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Stable isotopes of C and H in methane fermentation of agriculture substrates at different temperature conditions

Abstract: Agricultural substrates (maize silage and cattle manure) were used to carry out methane fermentation process in bioreactors under laboratory conditions. Identical mixtures of these substrates were incubated for 43 days at 20, 30 and 40ºC to determine how different temperature conditions affect the $\delta^{13}C(CH_4)$, $\delta H(CH_4)$, and $\delta^{13}C(CO_2)$ values. To ensure correct anaerobic digestion, the following parameters of the organic substrates and fermentation solutions were monitored: total organic carbon (TOC), volatile solids (VS), volatile fatty acids (VFA), chemical oxygen demand (COD) and carbon to nitrogen ratio (C/N). The variants with higher incubation temperature yielded higher amounts of biogas (20ºC=84.5, 30ºC=101.8 and 40ºC=133.3 dm^3/kg VS). In the case of gas products of methane fermentation, it was observed that the higher temperature of incubation affects the depletion in heavy isotopes. At 20ºC, 30ºC, and 40ºC mean values of $\delta^{13}C(CH_4)$ reached −26.4, −29.7, and −35.4‰, respectively. Mean values of $\delta^2H(CH_4)$ were −311.6, −354.0, and −398.5permil, and of $\delta^{13}C(CO_2)$ +8.9, +3.7, and −2.3‰, respectively. Moreover, the apparent fractionation coefficient $\alpha^{13}C(CO_2$-$CH_4)$ were calculated, which decreased when the temperature increased. This isotopic tool was used to identify acetoclastic reaction as a dominant methanogenesis pathway. Observed changes in the isotopic composition of gaseous products obtained at different incubation temperatures may indicate decomposition of different carbon sources (e.g. lactate, propionate) to acetate and its fermentation by acetoclastic methanogens. It is possible that this was also related to the observation of the various metabolic models due to the varied methanogenic community composition.

1 Introduction

Anaerobic digestion (AD) is a microbiological decomposition of high molecular organic compounds yielding, among others, methane and carbon dioxide [1, 2]. A specific community of hydrolysing and acetogenic microorganisms as well as acetoclastic methanogens (Archaea), is responsible for the methane fermentation process [3]. Such processes are observed in many environments, eg. in marshes, wetlands, river sediments, in many coal basins, on landfills, as well as in the digestive tract of ruminants [16, 47].

AD depends on a number of physical and chemical factors, including chemical composition of the substrate, nutrient concentration, fermentation temperature, pH, volatile fatty acid composition, total nitrogen content, toxic substance content, hydration, salinity and dissolved oxygen level. These factors may stimulate or inhibit the speed and efficiency of the biological conversion of organic matter [4–7].

Research in the area of isotope geochemistry, including isotopic fingerprints of carbon and hydrogen, are commonly used to distinguish gas origin (thermogenic, biogenic), and to distinguish the main methane generation pathways [45, 46]. In natural environment methane production usually follows two main paths (apart from others) [8]. It is either a product of a biological decomposition of acetic acid by heterotrophic microorganisms [9] or

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reduction of carbon dioxide by autotrophic microorganisms [10]. According to Fey et al. [11], in natural environment, methane production is most often observed via acetoclastic methanogenesis, and then via hydrogenotrophic methanogenesis. Whiticar et al. [12] noticed that the isotopic composition of carbon in the methane generated during decomposition of acetic acid ranged from −60‰ to −33‰, while for the methane produced from carbon dioxide the value of δ^{13}C varied from −110‰ to −60‰. Stable isotope analysis of carbon and hydrogen in raw materials and fermentation products might be useful to identify metabolic pathways of the fermentation [13, 14]. It is also a tool used to monitoring and describing the AD process state [15]. The isotopic analysis is important in studies on methane fermentation as it improves our understanding of complex biological processes. It allows for determining the efficiency of the methanogenesis and enables preliminary identification of carbon sources preferred by the methane-producing microorganisms [16].

Research on the interactions between bacterial methanogenesis and methanotropy are still in the area of interest among geochemists and microbiologists [16, 35, 45, 46]. This article outlines a way to identify methanogenic pathways in a precise manner using techniques related to stable isotope mass spectrometry. Moreover, it is possible to track changes in biogas production and the isotopic composition of substrates and products depending on the temperature used during methane fermentation. In these experiments agricultural raw materials such as maize silage and cattle manure served as substrates for experimental methane fermentation in bioreactors under controlled laboratory conditions. Bioreactors contained identical proportions of substrates, but were run under different temperature (20, 30 and 40ºC).

2 Materials and methods

2.1 Basic laboratory analyses

Laboratory analyses of the raw materials and tap water preceded the fermentation experiments. Determination of total solids (TS) followed PN-EN 12880:2004 standard [36], and of volatile solids (VS) PN-EN 12879:2004 standard [37]. Total nitrogen was measured by Kjeldahl method [39] and ammonium nitrogen by photometric method (Photo-Lab 6600 UV-VIS) [43]. Total organic carbon (TOC) was determined with a dry combustion method [44]. Chemical oxygen demand (COD) was assessed as per PN-ISO

6060:2006 standard [38], and the content of volatile fatty acids (VFA) by extraction coupled with gas chromatography [40]. Among the analyzes regarding the tap water (TW), determination of the ammonium ion ($NH4+$) was carried out in accordance with the PN-ISO 7150: 1: 2002 [41] standard, free and combined chlorine according to the PN-EN ISO 7393-2: 2011 [42] standard.

