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Preliminary Tests of Biogas Microbiological Purity in Order to Asses a Possibility of Its Input into a Gas System

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1. Introduction

The need to meet the conditions of EU directives, requiring the increase in RES energy consumption and reduction of uncontrolled methane emissions from a variety of industries, including waste management sector and agriculture, results in growing interest in biogas technologies. Development of the domestic biogas market is encouraged by innovative research programs aimed at creating optimal conditions for the development of installations generating agricultural biogas. In the countries where biogas technologies have been extensively used for many years (e.g. about 4 thousand biogas plants in Germany), apart from converting the biogas into electricity and heat, there are also installations cooperating with the biogas plants that use biogas to produce biomethane injected later on into the gas network or used as car fuel. The main reasons for increased interest in this technology of converting biogas into energy is its relatively high energy efficiency, compared to the local electricity and heat production, especially if there are local problems with heat reception. Growing interest in this type of applications is also expected in Poland.

Normally, after treating the biogas and adjusting its parameters to those of the natural gas, and before injecting it into the gas distribution network, it undergoes specific tests to establish the physical and chemical parameters determining its interchangeability with methane-rich natural gas (composition, calorific value, Wobbe index, moisture content). Liter-

ature data describe bacteriological research in this field. These analyses are especially justified in the case of biomethane, produced on the basis of agricultural biogas, considering new corrosion mechanisms revealed in various studies (so far neglected in pipeline corrosion protection). Methanogen-induced anaerobic biodegradation [11], resulting in biomethane production, is a very important biotechnology from the economic point of view. Currently it facilitates waste reduction in the environment and is an important energy source.

2. Biogas microorganisms

Microorganisms that include aerobic and anaerobic bacteria, fungi and other microorganisms found in soil, water, air and the transported fuel can also cause damage to the pipeline walls. It facilitates further corrosion processes. Bacteria present in the pipelines can form so called biofilm, i.e. a layer composed of the bacterial cells and their metabolic products. Microbial adhesion and development in such a specific environment results in biocorrosion. In the presence of oxygen, wherever the metal parts are in contact with water, algae, ferruginous and aerobic bacteria can develop. Aerobic bacterium *Acidithiobacillus* is an especially important corrosion factor, as it is able to lower the pH down to 2.0, and consequently destroy the protective coatings. These bacteria multiply in the thin layer of water covering the metals, and speed up normal corrosion process by changing the electrochemical properties of the metal surface. An important element among the factors responsible for the corrosion is the biological process of sulfate reduction. Sulphate-reducing bacteria (SRB) producing biogenic H_2S in the transmission systems can induce a very dangerous phenomenon of hydrogen sulphide corrosion and biofilm formation. Considering the fact that knowledge concerning the presence of microbiological contaminants in biomethane is important when making decisions about using this type of gas in the natural gas distribution system, it seemed justified to analyze biogas and biomethane samples originating from different types of technological processes, and based on various substrates.

The substrates for biogas production involve for example sugar beet processing waste [2], wheat straw [5], fat-rich food waste [7], kitchen waste [4], fruit and vegetable waste [1] and many others. Studies on

biogas have been conducted in the world for several years. Many works in this field analyzed biogas microbial diversity [8, 13, 15, 16]. It was found that aerobic and anaerobic bacteria, and mould spores can exist in biogas. In some biogas samples there were even 10 to 100 CFU of aerobic bacteria per cubic meter [10]. In some cases, biogas can also contain SRB that increase hydrogen sulfide concentration in the environment [3]. Studies on biogas [8, 12], published in 2008 and 2009, revealed the presence of metylotrophic bacteria and identified 69 bacterial operational taxonomic units (OTU) and 25 archaeal OTU, including, among others, bacteria of the genera: *Methanosarcina, Methanospirillum* and *Methanoculleus*. Thermofilic strains of *Mathanosarcina* spp. were also isolated from full-scale thermofilic biogas plants treating animal manures [9]. One of the six isolates was *Methanosarcina thermophila* TM-1(T).

