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The quantity and quality of methanogenic microorganisms based on rRNA analysis and their relation to methane production

Maciej Walczak and Maria Swiontek Brzezinska*

Department of Environmental Microbiology and Biotechnology, Nicolaus Copernicus University, Gagarina 9, 87-100 Torun, Poland.

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Biogas plants continuously convert biological wastes mainly into a mixture of methane, CO_2 and H_2O a conversion that is carried out by a consortium of bacteria and archaea. The microbial community structure of the fermentation process was evaluated by using oligonucleotide probes complementary to conserved sequences of the 16S rRNAs of phylogenetically defined groups of methanogens. At the beginning of fermentation, Eubacteria are the main group and the count of Archaea then constitutes only an insignificant percentage of the population of microorganisms. During the process of fermentation, we recorded a very significant increase in methanogenic microorganisms after 40 days of the process in progress. It is reflected in the quantity and the composition of released biogas.

Key words: Methane fermentation, methanogenic microorganisms, phylogenetic diversity, rRNA.

INTRODUCTION

Anaerobic digestion is an effective way to treat organic wastes and biomass with the production of methane, a valuable energy source. Anaerobic digestion involves a series of symbiotic reactions that can be grouped into acidogenesis and methanogenesis. Because methanogenesis is usually the rate-limiting step in the overall process, the appropriate control of the methanogenic phase has been a key factor in the successful operation of anaerobic processes (Yu and Hwang, 2006). Therefore, methanogens, the sole mediators of methanogenesis, have received particular attention in engineered environments (Karakashev et al., 2005). Biogas plants may play an important role in providing society with a sustainable mix of energy (Mata-Alvarez et al., 2000). Such plants convert biomass and biological waste into the so-called biogas, that is a mix of CH_4, CO_2 as well as H_2O containing in addition traces of N_2, H_2S. The methane is later converted into energy. The understanding of the microbiology of such continuously operated Industrial plants is still very rudimentary. The microbal community of an anaerobic digester is composed of large number of different organisms belonging to the Bacteria and Archaea domains. These microorganisms represent the major phylogenetic groups within the anaerobic digester community, and include such physiological groups as hydrolytic bacteria, fermentative acidogenic bacteria, acetogenic syntrophic bacteria, hydrogenotrophic and acetotrophic methanogens, and sulphate reducing bacteria. Methanogens are strict anaerobes which share a complex biochemistry for methane synthesis as part of their energy metabolism. It is widely accepted that methanogens are difficult to isolate as some members require long incubation periods for growth and some are sometimes difficult to separate from their syntrophic partners. In addition, a large majority of microbes including methanogens have evaded isolation as they are

*Corresponding author. E-mail: swiontek@umk.pl. Tel +48 56 611 25 22. Fax + 48 56 611 4443.

Table 1. The chemical composition of silage maize biomass.

Factor	Value
FAO coefficient	550
Dry mass	37.05%
Mineral compounds	2.3%
Organic compounds	82%
Lignin	1.43%
Cellulose	18.39%
Hemicellulose	19.59%
Carbon	42.03%
Nitrogen	1.77%
Hydrogen	5.98%

not amenable to laboratory cultivation due to our incomplete knowledge of their growth requirements.

Morphologically, the methanogens exhibit a wide variety of shapes and sizes, including rods, regular and irregular cocci, long-chained rods, spirilla, sarcina and irregular unusual flattened plates. Motility is sometimes present. Some species can aggregate in clusters. Several species of methanosarcina and methanosaeta contain gas vacuoles. The Gram reaction can be positive or negative even within members of the same genus (Beveridge and Schultze-Lam, 1996).

For better understanding and control of this diverse community, the groups must be analysed quantitatively. Because of difficulties with cultivation of the methanogenic microorganisms, the quantification of them we used fluorescence *in situ* hybridization (FISH) with rRNA-targeted fluorescent oligonucleotide probes (Manz et al., 1999; Amann et al., 1995). Culture-independent molecular techniques, particularly those based on 16S rRNA gene fragments, are powerful tools with which to investigate microbial communities in various environments. This technique is effective in detecting microbial community shifts and in identifying the phylogenetic affiliations of microbial populations in mixed culture systems.

