



## **BIODIVERSITY OF MICROORGANISMS ISOLATED FROM SELECTED SUBSTRATES USED IN AGRICULTURAL BIOGAS PLANTS VERSUS THE QUANTITY AND QUALITY OF OBTAINED BIOGAS**

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### ***Summary***

Apart from the basic input, the right course of fermentation process is determined by the appropriate microorganism populations and the parameters such as: pH, particle size, ionic strength (salinity) and concentration of nutrients and toxic compounds.

Three microorganism groups are involved in the process of anaerobic transformation of organic substances in fermentation gas: acid forming bacteria, acetate bacteria and methanogenic bacteria. The first two phases are dominated by the bacteria which are both obligate, facultative and absolute anaerobes (*Clostridium* spp., *Bifidobacterium* spp., *Streptococcus* spp., *Enterobacter* spp., *Bacillus* spp., *Pseudomonas* spp., *Aerobacter* spp., *Alcaligenes* spp, *Escherichia* spp, *Lactobacillus* spp, *Micrococcus* spp. czy *Flavobacterium* spp.) [Nimmrichter, Kuebler, 1999]. Their number during the mesophile fermentation is estimated for between  $10^8$ - $10^9$  per 1ml [Hartman, 1999]. The rate of bacteria growth at both phases fluctuates from 5 hrs, in the presence of carbohydrates to 72 hrs during fat decomposition [Heidrich, Nieścier, 1999].

Presented research aimed at determining the quantity and quality of biogas depending on the microbiological environment in fractions originating from agriculture and agro-food industry. To achieve the objective of research, was evaluated the number and biodiversity of microorganisms that inhabit selected agricultural raw materials used in the production of biogas. A very important aspect of the study was to investigate the species composition of the bacteria and fungi population at different stages of

fermentation process. Isolation of microorganisms from selected agricultural raw materials that stimulate the production of biogas could contribute in the future to optimize the process of its obtaining.

The following substrates were used in the investigations: distillery's grain, ensilaged beet pulp, rapeseed cake from biofuel manufacturing, apple pulp, fresh brewer's grains (wet), corn silage harvested by silage harvester without grain squeezer, corn silage harvested by silage harvester combined with grain squeezer, cellulose from paper industry.

Results of analysis of biogas yield in relation to dry mass revealed the highest productivity of the input from waste cellulose from paper industry at low productivity of biomass from agri-food industry. A delay in biogas volume increment visible in the course of fermentation of inputs from agri-food industry biomass is caused by the pasteurization of the mass which lacks microbiological environment, where microorganisms of methane fermentation multiply very slowly. The strongest inhibition of growth and delay in biogas formation was observed in the inputs made on the basis of distillery's grains and fresh brewers' grains. A normal productivity of biogas generation was obtained for the inputs prepared from cellulose and corn silage harvested by silage harvester equipped with grain squeezer.

**Key words:** biogas, agricultural biogas plants, waste from agri-food industry, microorganism

## INTRODUCTION

During metabolism facultative bacteria may use oxygen accidentally introduced to the system with added substrate and therefore create the environment appropriate for obligate anaerobes. Optimum conditions for the acid forming microorganisms are pH c.a.6 and the temperature c.a. 30°C [Thome-Kozmiensky, 1995]. Acetate bacteria [*Syntrophomonas* spp. and *Syntrophobacter* spp.) transform the products of acid phase (butyric and methylacetic acid and alcohols) into acetates and hydrogen which may be used by methanogenic bacteria. They can grow only when hydrogen is used by hydrogenotrophic bacteria. This cooperation between the microorganisms forming hydrogen and using up hydrogen was determined as "interspecies hydrogen transfer) [Boone, Mah, 1987]. Hydrogen may be removed from the environment also by means of homoacetogenic bacteria during the process of acetate formation from CO<sub>2</sub> and H<sub>2</sub>. However, under typical fermentation conditions the process does not take place due to thermodynamic reasons. Syntrophy between the organisms generating and using up hydrogen allows for the growth and activity of these microorganisms. However, its

absence makes impossible the reactions which supply the energy for the growth of both species [Jędrzak, 2008]. Bacteria of the acetate formation phase, similar as bacteria of acidification phase, are characterized by a long generation time, e.g. the time of growth of *Syntrophomonas wolfei* bacteria using butyric acid was 3 days, whereas for *Syntrophobacter wolinii* using methylacetic acid even about 7 days. Acetategenes are greatly sensitive to changes of the environment and require long adjustment periods for new conditions [Janosz-Rojczyk, 2004].

