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Inhibitory Effects of Phenolic Monomers on Methanogenesis in Anaerobic Digestion

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Authors' contributions

This work was carried out in collaboration between all authors. Author KK designed the study and performed the experimental work. Author LB performed the statistical analysis. Author PCS wrote the protocol. Author PTM wrote the first draft of the manuscript, managed the analyses of the study. Author KM made the literature searches. All authors read and approved the final manuscript.

Research Article

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ABSTRACT

Aims: The purpose of this study was to evaluate the inhibitory effects of phenolic monomers on methanogenesis in anaerobic digestion and to assess the effect of hydroxyl groups' number of phenolic monomers (aromatic structure) on inhibition of methane production by acetoclastic methanogens (archaea).
Study Design: Anaerobic digestion of pig manure, anaerobic toxicity essay, The effect of the hydroxyl group's number on the methanogenic toxicity as exhibited by monomeric tannins, Correlation of the methanogenic toxicity (IC_{50}) with aromatic compounds hydrophobicity (logPoct), Correlation of the methanogenic toxicity (IC_{50}) with Cresols boiling point (bp).
Place and Duration of Study: Department of Chemistry, University of Kinshasa (DR Congo), between September 2011 and May 2012.
Methodology: The toxicity to acetoclastic methanogenic bacteria was performed with the standard method of serum bottles; digested pig manure was utilized as inoculums and acetate as substrate. The methane gas volume produced was measured by serum bottles liquid displacement systems (Mariotte flask system).
Results: The results of this study indicate that an increase in the number of hydroxyl groups on the aromatic compound was associated with a decrease in the compound's

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toxicity to methanogens (archea). The toxicity of various phenolic monomers are decreasing in the following order: pyrogallol < hydroquinone < resorcinol < phenol < benzene with 3172, 2745, 1725, 1249 and 209 mg/l IC_{50} values respectively. A significant negative linear correlation between the toxicity of phenolic monomers together with the reference compound (benzene) and their hydrophobicity was found. Moreover, a high positive linear correlation has been found between the IC_{50} values of phenolic monomers and their boiling temperatures.

Conclusion: The obtained results indicate that relationships exist between the phenolic monomers structure and their inhibitory effects in methane biosynthesis. The analysis of experimental results suggests that an increase in the number of hydroxyl groups on the aromatic compounds was associated with a decrease in the phenolic monomers toxicity.

Keywords: Phenolic monomers; tannin; methanogenesis; anaerobic digestion; toxicity; methanogens; archea; methane.

1. INTRODUCTION

Tannin is forth most abundant biochemical compounds in terrestrial biomass, following cellulose, hemicellulose and lignin. In rapidly cycling soft tissues such as leaves and needles, however, tannin is often more abundant than lignin [1] and, therefore, an important component of the carbon cycle. Tannin consists of two types, condensed and hydrolysable tannin. The former exists as oligomers and polymers of three-ring flavanols, while the latter is made up of gallic acid or its derivatives often esterified to polyols such as glucose [2] Phenolic monomers are naturally present in the environment as degradation products of tannins [2].

Anaerobic digestion involves the degradation and stabilization of organic materials under anaerobic conditions by microbial organisms and leads to formation of biogas (a mixture of carbon dioxide and methane, a renewable energy source) and microbial biomass. Anaerobic treatment provides a method of reducing pollution from agricultural and industrial operations while at the same time offsetting the operations' usage of fossil fuels. As one of the most efficient and least expensive waste treatment technologies for developing country such as DR CONGO, anaerobic digestion offers numerous significant advantages, such as low sludge production, fertilizer production , energy recovery and deforestation reduction [3,4,5].

Inhibitory substances are often found to be the leading cause of anaerobic reactor upset and failure since they are present in substantial concentration in wastewaters and organic solid wastes. A wide variety of substances have been reported to be inhibitory to methanogenic archaea. Among these inhibitory compounds, organic compounds are mentioned and more especially aromatic compounds [6,7].

Aromatic compounds are present in natural environments as degradation products of lignin, tannins, phenolic amino acids, pigments and other aromatic plants components. Human activities also contribute to the presence of aromatic compounds in the environment. Waste incineration, mining and the discharge of wastewater steams generated petrochemical factories, paper manufacturing and chemical industries, among others are important sources of aromatics pollution [4,6,3].

