# Quantitation of the Inhibition Effect of Model Compounds Representing Plant Biomass Degradation Products on Methane Production

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During the steam explosion pretreatment of plant biomass, degradation products are generated, and some of these have inhibitory activity against biogas production. The aim of this study was to investigate and quantify the effect of selected model inhibitory compounds on methane production. The results showed no significant inhibition of methane production by furfural at concentrations below 1 g/L. In addition, the microbial community was able to restore biogas production inhibited by this compound after a certain time. 5-hydroxymethylfurfural was evaluated as a more potent inhibitor, with a significant effect above 0.2 g/L. Both compounds were more effective inhibitors with cellulose as the carbon substrate, probably reflecting higher sensitivity of the cellulolytic step in biogas production. No significant inhibition was observed for the phenolic compounds tested, gallic and tannic acids, at concentrations of up to 2 g/L. Thus, the compounds investigated should not represent a problem for the biogas production involving steam explosion preprocessed plant biomass.

*Keywords: Biogas; Furfural; 5-Hydroxymethylfurfural; Gallic acid; Tannic acid*

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## INTRODUCTION

Biogas production by anaerobic fermentation is an economically viable alternative to fossil fuel resources. The principal feed or substrate for industrial biogas units is plant biomass, most often wheat or corn silage. Alternative materials include manure and waste from food or wood processing industries, albeit usually in combination with already mentioned types of plant biomass, which, in any case, represents at least about 50% of the feed available for processing in biogas plants. The main reasons for the dominance of plant biomass for biogas production include high unit yields of biogas, verified technologies for the plant biomass production, good conservation and processing, and, of course, favorable cost and general availability.

Plant biomass is predominantly composed of cellulose, hemicellulose, and lignin. These natural polymers interact closely together and create a complex matrix that is rather resistant to biotic and abiotic degradation, especially under anaerobic conditions (Adney *et al.* 1991; Prochazka *et al.* 2012). Cellulose constitutes a major component of plant biomass, consisting of β(1→4) glycosidic bonds connected D-glucose units. Cellulose chains are

assembled into fibrils that possess a semi-crystalline structure, and these are interconnected with hemicelluloses and lignin at the higher level of structural hierarchy (Hendriks and Zeeman 2009). Hemicelluloses are complex polysaccharides that usually comprise pentoses, but hexoses and sugar acids are also present. The third major component of plant biomass lignin has a rather complex structure, composed of various interconnected phenol propane units. Of these three components, lignin is the most resistant to the enzymatic attack (Paulova *et al.* 2015). Indeed, this very resistance exhibited by the lignocellulose complex constitutes a major obstacle to exploit the potential of plant biomass in biotechnology, as it prolongs processes and simultaneously decreases yields.

Due to these reasons, physico-chemical pretreatment of the biomass is usually included prior to the main biotechnological process to disrupt the organized structure of the lignocellulose complex (Fan *et al.* 2006). The steam explosion method is the most often used pretreatment (Galbe and Zacchi 2007; Bruni *et al.* 2010). In this process, the biomass slurry is fed to a channel, where it is heated and pressurized with external hot steam. Upon leaving the channel, a pressure drop causes disruption to the structure of the lignocellulose complex. The three principle parameters that have to be controlled are temperature, pressure, and retention time. The mentioned procedure was used with positive results for the preprocessing of swine slurry (Ortega-Martineza *et al.* 2016), vinegar residue (Feng *et al.* 2016), or corn stover (Ji *et al.* 2016), where a significant increase of methane production was observed. Other authors reported improvements in the process kinetics (Theuretzbacher *et al.* 2015; Rincón *et al.* 2016). A more complex and somewhat skeptical view was brought by Dereix *et al.* (2006) showing that despite the increase in methane yield by about 50% the energy requirements of the steam-explosion process were substantially higher than the additional energy produced.