Studies carried out in Germany in 2008 [15] showed that biogas contained such bacteria as: *Thermoacetogenium* sp., *Anaerobaculum* sp., *Clostridium* sp., *Lactobacillus* sp. and *Entrococcus faecalis*. Bacterial and archaeal consortia continuously transform organic waste mainly into a mixture of methane, CO_2 and H_2O. Other experiments [6], carried out in 2007, proved the presence of one hundred and forty clones, within the Archaea domain, all clones showed a close relationship to methanogenic species. Major bacterial groups represented in the clone library belonged to the class Clostridia of the phylum Firmicutes, the class Deltaproteobacteria of the phylum Proteobacteria, the class Bacilli of the phylum Firmicutes and members of the phylum Bacteroidetes. Some studies were conducted to identify an indicator organisms in evaluating the pathogen-reducing capacity of biogas plants [14].

3. Material and methods

Microbiological tests were conducted in Oil and Gas Institute (Kraków) for fourteen biogas samples. For collection of one sample, a set was used containing two sterile glass scrubbers filled with saline solution, connected with sterile rubber hose. Two 500 milliliter Dreschel sterile bottles, filled with 300 ml of sterile saline were joined with sterile rubber tubing creating a system connected to a gas valve. The samples of biogas were tested in duplicate (samples A1, A2; B1, B2 etc.). After collecting a sample, that is pumping a specific amount of gas through the

above glass set, physiological fluid from both containers was mixed and the number of bacteria contained therein was determined, using the method of plating onto Petri dishes.

The system of two bottles was filled with biogas, the volume of which is shown in Table 1. Table 2 shows the number of bacteria per cubic meter. Tests were carried out with different volumes of biogas, due to the widely different pumping (flow) rate of the test gas under specific conditions and technical possibilities of the sampling in specified measurement point. Then, the results were converted into 1 cubic meter.

The collected material was analyzed for:

- total number of aerobic bacteria – 1 ml of the collected sample and 1 ml of dilutions in the range of 10^{-1}–10^{-5} were plated on agar enriched with readily assimilable carbon source, and incubated at 30°C for 3 days, before reading the results.
- total number of anaerobic bacteria – 1 ml of the collected sample and 1 ml of dilutions in the range of 10^{-1}–10^{-5} were plated on Brewer medium, and incubated at 30° C for 7 days, before reading the results.
- presence of fungi – 1 ml of the collected sample and 1 ml of dilutions in the range of 10^{-1}–10^{-5} were plated on Czapek-Dox medium, and incubated at 30°C for 7 days, before reading the results.
- presence of *E. coli* – 100 ml of the liquid was filtered through 0.45 μm filter in a filter apparatus, then the filter was transferred to m-Endo Les (Merck) selective medium, and incubated at 37°C for 24 h, before reading the results.
- presence of *Enetrococcus fecalis* – 100 ml of the liquid was filtered through 0.45 μm filter in a filter apparatus, then the filter was transferred to *Enterococcus* Selective Agar (Fluka), and incubated at 37°C for 48 h, before reading the results.
- presence of sulfite reducing *Clostridium* – 100 ml of the liquid was filtered through 0.45 μm filter in a filter apparatus, then the filter was transferred to TSC Agar, and incubated at 37°C for 72 h in anaerobic condition.

As the biogas contained sulfur compounds (mercaptans, dimethyl sulphide, COS – carbon oxysulfite), we also investigated microbial growth on sulfur oxidising bacteria isolation media:

- *Acidithiobacillus ferrooxidans* – 1ml of each sample was added to 50 ml of selective *Leptospirillum* medium and incubated for 30 days at 30°C.
- *Acidithiobacillus thiooxidans* – 1ml of each sample was added to 50 ml of selective *Acidith. thiooxidans* medium and incubated for 30 days at 30°C.
- *Thiobacillus thioparus* – 1ml of each sample was added to 50 ml of selective *Th. thioparus* medium and incubated for 30 days at 30°C.
- *Thiobacillus denitrificans* – 1ml of each sample was added to 50 ml of selective *Th. denitrificans* medium and incubated for 30 days at 30°C.