This technique makes it possible to identify cells through detecting fluorescence originating from marked oligonucleotide probes, which underwent hybridization with a complementary nucleic acid occurring in a given cell. Oligonucleotides used in phylogenetic investigations permit not only to detect presence of bacteria from specific groups, but also to determine their abundances in samples (Llobet-Brossa et al., 1998). Additionally, this technique provides a reliable measure of activity or, at least, potential activity of cells, because ribosomes undergo hybridization, and their amount depends on metabolic activity of a cell (Walczak and Swiontek Brzezinska 2010).

The purpose of this study was to determine the numbers of methanogenic microorganisms and their relation to methane production.

MATERIALS AND METHODS

Experimental

Methane fermentation was conducted in five laboratory fermenters with the capacity of 10 L, equipped with stirring blades. Mixing was conducted periodically for 5 min/h.

Maize silage constitutes the raw material for fermentation. The maize came from an experimental cultivation intended as biomass for methane fermentation. The chemical composition of silage maize biomass was: FAO coefficient of 550, dry mass of 37.05%, mineral compounds of 2.3%, organic compounds of 82%, lignin of 1.43%, cellulose of 18.39%, hemicellulose of 19.59%, carbon of 42.03%, nitrogen of 1.77% and hydrogen of 5.98% (Table 1).

At T_0 time, the composition of fermentation feedstock was as follows: maize silage biomass of 1800 g; water of 6 L; inoculum of 0.5 L (post-fermentation liquid from previous processes of methane fermentation constituted the inoculum). The fermentation was conducted in thermally stable conditions (T=36°C) for the period of 90 days. The process was conducted at the Department of Chemical Proecological Processes, the Faculty of Chemistry, NCU in Toruń.

After all the components were placed in the fermenters, anaerobic conditions formed spontaneously after 24 h, which was confirmed through measuring the oxygen concentration with a dissolved oxygen meter (Hanna Instruments). The resulting biogas was directed to a glass tube flow meter equipped with a valve for connecting a methane detector. The composition of biogas was determined by means of multigas monitor (Gas Data). Sampling of fermentation liquid was done with a sterile syringe. A single collection amounted to ca. 30 ml of the liquid. Then, a relevant volume of the collected liquid was filtered through a polycarbonate filter with pores of 0.22 µm in diameter. This volume was dependant on the bacteria count and was determined individually for each sample. Filters with the biomass of microorganisms retained on their surface were subjected to the microbiological analyses.

Determination of the total number of microorganisms

The total number of microorganisms was determined with the method of direct counting under an epifluorescence microscope (Nicon Eclipce T300). For the determination purpose, microorganisms retained on the filter surface were stained with the pigment 4',6-diamino-2-phenylindole (DAPI).

Determination of the number of alive and dead microorganisms

In order to perform this determination, microorganisms retained on the filter surface were subjected to LIVE/DEAD staining. In the procedure of staining and identification, the diagnostic set LIVE/DEAD (Invitrogen) was applied (Davies, 1991).

Determination of the number of methanogenic microorganisms

Determination was performed with the method of fluorescence *in situ* hybridization (FISH) (Amann et al., 1990; Raskin et al., 1995). In the process of hybridization, oligonucleotide probes were applied, complementary to rRNA of the major phylogenetic groups of methanogenic microorganisms. The following oligonucleotide probes were used: *Methanosarcinales* (Raskin et al., 1994**)** 5'-GGCTCGCTTCACGGCTTCCCT-3', *Methanomicrobiales* (Crocetti et al., 2006) 5'-CGGATAATTCGGGGCATGCTG-3', *Methanobacteriales* (Raskin et al., 1994) 5'-ACCTTGTCTCAGGTTCCATCTCC-3', *Methanococcales* (Raskin et

Table 2. The amount and the composition of released biogas, as well as the composition of populations of methanogenic microorganisms.