Bauer *et al.* (2009) studied the effect of steam explosion pretreatment on the methane yield from wheat straw, finding that at 10 min at 160 °C or 15 min at 180 °C, the treatment increased the specific methane yield by up to 14%, or 20%, respectively. Surprisingly, at 20 min at 200 °C, the positive effect on methane production was no longer observed. Treatment of plant biomass involving relatively high temperatures under elevated pressure can give rise to toxic degradation products derived from plant biomass constituents; indeed, such compounds may subsequently inhibit the key enzymes of the process. Examples of potentially inhibiting substances include phenolic compounds, particularly vanillin, gallic acid, and tannic acid, which arise through lignin decomposition, as well as the dehydration products of pentoses and hexoses, *i.e.* furfural and 5-hydroxymethylfurfural, respectively, from corresponding polysaccharides (Palmqvist and Hahn-Hagerdal 2000). The effects of furfural and 5-hydroxymethylfurfural have been studied, to some extent, in connection with biotechnological production of bioethanol or hydrogen from lignocellulose biomass (Oliva *et al.* 2006; Lu *et al.* 2007; Bellido *et al.* 2011; Paulova *et al.* 2012). Inhibition of at least three enzymes of the central carbon metabolism has been proven (Modig *et al.* 2002). The concentrations of these compounds in the pretreated biomass were over 1 g/L in some cases, and in an extreme instance even exceeded 3 g/L. Nevertheless, it was shown that for the production of hydrogen the threshold of the inhibition effect was about1 g/L. As a representative of the group of phenolic inhibition compounds derived from lignin, gallic acid was tested for the inhibition effect on methane production by Mousa and Forster (1999). The compound did not exhibit any significant effect in concentrations of up to 20 mg/L, whereas 50 mg/L caused about a 15% decrease in methane content of the biogas. The same study also showed

that supplementation with an additional, easily metabolizable carbon substrate (glucose) effectively restored methane production, likely through promoting degradation of the inhibiting compound. In another study (Hernandez and Edyvean 2008), caffeic acid and gallic acid were selected as model phenolic degradation products of lignin, and their inhibition effect on the production of methane was significant at concentrations of about 1.0 g/L.

In this study, the inhibition effect of furfural and 5-hydroxymethylfurfural, as model cellulose and hemicellulose degradation products, and gallic and tannic acids as model lignin degradation products were investigated in an experimental system mimicking general conditions at an actual industrial biogas plant. The aim was to evaluate the potential risks of the steam explosion pretreatment of plant biomass on methane production, with respect to the possible formation of inhibiting degradation products and their concentrations.

## EXPERIMENTAL

### Microbial Inoculum

Digestate containing the microbial community was sampled at the biogas plant (České Budějovice, Czech Republic), which mainly utilizes corn straw and grass silage as the feedstock for anaerobic fermentation at 39 °C. The sludge was sieved (1 $mm^2$) to remove corn grains and other solid particles and was suspended in mineral salt medium ($MgSO_4 \cdot 7H_2O$, 22.5 g/L; $CaCl_2$, 27.5 g/L; $FeCl_3 \cdot 6H_2O$, 0.25 g/L; $(NH_4)_2SO_4$, 10.0 g/L; $KH_2PO_4$, 8.2 g/L; $K_2HPO_4$, 21.8 g/L; $Na_2HPO_4 \cdot 12H_2O$, 44.7 g/L; $H_3BO_3$, 0.75 g/L; $FeSO_4 \cdot 7H_2O$, 3.00 g/L; $ZnSO_4 \cdot 7H_2O$, 0.10 g/L; $MnSO_4 \cdot 4H_2O$, 0.50 g/L) to reach the dry-weight concentration of 1 g/L. After dilution, the sludge was acclimated 7 days at 39 °C.

The basic characteristics of the digestate after sampling and after acclimation are summarized in Table 1 to document that the diluted and acclimated media from all three sampling instants had similar properties at the beginning of the experiments.

