] *C_2 = acetic acid, C_3 =propionic acid, C_4 = butyric acid.*
[c] *Approximately 50% inhibitory concentration estimated from reported data.*
[d] *The number in the parenthesis indicates the inhibition observed at the reported cocentration.*

aquatic organisms. Resin acids and other resin constituents have been identified as important fish toxins. Leach and Thakore reported a LC_{50}(96h) for various resin acids and LCFA ranging from 0.3 to 5.0 mg/L with Rainbow trout as a test organism.

The methanogenic toxicity of resin constituents has been studied to a lesser extent. Some toxicity data available in the literature are summarized in Table 3. In this table one can observe that mostly the effects of LCFA on methanogenic mixed cultures have been investigated whereas very little data are available concerning the effects of other resin components in anaerobic environment. The purpose of this study was to evaluate the inhibitory effect of various representative wood resin compounds on the activity of methanogenic bacteria.

II. MATERIALS AND METHODS

1. Biomass

The granular sludge used in these experiments was obtained from a full-scale upflow anaerobic sludge blanket reactor treating distillery wastewater. The sludge was elutriated and stored 4℃ under nitrogen gas. The sludge used was not acclimated to the toxic compounds prior to the toxicity assays.

2. Basal medium

The inorganic macro- and micro-nutrients were supplied to the assay media as five-fold concentrated solution. After dilution the basal medium used in the anaerobic toxicity assay contained; $NaHCO_3$(400mg/ℓ), NH_4Cl(280mg/ℓ), $CaCl_2 \cdot 2H_2O$(10mg/ℓ), K_2HPO_4(250mg/ℓ), $MgSO_4 \cdot 7H_2O$(100mg/ℓ), yeast extract (100mg/ℓ), H_3BO_3(0.05mg/ℓ), $FeCl_2 \cdot 4H_2O$(2mg/ℓ), $ZnCl_2$(0.05mg/ℓ), $MnCl_2 \cdot 24H_2O$(0.05mg/ℓ), $(NH_4)_6MoO_{24} \cdot 4H_2O$(0.05mg/ℓ), $AlCl_3 \cdot H_2O$(0.09mg/ℓ), $CoCl_2 \cdot 6H_2O$ (2mg/ℓ), $NiCl_2 \cdot 6H_2O$(0.05mg/ℓ), $CuCl_2 \cdot 2H_2O$ (0.03mg/ℓ), $Na_2SeO_3 \cdot 5H_2O$(0.1mg/ℓ), EDTA(1mg/ℓ), resazurin(0.2mg/ℓ) and 36% HCl (0.001㎖/ℓ). All chemicals were of analytical grade. The yeast extract was supplied by Gist-Brocades.

3. Analyses

Volatile fatty acid(VFA) were determined with gas chromatograph equipped with Hewlett-Packard FFAP capillary column. The temperature of the column, the injection port and the flame ionization detector were 130, 220 and 240℃, respectively. Nitrogen saturated with formic acid was used as carrier gas at a flow rate of 50㎖/min. COD and volatile suspened solids(VSS) were determined according to Standard Methods[4].

4. Anaerobic toxicity assay

Methanogenic activity measurements were performed in 0.6L glass serum bottle flasks. Distilled water, granular sludge(1.5gVSS/ℓ) and known amounts of toxicant were transferred to the flasks containing 100mL of the concentrated nutrient solution.

The toxicant concentrations supplied were chosen to provide an inhibition of the methanogenic activity ranging from 0 to 100%. Substrate controls were based on assays where no toxicant was added. Subsequently, all serum flasks were supplied with 4 g COD/ℓ of a neutralized VFA solution, containing 100:100:100g acetate:propionate:butyrate per kg, that served as substrate. Finally, distilled water was added to complete a medium volume of 500㎖. The liquid was flushed with nitrogen gas and the flasks were sealed with a butyl rubber septum and a screw cap, and then incubated in a temperature controlled room at 35±2℃. All experiments were mechanically shaken unless otherwise indicated. Methane production was monitored periodically during the 14days that followed with modified Mariotte flask.

These flasks were filled with a 3% NaOH solution which served to remove the carbon dioxide contained in the biogas.

