Fermentation Technology

Ngoma et al., Ferment Technol 2015, 4:1
http://dx.doi.org/10.4172/2167-7972.1000113

Research Article Open Access

Impact of Different Plant Secondary Metabolites Addition: Saponin, Tannic Acid, Salicin and Aloin an Glucose Anaerobic Co-Digestion

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Received date: Aug 25, 2014, **Accepted date:** Apr 04, 2015, **Published date:** Apr 08, 2015

Abstract

Vegetal waste and some wastewater of agro-food industries contain plant secondary metabolites (PSMs). It was showed in nutritional researches that these substances such as saponins and tannins reduced the methane production in the rumen. To our knowledge no study was done in the waste treatment domain to evaluate the inhibitory effect of the principal glycosidic metabolites from the wastewater or vegetal waste on their own methane-producing anaerobic digestion. Therefore in this paper BMP tests were carried out at 30°C with four commercial PSMs (CPSMs) in mixture with glucose monohydrate (Gl) used as control sample. These CPSMs were saponin from *Quilaja Saponaria* Molina Pract (Sap), tannic acid (Tan), salicin (Sal) and aloin from Curacao Aloe (Alo) representing respectively saponins, tannins, alcoholic glycosides and anthraquinones sources. Acidogenesis and acetogenesis were recorded for all the mixtures of Gl and CPSMs; however their conversion rates decreased with the increase of the concentrations of CPSMs. By contrast, the methanogenesis was inhibited at concentrations of CPSMs above 0.3 g/l. The inhibition degree for aromatic compounds on the anaerobic biodegradation of Gl seemed directly to depend on the numbers of benzene rings in the medium and the synergism. Thus, the highest inhibition of the biogas production from Gl was recorded for Alo, followed by Sap, Tan and Sal. However, the highest inhibition of the methane production from Gl was recorded with Sap, Alo, Tan and Sal. It was supposed that the toxicity potentials of these PSMs on the own biomethanization would be in following decreasing order: Sap or Alo, Tan and Sal.

Therefore, the concentration of PSMs alone or in mixture in a digester should be below 0.3 g/l. for a better methanization.

Keywords: Anaerobic digestion; Biogas; Methane; Inhibition effect; Plant secondary metabolite

Abbreviations

Alo: Aloin from Curacao Aloe (ᵹ50%); BMP: Biochemical Methane Potential; C/N: Carbon/Nitrogen Ratio; DW: Dry Weight; CPSM: Commercial Plant Secondary Metabolite; Gl: Glucose Monohydrate (Contrôle Sample; GC: Gas Chromatography; HPLC: High Performance Liquid Chromatography; ND: Not Determined; PSM: Plant Secondary Metabolite; Sal: Salicin (99%); Sap: Saponin From Quilaja Molina Pract; MD : Mean Deviation; Ta: Tannic Acid; UV: Ultraviolet; VFA: Volatile Fatty Acid

Introduction

Plants produce different kind of secondary metabolites to protect against microbial and insect attacks [1,2]. These plant secondary metabolites (PSMs) considered as bioactive compounds were used for long times in medicine and preservation of foods [2]. As a consequence, these compounds can be present in wastewater coming from agro-alimentary, pharmaceutical and chemical industries or in vegetal wastes. The water-soluble PSMs such as saponins, polyphenols, alcoholic glycosides and bound quinones (anthraquinones) may inhibit directly the present microorganisms in the effluents. Therefore, anaerobic biodegradation processes of these solid wastes and industrial effluents may be limited by inhibition of the methanogenic archaea since they are very sensitive to this molecule type. The production of biogas can be low or nil and the organic matter contained in the effluent is not reduced. Also these compounds potentially reduce the ability to produce biofuels by biomass fermentation [3]. These solids or effluents poured in the nature can be the basis of pollution.

The saponins, tannins and anthraquinones have been shown to be toxic for microorganisms and to suppress methane production in animal nutrition researches [2,4-6] and also in a recent study on the waste treatment achieved by Mambanzulua et al. [7]. However, to our knowledge, few works are published on the evaluation of methanogenic inhibition process by PSMs in anaerobic digestion processes treating wastes. Consequently, there is a lack of information about the toxic effects and about potential inhibition. Some publications about non glycosylated phenolic monomers are focused on the evaluation of the inhibitory effects of aromatic structure on methanogenesis [3,8,9]. There are also some inconsistencies about methane inhibition in for instance studies on saponins [2]. Besides, the information available on methanogenic fermentation of phenolic compounds is fragmented and sometimes contradictory. It also lacks a consistent use of units for reporting biomass concentration. That does not allow compare the anaerobic degradation of these compounds [3].

In the recent decade a limited number of studies has been published on the effect of PSMs on the enteric methanogenesis [2]. However, these studies have been based on the reduction of methane production by adding PSMs in diet. Less attention has been given to the correlation of PSMs structure and their toxic effects on the population of anaerobic bacteria. However, the knowledge of the PSMs structures effect on the inhibition of biogas biosynthesis is essential in predicting the impact of these xenobiotics on methanization and wastewater treatment. Thereby preventing potentially costly upsets of treatment plant operations, a better understanding of the structure-toxicity relationships will enable the application of anaerobic technologies to solid waste and wastewater containing PSMs.

The literature on anaerobic digestion shows considerable variation in the inhibition or toxicity levels reported for most substances. The major reason for these variations lies in the complexity of anaerobic digestion processes where mechanisms such as nature of inoculum and substrate, antagonism, synergism, acclimation and complexing could significantly affect the inhibition phenomenon [10].

The present paper aims to study the impact of glycosidic PSMs on the anaerobic co-digestion with the glucose for methane production and also their effects on the biogas biosynthesis according to their chemical structures. Therefore, biochemical methane potential (BMP) tests according to Owen et al. [11] were carried out with four commercial PSMs (CPSMs): saponin, tannic acid, salicin and aloin. Except saponins, all others are aromatic substances. Biogas volume and composition and Volatile Fatty Acids (VFAs) productions were monitored. Finally, an objective comparison of the inhibition degrees and the biochemical methane potentials of these anti-microbial molecules were determined.

Materials and Methods

Characters of substrates

The saponin from Quilaja Saponaria Molina Pract (Sap), tannic acid (Tan), salicin at 99% (Sal) and aloin from Curacao Aloe at nearly 50% (Alo) were used as references for the saponins, tannins, alcoholic glycosides and anthraquinones, respectively. All the CPSMs were obtained from Sigma-Aldrich, St-Louis, USA; except, Sap that comes from Acros Organics, Geel, Belgium. Their solubilities in the water, their molecular formulas, their molecular weights and their chemical structures are reported in Table 1 and Figure 1 [12-14].