Sample/day of fermentation	Volume of biogas (ml/d)	CH_4 (%)	H_2S (ppm)	H_2 (ppm)	NH_3 (ppm)	Methanogenic microorganism (numbers of x 10^7 /ml)			
						Methano-bacteriales	*Methano-coccales*	*Methano-microbiales*	*Methano-sarcinales*
T_0 / 0	116	0	0	0	1	0	1.8	0	8.2
T_1/ 42	2050	10	3	0	0	1.8	19.9	17.6	4.7
T_2/ 70	4300	49	0	22	0	7.1	14.1	39.3	13.5
T_3/ 88	1750	50	0	20	0	20.5	65.6	97.3	105.5

al., 1994) 5'-GCAACATAGGGCACGGGTCT-3'. Also, Eubacteria and Archaea were included in the phylogenetic analysis. For Eubacteria (Amann et al., 1995), we used oligonucleotide probes 5'-GCTGCCTCCCGTAGGAGT-3' and Archaea (Raskin et al., 1994) 5'-GTGCTCCCCCGCCAATTCCT-3' Termini 5' of oligonucleotides were marked with the fluorescent dye Cy3. Conditions of the hybridization process for particular phylogenetic groups were different. Fixed prokaryotic cells were hybridized by application of 20 µl hybridization buffer [20% (vol/vo)] formamide for probe *Methanomicrobiales*, 30% (vol/vol) formamide for probe *Methanobacteriales*, 45% (vol/vol) formamide for probes *Methanosarcinales* and *Methanococcales*, 35% (vol/vol) formamide for probes Eubacteria and Archaea, 0.9 M NaCl, 0.01% sodium dodecyl sulfate, 20 mM Tris/HCl, and pH 7.4. For detection of prokaryotes, 30 ng of Cy3-labeled and 50 ng of fluorescein- labeled probes were used. After 2 h incubation in an isotonically equilibrated humid chamber at 46°C, the labeled oligonucleotides were gently removed by incubating the slides with washing buffer (20 mM Tris/HCl, pH 7.4, 0.01 % sodium dodecyl sulfate, 5 mM EDTA, 0.2 M NaCl *Methanosarcinales,* 0.1 M NaCl for *Methanobacteriales*, 0.03 M NaCl for *Methanosarcinales* and *Methanococcales* and 0.08 M NaCl for Eubactera and Archaea) at 48°C for 15 min.

Microscopic analysis

In all microscopic analyses, the epifluorescence microscope (Nicon Eclipse T300) with digital registration of images was used. During the analysis of preparations, each time, 40 randomly selected fields of view were analysed. On the obtained images, labelled cells were counted; an average value was calculated and then expressed per number of cells in the unit volume of the fermentation liquid.

Statistical analysis

All results were statistically analysed with the analysis of variance applying the software Statistica 6.0.

RESULTS

The volume of released biogas was significantly increased from the 15th day to the 60th day of the process ($p<0.000$). After the 80th day, the volume of biogas started considerably decreasing. However, the content of methane in the gas increased after 35 days of the process. Obtained results related to the dynamics of methanogenic microorganisms ($p<0.000$), as well as the volume and parameters of biogas, indicate that the intensive methanogenic processes started relatively late (Table 2). The Tables and the fgures show average values for 5 fermenters.

During the fermentation, an increase in the total number of microorganisms was observed (Figure 2). However, between sample T1 and T2 that is between the 40th and the 70th day of fermentation, reduction in the total number of microorganisms was observed in volumes from 44.5 x 10^8/ml to 39.2 x 10^8/ml. At the same time, increased content of dead cells was recorded in relation to live ones (Figure 2) whereas, in subsequent measurement, both the total number of microorganisms cells, as well as the content of live cells increased significantly ($p<0.000$).