2.2 Design of anaerobic digestion experiments

Maize silage and cattle manure were used for fermentation experiments. The research was conducted in three Sartorius Biostat A Plus bioreactors with a working capacity of 6.5 dm^3, and the ability to control physical and chemical parameters (pH, temperature, oxygen content and redox potential). Raw materials (200 g maize silage + 200 g cattle manure) were placed in the bioreactors filled with 4.6 dm^3 of tap water and set to three different temperatures (20°C, 30°C, 40°C). Experiments were named FA=20°C, FB=30°C and FC= 40°C. The fermentation chambers were closed with a tight lid preventing gas exchange. Incubation was carried out for 43 days. The temperature was maintained by a heating mantle and a cooler, connected to each fermentation chamber. Methane fermentation parameters (temperature and solution mixing speed) were set using PC-based operation software and controlled by μDCU software installed on the control unit. The chamber content was stirred with a mechanical paddle at 60 rpm to maintain a constant temperature of the entire batch. The pH value was monitored with Hamilton Easyferm plus K8 325 process electrode, and oxygen content with Hamilton Oxyferm FDA 325 process electrode.

To determine qualitative and quantitative composition of the gas released in the bioreactors, we measured the volume of the generated biogas (overpressured gas) with a syringe each day and collected samples for chromatographic and isotopic analysis. We used a flame-sterilized syringe needle. The gas was collected into sealed glass ampoules (20 cm^3) with a PTFE septum, filled with redistilled water and stored in a refrigerator at 4°C. Concentration of methane in the biogas was determined using gas chromatograph AGILENT 7890A in Laboratory of Oil and Gas Geochemistry at the Oil and Gas Institute in Krakow, equipped with FID, TCD detectors. For chromatographic analysis at least 1 cm^3 of the biogas was used. Analytical error of the tests was ± 1%.

2.3 Isotopic analysis

Preparation of the substrates and post-digestion residues for measurement of carbon isotopes (δ^{13}C) from organic matter was carried out offline on a vacuum line. The samples were placed in quartz vials with copper oxide and burned at $900°$C. The combustion products were cryogenically separated to obtain pure CO_2 [14], which was subjected to isotopic ratio measuring with a mass spectrometer. The results were normalized to international V-PDB standards. The measurement error was 0.3‰.

Gas products (headspace gas) were analyzed for δ^{13}C(CH$_4$), δ^{13}C(CO$_2$), and δ^{2}H(CH$_4$). Gas samples were prepared offline on the vacuum line. In order to separate the biogas components, approximately 4-5 cm^3 of the gas were injected on the vacuum line through a teflon septum. Next, carbon dioxide and water (moisture from the biogas) were frozen on a spiral trap using liquid nitrogen. The biogas fraction not frozen in the presence of liquid nitrogen (e.g. methane), was adsorbed on molecular sieves cooled with liquid nitrogen. The methane deposited on the sieves was heated up to room temperature, dosed in aliquots and burned in a furnace with copper oxide at $900°$C. The gases resulting from the combustion of methane (CO_2 and H_2O) were frozen in a spiral trap cooled by liquid nitrogen. Then, using a mixture of ethanol and dry ice, CO_2 was cryogenically separated from H_2O. The separated CO_2 was frozen in a glass ampoule. As a next step, the spiral trap was heated to $500°$C and H_2O from methane combustion was collected into another glass ampoule using liquid nitrogen. Finally, pure CO_2 held in a spiral freezer was collected into a glass ampoule. After that, H_2O was gathered in the last glass ampoule and reduced using pre-prepared activated zinc [17]. The CO_2 and H_2O from methane combustion and CO_2 as the primary biogas component were subjected to mass spectrometry. Composition analysis of δ^{13}C(CH$_4$), δ^{2}H(CH$_4$) and δ^{13}C(CO$_2$) was carried out on Thermo Scientific Delta V Advantage IRMS mass spectrometer. Measurement error for δ^{13}C was 0.1‰ and for δ^{2}H 2‰. The results for C and H isotope ratios were normalized to international standards Vienna Pee Dee Belemnite (V-PDB) and Vienna Standard Mean Ocean Water (V-SMOW), respectively.

3 Results

3.1 Characterization of raw materials and fermentation solutions

Table 1 presents the results of basic laboratory analyzes of raw materials and post-digestion residues (fermentation solutions). TOC of maize silage (MS) and cattle manure (CM) reached 56.8% and 42.5%, respectively. In the pre-fermentation solution (F) TOC measured in a 1:1 mixture of MS and CM was 53.6%, while in the fermentation solution after the incubation experiment at $20°$C the final TOC was 34.3%, at $30º$C (FB) it was 33.9%, and at $40°$C (FC) 33.5%.