CPSMs	Molecular formulas	Molecular weights (g)	Water solubilities (g/l)
Saponin	ND	ND	ND
Tannic acid	$C_{76}H_{52}O_{46}$	1701.2	28.6
Salicin	$C_{13}H_{18}O_7$	286.3	40.0
Aloin	$C_{21}H_{22}O_9$	418.4	ND

Table 1: Water solubilities, molecular formulas, molecular weights and chemical structures of the different CPSMs tested.

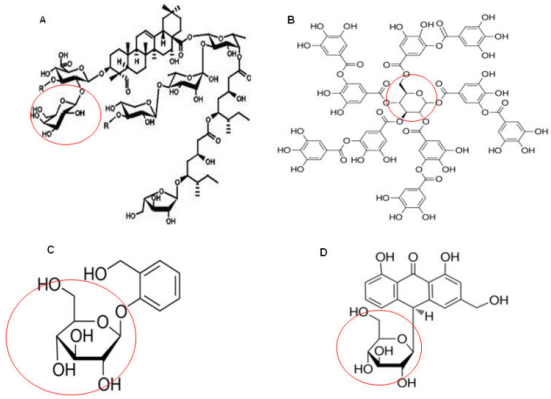

Figure 1: Molecular structure of sap (a), Tan (b), Sal (c) and Alo (d)

Identification of saponins, tannins and total polyphenols in Alo

The purity of Alo was poor; reason why in saponins, tannins and water-soluble total polyphenols were analyzed. The tannins were qualitatively determined in the aqueous extracts of Alo by qualitative colorimetry after the following reactions [15-17]. The aqueous extracts were obtained after steeping under magnetic agitation of 1 g of Alo in 16 ml of distilled water during 30 minutes and filtration on membrane [15-17]. The tannins were determined by adding 1 ml of $FeCl_3$ 2% (Burton reagent) to 2 ml of aqueous extracts. The presence of tannins was indicated by a greenish red coloration with or without precipitate.

The concentration of total polyphenols was quantitatively determined according to a procedure derived from Singleton and Rossi [18]: in a 25 ml vial, 0.5 ml of 1% Alo aqueous extracts reacted for 3 min with 0.5 ml of Folin-Ciocalteu reagent (VWR Prolabo). After addition of 4 ml of sodium carbonate solution (1M), the mixture was brought to volume with demineralized water and homogenized. The absorbance was read at 765 nm after incubation at room temperature for 2 hours in the dark. Gallic acid was used as a reference standard.

The saponins were determined by agitating vigorously 5 ml of aqueous extracts in a test tube and formation of persistent foam of at least 1 cm height during 15 minutes. This test is a semi quantitative [19].

Biogas and methane

The BMP assays of the CPSMs were determined following the procedure described by Rodriguez et al. [20] and Wang et al. [21]. The tests were carried out in duplicate in 250 ml sterile glass serum bottles filled with 150 ml of mixture. This mixture contained 125 ml of phosphate-carbonate buffer solution (pH adjusted to 7.2 with KOH 5N), 25 ml of sludge, 0.25 g of glucose monohydrate (Gl) and the concerned CPSM at different concentrations. The different CPSMs were used for tests with 50 mg, 250 mg, 500 mg, 1000 mg and 2000 mg. The glucose monohydrate (Gl) was used as a positive control sample and a second addition of 0.25 g was done after the 100th day, by adding 2 ml of a 125 g/l aqueous solution by syringe injection

through the septum. It is to note that 2 ml of the same Gl aqueous solution was also added by the same way in each BMP test with CPSM sample after the 100_{th} day. This Gl addition was done to confirm the biomethanization inhibition. Each blank sample consisted of 25 ml of the anaerobic sludge inoculum and 125 ml of phosphate-carbonate buffer solution. No energetic substrate was added to the blank samples. The minerals elements and vitamins were not added considering that those substances should be present in the sludge. This anaerobic sludge contained 14.18 g DW/l and had a C/N ratio of 2.63. It was collected from a stirred anaerobic digester used in Walloon Center of Industrial Biology for BMP assays of different agro-food organic wastes.

When the sample bottles were filled, they were capped tightly with rubber septa and sealed with aluminum seals, and nitrogen was passed into the bottles to flush out air and other gases before the incubation [7,22]. The bottles were then incubated at 30°C. The composition and the volume of biogas produced were periodically measured during 230 days according to the method of CO_2 absorption by KOH, described by Hiligsmann et al. [22].

H_2, and CO_2 were determined using a method described by Hamilton et al. [23] and separation was achieved using a Hewlett Packard 5890 Series II gas chromatograph (GC; Agilent Technologies, Santa Clara, CA, USA) equipped with a 30 m long, 0.32 mm id Alltech GAS PRO GSC column (Grace, Deerfield, IL, USA) in series with a 20 m long, 0.25 mm id Chrompack CARBOPLOT P7 column (Agilent Technologies) and a thermal conductivity detector. The carrier and reference gas was N_2 and a mixture of H_2 (80%) and CO_2 (20%) was used to determine the fraction of H_2 in the biogas produced. The GC injection port, the thermal conductivity detector chamber, and the oven were maintained at 90, 110 and 55°C, respectively.

The volume of biogas or of methane produced from a CPSM was determined by subtracting from the whole volume of the mixture, the volume of biogas or of methane produced from Gl. The yields of biogas or of methane were calculated by dividing the measured volume of biogas or of methane by its volatile solid.

The inhibition degree of biogas or of methane production of a CPSM was determined by comparing the volume of biogas or of methane produced from Gl with that from the mixture containing Gl and CPSM. For a CPSM achieving a production of biogas or of methane of A ml from its mixture with Gl and B ml from the Gl alone, the inhibition degree would be $[(A-B)/B] \times 100\%$. A positive percentage means a gain of volume of biogas or of methane produced from the mixture containing Gl and CPSM compared to that of Gl.

Analysis of glucose, ethanol and volatile fatty acids (VFAs)

The evolution of glucose, ethanol and VFAs concentrations in the samples was analyzed by HPLC during the anaerobic digestion. The culture media of samples were centrifuged at 13000 g for 10min and the supernatants were filtered through a 0.2 µm cellulose acetate membrane (Sartorius Minisart). The glucose, ethanol, formate, acetate, propionate, butyrate, lactate and succinate were analyzed using a HPLC equipped with a differential refraction index detector as formerly described by Masset et al. [24] .

Results

Saponins, tannins and total polyphenols in Alo

The chemical analysis of Alo sample showed that it contained tannins and 232 mg of total polyphenols/g but no saponin was detected.