3. Results of biogas microbiological tests

Results presented in the following table indicate that microbial growth in the tested samples was basically very weak. An analysis of total aerobic bacterial count in A1 sample revealed 1.6 CFU per milliliter of saline. Second sample (A2), collected from the same place Opole bio0205, yielded insignificant fungal growth of 1 CFU per milliliter. The results for the next sample Jasło bio0224 B1 were similar to those found for the previous sample and amounted to 1.2 CFU per milliliter of saline, and the number of anaerobic bacteria was 1 cfu/ml. No growth was observed for B2 sample on the media for determining the total count of aerobic and anaerobic bacteria, but sulfur using bacteria were found on *Thiobacillus thioparus* medium. These bacteria were also present in B1 sample. Biłgoraj bio0222 sample did not contain anaerobes, and there was no growth on the medium for determining total anaerobic bacterial count. This sample yielded 10 CFU per milliliter (C1 sample) and 1 CFU per milliliter (C2 sample) of fungi. C1 and C2 samples comprised bacteria growing on the medium for *Thiobacillus denitrificans.* In D1 and D2 bio0227 samples 1 CFU per milliliter of aerobic bacteria were found, as well as some bacteria growing on the medium for *Thiobacillus thioparus.* Tarnów bio0202 samples comprised 1 CFU per milliliter (E1 sample) and 2 CFU per milliliter (E2 sample) of fungi, and no other bacterial groups were present. Rybnik bio0196 F1 sample was free from microorganisms, while in F2 sample 1 CFU per milliliter of aerobic bacteria and fungi were detected. In general it should be concluded that the analyzed 12 samples are the material of high microbiological purity and they do not contain dangerous microorganisms. Only one sample collected in

Krosno (G2) contains bacteria *E.coli* and *Enterococcus faecalis*. The total amount of anaerobic bacteria in this sample was 3 CFU per milliliter. It should also be emphasized that no sulfite-reducing *Clostridium* was found in the tested samples. DNA sequences of bacteria isolated from biogas (the analysis in CB DNA Poznań) shown that in tested samples were *Thiobacillus sp.* (incubated in liquid medium) and *Micrococcus luteus* (plated on enriched agar).

Table 1. The results of microbiological tests of biogas samples
Tabela 1. Wyniki badań mikrobiologicznych prób biogazu

Sample name		Volume of passing biogas (cubic meters)	Total count of aerobic bacteria (CFU per milliliter)	Total count of anaerobic bacteria (CFU per milliliter)	Fungi (CFU per millliliter)	*E.coli* (CFU per milliliter)	*Enterococcus fecalis* (CFU per milliliter)	Sulfite-reducing *Clostridium* (CFU per milliliter)	*Acidith.ferroxidans* (per 1 ml of liquid culture – qualid test	*Acidith.ttiooxidans* (per 1 ml of liquid culture-qualit.test)	*Th.thioparus* (per 1 ml of liquid culture-qualit.test)	*Th.denitrificans* (per 1 ml of liquid culture-qualit.test)
Opole bio0205	A1	0.17	1.6	<1	<1	<1	<1	<1	–	–	–	–
	A2		<1	<1	1	<1	<1	<1	–	–	–	–
Jasło bio0224	B1	1.55	1.2	1	<1	<1	<1	<1	–	–	+	–
	B2		<1	<1	<1	<1	<1	<1	–	–	+	–
Biłgoraj bio0222	C1	0.22	<1	<1	10	<1	<1	<1	–	–	–	+
	C2		<1	<1	1	<1	<1	<1	–	–	–	+
Piła bio0227	D1	0.06	1	<1	<1	<1	<1	<1	–	–	+	–
	D2		1	<1	<1	<1	<1	<1	–	–	+	–
Tarnów bio0202	E1	0.84	<1	<1	1	<1	<1	<1	–	–	–	–
	E2		<1	<1	2	<1	<1	<1	–	–	–	–
Rybnik bio0196	F1	1.99	<1	<1	<1	<1	<1	<1	–	–	–	–
	F2		1	<1	1	<1	<1	<1	–	–	–	–
Krosno bio0236	G1	1.90	<1	1	<1	<1	<1	<1	–	–	–	–
	G2		<1	3	<1	1	1	<1	–	–	–	–

After conversion to 1 cubic meter of biogas (Table 2) results showed that the total number of aerobic bacteria in five samples ranges from 0.5 to 16.7 CFU per cubic meter of tested gas. In the remaining nine samples, there were no bacteria found. Number of anaerobic bacteria in the three samples was from 0.5 to 1.6 CFU per cubic meter of gas. In the remaining eleven samples no isolated group of bacteria has been observed. Fungi were isolated from six samples of the fourteen surveyed, in the number from 0.5 to 45.5 CFU per cubic meter.