The presence of synthetic aromatic compounds in the environment may create serious public health and environmental problems. Some aromatics compounds are mutagenic or

carcinogenic and some may bioaccumulate. Additionally, man-made aromatics are often resistant to biodegradation and toxic to microorganisms [3,8].

Indeed, although the anaerobic biodegradability of aromatic compounds has been extensively studied, less attention has been given to the correlation of aromatic compounds structure and their toxic effects on the community of methanogenic archaea [6,8] Such knowledge is essential in predicting the impact of these xenobiotics on anaerobic waste and wastewater treatments, thereby preventing potentially costly upsets of treatment plant operations. A better understanding of the structure-toxicity relationships will make feasible the application of anaerobic technologies to waste and wastewater containing aromatics compounds [8].

At our knowledge, few works are published on the methanogenic toxicity of aromatic compounds. Most of these works were performed with the granular sludge as inoculums from an industrial anaerobic reactor called "Upflow Anaerobic Sludge Bed" (UASB-reactor). Generally, the digesters are heated to about 30ºC and more, but in this work digested pig manure from a laboratory scale digester was used as inoculum and the experiment was conducted at room temperature of an African tropical country (27±1ºC) [3].

The purpose of this study was to evaluate the inhibitory effects of phenolic monomers on methanogenesis in anaerobic digestion and to assess the effect of hydroxyl groups' number (aromatic structure) on inhibition of methane production by acetoclastic methanogens (archaea).

2. MATERIALS AND METHODS

2.1 Biomass

Pig manure from DAIPN farm of Nsele/KINSHASA (DR CONGO) was digested in laboratory scale digester during about six months. The digested pig manure (sludge) was utilized as inoculums in our anaerobic toxicity tests. The digested pig manure was not previously acclimated to any aromatic compounds.

Characteristic of inoculums: total suspended solids (TSS) concentration 91.10 g/l, volatile suspended (VSS) concentration 56.59 g/l, specific acetoclastic methanogenic activity 163.40 -210.81 mg COD-CH_4/g VSS .d (27±1ºC).

2.2 Stock Solutions

2.2.1 Stock substrate solution

The stock solution of the substrate is composed of acetic acid neutralized to pH = 7 with NaOH solution. It is at the concentration of 100 g COD-CH_3COOH /l (chemical oxygen demand per liter);

2.2.2 Stock solution 1

Macro-nutrients: NH_4Cl (170g/l); KH_2PO_4 (37 g/l); $CaCl_2.2H_2O$ (10 g/l); $MgSO_4.4H_2O$ (37 g/l) [3];

2.2.3 Stock solution 2

Trace elements: $FeCl_3.4H_2O$ (2000mg/l); $CoCl_2.6H_2O$ (2000 mg/l); $MnCl_2.4H_2O$ (500 mg/l); $CuCl_2$ (50 mg/l); H_3BO_3 (50 mg/l); $(NH_4)6Mo7O_2.4H_2O$ (90 mg/l); Na_2SeO_3. 5 H_2O (100 mg/l); $NiCl_2$. 6 H_2O (50 mg/l mg/l); EDTA (1000 mg/l) ; HCl 36% (1 mg/l); yeast extract(200 mg/l) resazurin (500 mg/l) [3];

2.2.4 Stock solution 3

Sulfide Na_2S (100 g/l) [7,8,3].

2.3 Aromatic Compounds

The used phenolic monomers included: phenol, resorcinol, hydroquinone and pyrogallol, which are natural occurring phenols. Otherwise benzene was utilized as reference aromatic compounds. All aromatic compounds were of high purity available (pure for analysis) supplied by MERCK.

2.4 Anaerobic Toxicity Assay

Specific acetoclastic methanogenic activity measurements were performed with 1l glass serum bottles sealed with butyl rubber septa.

Add to each serum bottle from the scale laboratory digester 1.5 g VSS of digested pig manure and add to this:

- Two ml stock solution 1;
- 1 ml stock solution 2;
- 2 drops stock solution 3;
- 40 ml stock substrate solution.

Fill the serum bottle to about 1000 ml with oxygen free tap water which is flushed with nitrogen gas for at least 15 minutes [3,9]. The flask were sealed with rubber septum cap and placed in a reciprocating shaker at 27±1ºC (room temperature).