Biogas production from mixtures containing Gl and CPSMs and CPSMs alone

The evolution of the biogas production was monitored in BMP tests carried out to assess anaerobic digestion of the mixtures containing Gl and CPSM or CPSMs alone. The results over 230 days of experimentation with addition of glucose in the samples after 100 days are presented in Figure 1. This addition was done in order to confirm the results recorded over the first period of 100 days. The total volumes of biogas after this first period were from 12.0 ± 7.8 ml for blank samples, 45.7 ± 27.1 ml for Gl samples, 81.9 ± 12.0 to 219.9 ± 20.0 ml, 45.7 ± 0.5 to 91.2 ± 8.0 ml, 48.4 ± 10.3 to 98.1 ± 0.0 ml, 99.7 ± 9.0 to 51.9 ± 3.3 ml for the mixtures containing 1.7 g/l Gl and 0.3 to 13.3 g/l of Sap, Tan, Sal and Alo, respectively. For day 230 of anaerobic digestion, the total volumes were 12.0 ± 7.8 ml for blank samples, 194.9 ± 42.7 ml for the Gl samples and from 215.4 ± 14.8 to 256.3 ± 1.3 ml, 211.2 ± 33.5 to 112.7 ± 19.0 ml, 222.9 ± 5.5 to 165.4 ± 0.0 ml and 181.7 ± 5.5 to 105.9 ± 2.8 ml for the mixtures containing 3.3g/l Gl and 0.3 to 13.3 g/l of Sap, Tan, Sal and Alo, respectively.

The results recorded after 230 days of anaerobic digestion of CPSMs alone are reported in Figure 2. The total volumes of biogas were from 20.5 ± 14.8 to 61.4 ± 1.8ml, 24.9 ± 0.0 to 0.0 ± 0.0 ml and 28.0 ± 5.5 to 0.0 ± 0.0 ml for Sap, Tan and Sal at concentrations of 0.3 to 13.3 g/l, respectively. No biogas production was recorded for Alo alone.

Hydrogen and methane production from the mixtures containing Gl and CPSM and CPSMs alone

The contents in hydrogen in the biogases from all the samples were 0 after 16 days of incubation. The results of methane production from the mixtures containing Gl and CPSM after 230 days of incubation are showed in Figure 3. The total volumes of methane produced during this period were 0.0 ± 0.0 ml for blank samples, 61.8 ± 24.1ml for Gl and from 3.5 ± 3.4 to 3.5 ± 2.7 ml, 88.2 ± 24.3 to 0.0 ± 0.0 ml, 88.1 ± 7.9 to 34.9 ± 0.0 ml and 54.9 ± 3.0 to 0.0 ± 0.0 ml for the mixtures containing 3.3g Gl /l and 0.3 to 13.3 g/l of Sap, Tan, Sal and Alo, respectively.

The results of methane production from the CPSMs alone after 230 days of incubation are shown in Figure 3.

The total volumes of methane produced were 0.0 ± 0.0 ml for Sap and Alo at all concentration, and from 0.0 ± 0.0 to 0.0 ± 0.0 ml and 26.4 ± 7.9 to 0.0 ± 0.0 ml for Tan and Sal at concentrations of 0.3 to 13.3 g/l, respectively.

Biogas and methane yields of the mixtures containing Gl and CPSM and CPSMs alone and evaluation of inhibitory effects of CPSMs

The biogas and methane yields related to different mixtures of Gl and CPSM and the inhibition degrees by CPSMs on biogas and methane production are reported in Table 2. By contrast the biogas and methane yields related to each CPSM are reported in Tables 3.

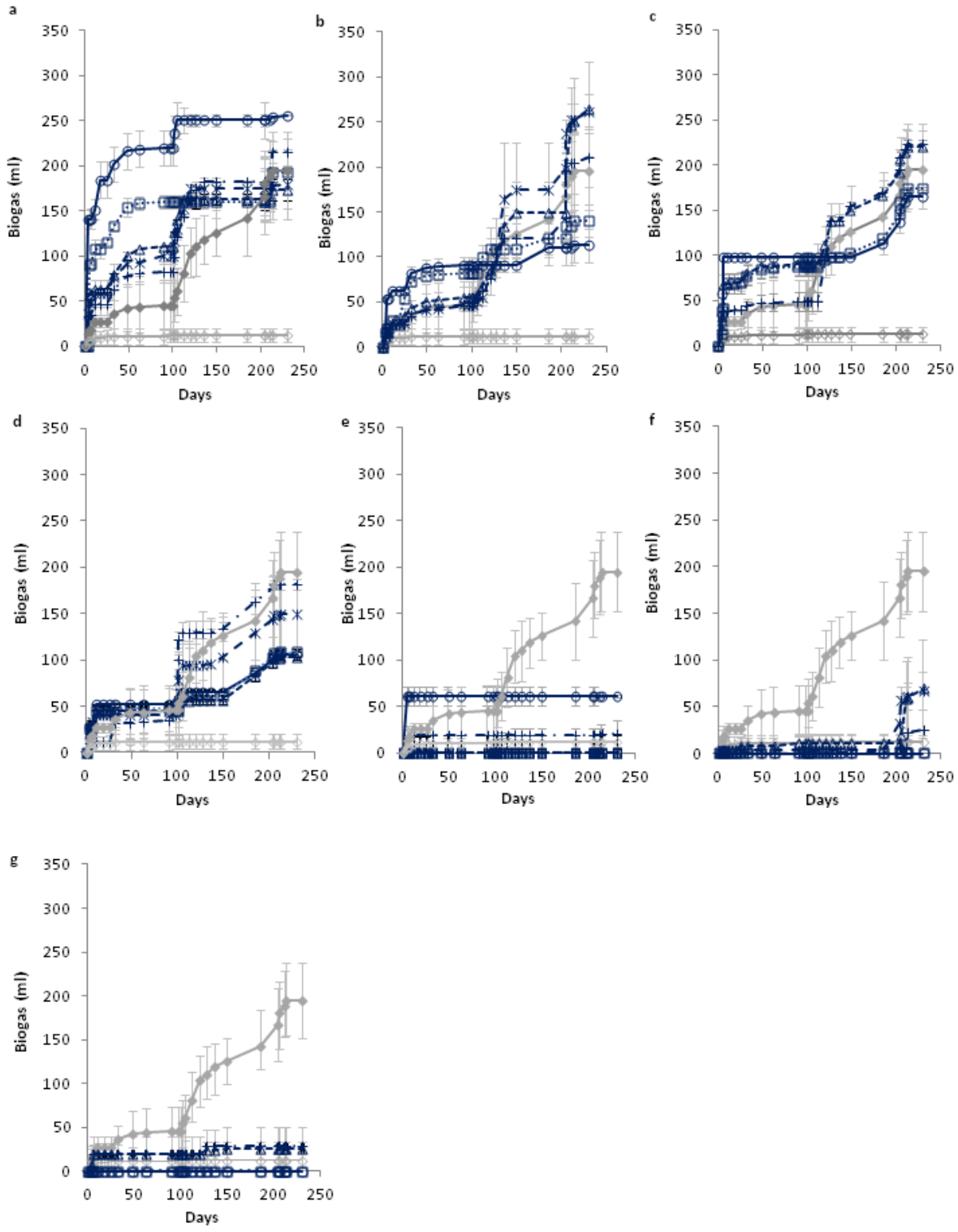

Figure 2: Biogas production (ml ± MD) from the mixtures containing Gl+Sap (a), Gl+Tan (b), Gl+Sal (c), Gl+Alo (d) and Sap alone (e), Tan alone (f), Sal alone (g) where the meaning of O : 13.3 g CPSM/l, : 6.7 g CPSM/l, Δ : 3.3 g CPSM/l, *: 1.7 g CPSM/ l,+: 0.3 g CPSM/l, : 3.3 Gl/l and ◊ : Sludge.