**Table 1.** Physicochemical Properties of Initial and Acclimated Digestate

| | Sampling I | Sampling II | Sampling III |
|---|---|---|---|
| Initial digestate | | | |
| ORP (mV) | -289 | -383 | -219 |
| pH | 7.8 | 8.9 | 8.2 |
| DW (g/L) | 5.70 | 3.00 | 28.5 |
| Acclimated digestate | | | |
| ORP (mV) | -208 | -253 | -225 |
| pH | 7.8 | 8.0 | 8.0 |
| DW (g/L) | 1.14 | 0.98 | 0.98 |
| %$CH_4$(acetate) | 62% | 68% | - |
| %$CH_4$(cellulose) | - | 49% | 30% |

ORP, oxidation-reduction potential; DW, dry weight; %$CH_4$, percentage of methane in biogas with the given substrate in the initial phase of the experiment.

## Production of Methane and Carbon Dioxide during Anaerobic Digestion

Biogas production experiments were performed in 300 mL flasks filled with 100 mL of the acclimated digestate, the appropriate carbon substrate (sodium acetate or cellulose) was added at the final concentration of 10 g/L, and the contents were purged with nitrogen. Afterwards, the tested inhibiting substances were added at given concentrations. At the beginning of the tests, the pH was 7.8 ± 0.5. After repeated purging with nitrogen, all flasks were sealed with stoppers equipped with gas-tight sampling valves and incubated at 39 °C. The contents of bottles were continuously stirred, and the gas phase of each flask was sampled several times a week. The gas pressure in the flasks was controlled by a digital manometer ( ISO 11734 (1995)). All experiments, including blank and control bottles, contained all the components except the tested compound or the carbon substrate, respectively, and were carried out in triplicate.

$CH_4$ and $CO_2$ production were determined by gas chromatography (Agilent GC 7890A equipped with a PORAPAK Q column and TCD detector, Santa Clara, USA; with helium as a carrier gas at 50 mL/min, $T_{injector}$ = 200 °C, $T_{owen}$ = 50 °C, $T_{det}$ = 220 °C) and expressed as the amount of carbon in the form methane produced per gram of carbon introduced m($CH_4$), the calculation were based on the ideal gas state equation (Drimal *et al.* 2006; Hubáčková *et al.* 2013). The concentrations of $CH_4$ and $CO_2$ were derived from the calibration curve obtained using the calibration gas mixture with certified composition (0.8% $CO_2$, 4% $CH_4$, 95.2% $N_2$, Linde 2016). At the end of the incubation dissolved inorganic carbon content was determined in all flasks (5000A TOC analyzer, Shimadzu, Tokyo, Japan). The pH and oxidation-reduction potential (ORP) were also determined to verify the validity of experiments.

The percentage of the net mineralization of the organic carbon ($D_t$) was estimated from the relation of the amount of carbon released in the form of $CH_4$ and $CO_2$ ($m_g$, mg) and dissolved innorganic carbon ($m_l$, mg), corrected by a blank, to a theoretical amount of the carbon introduced as the substrate (cellulose or acetate) and the inhibiting compound eventually ($m_v$, mg), and expressed in terms of the percentage of anaerobic biodegradation, as follows:

$$\mathrm{D_t} = \frac{m_g + m_l}{m_v} \times 100\ \% \quad (1)$$

## Mathematical Model

Methane production during the growth of microbial biomass was expressed according to a previously published model of inhibited biomass growth (Rial *et al.* 2011), where the amount of the produced methane is proportional to the biomass and the initial methane concentration is zero (Eq. 2),

$$m(CH_4) = k\left[\frac{X_0}{1+\exp(c-\mu_m t)} - X_0\right] \quad (2)$$

where $\mathrm{c} = \ln\left(\frac{X_m}{X_0} - 1\right)$;

*m(CH₄)* is the mass of the methane produced; *k* is the proportionality constant between biomass and methane production; $X_0$ is the biomass at the beginning of the experiment; $X_m$

is the asymptotic maximum of biomass; and $\mu_m$ is the the biomass increase per biomass unit and time unit (dimensions $t^{-1}$).