The required quantity of inhibitory compound was added to each flask to provide the toxic concentration to be investigated. No toxicant was added to the controls. The toxicant concentrations were chosen as to cause an inhibition of the acetoclastic methanogenic activity ranging from 0-100 % [10,3,7]. The concentrations of inhibitors used in the anaerobic toxicity assay are given in the Table 1.

The specific methanogenic activity was calculated from the slope of the cumulative methane production versus time curve and the quantity of VSS. The compound concentration that caused 50% inhibition of the methanogenic activity was referred to as “50% IC”. All specific methanogenic activity measurements were conducted in triplicate. To determine the degree of inhibition, the specific methanogenic activities of the control and samples containing inhibitory compounds were determined [3,11,12].

Table 1. The inhibitory concentrations of phenolic monomers used in anaerobic toxicity assay

Nº	Compounds	Concentrations (mg/l)					
		1	2	3	4	5	6
1	Benzene	0	150	300	450	600	750
2	Phenol	0	500	1,220	2,500	3,500	4,500
3	Resorcinol	0	500	1,000	2,500	3,500	4,500
4	Hydroquinone	0	1,000	3,000	5,000	8,000	10,000
5	Pyrogallol	0	1,000	3,000	5,000	8,000	10,000

2.5 Methane Gas Measurement

The methane gas volume produced was measured by serum bottle liquid displacement systems (Mariotte flask system) as previously described [6,11,13]. The liquid used is a strong solution of NaOH or KOH ranging from 15 to 50 mg/l. As the biogas passes through these high pH solutions, the CO_2 of biogas is converted to carbonate and absorbed into the liquid. Only the methane passes through the solution and an equivalent volume is pushed out of the mariotte flask. The displaced liquid is measured in a graduated cylinder as a volume [6,9,11,13,].

3. RESULTS AND DISCUSSIONS

3.1 Inhibition of Specific Methanogenic Activity

All concentrations of aromatic compounds examined exerted an inhibitory effect on the specific acetoclastic methanogenic activity.

Fig. 1 shows the decrease in specific methanogenic activity with the increasing of the concentration of hydroquinone. The IC_{50} is calculated as the concentration of hydroquinone corresponding to 50% of inhibition.

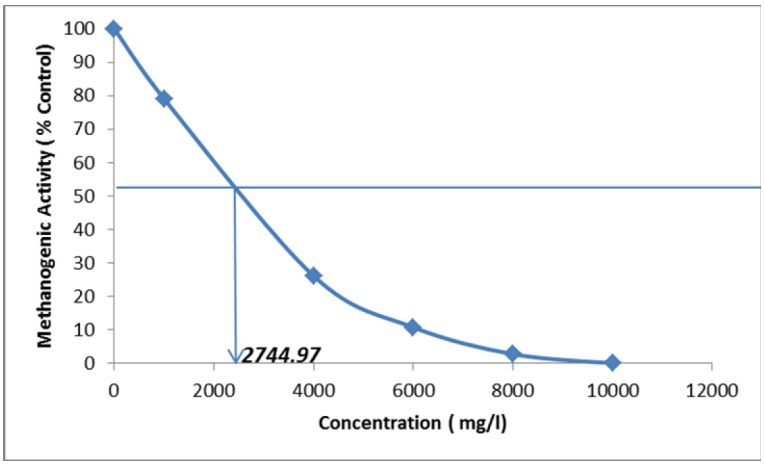

Fig 1. Methanogenic activity of digested pig manure exposed to hydroquinone as a function of Hydroquinone concentration. The method used for calculating the 50% IC is illustrated in this figure

3.2 Effect of the Hydroxyl Group's Number on the Methanogenic Toxicity

The inhibitory effect of phenolic monomers (natural occurring phenols): phenol, resorcinol, hydroquinone, pyrogallol, and reference compound (benzene) on the activity of acetoclastic methanogenic bacteria was studied at various levels, from concentrations that were nontoxic to those that were completely inhibitory to acetoclastic methanogenic activity, as typified by the experiment with hydroquinone in Fig. 1, Table 1 summarizes the 50% inhibiting concentrations (IC_{50}) of phenolic monomers evaluated in this study, ranked in decreasing order of toxicity.