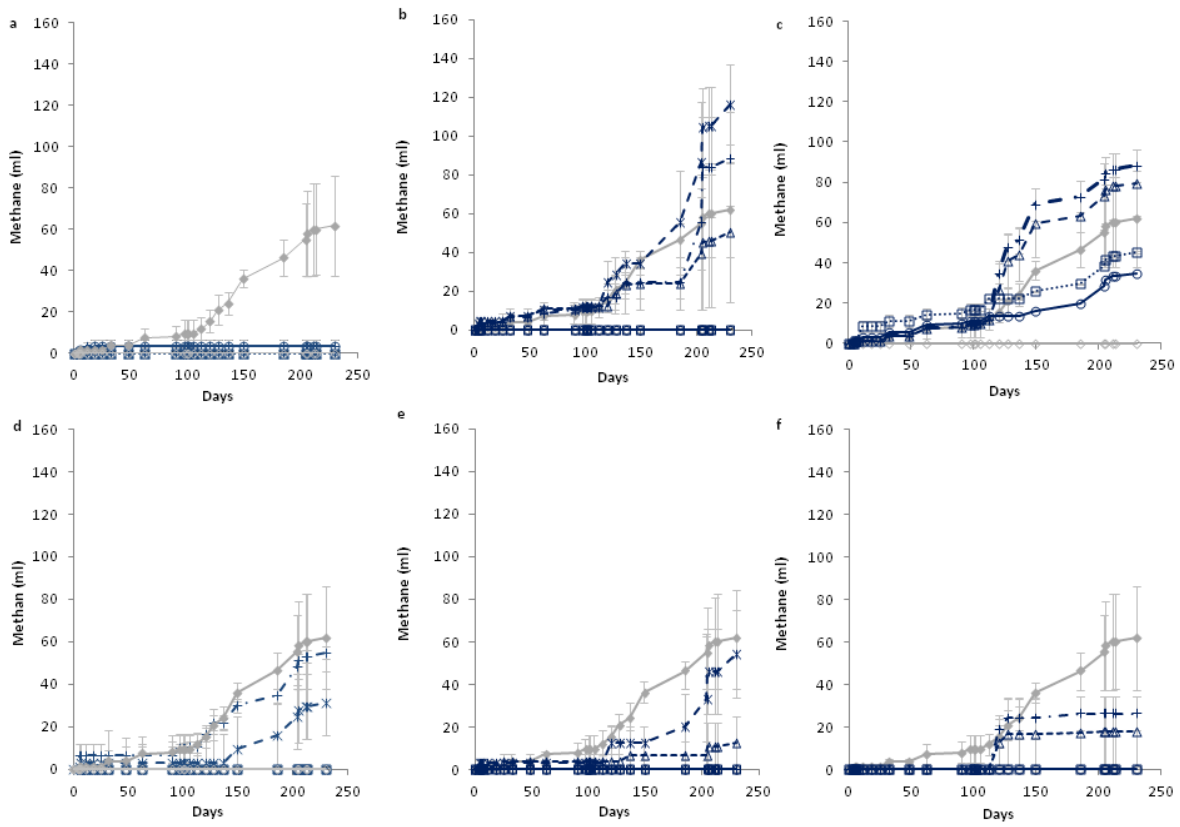

Figure 3: Methane production (ml ± MD) from the mixtures containing Gl+Sap (a) , Gl+Tan (b), Gl+Sal (c), Gl+Alo (d), Tan alone (e), Sal alone (f) where the meaning of O : 13.3 g CPSM/l, : 6.7 g CPSM/l, Δ : 3.3 g CPSM/l, *: 1.7 g CPSM/ l,+: 0.3 g CPSM/l, : 3.3 Gl/l and ◊ : Sludge.

Concentrations of samples		Biogas yields	Methane yields	Biogas inhibition	Methane inhibition
g Gl/l or g Gl/l + g CPSM/l	g C/l	(ml/g VS)	(ml/g VS)	Degrees (%)	Degrees (%)
3.3 g Gl/l	1.3	474.8	171.8		
3.3 g Gl/l+0.3g Sap/l		418.7	12.5	10.7	-94.3
3.3 g Gl/l+1.7g Sap/l		290.5	0	-7.8	-100.0
3.3 g Gl/l+3.3 g Sap/l		185.7	0	-10.7	-100.0
3.3 g Gl/l+6.7g Sap/l		145.5	0	-0.9	-100.0
3.3 g Gl/l+13.3 g Sap/l		103.0	11.7	31.6	-100.0
3.3 g Gl/l+0.3g Tan/l	1.5	444.9	204.5	8.4	42.7
3.3 g Gl/l+1.7g Tan/l	2.2	375.6	182.4	34.4	88.1
3.3 g Gl/l+3.3 g Tan/l	3.1	316.7	86.7	35.6	-18.3
3.3 g Gl/l+6.7g Tan/l	4.9	105.8	0.0	-28.1	-100.0
3.3 g Gl/l+13.3 g Tan/l	8.5	52.7	0.0	-42.2	-100.0
3.3 g Gl/l+0.3g Sal/l	1.5	415.3	174.7	14.4	42.6
3.3 g Gl/l+3.3 g Sal/l	3.1	245.4	96.0	13.1	28.9

3.3 g Gl/l+6.7g Sal/l	5.0	116.6	0.0	-10.9	-26.7
3.3 g Gl/l+13.3 g Sal/l	8.6	66.2	0.0	-15.1	-43.5
3.3 g Gl/l+0.3g Alo/l	1.5	340.4	105.3	-6.8	-11.2
3.3 g Gl/l+1.7g Alo/l	2.3	256.9	61.7	-23.5	-49.5
3.3 g Gl/l+3.3 g Alo/l	3.3	104.2	0.0	-47.4	-100.0
3.3 g Gl/l+6.7g Alo/l	5.4	74.5	0.0	-44.4	-100.0
3.3 g Gl/l+13.3 g Alo/l	9.3	43.5	0.0	-45.6	-100.0

Table 2: Biogas and methane yields of the mixtures containing 3.3 g Gl/l and CPSM (Sap, or Tan, or Sal, or Alo) at concentrations of 0.3 to 13.3 g/l and calculated inhibition degrees after 230 days.