After re-parametrization (Rial *et al.* 2011), the model equation for the methane production is as follows,

$$m(CH_4) = \frac{m(CH_4)_{MAX}}{1+\exp\left[2+\frac{4\nu_{MAX}}{m(CH_4)_{MAX}}(\lambda - t)\right]} - \frac{m(CH_4)_{MAX}}{1+\exp\left[2+\frac{4\nu_{MAX}}{m(CH_4)_{MAX}}\lambda\right]} \quad (3)$$

where $m(CH_4)_{MAX}$ is the asymptotic maximum of methane production; $v_{MAX}$ is the maximal rate of methane production; and $\lambda$ is the length of lag phase. Fitting and estimation of parameters from the experimental results were performed by sum of square minimisation nonlinear method (quasi-Newton) using the macro 'Solver' of Microsoft Excel.

## RESULTS AND DISCUSSION

The inhibition effect of selected model compounds was studied in experiments simulating biogas production from corn biomass, as is often the case at industrial scale biogas production plants. Semiliquid digestate from such a process was utilized as the microbial inoculum. Two groups of experiments were carried out with two different carbon substrates. The first one, with cellulose, comprised all phases of the process, *i.e.* hydrolysis, acetogenesis, and methanogenesis, whereas the other set of experiments with sodium acetate studied solely the methanogenesis. Furfural and 5-hydroxymethylfurfural were selected as model inhibition compounds formed as dehydration products during thermal treatment of carbohydrate-based plant biomass constituents (Barakat *et al.* 2012; Chiaramonti *et al.* 2012); gallic and tannic acids are phenolic model inhibition compounds derived during the degradation of plant lignin (Hernandez and Edyvean 2008; Barakat *et al.* 2012).

### Inhibition Effect of Furfural and 5-Hydroxymethylfurfural on Biogas Production

The inhibition effects of furfural and 5-hydroxymethylfurfural were studied in concentrations of up to 2 g/L, which lie at the higher limit of expected concentrations in a real process (Bellido *et al.* 2011). The data obtained on methane production were fitted with the described mathematical model and the parameters of the model, *i.e.* the length of the lag phase, maximal methane production rate, and asymptotic maximal methane production level calculated (Table 2). Subsequently, these parameters were used to compare inhibition effects among the individual experiments. During the experiment with sodium acetate as the carbon substrate, furfural concentrations under 1 g/L exhibited a stimulating effect on methane production; hence, the compound could probably be utilized as an additional carbon substrate under such conditions (Fig. 1A). Even at 1.0 g/L, it was possible to discern a noticeable inhibition effect, and the effect was proportionally more pronounced at 2.0 g/L. Inhibition by furfural was manifested primarily through an increase in lag phase prior to the onset of methane production (Fig. 2). However, it seemed that even at the highest furfural concertation tested, the compound was metabolized, *i.e.* detoxified. After a certain time, methane production was substantially restored with a comparable maximal production rate to the control experiment without the inhibiting compound. The

same was generally applicable for experiments with cellulose as the carbon substrate (Fig. 1C), except that methane production inhibition was exhibited as early as at 0.5 g/L and then strongly at 1.0 g/L. Thus, it is likely that some processes preceding methanogenesis in the path of cellulose utilization are slightly more sensitive to furfural inhibition than methanogenesis itself.

5-hydroxymethylfurfural was found to have a slightly stronger inhibition effect than furfural, showing a noticeable influence even at 0.5 g/L (Fig. 1B). In contrast to furfural, hydroxylmethylfurfural decreased the maximal methane production rate and the level of maximum methane production (Fig. 2), presenting no evident tendency to restore methane production after a certain period. This suggested that hydroxymethylfurfural was not readily removed from the system and caused permanent inhibition of the process. As in the case of furfural, methane production from cellulose was noticeably more inhibitor-sensitive than acetate as the carbon substrate, with an inhibition appearing even at 0.2 g/L 5-hyroxymethylfurfural (Fig. 1 D). The latter finding confirms that methanogenesis was not a critical step in biogas production with respect to sensitivity to these inhibitory compounds. Rather, the critical step was cellulolysis or acetogenesis.