Table 2. The IC_{50} values observed in the study for the monomeric tannin compounds and reference aromatic

Nº	Compound	IC_{50} (mg/l)	logPoct	Boiling point (ºC)	Number of hydroxyl groups
1	Benzene	209±6.32	2.13	80.10	0
2	Phenol	1249±29.59	1.47	181.70	1
3	Resorcinol	1725±5.27	0.80	178.00	2
4	Hydroquinone	2745±97.80	0.59	285.00	2
5	Pyrogallol	3173±91.02	0.97	309.00	3

The obtained results indicate that relationships exist between the hydroxyl group's number of phenolic monomers and their inhibitory effects on methanogens.

According to the Table 2, the toxicity of phenolic monomers increases in the following order: pyrogallol < hydroquinone < resorcinol < phenol < benzene.

In this sequence of toxicity, benzene is the most toxic compound, while pyrogallol is the least toxic. Thus, the substitution of a hydroxyl group (-OH) on the benzene ring giving the phenol, has the effect of reducing the toxicity of the benzene ring and the addition of a second hydroxyl group to phenol to form bifunctional compounds (hydroxyphenols) which are the isomeric compounds: resorcinol and hydroquinone; decreases the toxicity of the obtained compounds compared with phenol. Pyrogallol, obtained by adding three hydroxyl groups (-OH) on benzene ring was the least toxic of the studied phenolic monomers. The analysis of experimental results suggests that an increase in the number of hydroxyl groups on the aromatic compounds was associated with a decrease in the phenolic monomers toxicity.

With few exceptions, the results obtained in this study are comparable to those obtained by Sierra and Lettinga (1989) for aromatics compounds. The addition of a functional group containing an oxygen or sulfur heteroatom to benzene, our reference compounds, decreased the benzene toxicity as it is the case of the -OH substitution.

This demonstrates sufficiently that the grafting of hydrophilic substituent on the benzene ring, make the obtained compound less toxic and the effects of hydrophilic (electron donors) group's number such as the hydroxyl group are additives [14,15].

However, this behavior is valid only when the two substituents have no electronic and steric interactions and when there is not the formation of intramolecular hydrogen bonds. This is possible, when the two substituents are in the para position relative to each other. Indeed, when the substituents are in ortho or meta position, interactions change the order of toxicity in one direction or another. In this case, resorcinol (meta isomer) is most toxic than

hydroquinone (para isomer). Besides, the logPoct of resorcinol is 0.80 while that of hydroquinone is 0.59, indicating that resorcinol is more hydrophobic than hydroquinone; therefore more toxic than the latter.This phenomenon can be interpreted by the fact that the toxicity of isomers varies with the position of functional groups that result in steric and electronic interactions, and also with the formation of intramolecular hydrogen bonds [14,15].

3.3 Correlation of the Methanogenic Toxicity with Aromatic Compounds Hydrophobicity

Correlations between toxicity and partition coefficient within series of organic contaminants structurally related have been reported by a number of research groups using fish, ciliate or microorganisms as tests organisms. Therefore, when comparing compounds that possess different types of substitutions, a perfect correlation with logPoct of the compound cannot be expected. A higher correlation could potentially be obtained by comparing compounds in homologous series [10,7]

To determine if the lipophilic character of phenolic monomers and reference aromatic compounds (benzene) tested could be correlated with their methanogenic toxicity, the logarithm of the IC_{50} values of these aromatics were plotted against the logarithm of the octanol-water partition coefficient (logPoct) of the aromatic compounds. Fig. 2 shows the correlation line between the methanogenic toxicity and partition coefficient logPoct for phenolic monomers and reference aromatic compounds (benzene).

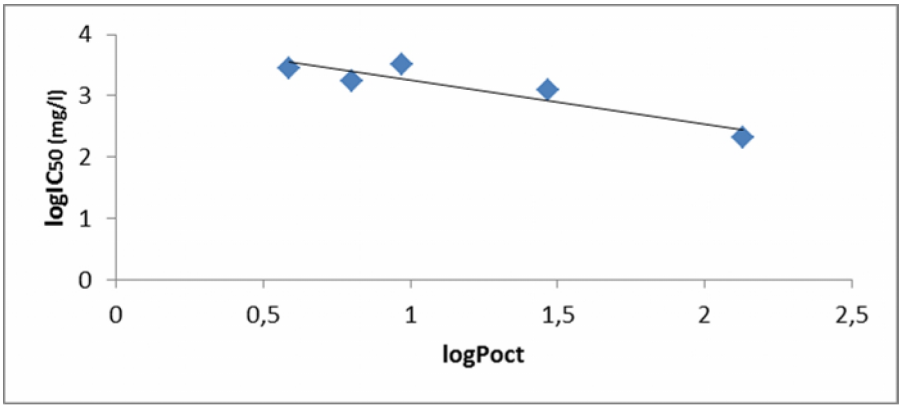

Fig. 2. Effect of hydrophobicity on methanogenic toxicity: methanogenic toxicity (IC_{50}) and partition coefficient (logPoct) correlation