Concentrations of CPSMs		**Biogas yields (ml/g VS)**	**Methane yields (ml/g VS)**
g SV/l	g C/l		
0.3g Sap/l		706.0	0.0
1.7g Sap/l		0.0	0.0
3.3 g Sap/l		0.0	0.0
6.7g Sap/l		0.0	0.0
13.3 g Sap/l		31.7	0.0
0.3g Tan/l	0.2	497.5	0.0
1.7g Tan/l	0.9	347.0	300.2
3.3 g Tan/l	1.8	243.5	24.9
6.7g Tan/l	3.6	0.0	0.0
13.3 g Tan/l	7.1	0.0	0.0
0.3g Sal/l	0.2	670.0	686.2
3.3 g Sal/l	1.8	101.0	68.6
6.7g Sal/l	3.7	0.0	0.0
13.3 g Sal/l	7.2	0.0	0.0
0.3g Alo/l	0.2	0.0	0.0
1.7g Alo/l	1.0	0.0	0.0
3.3 g Alo/l	2.0	0.0	0.0
6.7g Alo/l	4.0	0.0	0.0
13.3 g Alo/l	8.0	0.0	0.0

Table 3: Biogas and methane yields of the different CPSMs after 230 days.

Analysis of glucose, ethanol and VFAs in the mixtures containing Gl and CPSM

The results of the concentrations of VFAs (succinic, formic, acetic, propionic and butyric acids), glucose and ethanol measured in the culture media after 7, 100 and 230 days of biodegradation are shown in Table 4.

Gl or Gl+CPSM	Metabolites(g/l) after 7 days							Metabolites(g/l) after 100 days							Metabolites(g/l) after 230 days					
	Glu	Su	Fo	Ac	Pro	Eth	But	Glu	Su	Fo	Ac	Pro	Eth	But	Glu	Su	Fo	Ac	Pro	But
3.3 g Gl/l	0.0	0.0	0.1	0.4	0.1	0.0	0.1	0.0	0.0	0.0	0.4	0.1	0.0	0.0	0.0	0.0	0.0	0.4	0.0	0.0
3.3 g Gl/l+0.3g Sap/l	0.0	0.0	0.2	0.0	0.0	0.0	0.0	0.0	0.0	1.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.5	0.1	0.0
3.3 g Gl/l+1.7g Sap/l	0.0	0.0	0.0	1.1	0.4	0.0	0.0	0.0	0.0	0.0	0.8	0.5	0.0	0.0	0.0	0.0	0.0	3.1	0.2	0.0
3.3 g Gl/l+3.3 g Sap/l	0.0	0.0	0.2	1.1	0.5	0,1	0.1	0.0	0.0	0.0	1.6	0.9	0.0	0.0	0.0	0.0	0.0	3.8	0.3	0.0
3.3 g Gl/l+6.7g Sap/l	0.0	0.4	0.0	1.8	1.3	0.0	0.2	0.0	0.0	0.0	2.0	1.0	0.0	0.0	0.0	0.0	0.0	4.5	0.3	0.0
3.3 g Gl/l+13.3 g Sap/l	0.0	1.0	0.0	2.1	0.4	0.8	0.6	0.0	0.0	0.0	2.1	0.5	0.0	0.7	0.0	0.0	0.0	8.2	1.2	0.2
3.3 g Gl/l+0.3g Tan/l	0.0	0.0	0.0	0.6	0.2	0.0	0.0	0.0	0.0	0.0	1.1	0.0	0.0	0.0	0.0	0.0	0.0	0.4	0.0	0.0
3.3 g Gl/l+1.7g Tan/l	0.0	0.2	0.6	0.5	0.0	0.3	0.0	0.0	0.0	0.0	1.2	0.1	0.0	0.0	0.0	0.0	0.0	0.4	0.0	0.0
3.3 g Gl/l+3.3 g Tan/l	0.0	0.2	0.7	0.5	0.0	0.2	0.0	0.0	0.0	0.0	1.2	0.4	0.0	0.0	0.0	0.0	0.0	3.9	0.5	0.1
3.3 g Gl/l+6.7g Tan/l	0.3	0.2	0.9	0.6	0.3	0.2	0.0	0.0	0.0	0.0	1.0	0.4	0.0	0.2	0.0	0.0	0.0	6.0	0.8	0.7
3.3 g Gl/l+13.3 g Tan/l	0.0	0.2	1.1	0.7	0.8	0.5	0.0	0.0	0.2	0.4	1.0	0.0	0.0	0.0	0.1	0.1	0.8	1.4	0.8	0.6
3.3 g Gl/l+0.3g Sal/l	0.0	0.0	0.8	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.1	0.1	0.0
3.3 g Gl/l+3.3 g Sal/l	0.0	0.0	0.2	0.7	0.4	0.0	0.0	0.0	0.0	0.0	0.2	0.6	0.0	0.1	0.0	0.0	0.0	1.2	0.1	0.0
3.3 g Gl/l+6.7g Sal/l	0.0	0.0	0.8	1.1	0.4	2.1	0.0	0.0	0.0	0.0	0.6	0.6	0.0	0.6	0.0	0.0	0.0	1.8	0.6	0.4
3.3 g Gl/l+13.3 g Sal/l	0.0	0.3	1.4	1.4	0.2	4.5	0.0	0.0	0.0	0.0	1.7	1.4	9.3	0.0	0.0	0.0	0.0	3.3	1.2	2.3
3.3 g Gl/l+0.3g Alo/l	0.0	0.0	0.6	0.5	0.2	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.2	0.0	0.0
3.3 g Gl/l+1.7g Alo/l	0.0	0.0	0.5	0.6	0.3	0.0	0.0	0.0	0.0	0.0	1.5	0.2	0.0	0.0	0.0	0.0	0.0	1.9	0.5	0.2
3.3 g Gl/l+3.3 g Alo/l	0.0	0.2	0.5	0.6	0.2	0.0	0.0	0.0	0.0	0.0	2.0	0.5	0.0	0.0	0.0	0.0	0.0	2.2	0.8	0.2
3.3 g Gl/l+6.7g Alo/l	0.0	0.2	0.5	0.7	0.2	0.0	0.3	0.0	0.2	0.0	2.4	0.2	0.0	0.3	0.0	0.2	0.0	2.2	0.8	0.7
3.3 g Gl/l+13.3 g Alo/l	0.5	0.3	0.6	0.9	0.3	0.0	0.1	0.4	0.4	0.2	2.4	0.1	0.0	0.8	0.0	0.3	0.3	2.4	0.1	0.9

Table 4: Glucose (Glu), Succinate (Su), Formate (Fo), Acetate (Ac), Propionate (Pro), Ethanol (Eth) and Butyrate (But) production during anaerobic digestion of the mixtures containing Gl+CPSM at concentrations of 0.3 to 13.3 g/l after 7, 100 and 230 days.