**Table 2.** Optimized Parameters of the Mathematical Model

| Acetate as the carbon substrate | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| $C_{FUR}$ (g/L) | $\lambda$ (d) | $m(CH_4)_{MAX}$ (mg/g) | $v_{MAX}$ (mg/(g•d)) | | $C_{HMF}$ (g/L) | $\lambda$ (d) | $m(CH_4)_{MAX}$ (mg/g) | $v_{MAX}$ (mg/(g•d)) |
| 0.0 | 5.0 | 213 | 11 | | 0.0 | 15 | 280 | 4.94 |
| 0.1 | 0 | 230 | 12 | | 0.1 | ND | ND | ND |
| 0.2 | 0.9 | 226 | 18 | | 0.2 | 7.7 | 324 | 6.39 |
| 0.5 | 2.7 | 200 | 24 | | 0.5 | 11 | 280 | 3.38 |
| 1.0 | 9.9 | 180 | 16 | | 1.0 | 4.4 | 103 | 0.91 |
| 2.0 | 23 | 126 | 18 | | 2.0 | ND | ND | ND |
| Cellulose as the carbon substrate | | | | | | | | |
| $C_{FUR}$ (g/L) | $\lambda$ (d) | $m(CH_4)_{MAX}$ (mg/g) | $v_{MAX}$ (mg/(g•d)) | | $C_{HMF}$ (g/L) | $\lambda$ (d) | $m(CH_4)_{MAX}$ (mg/g) | $v_{MAX}$ (mg/(g•d)) |
| 0.0 | 1.9 | 121 | 4.58 | | 0.0 | 0.0 | 28.2 | 0.68 |
| 0.1 | 0.1 | 132 | 5.01 | | 0.1 | ND | ND | ND |
| 0.2 | 3.1 | 114 | 4.28 | | 0.2 | 0.0 | 12.9 | 18.0 |
| 0.5 | 2.7 | 106 | 4.35 | | 0.5 | 3.0 | 4.42 | 17.6 |
| 1.0 | 23 | 140 | 4.95 | | 1.0 | 20 | 2.57 | ND |
| 2.0 | ND | 0 | ND | | 2.0 | ND | ND | ND |

$\lambda$, length of lag phase; $m(CH_4)_{MAX}$, asymptotic maximum of methane production; $v_{MAX}$, maximal rate of methane production; d, day; $c_{FUR}$, concentration of furfural; $c_{HMF}$, concentration of 5-hydroxymethylfurfural.

The reactions were characterized at the end of the incubation period, as summarized in Table 3. In all experiments, the pH and concentration of dissolved inorganic carbon indicated that adding an inhibiting compound did not cause a shift in metabolic processes from methanogenesis. The amounts of dissolved inorganic carbon were comparable in every incubation, which confirmed that the contents of gaseous-endmetabolites, which were determined in the head space of the incubation vessels, are applicable for interpreting the development of metabolic processes.