Changes observed in the ratio of Eubacteria to Archaea (Figure 1) are characteristics of transformations taking place during methane fermentation. At the beginning of fermentation, Eubacteria form the main group and the count of Archaea constitutes only an insignificant percentage of the microorganisms' population. These relationship changes during the process to the advantage of Archaea and at the end of the process, the numbers of Archaea were higher than for Eubacteria (Figure 1). The differences between the total number of Eubacteria and Archaea (Figure 1) and the total number of microorganisms determined with DAPI staining result from the fact that during FISH process, dead or/and inactive cells were not hybridized and were therefore not detected with FISH method.

During the conducted methane fermentation, the count of individual groups of methanogens differed considerably in particular phases of the process (Table 2 and Figure 3). The very significant increase in all methanogenic microorganisms was recorded after 70 days of the process in progress ($p<0.000$). It is reflected in the quantity and the composition of the released biogas (Table 2 and Figure 4).

The analysis of phylogenetic composition of methanogenic microorganisms proved that at the beginning of the process (T0) the largest was the autotrophic group *Methanosarcinales* (8.2 x 10^6/ ml) (Table 2 and Figure 3). In the 40th day of fermentation (T1), the *Methanosarcinales* number fell to 4.7 x 10^6/ml meanwhile the numbers of methanogens from other groups increased. The biggest increase pertained to *Methanococcales* and *Methanomicrobiales*. After the 40th day of fermentation, the number of methanogens begun to rise further, excluding *Metnaonococcales*. The number of that group of microorganisms in the 70th day of fermentation was

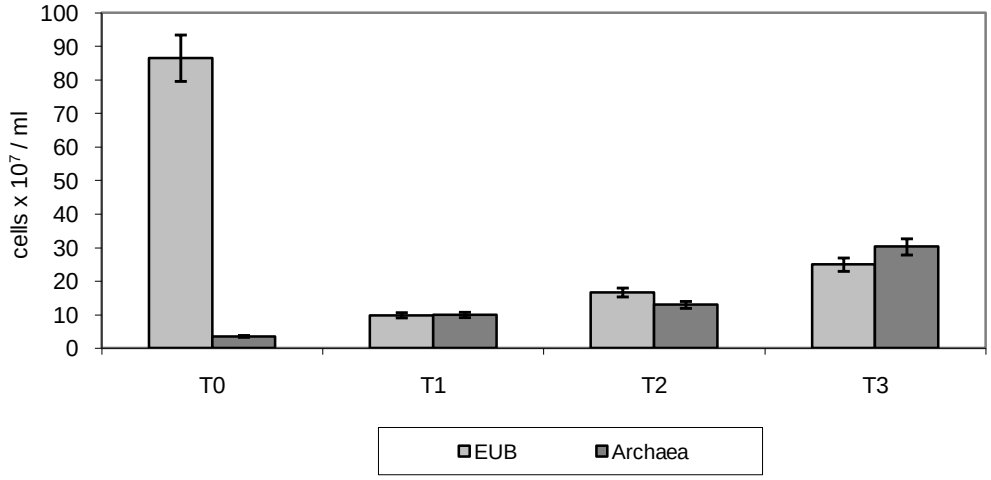

Figure 1. The count of Eubacteria and Archaea during the methane fermentation. Sample/day of fermentation: T0/0, T1/42, T2/70, T3/88.