In order to evaluate the residual activity of the sludge after exposure to the toxic compound, on day of 14 all serum bottle were provided with second substrate feeding lacking the toxicant. The supernatants were carefully decanted to avoid losses of

methanogenic sludge and replaced, while maintaining nitrogen gas flushing in the head space, with a nutrient supplemented medium containing 4g VFA-COD/ ℓ. The bottles were reincubated again for 1 to 2 weeks. The specific methanogenic activity, expressed as the amount of methane produced by 1g of sludge VSS per day, was calculated from the slope of the methane production versus time curve and the quantity of VSS. The methanogenic activities for each toxicant concentration were calculated in the time interval corresponding to the maximum control activity. The inhibited activity was expressed as percentage of the control activity, and it is abbreviated as(%ACT).

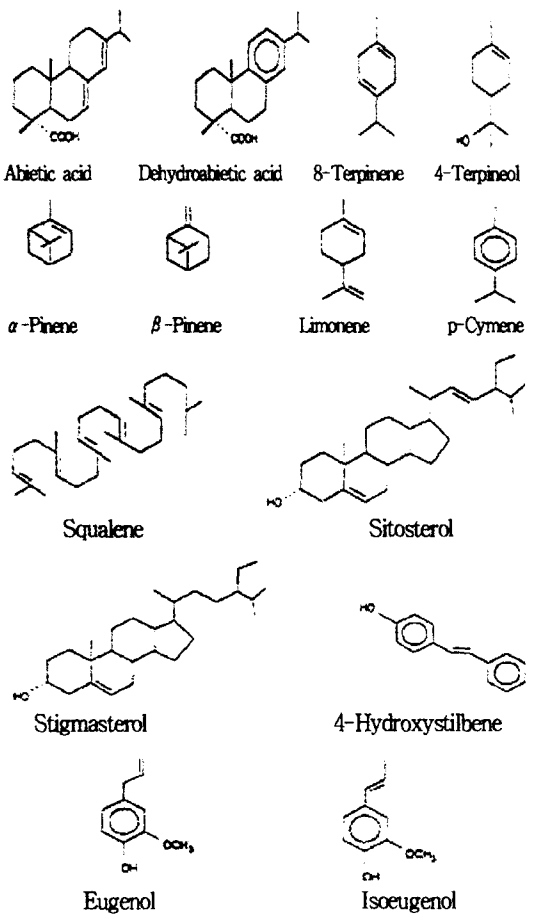

Fig. 1. Chemical structure of the wood resin compounds investigated in this study.

The percentage inhibition(% I) was defined as : % I = 100- % ACT. The compound concentrations that caused 50% and 80% inhibition of the methanogenic activity are referred to as 50% IC and 80% IC, respectively.

Chemical structure of the wood resin compounds investigated in this study illustrated figure 1.

III. RESULTS

The effect of several individual resin constituents on the activity of methangenic bacteria were evaluated in this study. Figure 2 shows an example of the cumulative methane production as a function of time obtained from a typical toxicity assay. The 50% and 80% IC, as determined in the first and second feedings of the assay, are listed in Table 4.

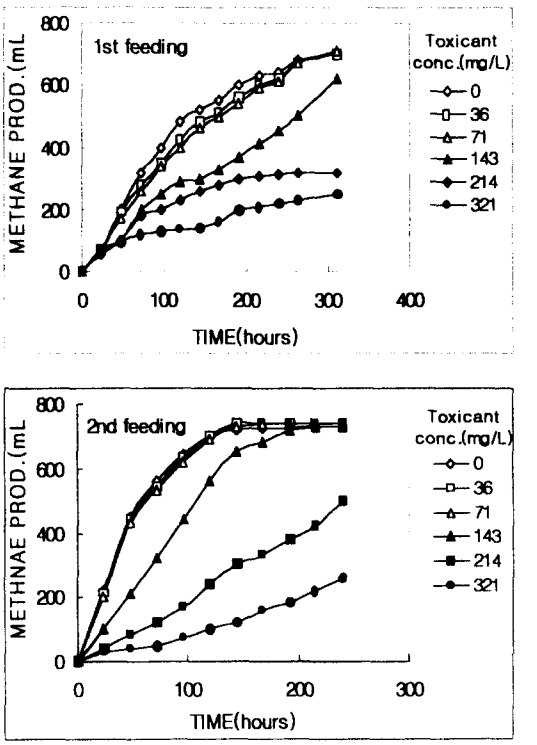

Fig. 2. The cumulative methane production during the methangenic toxicity assay of abietic acid.