**Table 3.** Selected Properties of the Individual Incubations at the End of the Experiments

| Acetate as the carbon substrate | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| $C_{FUR}$ (g/L) | pH | IC (g/L) | $D_t$ (%) | $CH_4$ (%) | | $C_{HMF}$ (g/L) | pH | IC (g/L) | $D_t$ (%) | $CH_4$ (%) |
| 0.0 | 8.38 | 1.73 ± 0.05 | 75 | 93 | | 0.0 | 8.36 | 1.74 ± 0.10 | 89 | 97 |
| 0.1 | 8.31 | 1.88 ± 0.04 | 78 | 93 | | 0.2 | 8.25 | 1.75 ± 0.09 | 87 | 94 |
| 0.2 | 8.29 | 1.78 ± 0.08 | 76 | 92 | | 0.5 | 8.05 | 1.69 ± 0.11 | 80 | 89 |
| 0.5 | 8.11 | 1.71 ± 0.12 | 70 | 89 | | 1.0 | 8.42 | 1.65 ± 0.01 | 56 | 24 |
| 1.0 | 7.97 | 1.83 ± 0.16 | 66 | 87 | | 2.0 | 8.18 | 0.0 | 0 | 0 |
| 2.0 | 7.21 | 1.41 ± 0.62 | 46 | 79 | | 4.0 | 8.21 | 0.0 | 0 | 0 |
| Cellulose as the carbon substrate | | | | | | | | | | |
| $C_{FUR}$ (g/L) | pH | IC (g/L) | $D_t$ (%) | $CH_4$ (%) | | $C_{HMF}$ (g/L) | pH | IC (g/L) | $D_t$ (%) | $CH_4$ (%) |
| 0.0 | 7.99 | 1.13 ± 0.02 | 42 | 57 | | 0.0 | 8.15 | 1.99 ± 0.07 | 51 | 39 |
| 0.1 | 8.15 | 1.10 ± 0.01 | 42 | 60 | | 0.2 | 8.09 | 2.12 ± 0.05 | 50 | 26 |
| 0.2 | 8.32 | 1.12 ± 0.02 | 40 | 57 | | 0.5 | 8.32 | 2.06 ± 0.05 | 47 | 8 |
| 0.5 | 8.13 | 1.10 ± 0.03 | 39 | 56 | | 1.0 | 8.24 | 2.35 ± 0.08 | 47 | 5 |
| 1.0 | 8.21 | 1.07 ± 0.36 | 37 | 67 | | 2.0 | 8.40 | 0 | 0 | 0 |
| 2.0 | 8.09 | 0.00 | 0 | 9 | | 4.0 | 8.15 | 0 | 0 | 0 |

$c_{FUR}$, concentration of furfural; $c_{HMF}$, concentration of 5-hydroxymethylfurfural; IC, concentration of dissolved inorganic carbon in aqueous phase; $D_t$, % of carbon mineralization; $CH_4$, the percentage of $CH_4$ in biogas

As the simpler of the two substrates, acetate exhibited a considerably high level of mineralization, which resulted in a higher content of methane in the biogas formed. Furthermore, the effective concentrations of inhibiting compounds gradually decreased the content of methane in the biogas produced, although a dramatic effect was only seen at concentrations strongly inhibiting methane production. The last performed experiments with 5-hydroxymethylfurfural and cellulose as substrate were performed with the inoculum from the third sampling instant. In these experiments the production rate of $CH_4$ and its content in biogas (Table 1) was somewhat lower witnessing probably a lower initial content of methanogens on the beginning of the experiment.