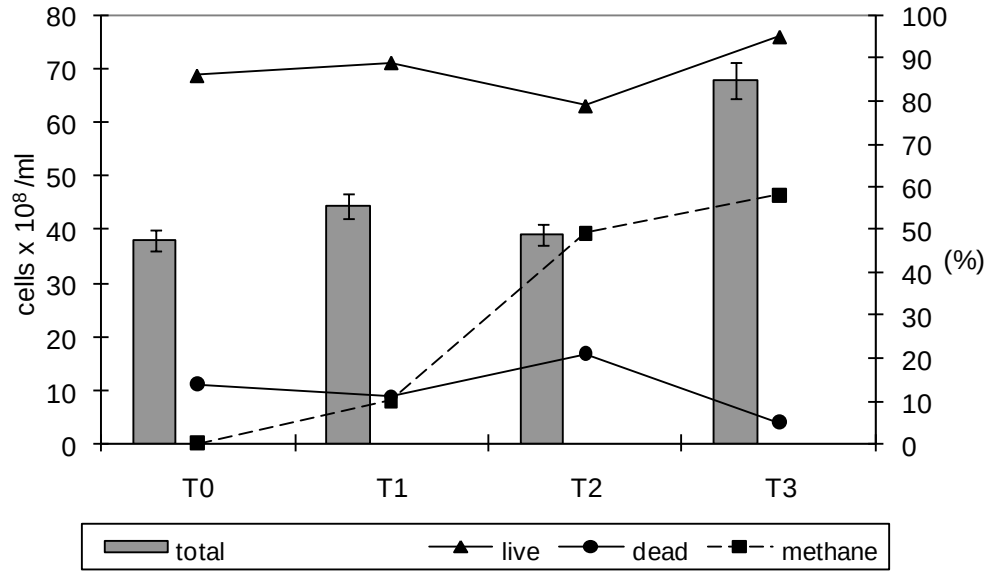

Figure 2. The total number (TN) of microorganisms in the fermentation liquid and the contribution of alive and dead cells. Sample/day of fermentation: T0/0, T1/42, T2/70, T3/88.

lower than before. After the 70th day of fermentation, a very rapid growth of all surveyed groups of methanogens was observed. This rapid growth of methanogenic microorganisms number coincides with considerable increase of methane content in biogas (beyond 50%) ($p<0.000$).

DISCUSSION

Processes leading to methane production in the anaerobic environment require cooperation between many species of microorganisms and result from interactions between organisms of different metabolism (Kubota et al., 2009). Obtained results related to the dynamics of methanogenic microorganisms, as well as the volume and parameters of biogas, indicate that the intensive methanogenic processes started relatively late.

Observed changes in numbers of Eubacteria and Archaea are characteristics of transformations taking place during methane fermentation and are similar to data described by Montero et al. (2008). It appears from this research that at the beginning of fermentation, Eubacteria form the main group and the count of Archaea constitutes only an insignificant percentage of the population of microorganisms. This relationship changes during the process to advantage of Archaea, however the count of Eubacteria basically always remains higher (Montero et al., 2008). Among the analysed phylogenetic groups, the most numerous at the beginning and at the end of