Table 4. The effect of he wood resin components investigated on the methanogenic activity

Compounds	First feeding		Second feeding	
	50% IC (mg/ℓ)	80% IC (mg/ℓ)	50% IC (mg/ℓ)	80% IC (mg/ℓ)
Resin acids				
abietic acid [a]	89	139	235	206
dehydroabietic acid[a]	43	105	123	141
Volatile terpenes				
α-pinene	105	230	180	290
β-pinene	110	210	165	390
δ-terpinene	42	67	112	94
4-terpineol	330	505	267	470
p-cymene	110	245	220	310
limone	90	175	150	250
Triterpenols				
β-sitosterol	NT[b]	NT	NT	NT
stigmasterol	NT[b]	NT	NT	NT
Triterpenes squalene	NT[c]	NT	NT	NT
Apolar phenols				
4-hydroxy-stilbene	20	25	42	56
isoeugenol[a]	96	182	235	252
eugenol	274	479	428	569

[a] *Toxicity data obtained in unshaken experiment.*
[b] *The compounds were non-toxic at the highest concentration tested, 1,300mg/ℓ.*
[c] *Non-toxic at the highest concentration tested, 1,000mg/ℓ.*

These toxicity data were obtained by interpolation of the %ACT versus concentration curves determined for each toxicant. Figure 3 illustrates the curves obtained for abietic acid, p-cymene, α-pinene and isoeugenol, respectively. An apolar phenol, 4-hydroxystilbene, was the most toxic compound stduied. The 50% IC and 80% IC of this compound in the first feeding were only 20 and 25mg/ℓ, respectively. Resin acids and monoterpenes had inhibitory concentrations in the same order of magnitude. The 50% IC for abietic acid and dehydroabietic acid were 43 and 89mg/ℓ, respectively. The 50% IC found for various volatile terpenes ranged 45 to 110mg/ℓ. The only monoterpenol tested, 4-terpenol, with 50%IC of 330mg/ℓ, was distinctly less toxic than the homologous terpene lacking the hydroxyl function group. Two polar lignin monomers, eugenol and isoeugenol, were also tested. These compounds had 50%IC of 274 and 96mg/ℓ, respectively. In contrast, the triterpenic conpounds tested did not cause any decrease of the methanogenic activity at the highest concentration tested. These corresponded to 1,300mg/ℓ and 1,000mg/ℓ for the sterols and squalene, respectively.

The residual methanogenic activities determined in the second feeding that followed the 2 week exposure to the toxicants were much lower than that of the control residual activity. The persistance of the inhibition beyond exposure indicates a damaging effect of the resin compounds on the sludge. However, partial recoveries of the methangenic activity were evident since higher 50%IC and 80% IC were observed in the second compared to the first feeding. Spruce crude resin was used as a raw material for evaluating the methanogenic inhibition caused by a natural resin mixture. Crude spruce resin extracts were obtained upon prolonged extractions of 2.5g resin in 1ℓ of water or diluted NaOH solutions. The solubility of resin components depended very much on the pH during extraction. This was indicated by the increasing COD concentrations in the filtered extracts of increasing pH values. Concentrations of 42, 121, 503, 3,153mgCOD/ℓ (1g resin = 2.8gCOD) corrresponded to solutions extracted at the pH values of 7, 9, 11, 12, respectively. The toxicity of the filtered extracts was determined after neutralization with HCl. The results of the toxicity experiments show that alkaline extracted natural wood resin was highly inhibitory to the activity of methanogenic bacteria. as shown in Figure 4.

IV. DISCUSSIONS

In this study we have demonstrated that various wood resin compounds are highly toxic to methane bacteria. The results show that apolar phenols, volatile