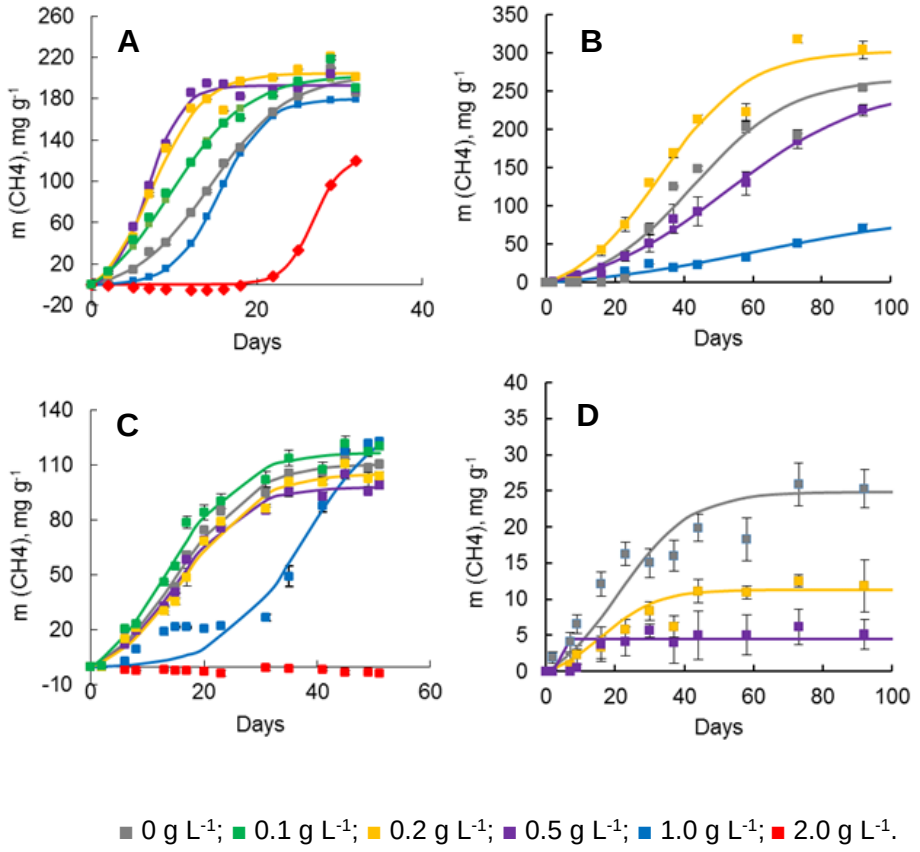


**Fig. 1.** Production of methane in the presence of various concentrations of furfural (A, C) and 5-hydroxymethylfurfural (B, D). The methane production is expressed per gram of carbon substrate *i.e.* sodium acetate (A, B) or cellulose (C, D). Solid lines represent described theoretical models fitted to the experimental data.

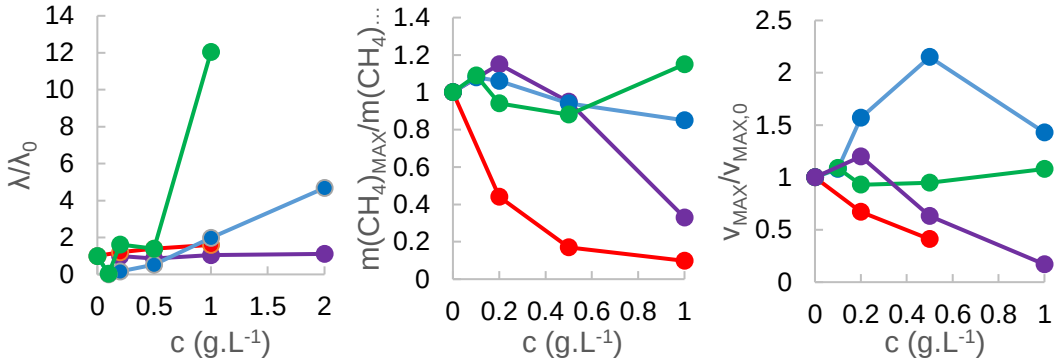


**Fig. 2.** Changes of model parameters: length of lag phase ($\lambda$), asymptotic maximum of methane production ($m(CH_4)_{MAX}$), and maximal rate of methane production ($v_{MAX}$) with the concentration of inhibiting compounds relative to the parameter value at zero concentration of the inhibiting compound ($\lambda_0$; $m(CH_4)_{MAX,0}$; $v_{MAX,0}$). ●, furfural +sodium acetate as substrate; ●,furfural+cellulose as substrate; ●, 5-hydroxymethylfurfural +sodium acetate as substrate; ●, 5-hydroxymethylfurfural +cellulose as substrate.