Methanogenic bacteria, classified to *Archaeobacteriales*, belong to absolute anaerobes. When oxygen appears (even  $0.01\text{mg}\cdot\text{dm}^{-3}$ ) methanobacteria are immediately inhibited, which leads to increase in organic acid concentrations and decrease in the environment pH. Methanogenic bacteria are greatly diversified morphologically and therefore specialize in assimilating and transforming determined kinds of substrates. Over 40 isolated methanogene strains may be generally divided into two groups, consumers of acetic acid and  $\text{H}_2/\text{CO}_2$ . Only few bacteria are able for acetate uptake (*Methanosarcina* spp. and *Methanothrix* spp., presently *Methanosaeta* spp.) but they do not assimilate formate. A majority of  $\text{H}_2/\text{CO}_2$  consumers is capable also of using formate. Both in nature and in fermentation chambers methanogenic bacteria occur in the form of bacilli (*Methanobacterium* spp.), spirals (*Methanospirillum* spp.) or granules (*Methanococcus* spp., *Methanosarcina* spp.). The time of methanogenic bacteria generation ranges from 15 to 85 hours. The minimum time of hydrogenotrophic methanogens growth is estimated for 6 hours, whereas for slowly multiplying acetate methanogens for c.a.72 hours [Jędrzak, 2008].

Biogas production takes place in the presence of numerous and diversified microorganism groups. Distillery's grain, corn silage, silage of beet leaves or beet pulp are naturally colonized by bacteria and fungi which affect the quality and quantity of biogas obtained during fermentation process. Moreover, it should be also noted that the kind and composition of the raw material used for biogas production may significantly affect biodiversity of the microorganisms capable of its colonization. Also during fermentation itself a natural succession of individual microorganism groups and their selection was observed [Chung and Hoitink, 1990; Chmiel, 1994; Hadar and Gorodecki, 1991; Hardy and Sivasithamparan, 1991; Phae et al.1990]. Often enough agricultural raw products are infected by bacterial and fungal pathogens, which leads to their spoiling and disturbs fermentation process. Moreover, secondary metabolites of pathogenic organisms, e.g. Mycotoxins produced by moulds may be found in such infected raw materials. Mycotoxins reveal multidirectional toxic effect, therefore their

presence in agricultural raw materials poses potential threat for the fermentation process [Kłosowski and Mikulski, 2010; Sieliwanowicz, 2003].

Knowledge about biomass utilisation for energy generating purposes, particularly biogas production is expanding but still remains inadequate, often incoherent and not unanimous among the specialists, agricultural advisors and farmers. It refers both to the input to the fermentation process, management of generated post-digestate and management of biogas. Most often agricultural biogas plants in Poland use appliances which produce biogas from the waste from livestock production (slurry, liquid manure, more rarely fresh manure). Another solution is biogas production from agricultural products, particularly from corn silage. Such approach to management of biomass surplus on farm leads to monoculture tillage. Therefore, it is necessary to seek waste biomass destined for gasification in agricultural biogas plants [Sikora, 2012].

## **METHODS**

Analyses were conducted at the laboratory of the Microbiology Department and at the Faculty of Power Production and Engineering, University of Agriculture in Krakow. Organic (agricultural) mass was obtained from a private farm focused on milk production. Biomass originating from agri-food industry came from Bio Alians Ltd. Enterprise. Analysis of the input samples for their microbiological biodiversity was performed at 2 stages of fermentation: initial and final.

### **Isolation of microorganisms**

Samples of agricultural raw materials and agri-food industry biomass (corn silage, cattle manure, cattle liquid manure, beet leaves silage, distillery's grains, beet pulp and brewers' grains will be supplied to the laboratory of the Microbiology Department in sterile containers. Each sample will be analysed using serial dilution method to state the presence of selected microorganism groups. Petri dishes with dilutions of the analysed raw materials will be covered with selected media and then placed in thermostats at the temperatures appropriate for growth of individual kinds of microorganisms. Developed colonies will be counted and inoculated to subsequent media to isolate pure strains.

The following microorganism species have been isolated: mesophilic and thermophilic bacteria, moulds, actinomycetes, *Bacillus* spp., *Clostridium* spp.,

*Azotobacter* spp., *Escherichia coli*, *Enterococcus faecalis*, *Staphylococcus* spp., *Salmonella* spp., *Shigella* spp.