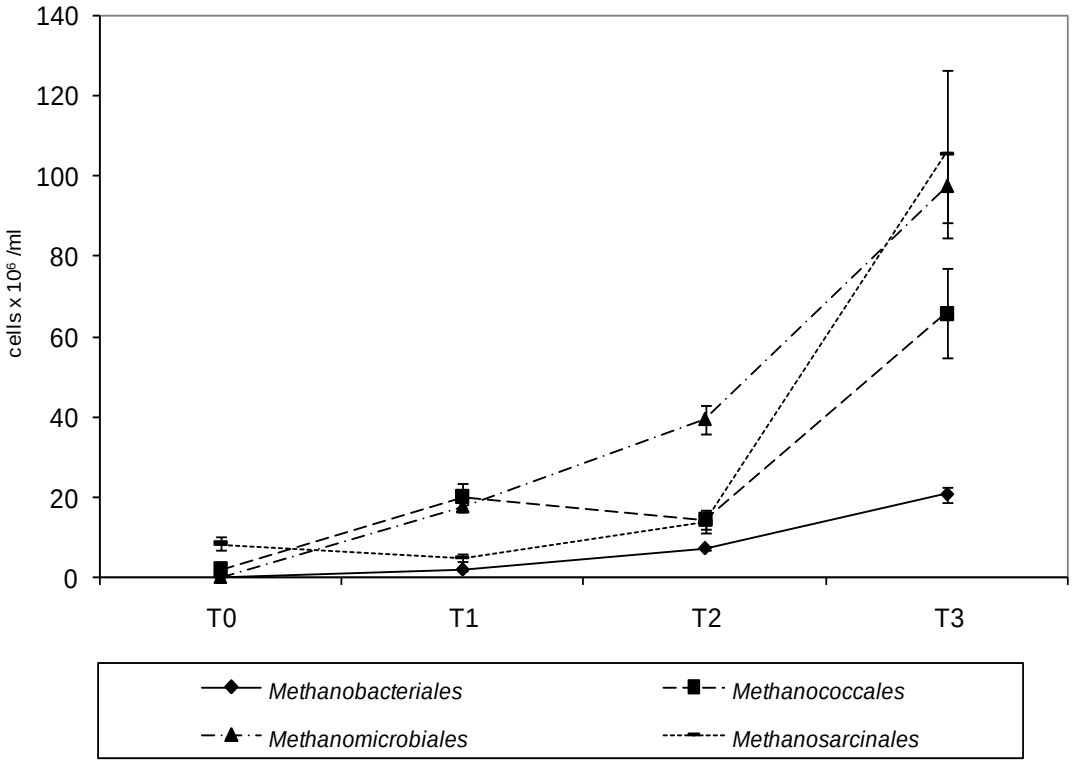

Figure 3. The dynamics of populations of methanogenic microorganisms during the methane fermentation in progress. Sample/day of fermentation: T0/0, T1/42, T2/70, T3/88.

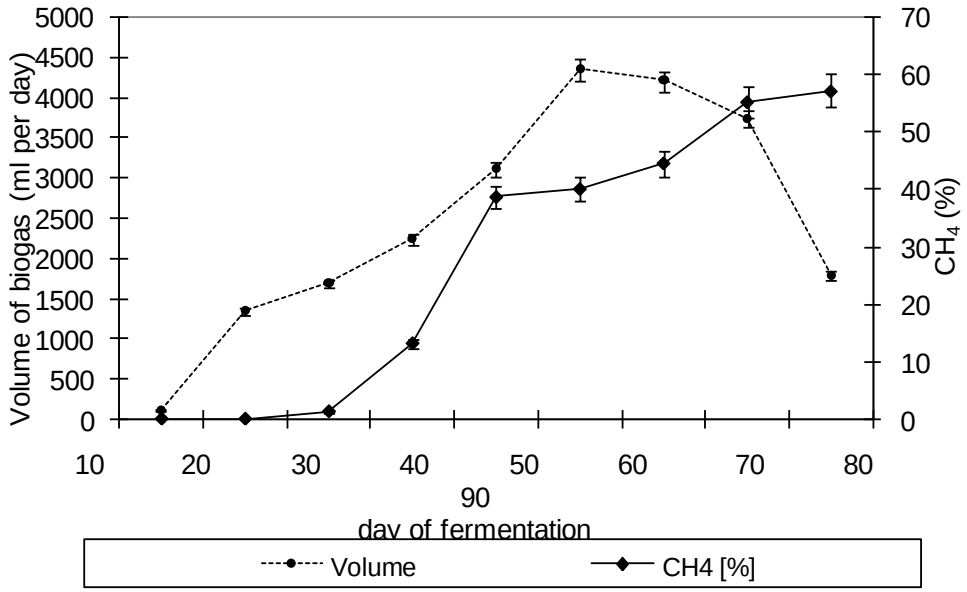

Figure 4. Biogas production and content of methane.

process was *Methanosarcinales.* These groups of micro-organisms are the only ones who use up acetate in the production of methane (Padmasiri et al., 2007). It appears from many researches (Hori et al., 2006; Lee et al., 2009) that their counts increase if the concentration of acetate increases in a reactor; the latter being a product

of decomposition of complex chemical compounds. Therefore, the count of *Methanosarcinales* at the beginning of the process is low and it increases only after a certain time, when bacteria of an acetogenic phase deliver acetate.

During the further fermentation phases, the count of this group of methanogens is generally different and depends on the availability of substrate. From the data presented in this paper, it appears that the count of *Methanosarcinales* in the initial phase of fermentation decreased. Therefore, it can be assumed that the inoculum contained significant quantities of microorganisms from this group, and then their number was decreased due to the absence of acetate in the fermentation tank. After the increase in the concentration of substrate (acetate), which resulted from the activity of acetogenic bacteria, the count of *Methanosarcinales* increased, and this increase proceeded till the end of the process, which might have resulted from the constant presence of acetate in the fermentation tank. Some isolated strains of *Methanosarcina* may also use hydrogen; however its significance as a substrate for this phylogenetic group in complex populations of microorganisms was not yet thoroughly researched.