Discussion

Biogas yields of the mixtures containing Gl and CPSMs and CPSMs alone

Generality: The biogas production from the blank samples, the control samples and the mixtures containing Gl and CPSM began in the first four days of incubation with the biogas yields of different mixtures of Gl and CPSM lower than that of Gl at 100_{th} day; except the biogas yields of the mixtures of 1.7 g Gl/l with 0.3 g Sap/l and with 0.3 g Alo/l (Figure 2). After the second addition of Gl in the mixtures of Gl and CPSM at the 100th day, the biogas yields of different mixtures increased (Figure 2 and Table 2). That could be explained by a positive effect of the co-digestion of Gl and CPSM due to nutrients released by Gl at this dose enabling microorganisms to degrade the CPSMs or/and Gl or/and to the growth and metabolism of certain microorganisms favored by this dose of Gl. In general, all the CPSMs exerted a beneficial effect on the biodegradation of Gl with a biogas gain only at low concentrations i.e. 0.3 g/l. Except Alo, which inhibited the Gl degradation at all concentrations utilized in this study and the mixture of Gl and Sap which produced again a biogas gain at 13.3 g/l (Tables 2).

Biogas yields of the mixtures of Gl and Sap and Sap alon=

During the first 100 days, the biogas yields decreased with the increase of Sap concentrations in different mixtures with Gl (Figure 2a).

However, after the second addition of Gl, yields of biogas after 230 days of incubation of different mixtures containing Gl and Sap increased comparatively at the 100th day but decreased with the increased of Sap concentrations. That could be due to a progressive inhibitory effect of Sap [25]. Biogas gains were recorded in the mixture of 3.3 g Gl/l and 13.3 g Sap/l after 230 days. That could be explained by

a synergic effect of both substrates Gl and Sap for the growth stimulation of some bacteria species e.g. *Prevotella ruminicola* or the enzymes production leading to partial degradation of Sap or improvement of the Gl biodegradation or of both substrates [25,26]. Indeed, Patra et al. [26] demonstrated a positive effect on feed digestibility at the low dose of sapogenins i.e. 48mg sapogenins/l present in 0.2 g Quilaja saponin/l. However, Sap inhibited partially Gl biodegradation from 1.7 to 6.7 g/l. This partial inhibition on the Gl digestion increased with the increase of the Sap concentrations from 1.7 to 3.3 g/l then decreased with the concentrations of the Sap from 3.3 to 6.7 g/l (Tables 2).

The biogas yield supposed of Sap alone at 0.3 g/l was higher than that of Gl and that of Sap at 13.3 g/l. Sap inhibited totally its own digestion from 1.7 g/l to 6.7g/l (Tables 3). Indeed, it is to be noted that saponin of Quilaja is composed of 24.2% of sapogenin (aglycone) and 75.8% of glycone. By considering this composition in the incomplete molecular formula of Sap in Figure 1, the sole content to the glycosyl group could not exceed 10% since it represents about an eighth of its glycone i.e. most of the biogas supposed produce by Sap alone at 0.3 g/l would come totally from its glycone (saccharide residues). However, the biogas supposed produce by Sap at 13.3 g/l would probably come from the glycosyl group of its glycone (Figure 1).

Biogas yields of the mixtures of Gl and Tan and Tan alon

As showed in Figure 2b and in Table 2, the biogas yields of the mixtures containing Gl and Tan decreased with the increase of Tan concentrations before the 100th day. After the second addition of Gl at the 100th day, the total biogas yields of different mixtures of Gl with Tan recorded after 230 days decreased also with the increase of the Tan concentrations comparatively to Gl biogas yield proving that Tan exerted a certain inhibition on the biodegradation (Figure 2b and Table 2).

However, biogas gains were recorded for the mixtures up to 3.3 g Tan g/l when comparing to the biogas volume produced from the Gl alone and seemed to be directly proportional to Tan concentrations from 0.3 to 1.7 g/l (Tables 2). That demonstrated a positive effect for the co-digestion at this concentration. By contrast, reductions of biogas yields were observed in these mixtures from 6.7 to 13.3 g Tan/l (Tables 2). Thus, these concentrations corresponded to those of partial inhibition of Tan on Gl digestion i.e. 3.6 and 7.1 g C/l and were higher than those of the gallic acid or phenols which were reported to inhibit partially gas production of sludge (inoculum) at concentrations between 0.8 and 1.6 g C/l of phenolic compounds [3].

Indeed, by comparison to Gl biogas volume, it could be concluded that the biogas produced by Tan alone at the 230th from 0.3 to 1.7 g/l would seem to result from the transformation of its ten carboxylate groups which represent 25.9% and its glycosyl group representing 10.6% ,without toxicity (Figure 1 and Table 3). However, biogas yields of Tan alone began to decrease at 3.3 g/l and became 0 from 6.7 g Tan/l (Figure 2f and Table 3).

Biogas yields of the mixtures of Gl and Sal and Sal alone

As showed in Tan case, the biogas yields of the mixtures of Gl with Sal decreased with the increase of the Sal concentrations and biogas gains were recorded at 0.3 and 3.3 g Sal/l (Figure 2c and Table 2). This gain was highest at 0.3g Sal/l. However, a reduction of biogas yield was noted for the mixtures with 6.7 and 13.3 g Sal/l when comparing to that of Gl (Table 2). That showed that a partial inhibition of the biogas production of the Gl by Sal from 3.7g C/l. Sal alone at 0.3g/l and 3.3 g/l would produce biogas volumes nearly equal with biogas indicating that the biogas yields decreased as the concentrations of Sal increased (Figure 2c and Table 2). The biogas yield at 0.3g Sal/l was better than Gl at 3.3 g/l. and this biogas would result from its hydroxymethyl group and its glycone constituted of a sole glycosyl group (Figure 1, Tables 2 and 3). However, Sal inhibited already partially its own biogas production at 3.3 g/l and completely at 6.7g/l (Table 3).

Biogas yields of the mixtures of Gl and Alo and Alo alone

The biogas yields of the mixtures containing Gl and Alo decreased with the increase of Alo concentration (Figure 2d and Table 2) (None of these mixtures produced an additional volume of biogas compared to that of Gl, in spite of the presence of glycone in the Alo which represented 42% of its molecular weight and although this content is higher than that of Tan (Table 2). In this work, Alo starded a partial inhibition on the biodegradation of Gl at 0.3 g/l up to 13.3 g/l. Consequently, Alo alone did not produce biogas (Table 3). That would be probably due to the synergy of toxicity effects of aloin (50%) and polyphenols (23%) such as tannins contained in Alo as suggested by Chen et al. [9].

Methane yields of the mixtures containing Gl and CPSM and CPSMs alone

Generality: Regarding cumulative methane production depicted in Figure 3, no methane was detected for the sludge. That showed that its biogas was essentially composed of carbon dioxide (Figure 2) since it did not contain either H2 after 16 days of incubation. By contrast, the BMP tests with Gl or mixtures of Gl with different CPSMs produced methane. However, the methane yields of these different mixtures decreased when the concentrations of the CPSMs increased (Table 2). Indeed, after 100 days, the methane yields of these different mixtures were similar to that of Gl; except the methane yields of the different mixtures of Gl with Tan and with Sal (Figure 3 and Table 2). Moreover, after day 230 of incubation, methane yields of these different mixtures were lower than that of Gl; except the methane yields of the mixtures of Gl with Tan at concentration of 0.3 and 1.7 g/l and with Sal at concentration of 0.3 g/l (Figure 3 and Table 2). That demonstrated that the high C/N ratio tending towards the infinity, did not inhibit the methanization but slowed its kinetic and the inhibition would be especially due to concentrations of CPSMs.