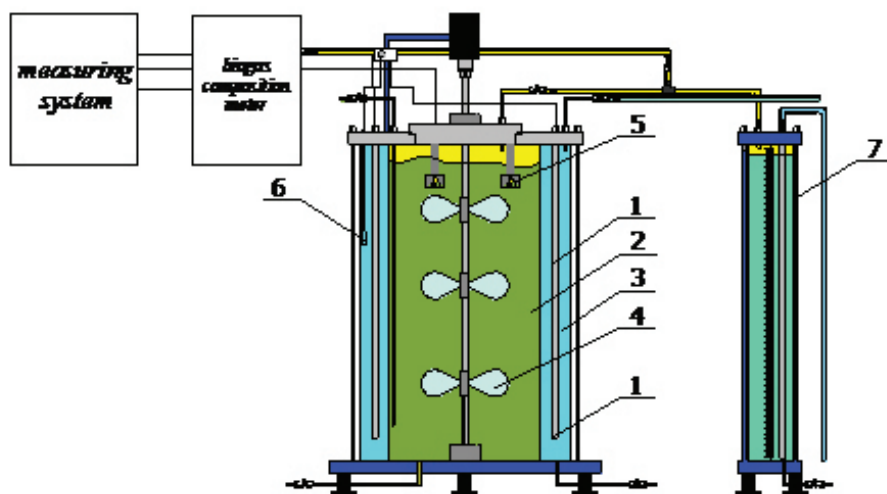
Isolation of mesophilic and thermophilic bacteria and moulds allowed for a general assessment of the number of microorganisms living in the analysed samples. Isolation and determining of *Bacillus* spp. and *Clostridium* spp. bacteria responsible for the first stage of methane fermentation (acid forming and acetate forming phase) testifies a potential of obtaining biogas from the analysed samples. Isolation and assessment of the number of actinomycetes and *Azotobacter* spp. – microorganisms which are indicators of soil fertility will supply information about potential application of selected agricultural raw products as organic fertilizers. Determining the microorganisms regarded as pathogenic: *E. coli*, *E. faecalis*, *Staphylococcus* spp., *Salmonella* spp., *Shigella* spp. will allow to decide if the processed raw materials are colonized by these pathogens, which may be dangerous for persons who have contact with them.

### **Identification of species**

Identification of species was conducted on the basis of microscopic observations and culturing on selected microbiological media using diagnostic keys [Domsch et al. 1980; Gilman, 1957; Holt, 1989; Marcinowska, 2003]. Pure strains were used to make bacteriological preparations, which stained using to Gram method, were observed under a microscope. Intravital microbiological preparations were made using Lugol's iodine and also observed under a microscope.

### **Methane fermentation**

Inputs with parameters presented in Table 1 were placed in a chamber (2) where fermentation parameters, i.e. the temperature, redox and pH are monitored by probes (5). The parameters are automatically saved with time interval on the computer hard disc of a measuring system. In the chamber the input will be stirred with a mechanical stirrer (4) in order to avoid the substrate delamination. The stirrer has a possibility of variable adjustment within the range from 0 to 400 rpm, it is equipped with three propellers with adjustable spacing which enables a change of the intensity of mixing zones in the fermenter.



Source: Author's own elaboration

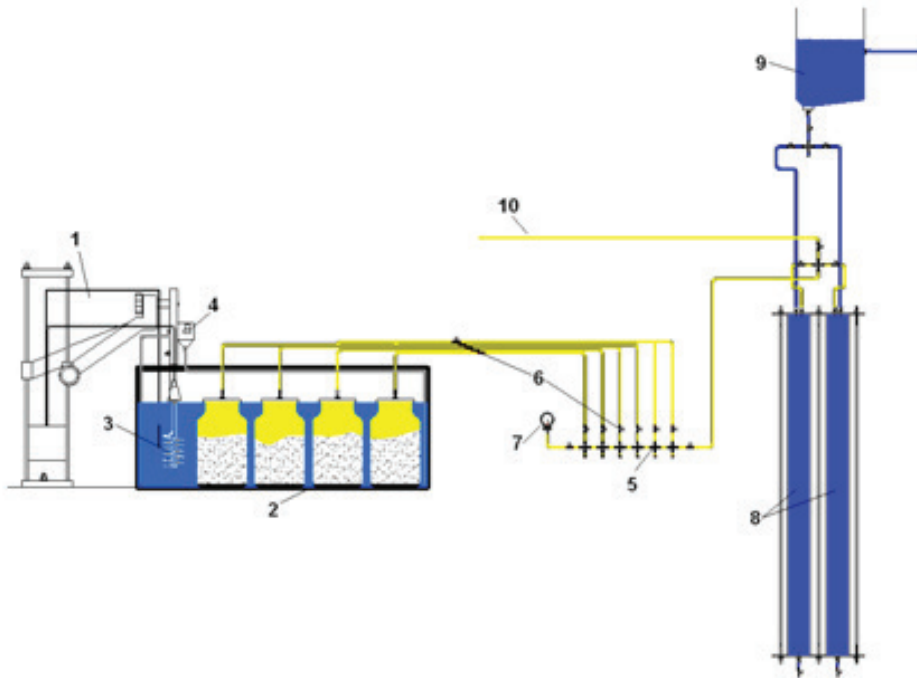
**Figure 1.** Diagram the of the test stand with 20-litre fermenter