According to Kubota et al. (2009), in most of the conditions prevailing in anaerobic reactors, *Methanosarcinales* are not able to compete for hydrogen with autotrophic methanogens. This assumption can constitute an explanation for the observations by Padmasiri et al. (2007) and Lee et al. (2009), who state that during the periods when the count of *Methanosarcinales* drops, the count of autotrophic methanogens increases and the other way round. In the presented studies, relationships similar to the aforementioned observations, proceeded only during the first 40 days of the process, when the count of *Methanosarcinales* decreased insignificantly, whereas the count of other groups increased. However, at the final stage of this process, the increase in the count was related to all analysed methanogens. These discrepancies could be caused by differences in the chemical composition of the fermentative mass or slightly different fermentation conditions, which determined the development of specific groups of microorganisms. The significance of autotrophic methanogens, as well as their competition in the population living in the bioreactor has not been studied yet so thoroughly as the bacteria using acetate (McMahon et al., 2001).

In the experiment performed by Padmasiri et al. (2007) concerning the fermentation of pig faeces, it was observed that *Methanobacteriales* formed the most abundant group at the beginning of the process, the number of which dropped in subsequent stages of the process, and among autotrophic methanogens - *Methanomicrobiales* dominated. The domination of this group was also observed during the fermentation of sewage sludge (Griffin et al., 1998; Hori et al., 2006; Raskin et al., 1994). Similar results were obtained in this paper, where *Methanomicrobiales* after the 40th day of the process were the most abundant group of autotrophic methanogens.

Montero et al. (2008) and Lee et al. (2009) in their studies on fermentation of miscellaneous resources (sewage sludge, synthetic glucose, whey) proved that the count of autotrophic groups of methanogens is higher at the beginning of the process, and after that, they are partially replaced by heterotrophic methanogenic microorganisms. During the processes studied in this paper, the count of *Methanosarcinales* was changing exactly in accordance with the described schema. At T0 time, these microorganisms dominated among methanogens, and their significant contribution could result from the introduced inoculum. In the subsequent sample (T1), their count and contribution in populations of methanogens significantly decreased, and after that (T2), their count increased, whereas the contribution in the whole population was still low. However, in the last sample (T3), the count of *Methanosarcinales* was the highest among the analysed methanogens and consequently their contribution in the population was also the highest.

From the research by Hori et al. (2006) it appears that the concentration of dissolved hydrogen is the main factor influencing the domination of autotrophic methanogens in the environment. The predominance of methanogenic bacteria that use up hydrogen could be caused by the availability of this substrate in high concentrations at the end of fermentation, which resulted in the development of these microorganisms. Furthermore, in each anaerobic environment, hydrogen is also used by other microorganisms, for example homoacetogenic bacteria or bacteria reducing sulphates, therefore microorganisms compete for this substrate. It should be assumed that conditions conducive to the development of methanogenic bacteria prevailed in fermenters, therefore they were winning the competition for hydrogen, and their count reached much higher values than in the experiments where a different substrate was subject to fermentation. The number of autotrophic methanogens can also be connected with the presence of syntrophic bacteria decomposing propionate and fatty acids, as well as interactions of these microorganisms (McMahon et al. 2001).

It appears from the above presented discussions that changes in the population size of microorganisms occur during the fermentation. Consequently, these changes may significantly affect the number of nascent biogas, its composition and the quality. On the one hand, the applied raw material is the cause of these changes, and on the other hand, the observed changes in the population of methanogens result from interactions of microorganisms, which unfortunately are seldom taken into consideration and studied.