Table 1 also presents δ^{13}C composition in the organic matter derived from the raw materials. It reached −12.8‰ for MS and - 23.3‰ for CM. The content of δ^{13}C in the organic matter from the fermentation mixture before the incubation (F) was −16.8‰ and post-fermentation residues from three temperature variants (FA $20°$C, FA $30°$C, FA $40°$C) ranged from −17.8 to −24.1‰, depending on the variant. The difference between the initial and final isotopic composition of the fermentation solutions ($\varDelta$13Cb-e‰), was in the range from 1.1‰ to 7.3‰.

VS content in MS and CM was 96.1% and 87.5%, respectively. VS of the fermentation solution before experiments (F) was 94.4%. After incubation it dropped to 71.4% (FA), 70.1% (FB), and 68.6% (FC) (see Table 1). Ammonium and organic nitrogen equalled 0.2 TS% and 1.5 TS% for MS, 0.9 TS% and 5.3 TS% for CM, and 0.7 TS% and 1.9 TS% for F, respectively. C/N ratio for the fermentation mixture (F) was 27.8. COD and VFA measured at the beginning and at the end of the incubation significantly decreased, particularly at higher incubation temperature (see Table 1).
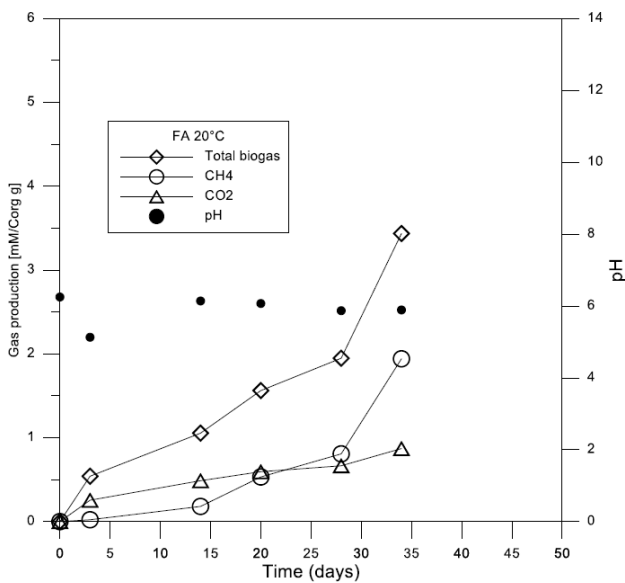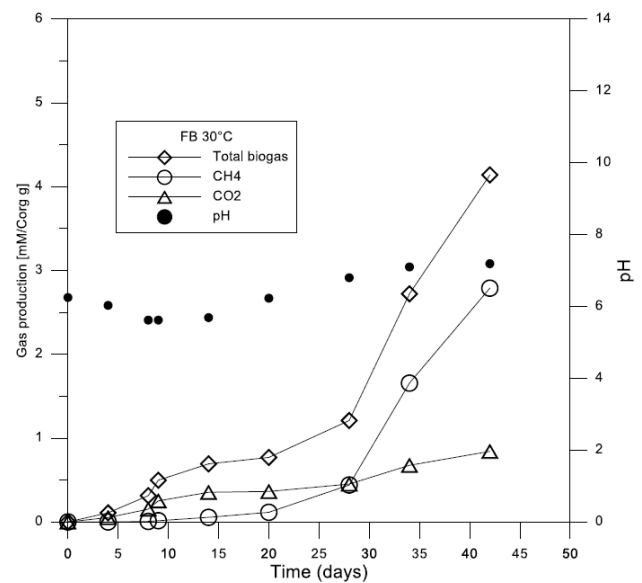
3.2 Biogas production

In each experiment pH ranged from about 5.1 to 7.9, depending on the methane fermentation stage. In the first days, acidification usually occurred and then the pH oscillated around neutral. As the experiment progressed, we observed a downward trend in oxygen content in the fermentation solution.

The yield of biogas production was 3.44 in FA variant, 4.14 in FB variant, and in FC was 5.42 mM of gas per gram of organic carbon (see Fig. 1). At higher experimental temperatures, the biogas production increased (FA=84.5, FB=101.8 and FC=133.3dm^3/kg VS), and the maximum of its production occurred sooner, than at the lowest temperature (FA=43 days, FB=36 days, FC=34 days).

Table 1: Initial and operating geochemical parameters of the experiments.