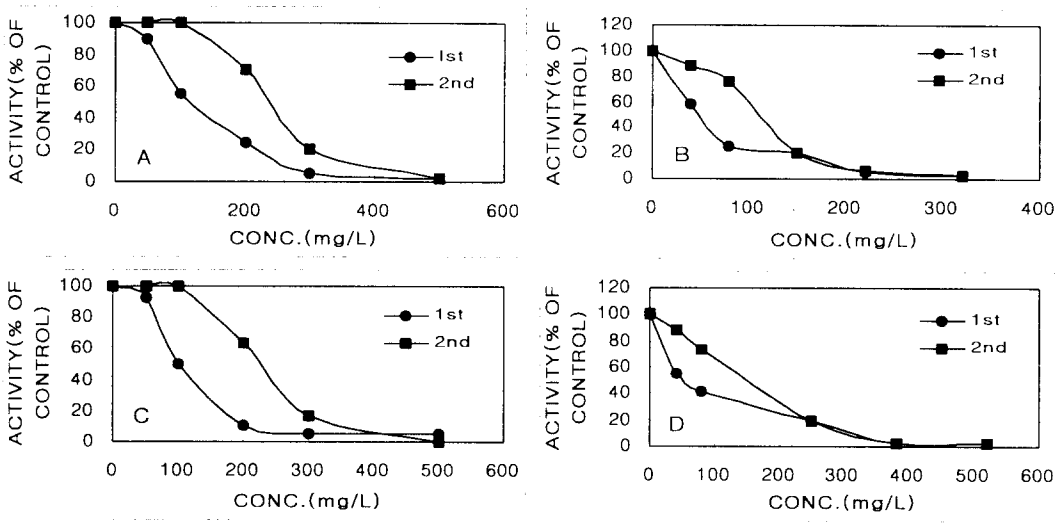

Fig. 3. The methanogenic toxicity of (A) abietic acid, (B) p-cymene, (C) α-pinene and (D) isoeugenol in the first and second VFA fed assay.

terpenes and resin aicds caused a 50% inhibition of the maximum specific methanpgenic activity at concentrations ranging from 20 to 110mg/ℓ. Eugenol and 4-terpineol with 50% inhibitory concentrations of 274mg/ℓ and 330mg/ℓ, respectively, were somewhat less toxic. The nontoxicity of several triterpenes was also established in this study. Compounds corresponding to this group were nontoxic at the highest concentration tested, 1,000 to 1,300mg/ℓ. These observations are supported by previous literature reports where the toxicity of a few individual resin constituents was studied. Our results are in good argument with those of Benjamin et al.[5] who reported the acetoclastic toxicity of eugenol and two monoterpenes, p-cymene and lomonene. The high toxicity of volatile terpenes was reported earlier by McNary et al.[6] in studies concerning the anaerobic digestion of citrus processing wastewaters.

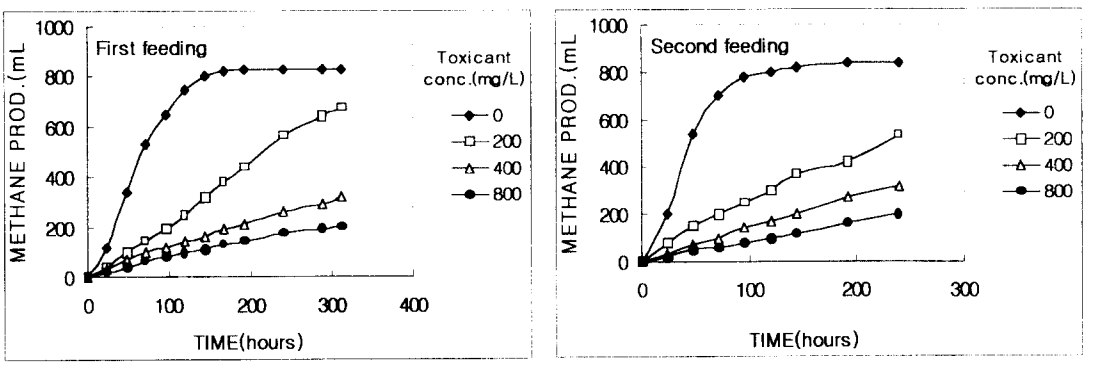

Fig. 4. The cumulative methane production during the methanognic toxicity assay of a crude spruce resin solution extracted at pH 12.

They observed that the presence of orange essential oil, composed mostly of limonene, at concentrations higher than 50mg/ℓ in the wastewater resulted in methanogenic inhibition. Terpenic essential oils extracted from numerous plants as well as various compounds isolated from the oils were also found to be inhibitory to the methanogenic activity of rumen bacteria[7]. Furthermore, Minami et al. have identified several monoterpenes and sesqui-terpenes as the major constituents of the woody oil that inhibited the anaerobic digestion of kraft evaporator condensate. Finally, at least two reports are available indicating that abietic acid, a resin acid, is highly inhibitory to methane bacteria[8].

LCFA are distinctly less toxic to methane bacteria than other resin constituents. The concentrations of various individual LCFA causing a 50% acetoclastic inhibition reported by Koster and Kramenr[9]. The toxicity of wood resin compounds to microorganisms other than methane bacteria is also indicated in the literature. Crane et al observed that cellulose digestion by ruminal bacteria was inhibited by the monoterpene hydrocarbons limonene and α-pinene[10]. The inhibitory effect of the terpenic juniper berry lils on ethanol fermentation has been noted by Veljkovic et al.[11] In other study, various terpenic compounds extracted from commercial species caused complete fungal growth inhibition[12]. Zemak et al. have also described the inhibitory effect of several resinous aromatic compounds on the growth of selected bacteria, yeast and fungi, isoeugenol was found to be the most potent inhibitor of the compounds tested in their experiments, concentrations that completely inhibited the growth of the organisms studied ranged from 50 to 250mg/ℓ[13].