The digester of the fermenter is equipped with a water jacket (3) where three cartridge heaters are placed (1), which are responsible for heating liquid. The measuring system equipped with a thermometer (6) PT100 is responsible for controlling the process temperature. The produced biogas is collected over the surface of the batch in the fermenter and in the container (7) of variable volume from which it is sucked in by the biogas composition measuring meter. This meter analyses the following parameters: moisture, temperature, pressure, methane  $\text{CH}_4$ , oxygen  $\text{O}_2$ , carbon dioxide  $\text{CO}_2$  and hydrogen sulphide  $\text{H}_2\text{S}$ . Biogas composition parameters which are measured are automatically saved on the computer disc of the measuring system.

Determination of the intensity of the biogas production in the remaining batches was carried out according to standard DIN 38414. Batch mixes were fermented in static conditions consisting in a single introduction of fraction to digesters and conducting the process to the end of fermentation.

Fermentation devices were installed in a container with regulated temperature forming a part of the test stand, which was additionally composed of a switch panel and the measuring system. Schematic representation of the test stand was presented in Figure 2. Devices for maintaining a constant temperature environment were mounted to a rack (1) located next to the container (2). Controlling took place by means of electronic thermostat ESCO ES-20 (unit switch 16A) with precision up to  $\pm 0.2$  °C resulting from hysteresis of a sensor. Temperature decrease by value exceeding 0.1 °C caused switching on a heater of 1500 W (3) power with a simultaneous start of the water pump Hanning DPO 25-205 (4) in order to ensure a uniform distribution of temperature in the whole chamber. After heating water to the temperature exceeding the set temperature by 0.1 °C the heater switched off and with a 30 seconds delay of then pump.

Source: Author's own elaboration



**Figure 2.** Diagram of the test stand with 2 litre fermenter

Separators combined in a row along with cut-off valves (6) and a manometer (7) which measures pressure in particular measuring branches constituted a switch board (5). Due to the use of such system for service of all fermenters, only one measuring system was enough. The system of measuring volume (8) was composed of two columns filled with water with drain valves and a container for filling up the liquid level in columns (9). Measuring system was combined with a switchboard and a biogas composition meter by means of a conduit (10) which was presented in figure 1.

## RESULTS

Results of the tested raw material pH assessments made at 2 stages of the process were presented in Table 1. The values assumed by the analysed samples pH ranged from 3.99 to 7.43. In all cases pH increased and its decline was noted only for brewers' grains and corn silage harvested with silage harvester without grain squeezer.

**Table 1.** Parameters of inputs to fermentation chambers

No	Name of material	pH A	pH B	% d.m.
Input 1	Distillery's grains	4.16	5.23	7.25
Input 2	Distillery's grains	3.99	5.15	7.94
Input 3	Beet pulp silage	4.45	5.23	26.28
Input 4	Rapeseed cake from biofuel production	5.31	5.36	67.25
Input 5	Apple pulp	4.26	4.59	21.56
Input 6	Fresh brewers' grains (wet)	5.64	5.01	29.64
Input 7	Corn silage harvested by silage harvester without grain squeezer	5.23	4.86	46.42
Input 8	Corn silage harvested by silage harvester with grain squeezer	4.46	4.71	44.65
Input 9	Cellulose from paper industry	7.35	7.43	65.37

Source: Author's own elaboration

A majority of actinomycetes and bacteria develop within a narrow pH range from 6.5 to 7.5. Moulds and yeasts prefer a low pH, between 4.0 and 6.0. In most of the analysed samples pH was acid, only in cellulose from paper



industry slightly alkaline pH was registered. The numbers of analysed microorganism groups from the investigated raw materials were shown in Table 2.