Table 2. The results of the microbiological tests of biogas samples per 1 cubic meter
Tabela 2. Wyniki badań mikrobiologicznych prób biogazu w przeliczeniu na 1 m^3

Sample name		Volume of passing biogas (cubic meters)	Total count of aerobic bacteria (CFU per milliliter)	Total count of anaerobic bacteria (CFU per milliliter)	Fungi (CFU per milliliter)	*E.coli* (CFU per milliliter)	*Enterococcus fecalis* (CFU per milliliter)	Sulfite-reducing *Clostridium* (CFU per milliliter)	*Acidith.ferroxidans* (per 1 ml of liquid culture – qualid test	*Acidith.tiooxidans* (per 1 ml of liquid culture-qualit.test)	*Th.thioparus* (per 1 ml of liquid culture-qualit.test)	*Th.denitrificans* (per 1 ml of liquid culture-qualit.test)
Opole bio0205	A1	1.00	9.4	<1	<1	<1	<1	<1	–	–	–	–
	A2		<1	<1	5.9	<1	<1	<1	–	–	–	–
Jasło bio0224	B1	1.00	0.8	0.6	<1	<1	<1	<1	–	–	+	–
	B2		<1	<1	<1	<1	<1	<1	–	–	+	–
Biłgoraj bio0222	C1	1.00	<1	<1	45.5	<1	<1	<1	–	–	–	+
	C2		<1	<1	4.5	<1	<1	<1	–	–	–	+
Piła bio0227	D1	1.00	16.7	<1	<1	<1	<1	<1	–	–	+	–
	D2		16.7	<1	<1	<1	<1	<1	–	–	+	–
Tarnów bio0202	E1	1.00	<1	<1	1.2	<1	<1	<1	–	–	–	–
	E2		<1	<1	2.4	<1	<1	<1	–	–	–	–
Rybnik bio0196	F1	1.00	<1	<1	<1	<1	<1	<1	–	–	–	–
	F2		0.5	<1	0.5	<1	<1	<1	–	–	–	–
Krosno bio0236	G1	1.00	<1	0.5	<1	<1	<1	<1	–	–	–	–
	G2		<1	1.6	<1	0.5	0.5	<1	–	–	–	–

/+/ – microbial growth on a specific medium
/–/ – no growth was observed

In comparison to corresponding research involving the isolation of bacteria from the biogas, in which 10 to 100 CFU of aerobic bacteria were found per cubic meter [10], in this study the number of bacteria was significantly reduced. Tests of a single measurement point have shown ca 17 CFU per cubic meter (in the remaining points the value was <10 CFU per cubic meter).

Conclusions

1. The research methodology applied allowed the evaluation of the microbiological condition of the samples of biogas.
2. Among the isolated microorganisms the largest group were fungi in the number from 0.5 to 45.5 CFU per cubic meter (occurring in about 40% of the samples tested).

3. In the test samples, there were no sulfite-reducing bacteria or bacteria of the genus *Acidithiobacillus*, and the bacteria *E. coli* and *Enterococcus faecalis* occurred sporadically, i.e. in one sample per 14 tested samples.
4. The results of this preliminary (pilot) experiments and its further steps are a useful source of information on the groups of microorganisms that may pose a potential threat to the proper functioning of the gas network. They will also help to assess the possibility of introducing biomethane into a gas grid.

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Wstępne badania czystości mikrobiologicznej biogazu pod kątem oceny możliwości jego wprowadzenia do sieci gazowniczej

Streszczenie

Badania mikrobiologiczne są jednym z elementów oceny jakości biogazu i stanowią uzupełnienie szczegółowych badań chemicznych tego medium. Badania parametrów biogazu, prowadzone pod kątem oceny możliwości ewentualnego wprowadzenia biogazu do krajowej sieci gazowniczej, stanowią ważny aspekt omawianej problematyki. Artykuł prezentuje wstępne badania laboratoryjne oraz przegląd doniesień literaturowych z tej dziedziny. Wyniki badań wskazują na wysoki stopień czystości mikrobiologicznej biogazu, w tym brak bakterii *E. coli*, *Enterococcus fecalis* i bakterii redukujących siarczyny, reprezentujących rodzaj *Clostridium*, w zdecydowanej większości przebadanych prób.