Methane yields of mixtures of Gl and Sap and Sap alone

Considering the BMP tests with Sap (Figure 3a), only the mixture containing 3.3 g Gl/l and 0.3 g Sap/l produced methane with a yield of 94.3% lower than that of Gl (Table 3). This value was higher than those reported by the studies in animal nutrition domain (10 - 25% with no precision on the saponins concentrations) [25,27-29]. That showed that the biogas produced from the mixture of 3.3 g Gl/l and 0.3 g Sap/l was essentially constituted of carbon dioxide since no H_2 was detected after 16 days of incubation. Thus, Sap inhibited totally the Gl methanization from 1.7 g/l (Tables 2 and 3) and the increase of methanization inhibition with the increase of Sap concentration was in accordance with others studies on saponins [25,30,31].

Indeed, Sap alone was supposed not produce methane by comparison to volumetric methane from Gl although having the glycone in its structure (Figure 1 and Table 3).

Methane yields of the mixtures of Gl and Tan and Tan alone

The methane yields of the mixtures of Gl and Tan decreased when the concentration of Tan increased in the medium (Figure 3b and Table 2). Methane gains were noted for the mixtures of Gl with Tan at concentrations of 0.3 to 1.7 g/l. By contrast, Tan inhibited the Gl methanization partially at concentration of 3.3 g/l and totally from 6.7 g/l (Table 2). This minimal inhibitory concentration was higher to that of tannins (0.7 g/l) reported by Gerardi [32].

Tan alone was supposed to not produce any methane at concentration of 0.3 g/l comparatively to the methane volume of Gl since this concentration would be insufficient for the methablism or the volume of methane produced would be undetectable with the method used (Table 3). However, from concentration of 1.7 g/l to 3.3 g /l, Tan alone supposed to produce methane with a partial inhibition at concentration of 3.3 g/l (Table 3). By referring to the structure of Tan in Figure1, it could suppose that this methane would result from the conversion of its glycosyl and carboxylate groups since its carboxylate groups would be transformed to acetate before its conversion to methane (Figure 3b). By contrast, Tan inhibited completely the methanization of itself from 6.7 g/l (Table 3). This inhibitory concentration was higher than that of phenolic compounds (0.8 and 1.6 g/l) according to Hermandez and Edyvean [3] maybe because tannic acid is a polymeric phenol [33] and also a glycosylated polyphenol.

Methane yields of the mixtures of Gl and Sal and Sal alone

The methane yields of the mixtures containing Gl and Sal decreased when the concentration of Sal increased however there were methane gains for the mixtures of Gl containing 0.3 to 3.3 g Sal/l (Figure 3c and Tables 2 and 3). Moreover, Sal completely inhibited methanization of Gl from the concentration of 6.7 g/l in the mixtures with Gl. Sal alone was supposed to produce methane with a highest yield at concentration of 0.3 g/l comparatively to the methane volume of Gl maybe since it possesses the highest content in glycosyl group among the CPSMs used in this work and its hydroxymethyl substitute would be transformed also to methane by methylotrophic methanogens (Figure 1, Tables 1 and 3). Furthermore, Sal alone inhibited partially its own methanization at concentration of 3.3 g /l and totally from 6.7 g/l. (Table 3)

Methane yields of mixtures of Gl and Alo and Alo alone

Regarding Alo, Figure 3d, Tables 2 show that whatever the Alo concentration, no additional methane production was detected for the mixtures with Gl comparatively to that of Gl alone. This suggests that Alo inhibited the methanization of Gl in any concentration, i.e. partially at concentration of 0.3g/l and totally from1.7g/l (Table2).

Concerning Alo alone, no methane production was recorded. (Table3) That demonstrated that Alo inhibited totally its own methanization from 0.3g/l. This phenomenon might be due to the synergy of inhibitory effects of aloin (50%) and polyphenols (23%) such as tannins contained in Alo as suggested by Chen et al. [9] although Alo possesses glycosyl group.

Evolution of glucose, ethanol and VFAs in the mixtures containing Gl and CPSM

Generality: In general, the total quantities of metabolites (glucose, ethanol, VFAs) produced during anaerobic co-digestion of Gl and CPSMs increased with the CPSM amounts in the bottles (Table 4). The VFAs concentrations were similar in the BMP tests carried out with the same CPSM content. That suggests that hydrolysis and acidogenesis processes were efficient whatever the organic matter as showed in Table 4. The maximum concentration of each VFA measured in the different media was directly proportional to initial substrate concentration and similar trends were recorded for each CPSM (Table 4). VFAs obtained from Gl alone and from all the mixtures of 3.3 g Gl/l and 0.3g CPSM/l stretched to convert to biogas after 230 days of anaerobic digestion (Table 4). The presence of residual amounts of VFAs in these samples after 230 days demonstrated that the high C/N ratio slowed the conversion; however the different anaerobic co-digestions of 3.3 g Gl/l and 0.3 g CPSM/l were faster than that of Gl alone (Table 4). That demonstrated that acidogenesis, acetogenesis and methanogenesis were efficient. After addition of Gl at the 100th day, it was noted accumulations of VFAs in the mixtures with the concentrations above 1.7 g CPSM/l at the 230th day. That showed that the hydrolysis and acidogenesis achieved themselves effectively but acetogenesis and/or metanogenesis were partially or completely inhibited by CPSMs since pH conditions were suitable, between 6.5 and 7.2. The methanogenesis was completely inhibited at CPSM concentration of 3.3 g/l.

Evolution of glucose, ethanol and VFAs in the mixtures of Gl and Sap

From the 7th day to the 100th day of incubation, the VFAs in bottles containing the mixtures of Gl and Sap were essentially converted to carbon dioxide proving the inhibition of methane production (Figure 3 and Table 4). That was confirmed by GC measurements since H_2 was not detected after 16 days of digestion in the biogas obtained from all mixtures. Furthermore as seen in Table 4, there was not an acetogenesis after the acidogenesis in the mixture of Gl and Sap at 0.3 g/l up to 100th day since formate was accumulated. That suggests that Sap would stimulate strains such as *Clostridium acetobutylicum* [34]. By contrast, after the second addition of Gl at the 100th day, acetogenesis started to develop as well as methane production with a weak yield and a presence of a few amount of propionate after 230 days. This small accumulation of VFAs could be due to the reduction of their conversion rates because of the high C/N ratio. It was observed also a classical acidogenesis and acetogenesis but there was not a methanogenesis in other media with concentration above 0.3 g Sap/l after 230 days (Table 14). It is also to notice that the amounts of VFAs were similar in the different mixtures of Gl and Sap from the concentration of 3.3 g Sap/l after 100 days of incubation. However, after the addition of Gl at the 100th day, these VFAs amounts were doubled at the 230th day; except in the mixture with 13.3 g Sap/l where the quantity of VFAs was tripled (Table 4). That confirmed the positive effect of Gl on the biodegradation of itself and on that of Sap relative to both concentrations with an additional biogas composed essentially of carbon dioxide (Figures 2 and 3, and Table 2). However, this observation about Sap contradicted most studies in animal nutrition reporting that saponins favor the increase of propionate production [25,30,35]. Besides, the VFAs concentrations recorded proved that these VFAs resulted from the conversion of Gl and the glycone of Sap. Thus, the inhibition of methanogenesis might be especially due to the toxicity of Sap exerted on the methanogens from the concentration of 1.7 g/l since the VFAs concentrations recorded would not be enable to inhibit alone the methanization completely according to Buffiere et al. [36].