No presence of *Salmonella* spp, *Shigella* spp., *Azotobacter* spp., *Staphylococcus* ssp. *E.faecalis* or coliforms and *E.coli* group was found. Taking into account that the temperature in the fermentation chamber is quite high (36°C), most microorganisms die under its influence. The microorganism which even then stay active considerably slow down their metabolism, which may indirectly affect the effectiveness of fermentation process. Worwąg et al. [2010] stated that during fermentation the number of microorganisms rapidly decreases in all tested raw material mixtures. Inhibitory effect of high temperature on growth of a majority of microorganisms stimulates research on selecting thermophilous organisms which may serve as starter consortia in biogas production [Szlachta 2009].

Considering the fact that in most cases pH was not optimal for bacteria growth, their quantity should be regarded as considerable. Additionally in some samples (3, 4 and 8), despite a increase in pH, a decline in total bacteria number was stated. The reaction of the analysed raw materials favoured mould development, however increased number was found only in four samples (1, 2, 5 and 9).

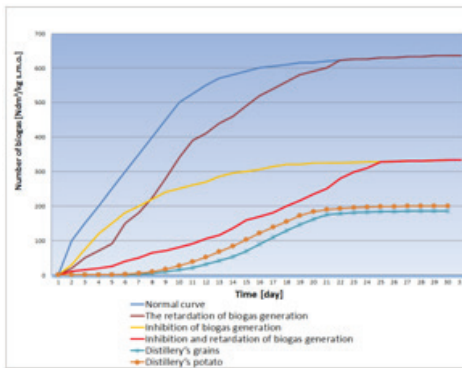
Actinomycetes presence was found only in two cases (8 and 9) and only at the in initial phase of fermentation process. *C.perfringens* was absent in three samples (4,6 and 8). In the other samples total number of *C. perfringens* grew. Because of a gradual using up of oxygen in the fermentation chamber leading to creation of anaerobic conditions, a growing number of *C.perfringens* seems fully justified. Research conducted by Worwąg et al. [2010] evidenced that pH is growing during fermentation, however the changes are slight and fluctuate depending on the day of measurement. pH of the raw materials analysed by Worwąg et al. [2010] fell within the range slightly higher than in the Authors' investigations, i.e. 6.6 – 7.8.

Investigations of fermentation process conducted in laboratory conditions allowed to compare the intensity of biogas generation, following the fermentation phases and an assessment of the analysed input loads effect on the processes of biochemical decomposition of organic mass. Parameters of the analysed fractions were presented in Table 1. Figures 3 – 6 show a summary amount of produced biogas and the intensity of biogas generation during fermentation. The amount and intensity of biogas emission are the parameters which evidence the process course.

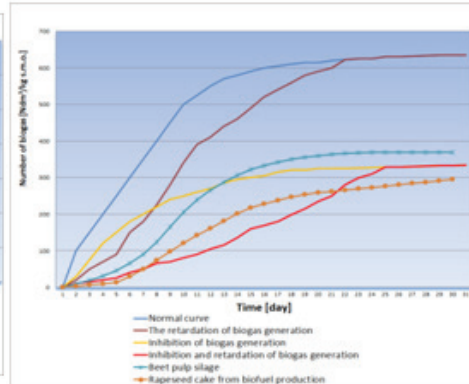
**Table 2.** Numbers of selected microorganism groups isolated from raw materials used in biogas plants

Microorganism	Number of cfu $\cdot$ cm <sup>-3</sup>	Input 1	Input 2	Input 3	Input 4	Input 5	Input 6	Input 7	Input 8	Input 9
Bacteria	A	560	33000	21000	10000	206000	81000	11777777	24757777	1546759
	B	16880	35183	13185	750	431500	111640	128208	15008	1562500
Fungi	A	340	497	294666	400	500	60	306000	164000	2150
	B	420	1710	0	0	94333	0	1820	1633	6600
Actinomycetes	A	0	0	0	0	0	0	0	6600	400
	B	0	0	0	0	0	0	0	0	0
<i>C. perfringens</i>	A	173	185	181	0	177	0	986	0	345
	B	200	195	200	0	225	0	1315	0	555