### Inhibition Effect of Tannic Acid and Gallic Acid

The inhibitory effects of the phenolic compounds, as model degradation products of plant lignin, were studied up to a concentration of 2.0 g/L. As shown in Fig. 3, even at the highest concentrations they were substantially incapable of inhibiting methane production from either of the two tested carbon substrates. However, in the experiments with acetate, the maximal methane production level was slightly lower for the two highest concentrations of gallic and tannic acid. Nevertheless, such high concentrations are not expected in real conditions. Indeed, the presence of phenol compounds also failed to demonstrate any apparent stimulating effect.

The selected parameters determined at the end of the incubation period (Table 2) confirmed that the basic chemical conditions in the processes were comparable regardless of the concentration of the inhibiting compounds. The moderate decrease in the level of mineralization at the highest concentration of gallic acid in the presence of both substrates could signal the onset of inhibition again at this rather extreme concentration of the compound.

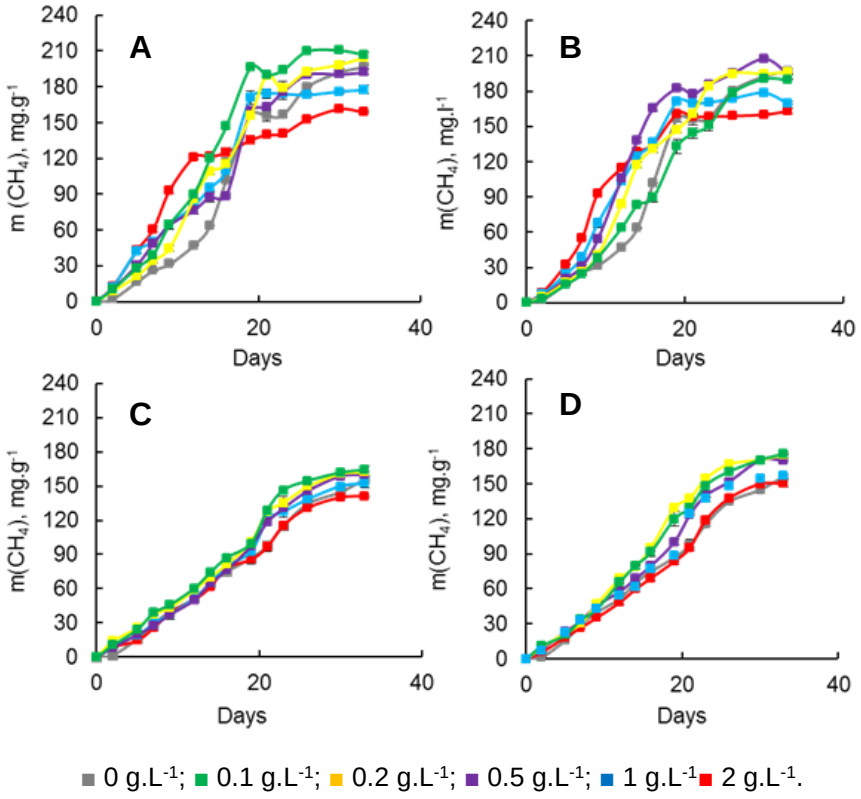


**Fig. 3.** Production of methane in the presence of various concentrations of gallic acid (A, C) and tanic acid (B, D). The methane production is expressed per gram of carbon substrate, *i.e.* sodium acetate (A, B) or cellulose (C, D).

## CONCLUSIONS

1. The study showed no noticeable inhibition of methane production by furfural at concentrations below 1 g/L

2. 5-hydroxymethylfurfural was a stronger inhibitor with a noticeable effect above 0.2 g/L.
3. Both compounds were more effective inhibitors with cellulose as the carbon substrate, potentially reflecting the higher sensitivity of the cellulolytic step in the process of biogas production.
4. No inhibition was observed for the tested phenolic compounds (gallic and tannic acid) at concentrations of up to 2 g/L.
5. The compounds investigated do not raise an issue for biogas production involving plant biomass preprocessed by the steam explosion method.

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