Evolution of glucose, ethanol and VFAs in the mixtures of Gl and Tan

Concerning the mixture of Gl and Tan, it was observed at the 100th day accumulations of VFAs; however they were lower than in Sap mixtures with Gl (Table 4). Table 4 also shows that during the first 100 days, all the stages of methanization were affected by the increase of the Tan concentration in the mixtures with Gl since the VFAs production did not proportionally increase with Tan concentrations. That explained why the yields of biogas were lower than those of the mixtures of Gl and Sap. This phenomenon was in accordance with that observed in the degradation of feed when the tannins are used as described in the literature [25,34,37,38]. However, it was observed higher methane yields than those of mixtures of Gl with Sap. The addition of Gl after 100 days of incubation seemed to stimulate the methanization in the mixture of Gl with 0.3 to 1.7 g Tan/l as in the mixtures of Gl and Sap. That was detectable thanks to the considerable reduction of VFAs with very small amount of residual VFAs at the 230th day. It was recorded accumulations of VFA in the mixtures of Gl with concentrations of Tan above 1.7 g/l indicating toxicity effects. The lowest quantity of VFAs in the mixture of Gl with 13.3 g Tan/l among the inhibitory concentrations; although Tan were totally soluble at this concentration according to Table 1, confirmed that even the hydrolysis of the substrates were affected by the toxicity of Tan since this concentration corresponds to 8.4 g/l in phenolic groups (aglycone) which are responsible of the inhibition of bacterial activity [25,39,40]. Then, methanogenesis was completely inhibited at 13.3 g Tan/l.

Evolution of glucose, ethanol and VFAs in the mixtures of Gl and Sal

The total amounts of metabolites observed at the 7th day decreased at the 100th day in the mixtures containing Gl and Sal with the highest methane yield at 0.3 g Sal/l. However in the mixture with 13.3 g Sal/l, there was an important accumulation of ethanol suggesting that big producers of the ethanol such as *Ruminococcus gnavus* or Bromiis [41] would be activated by these substrates in co-digestion (Table 4). After the second addition of Gl in the mixtures containing Gl and Sal at the 100th days of incubation, the acidogenesis and acetogenesis were stimulated since the ethanol would be converted to butyrate, propionate and acetate, leading to a reduction of total amount of metabolites accumulated at the 230th day of the anaerobic degradation; however without production of methane for the mixtures of Gl with 6.7 and 13.3 g Sal/l (Figure 3 and Tables 2 and 4).

Evolution of glucose, ethanol and VFAs in the mixtures of Gl and Alo

By considering Figure 2d and Table 4 , it is clearly demonstrated that during the first 100 days, the anaerobic digestion of the mixtures of Gl and Alo was slow that could be due to the high C/N ratio and especially to the synergic inhibitor effect of alo in and polyphenols contained in Alo. It was noted accumulations of VFAs coming from the conversion of Gl and of the glycone of Alo after 100 days of incubation in the mixtures of Gl and Alo at all concentration; except at 0.3 g Alo/l. where quite little acetate was recorded but without a supplementary production of biogas. This effect was similar to that observed in the study on the anaerobic degradation of *Manguifera Indica* leaves from 13.3 g/l containing anthraquinones and other PSMs [7]. Furthermore, the poverty in VFAs by comparison to others CPSMs and the presence of residual glucose at the 100th day in the media at the concentration of 13.3 g Alo/l, indicated that the hydrolysis, acidogenesis and acetogenesis were slowed and that methanogenesis was not effective. Gl added in the mixtures of Gl with Alo after the 100th day was completely consumed at the 230th day and the amounts of metabolites (VFAs and the other) were slightly greater than that at the 100th day and there was no glucose in the media. That suggests that only the acidogens bacteria would be stimulated in the mixtures of Gl with 3.3 to 13.3 g Alo/l that would explain why there were not production of methane.

Conclusion

In this paper, BMP tests were carried out with the glycosidic PSMs frequently released in water by vegetal wastes and present in certain industrial effluents. Except for the mixtures of Gl and Alo, the anaerobic co-digestion of these bioactive substances at the concentration of 0.3 g/l with Gl was achieved without inhibition in media with C/N ratio tending toward the infinity, compared to the digestion of Gl alone but with a slowing. Furthermore, the amount of biogas produced from each CPSM in this concentration seemed to be resulted from its glycone. During the biodegradation, each CPSM had its metabolic pathways; thus, Sap, Tan, Sal and Alo favored specifically the production of propionate, formate, ethanol and butyrate, respectively. It is necessary to note that the stimulating effects of biodegradation by Gl or CPSM would be relative to the ratio Gl/PSM which changed according to the structure of the CPSM.

Indeed, it was to notice that the inhibition was amplified by the concentrations of CPSM i.e. with the content of aglycone in the media and the synergism Reason why, glycosylated phenolic compounds would be less toxic then non-glycosylated. Thus, the highest inhibitor effect on the digestion of Gl was recorded with Alo followed by Sap, Tan and Sal. By contrast, the highest inhibitor effect on the methane production from the Gl was recorded with Sap followed by Alo, Tan and Sal. The inhibition potential of each on its own biomethanization according to the calculations would be in the following decreasing order: Sap=Alo>Tan>Sal.

Therefore, it would be very important to avoid these chemical compounds alone or mixed in a bioreactor at concentrations equal or superior to 0.3 g/l for a good anaerobic digestion. Otherwise, the anaerobic digestion of PSMs can be considered as a bio-refinement way for producing cyclic hydrocarbons or aromatic compounds or VFAs

In-depth further investigations will be carried out on the anaerobic digestion of glycosidic PSMs alone for methane production, on the synergic effect of these bioactive compounds in anaerobic digestion and on the impact of glucose on PSMs anaerobic co-digestion, especially on that of saponins.

Acknowledgement

The authors wish to thank the Belgian Technical Cooperation (BTC) for providing financial assistance to Philippe Mambanzulua Ngoma.

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