Sample	Maize silage (MS)	Cattle manure (CM)	Tap Water (TW)	F (MS:CM mixture, 1:1)	FA 20°C	FB 30°C	FC 40°C
TOC [%]	56.8	42.5	n.a.	53.6	34.3	33.9	33.5
Δ TOC [%]	n.a.	n.a.	n.a.	n.a.	19.3	19.8	20.1
δ^{13}C [‰]	−12.8	−23.3	n.a.	−16.8	−17.8	−22.8	−24.1
Δ^{13}C [‰]	n.a.	n.a.	n.a.	n.a.	1.1	5.9	7.3
VS [%]	96.1	87.5	n.a.	94.4	71.4	70.1	68.6
Δ VS [%]	n.a.	n.a.	n.a.	n.a.	23.0	24.3	25.8
N am [TS%]	0.2	0.9	n.a.	0.7	n.a.	n.a.	n.a.
N org [TS%]	1.5	5.3	n.a.	1.9	n.a.	n.a.	n.a.
C/N	38.4	8.1	n.a.	27.8	n.a.	n.a.	n.a.
COD [mg O_2/kg TS]	n.a.	n.a.	n.a.	42186	5564	5464	3594
VFA [mg/l]	n.a.	n.a.	n.a.	1504	194	49	124
VFA[mg CH_3COOH/l]	n.a.	n.a.	n.a.	1337.6	28.2	4.8	3.9
pH	3.7	7.4	7.5	n.a.	n.a.	n.a.	n.a.
(NH_4^+) [mg/l]	n.a.	n.a.	<0.05	n.a.	n.a.	n.a.	n.a.
Free chlorine [mg/l]	n.a.	n.a.	<0.03	n.a.	n.a.	n.a.	n.a.
Combined chlorine [mg/l]	n.a.	n.a.	0.13	n.a.	n.a.	n.a.	n.a.
EC [μS/cm]	n.a.	n.a.	658	n.a.	n.a.	n.a.	n.a.

n.a. – not analyzed

Figure 1: Biogas production over time in the FA agriculture substrate incubation experiment.

Figure 2: Biogas production over time in the FB agriculture substrate incubation experiment.
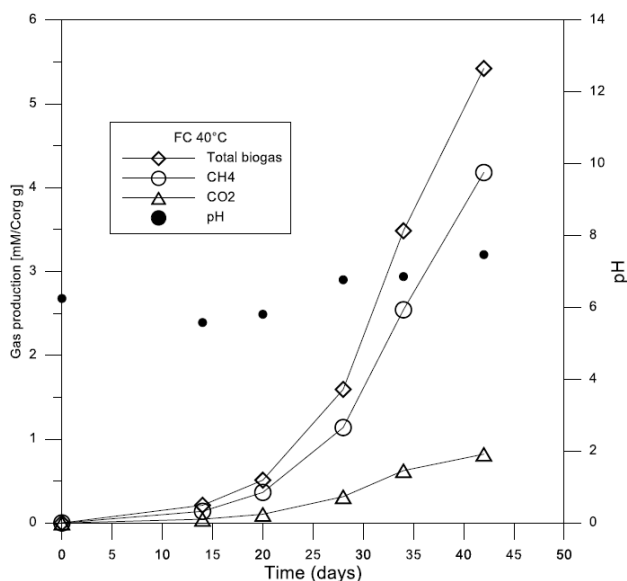
Figure 3: Biogas production over time in the FC agriculture substrate incubation experiment.

Figure 4: Time-dependent values of $\delta^{13}C(CH_4)$, $\delta^{13}C(CO_2)$, $\delta^2H(CH_4)$ and pH in FA experiment.

Gas chromatography first detected methane presence on the third day of FA experiment and it was 0.02 $[mM]/C_{org}[g]$. Methane concentration was the highest on 42nd day of FC experiment, when it reached 1.64 $[mM]/C_{org}[g]$. In all experiments, methane concentration increased around 14th day of the incubation and grew until the last day of the observation. Methane concentration in all experiments was in the range from 0.01 to 1.64 $[mM]/C_{org}[g]$ (see Tab. 2). An average concentration of methane in all headspace gas samples from the experiments presented in $[mM]/C_{org}[g]$ and it was 0.39 for FA variant, 0.35 for FB, and 0.84 for FC. Carbon dioxide concentration fluctuated from 0.01 to 0.31 $[mM]/C_{org}[g]$ in all experiments (see Tab. 2). An average concentration of CO2 in all headspace gas samples from the experiments presented in $[mM]/C_{org}[g]$ and it was 0.17 for FA variant, 0.11 for FB, and 0.16 for FC.

3.3 Isotopic analysis of headspace gases

Isotopic composition of methane and carbon dioxide in the collected biogas changed over time. In the FA variant (see Fig. 4), at 20°C, $\delta^{13}C(CH_4)$ ranged from −20.1‰ at the beginning of the study to −30.0‰ at the end of the study. Negative trend and decrease in value by nearly 10‰ from 3th day of incubation to 34th day with one episode of enrichment in heavy carbon isotopes on 28th day of incubation has been observed. The value of $\delta^2H(CH_4)$ varied from −370.3 to −253.6‰. Negative trend and decrease in value
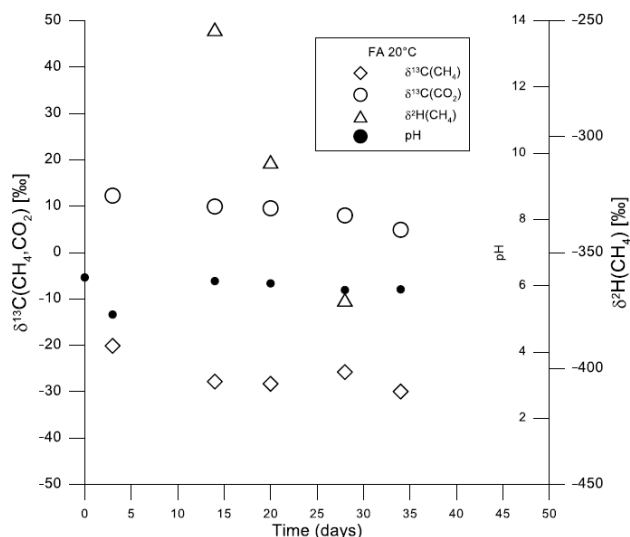
by nearly 117‰ from 14 day of incubation to 28 day has been recorded. Contrary to that, $\delta^{13}C(CO_2)$ values ranged from +4.9 to +12.3‰ and also a negative trend and a decrease in the value of 7.4‰ from the 3rd day of incubation to the 34th day has been observed.