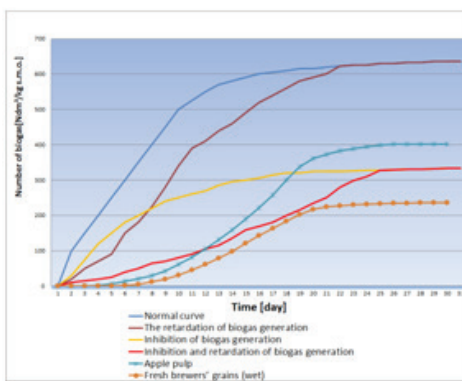
A – Input; B – post-digestate; 1-9 successive samples  
 Source: Author's own elaboration



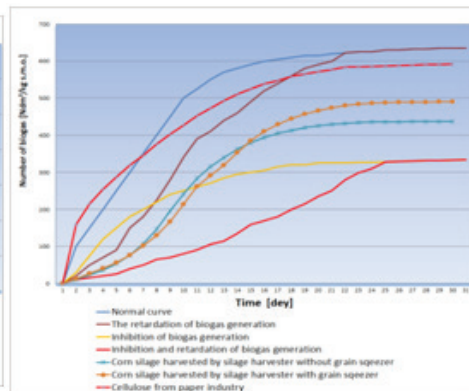
**Figure 3.** Effectiveness of biogas from distillery's grain



**Figure 4.** Effectiveness of biogas from beet pulp and rapeseed cake silage



**Figure 5.** Effectiveness of biogas from apple pulp and wet brewer's grains



**Figure 6.** Effectiveness of biogas from corn silage and cellulose

Figure 3 depicts a summary amount of biogas emitted in the process of methane fermentation of distillers grains and pulp charges. Hydrolysis occurs in the first stage of the fermentation process, by which carbohydrates, protein and fats are decomposed into simple organic compounds, i.e. amino acids, sugars and fatty acids. The process of hydrolysis is prolonged and fermentation is delayed. The observations made on the 12<sup>th</sup> day of the research showed biogas volume increment. The delay in the fermentation process was the result of low biodi-

versity of microorganisms in the charges (chart 3). The stillage-based charges produced 195 Ndm<sup>3</sup>/kg of dried organic mass (satisfactory amount). Figure 4 illustrates the process of summary biogas emissions from beet pulp and rapeseed oilcake charges. In the case of beetroot silage-based charge, the hydrolysis stage is shorter and the delay in the fermentation process equals 4 days, whereas the delay in the fermentation process of rapeseed oilcake charge is 6 days. Shorter delay in biogas increment of beet pulp silage results from larger number of bacteria in the charge mass (chart 3). The amount of biogas obtained from rapeseed oilcake equaled to 300 Ndm<sup>3</sup>/kg of dried organic mass. Figure 5. illustrates summary biogas emission from apple marc and wet grain stillage charges. The fermentation process was approximate to the inhibition curve and to biogas emission delay. The delay in the fermentation process was the result of low biodiversity of microorganisms in the charge mass. Figure 6. presents summary biogas emission curves from paper cellulose and corn silage charges prepared with the use of diverse methods. Those charge masses demonstrated higher microorganism biodiversity that influenced the process of fermentation, which is approximate to the curve illustrating standard fermentation process from paper cellulosebased charge.

Results of analysis of biogas yield in relation to dry mass definitely indicated the best efficiency of input No 9, at low effectiveness of biomass from agri-food industry (Fig.3,4,5) during the course of fermentation of agri-food industry inputs and a visible delay of biogas volume increment, which is caused by pasteurization of the mass where microbiological environment is absent, so the microorganisms of methane fermentation multiply poorly. The greatest inhibition of the increment and delay in biogas generation during fermentation was observed for the input prepared from distillery's grains and fresh brewer's grains. Normal course of biogas generation effectiveness was obtained for inputs No 8 and 9.

## CONCLUSIONS

The kind and composition of the raw material used for biogas production may significantly affect biodiversity of the microorganisms capable of its colonization. A natural succession of individual microorganism groups takes place during fermentation process, whereas their selection depends on the fermentation process parameters. No microorganisms pathogenic for people were found

in the substrate samples, apart from *Clostridium perfringens* – the sulphide reducing bacteria. Effectiveness of biogas obtained from the inputs containing a considerable number of bacteria, i.e. more than  $1\text{M cfu cm}^{-3}$ , is characterised by a normal course of methane fermentation curve. The quantity of fungi, actinomycetes and gas bacillus did not have any influence in the intensity of biogas emission during fermentation. From the input loads in which increased number of bacteria was registered, biogas with 50-60%  $\text{CH}_4$  and  $\text{H}_2\text{S}$  was obtained on the level of 30 ppm.

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