The factors determining the methanogenic toxicity of wood extractives are not yet fully described but the hydrophobic character of these compounds probably plays an important role. Lipid solubility and surface activity have been previously correlated with the antimicrobial actions of lipophilic organic compounds.

The inhibitory effect of LCFA has been attributed to their adsorption on the bacterial cell walls. Destruction of cell membrane, disturbance of cell division by changes in surface tension and non-defined chemical interactions are postulated as possible toxicity mechanism[14].

Structural features of volatile terpenes, resin acids and apolar phenols associated with increasing hydrophobicity include aliphatic side chains on aromatic or alicylic hydrocarbons, the presence of benzene functional groups and a general lack of polar substituents. Likewise, an increase in alkyl substitutions and the presence of benzene groups has been reported to be associated with an increasing toxicity of organic compounds[15]. Triterpenic compounds, though highly hydrophobic, did not cause methanogenic inhibition at relatively high concentrations. A distinct feature of triterpenes compounds as compared to the very toxic volatile terpenes is their higher molecular weight. Mocular weight has been found to be an important factor in determining the toxicity of resinous compounds.

Anderson and Scheffer observed that polymerization of aromatic terpenes was associated with a decrease in toxicity towards fungi. Monomeric aromatic compounds have been found to be more effective inhibitors than their dimeric homologs[16].

Anaerobic biotransformation of organic toxins, that may result in less inhibitory products or cause complete mineralization, also have an important role in determining the methanogenic inhibition of resinous compounds. The toxicity of LCFA is known to be largely affected by the anaerobic biodegradability of these compounds. Low concentrations of LCFA, which are readily biodegraded in anaerobic environment, have a stimulatory effect on the methanogenic activity.

Furthermore, an intermediate recovery of the methanogenic activity has been observed in experiments inhibited by LCFA when the anaerobic degradation of LCFA occurred[17]. As opposed to LCFA, typical resin components like terpenic hydrocarbons and resin acids are believed to be recalcitrant or slowly

biodegradable in anaerobic environments. In agreement with this idea, a poor removal of resin acids has been observed during anaerobic treatment of bleaching wastewaters. Furthermore, Schink suggested in this study on the anaerobic metabolism of unsaturated hydrocarbon, that factors such as branched carbon chains, hydrocarbon rings and lack of polar functional groups, like carboxyl and hydroxyl groups, prevent anaerobic microbial mineralization. In any case, it should be noted that partial degradation of squalene, a triterpenic compound, has been reported[18].

Natural wood resin are composed of a variety of fatty constituents as listed in Table 1. These components are hydrophobic and poorly soluble in water at neutral to acid pH values. In alkaline conditions, resin solubility increased due to the solubilization of acidic compounds, like resin acids and LCFA. A higher solubility crude spruce resin extracted in alkaline solutions as compared to neutral aqueous solutions was observed in our experiments. The toxicity results showed that alkaline aqueous extracts of natural spruce resin inhibited the methanogenic activity of the granular sludge used in this study.

Extracts concentrations causing a 50% inhibition corresponded to 130mgCOD/ℓ. The increased solubility of resin at high pH, indicates that resin toxicity will be more important in forest industry wastewaters generated from pulping or bleaching processes that use alkaline conditions. Additionally, resin toxicity is more likely greater in wastewaters derived from soft- as compared to hardwoods. Coniferous wood resin contains high proportions of resin acids and monoterpenes whereas deciduous wood mainly contains triterpenes, but does not include resin acids not monoterpenes.

V. CONCLUSIONS

This study indicates that wood resin is highly toxic to methane bacteria. Extracts of crude spruce resin resulted in high methanogenic toxicity. The 50% inhibiting concentration was 50 mg/ℓ. The most toxic constituents of wood resin are resin acids, volatile terpenes and apolar phenols, which caused 50% inhibition of the maximum specific methanogenic activity at concentrations ranging 20 to 274mg/ℓ. In contrast, triterpenes were nontoxic at the highest concentrations tested, 1,000 to 1,300mg/ℓ.

These results suggest that the potential toxicity of wastewaters containing wood resin constituents or related compounds should not be neglected when evaluating the feasibility of anaerobic wastewater treatment process.

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