In FB variant (see Fig. 5), where the incubation was carried out at 30°C, $\delta^{13}C(CH_4)$ values varied from −41.3 to −19.9‰, $\delta^2H(CH_4)$ values ranged from −379.4 to −294.5‰, and $\delta^{13}C(CO_2)$ values varied from −10.0 to +13.9‰. In the case of $\delta^{13}C(CH_4)$, a negative trend and a decrease in value by almost 15‰ from the 8th to 28th day of incubation has been observed, followed by a change in the trend and enrichment of the values in heavy isotopes by over 20‰ to 42nd of incubation. Regarding changes in the $\delta^2H(CH_4)$, a negative trend has been observed from 8th to 28th day of incubation and a decrease in value by 46‰, followed by a change in the trend and enrichment the values in heavy isotopes of carbon by 9.5‰ up to the 42nd day of incubation. In the case of $\delta^{13}C(CO_2)$, also a negative trend and a decrease in value by nearly 24‰ from 4 to 20 days of incubation has been observed, followed by a change in the trend to positive and enrichment the values in heavy carbon isotopes by 6.5‰.

The FC variant (see Fig. 6), in which methane fermentation proceeded at the highest temperature of 40°C, was characterized by $\delta^{13}C(CH_4)$ values varying from −43.7 to −26.8‰. $\delta^2H(CH_4)$ values ranged from −410.4 to −380.0‰, and $\delta^{13}C(CO_2)$ from −8.3 to +2.5‰. In this experiment, in the case of $\delta^{13}C(CH_4)$ and $\delta^{13}C(CO_2)$, a clear negative trend and a decrease in the value of nearly 12‰ and 11‰ respectively from the 14th to 28th day of incuba-

Table 2: Characteristics of gaseous products from incubation experiments of agriculture substrates at different temperatures.

	FA 20°C	FB 30°C	FC 40°C
Biogas yield [mM]/C_{org}[g]	3.44	4.14	5.42
CH4 [mM]/C_{org}[g]	0.02 – 1.13	0.01 – 1.22	0.14 – 1.64
CO2 [mM]/C_{org}[g]	0.07 – 0.26	0.01 – 0.22	0.04 – 0.31
$\delta^{13}C(CH_4)$ [‰]	−30.0 to −21.1	−41.3 to −19.9	−43.7 to −26.8
$\delta^2H(CH_4)$ [‰]	−370.3 to −253.6	−379.4 to −294.5	−410.4 to −380.0
$\delta^{13}C(CO_2)$ [‰]	+4.9 to +12.3	−10.0 to +13.9	−8.3 to +2.5
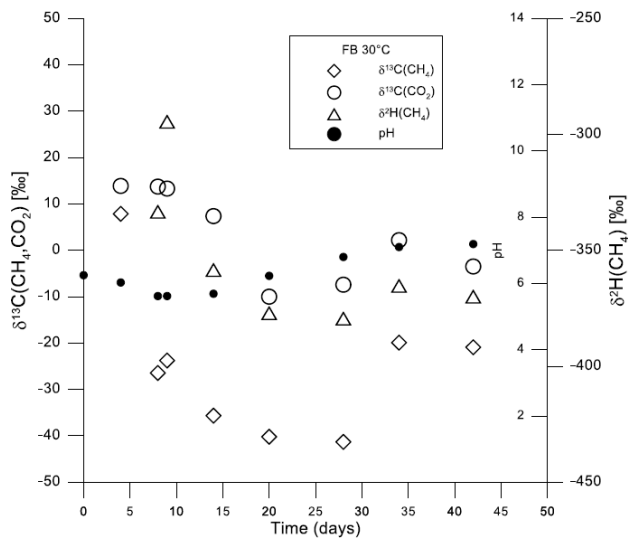
Figure 5: Time-dependent values of $\delta^{13}C(CH_4)$, $\delta^{13}C(CO_2)$, $\delta^2H(CH_4)$ and pH in FB experiment.
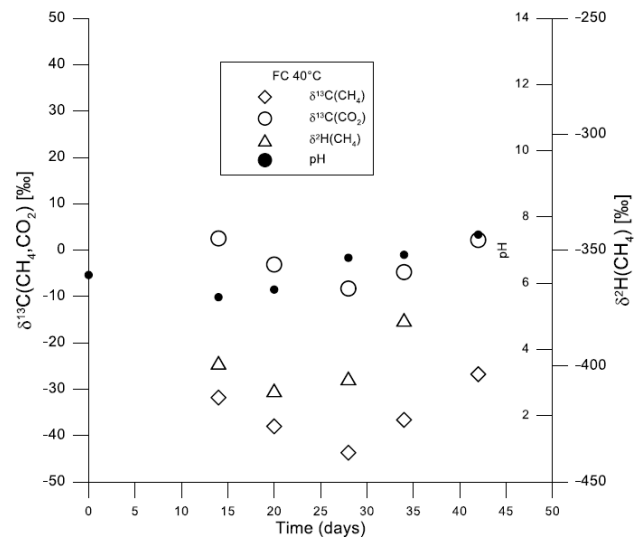
Figure 6: Time-dependent values of $\delta^{13}C(CH_4)$, $\delta^{13}C(CO_2)$, $\delta^2H(CH_4)$ and pH in FC experiment.