It can be notice that there is a significant correlation between the toxicity of these aromatic compounds and their hydrophobicity (R=0.92087). The diffusion of a molecule across a membrane depends on the permeability of the membrane. However, the membrane permeability is a function of the partition coefficient logPoct (hydrophobicity). So the more hydrophobic a molecule is, the higher is its membrane permeability and the greater is its toxicity [17,18].

Indeed, a substitution on the aromatic ring which tends to make the molecule lipophilic (hydrophobic) increases the affinity for membrane phase therefore the permeability of the membrane to this compound. The massive compound diffusion in methanogens thus

increases the toxicity for these microorganisms causing damage to subcellular components. This contributes to the decrease in methanogenic activity [8].

This causes that the more hydrophobic is the molecule, the more readily it crosses the cell membrane and becomes highly toxic and inversely [14,17].

3.4 Correlation of the methanogenic toxicity (IC_{50}) with Cresols boiling point (BP)

We tried to know if any correlation exists between the toxicity of phenolic monomers and their boiling temperatures. The inhibitory effects of phenolic monomers have been assessed to study whether relationships between phenolic monomers boiling point and methanogenic toxicity would be established. The IC_{50} values of phenolic monomers were plotted versus theirs boiling temperatures. Fig. 3 shows the correlation line between the methanogenic toxicity and boiling points for phenolic monomers.

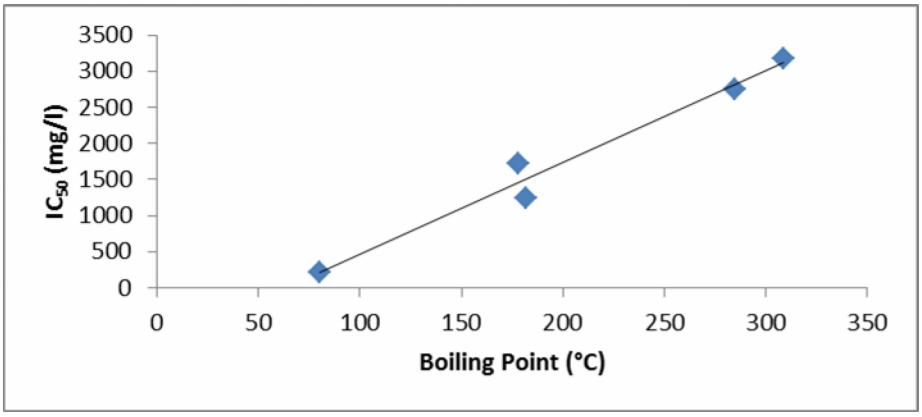

Fig. 3. Methanogenic toxicity (IC_{50}) with phenolic monomers boiling point (ºC) correlation

A positive linear correlation has been observed between the IC_{50} values of phenolic monomers and theirs boiling temperatures (R = 0.98971).

At our knowledge, it is the first time that a correlation between the aromatic compounds methanogenic toxicity and their boiling temperatures is found. Several of our unpublished data show that there are also such correlations in several series of the aromatic compounds structurally related. We noticed that the order of toxicity of aromatic isomers can be predicted from their boiling temperature in agreement with this correlation. For example one might predict that the catechol is more toxic than resorcinol and hydroquinone as is the case with cresols and chloroanilines (Kayembe et al., unpublished data).

4. CONCLUSION

The results of this study indicate that an increase in the number of hydroxyl groups on the aromatic compound was associated with a decrease in the compound's toxicity to methanogens. This demonstrates that the grafting of hydrophilic substituent on the benzene

ring, makes the obtained compound less toxic and the effects of hydrophilic (electron donors) group's number such as the hydroxyl group are additives.

A significant negative linear correlation between the toxicity of phenolic monomers with the reference aromatic compound (benzene) and their hydrophobicity was found. Moreover, a positive linear correlation has been found between the IC_{50} values of phenolic monomers and their boiling temperatures.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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