tion has been observed, and then change the trend to positive and enrichment the value in heavy isotopes by nearly 17‰ and 10.5‰, respectively, until the 42nd day. Values of $\delta^2H(CH_4)$, changed in a similar way, from the 14th to the 20th day of incubation, a negative trend and a drop in value by almost 12‰ has been observed, and then the trend changed to positive and enrichment in heavy isotopes by over 30‰, up to 34th day of incubation has been observed.

4 Discussion

Maintaining kinetic equilibrium at individual fermentation stages is important for proper course of anaerobic digestion. For this reason, it is crucial to ensure optimal starting conditions for fermentation e.g. by providing the right composition of fermentation solutions with an optimal amount of nutrients for microorganisms [18]. In our experiment, C/N ratio of the substrates was 27.8 (see Table 1), which is optimal for methane fermentation [19–21].

The other vital parameters of anaerobic digestion include VFA and COD. During the process, the production of volatile fatty acids increases and intensifies the biogas generation. This enhances fermentation of the substrates [22, 23].

In properly run AD, VFA are removed from each kg of COD loads, and the biogas results from microbial activity. High concentration of VFA may inhibit anaerobic digestion [24].

A comparison of VFA and COD values at the beginning and at the end of the experiment (see Table 1) showed a significant decrease in their concentration. At 20°C VFA and COD dropped by 86.8 and 87.1%, respectively, at 30°C the decrease was 87.0 and 96.7%, and at 40°C it peaked at 91.5 and 91.8%. Data analysis indicates that the use of a higher temperature of AD is associated with a decrease in the concentration of VFA and COD as well as with increase of the yield of biogas production (FA=84.5, FB=101.8 and FC=133.3 dm^3/kg VS).

The TOC value decreased from 53.6% (SD 0.42) to FA = 34.3% (SD 0.27), FB = 33.9% (SD 0.87), FC = 33.5 (SD 0.14) at the end of incubation. This indicated partial consumption of organic carbon derived from the substrates during the AD.

The influence of the higher process temperature on biodegradation of organic carbon from the substrates was found. The degree of organic carbon consumption, determined on the basis of TOC, varied from 19.3 to 20.1% for the lowest and the highest temperature (see Table 1). To prove the above statement, ΔVS was calculated and it also showed partial consumption of the substrates during fermentation. Results varied from 23.0 to 25.8% between experiments (see Table 1).

Plant materials most often used for anaerobic digestion include agricultural substrates such as maize silage and other plant silages, slurry, manure, or decoction distillers. Analysis of literature provides information about carbon isotope composition of the above components (see Table 3). In comparison with other raw materials, maize silage (C4 photosynthesis plant product) is significantly more abundant in heavy isotopes of carbon and in our study δ^{13}C value was −12.8‰, which is comparable with literature indications [25, 26]. Other raw materials used in AD in this study (cattle manure) show that the composition of the carbon isotope was −23.3‰ (see Table 1).

Table 3: Isotopic composition of substrates most frequently used for AD.

Substrate	δ^{13}C [‰]	Reference
Maize silage	−11.8	[25]
Grass silage	−29.6	[25]
Slurry	−27.0	[28]
Cattle manure	−26.0	[29]
Distillery decoction	−26.9	own data

In Table 1 shows the isotopic composition of the fermentation mixture measured at the beginning (F) and residue after the incubation at the end (FA, FB, FC) of AD. Noticeable changes in δ^{13}C as a result of microbial activity at different temperatures were observed. In each experiment the residue after the incubation was depleted in heavy carbon isotopes, which was similar to a report published by Bucha et al. [29].

At the highest temperature (FC=40ºC) the difference between the initial and final isotopic composition (Δ13Cb-e) of the fermentation solution was over 7‰, while at the lowest temperature (FA=20ºC) it was only 1‰ (see Table 1). We expected a reverse tendency, i.e. enrichment of the

residue in heavy carbon isotopes as light isotopic compounds reacts faster than heavier. However, this process is often observed in case of degradation of single substrate in closed system. In case of our incubation experiments, the system was closed, but the organic substrates consist of variety of compounds e.g. lignin, cellulose, other constituents of plant organic matter. It is possible that only the heavier fraction of the substrate organic matter was decomposed, resulting in depletion in heavy carbon isotopes. This should be studied on a molecular level by means of e.g. GC-IRMS, GC-MS and HPLC.

Our results of isotopic analysis of carbon in methane were between −43.7‰ (FC) and −19.9‰ (FB), which indicated that methane originated from the decomposition of acetic acid and/or oxidation processes [12]. Also, stable carbon isotopes in the products were significantly enriched in heavy isotopes, which may be caused by bacterial methane oxidation during incubation [45]. David L. et al. [30] indicated importance of the isotopic distribution when characterizing CH_4 source. Therefore, we postulate that this finding is probably due to a strong enrichment of the substrate mixture with ^{13}C (F) (see Table 1), and thus carbon sources from which methane was formed [31, 32]. The isotopic composition of the primary organic matter from which methane is produced, affects its final composition in terms of isotope distribution [1, 45].

The value of δ^2H in the methane generated as a result of CO_2 reduction varies from −250‰ to −150‰, and via the fermentation of acetic acid from −400‰ to −250‰ [12, 33]. Our results of isotopic analysis of hydrogen in methane ranged from −410.4‰ (FC) to −253.6‰ (FA). As mentioned above, these outcomes are indicative of methane formation via acetoclastic reaction.

To confirm acetoclastic methanogenesis we calculated the isotopic fractionation coefficient between CH_4 and CO_2 using a formula proposed by Whiticar [12]:

αC = α^{13}C(CO_2-CH_4)= (δ^{13}C(CO_2) + 1000)/(δ^{13}C(CH_4) + 1000)

value <1.06: pathway - acetic fermentation
values> 1.06: pathway - CO_2 reduction

The α C mean values from the experiments FA-FC ranged between 1.036 and 1.034, indicating predominance of the acetic pathway (see Table 4).

As reported in the literature, in the freshwater sediments the temperature is not a direct factor affected the methanogenesis pathways [34]. The isotopic composition of CH_4 is mostly dependent on carbon sources, their availability and microbial processes. However, in our experiments at higher AD temperature the isotope signal from the tested gas components is richer with light isotopes (Table 4). Szynkiewicz et al. [33] indicate that
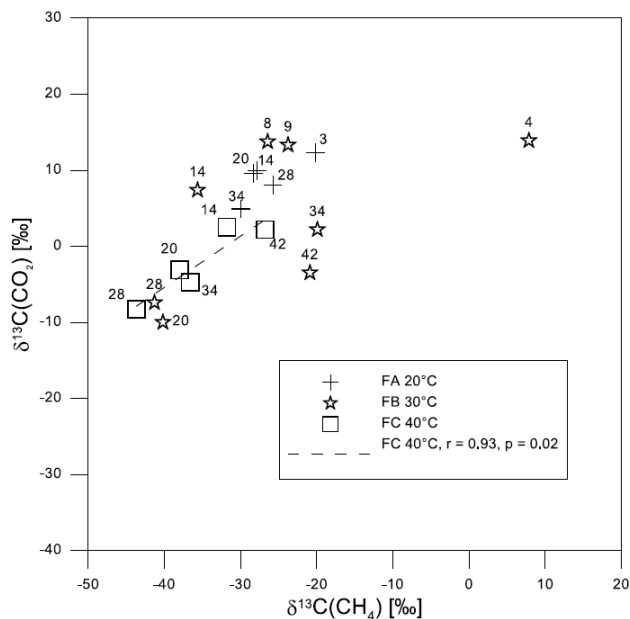
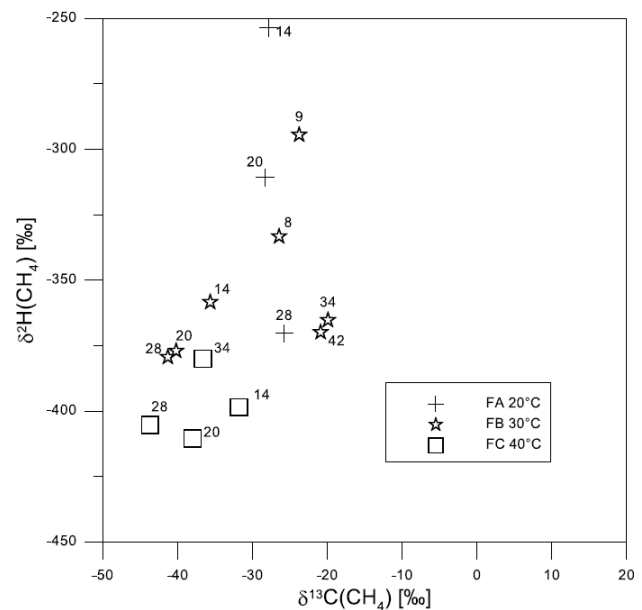**Figure 7:** Variability of $\delta^{13}C(CH_4)$ and $\delta^{13}C(CO_2)$ values in FA, FB and FC experiments (all days).

Figure 8: Variability of $\delta^{13}C(CH_4)$ and $\delta^2H(CH_4)$ values in FA, FB and FC experiments (all days).

temperature changes affect the activity of various groups of methanogenic microorganisms, and thus changes in the dominance of methanogenesis pathways can be observed. In case of our experiments, the role of acetoclastic methanogenesis pathway was dominant in all temperature conditions, but the lower $\alpha^{13}C_{CO2-CH4}$ fractionation factor was observed at 30ºC and 40ºC ($\alpha^{13}C_{CO2-CH4}$= 1.033 and 1.034, respectively), than at 20ºC ($\alpha^{13}C_{CO2-CH4}$= 1.036). It should be pointed that maize silage is a substrate rich in lactic acid, which can be used for acetate production. Detman A., et al. [35] suggested that the energy output on lactate degradation to the substrates for methanogenesis is the lowest in comparison to e.g. oxidation of acetate, butyrate, and propionate. So, attractiveness of lactate as an intermediate during AD is the highest. In acetogenesis lactate is oxidized mainly to acetate, which is used for methane production in acetotrophic pathway of methanogenesis.

To better understand the processes occurring during methanogenesis in the systems composed of many substrates, a correlation between $\delta^{13}C(CH_4)$ and $\delta^{13}C(CO_2)$ and between $\delta^{13}C(CH_4)$ and $\delta^2H(CH_4)$ (see Figure 7 and 8) was also analysed.

Analyzing the correlation graph (see Figure 7), it is clearly visible that with the application of a higher incubation temperature, the isotopic composition of both gases presents increasingly lighter isotope values.

Isotopic composition of methane and carbon dioxide shows the general tendency of enriching in time, both gases in the light carbon isotopes, in each temperature variant of the experiment. Surprisingly in the case of FB and FC experiments, after the 30 days of incubation, we can observe a sudden enrichment of both gases in heavy carbon isotopes. This may indicate the substrate consumption and switching the carbon source which has been decomposed during AD process [8, 13]. The highest correlation coefficient r = 0.93 occurred for the FC variant and there are statistically significant differences p = 0.02 (see Figure 7). This proves stability of the process at 40°C as well as may also indicate that the process was carried out with one path of methanogenesis.

Interpretation of correlation between $\delta^{13}C(CH_4)$ and $\delta^2H(CH_4)$, it's more complicated (see Figure 8). Nevertheless, a similar tendency to the one described above can be noticed. It can be seen that the values of carbon and hydrogen in methane, with increasing temperature of incubation are more depleted in heavy isotopes. In addition, particularly for the experiment FB and FC, it can be observed depletion of methane in heavy isotopes of carbon and hydrogen in the early days of incubation until the about 30 days, where the values abruptly change their course. The authors explain this by switching the source of carbon in digestion residues from which methane was generated.

Table 4: Mean values of the isotopic composition of headspace gas.

Experiment	$\delta^{13}C(CH_4)$ [‰]	$\delta H(CH_4)$ [‰]	$\delta^{13}C(CO_2)$ [‰]	$\alpha^{13}C_{CO2-CH4}$
FA=20ºC	−26.4	−311.6	8.9	1.036
FB=30ºC	−29.7	−354.0	3.7	1.033
FC=40ºC	−35.4	−398.5	−2.3	1.034

5 Conclusions

In the course of a well-designed AD, the yield of biogas production rose along with temperature increase (FA 20ºC=3.44, FB 30ºC=4.14, and FC 40ºC=5.42 [mM]/C$_{org}$[g]). At the highest temperature of anaerobic digestion the consumption of organic carbon from the fermentation mixture was also the highest and amounted to 20.1% for TOC and 25.8% for VS.

Isotopic composition of maize silage was enriched in ^{13}C. This could affect the isotopic composition ratio of the gases generated during AD. We observed enrichment of the fermentation mixture and the gas products in ^{13}C. The isotopic composition of carbon in methane depends on the degree of selective decomposition of substrates and the share of individual components in total methane production.

At the highest experimental temperature we noted the highest isotopic fractionation of the fermentation mixture of more than 7‰, as compared with its initial and final isotopic composition.

Isotopic studies allow us to identify methanogenic pathways with high probability. Higher AD temperature resulted in the enrichment of gas products in light carbon and hydrogen isotopes. Our findings indicate that methane was generated in the acetoclastic pathway. The calculated carbon isotopic fractionation coefficient between CH$_4$ and CO$_2$ also indicated this type of methanogenesis.

It was observed that during the fermentation of acetic acid, as the temperature of the experiment increased, the isotopic fractionation between CH$_4$ and CO$_2$ was lower.

We found no strong correlations between $\delta^{13}C(CH_4)$ and $\delta^{13}C(CO_2)$, for bioreactors FA and FB, which may reflect complexity of the AD process. The correlation coefficient value turned out to be significant only for the highest incubation temperature (r= 0.93).

Performing an isotopic mass balance could provide additional insight and will be the subject of our future research. Moreover, as the biomass we used for AD consists of many potential and complex carbon sources, the experiments should be supplemented with molecular level studies involving simple carbon sources characteristic of agricultural biomass as well as research in the field of genomics.

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