REFERENCES

Amann RI, Krumholz L, Stahl DA (1990). Fluorescent-oligonucleotide probing of whole cells for determinative, phylogenetic and

environmental studies in microbiology. J. Bacteriol. 172: 762-770.
Amann RI, Ludwig W, Schleifer KH (1995). Phylogenetic identification and in situ detection of individual microbial cells without cultivation. Microbiol. Rev., 59: 143–169.
Beveridge TJ, Schultze-Lam S (1996). The response of selected members of the archae to the Gram stain. Microbiology, 142: 2887-2895.
Crocetti G, Murto M, Bjornsson L (2006). An update and optimisation of oligonucleotide probes targeting methanogenic Archaea for use in fluorescence in situ hybridisation (FISH). J. Microbiol. Meth., 65: 194–201.
Davies Ch M (1991). A comparison of fluorochromes for direct viable counts by image analysis. Lett. Appl. Microbiol., 13: 58-61.
Griffin M E, Mc Mahon KD, Mackie RI, Raskin L (1998). Methanogenic population dynamics during start-up of anaerobic digesters treating municipal solid waste and biosolids. Biotechnol. Bioeng., 57: 342-355.
Hori T, Haruta S, Ueno Y, Ishii M, Igarashi Y (2006). Dynamic transition of a methanogenic population in response to the concentration of volatile fatty acids in a thermophilic anaerobic digester. Appl. Environ. Microbiol., 2: 1623-1630.
Karakashev D, Batstone DJ, Angelidaki I (2005). Influence of environmental conditions on methanogenic compositions in anaerobic biogas reactors. Appl. Environ. Microbiol., 71:331–338.
Kubota K, Ozaki Y, Matsumiya Y, Kubo M (2009), Analysis of relationship between microbial and methanogenic biomass in methane fermentation. Appl. Biochem. Biotechnol., 158: 493-501.
Lee C, Kim J, Hwang K, O'flaherty V, Hwang S (2009). Quantitative analysis of methanogenic community dynamics in three anaerobic batch digesters treating different wastewaters. Water Res., 43: 157-165.
Llobet-Brossa E, Rossello´-Mora R, Amann R (1998). Microbial community composition of Wadden Sea sediments as revealed by fluorescence in situ hybridization. Appl. Environ. Microbiol., 64: 2691–2696.
Manz W, Wendt-Potthoff K, Neu TR, Szewczyk U, Lawrence JR (1999). Phylogenetic composition. spatial structure and dynamics of lotic bacterial biofilms investigated by fluorescent in situ hybridization and confocal laser scanning microscopy. Microbiol. Ecol., 37: 225–237.
Mata-Alvarez J, Mace S, Llabres P (2000). Anaerobic digestion of organic solid wastes. An overview of research achievements and perspectives. Biores. Technol., 74: 3–16.
McMahon KD, Stroor PG, Mackie RI, Raskin L (2001). Anaerobic codigestion of municipal solid waste and biosolids under various mixing conditions – II: microbial population dynamics. Water Res., 7: 1817- 1827.
Montero B, Garcia-Morales JL, Sales D, Solera R (2008). Evolution of microorganisms in thermophilic-dry anaerobic digestion. Biores. Technol., 99: 3233-3243.
Padmasiri S I, Zhang J, Fitch M, Norddahl B, Morgenroth E, Raskin L (2007). Methanogenic population dynamics and performance of an anaerobic membrane bioreactor (AnMBR) treating swine manure under high shear conditions. Water Res., 41: 134-144.
Raskin L, Poulsen LK, Noguera DR, Rittmann BE, Stahl DA (1994). Quantification of methanogenic groups in anaerobic biological reactors by oligonucleotides probe hybridization. Appl. Environ. Microbiol., 4: 1241- 1248
Raskin L, Zheng D, Griffin ME, Stroot PG, Misra P (1995). Characterization of microbial communities in anaerobic bioreactors using molecular probes. Antonie van Leeuwenhoek, 68: 297–308.
Walczak M, Swiontek Brzezinska M (2010). Phylogenetic diversity and abundance of bacteria from surface microlayer and subsurface water in eutrophic lake. Pol. J. Ecol., 1: 177-186.
Yu Y, Kim J, Hwang S (2006). Use of real-time PCR for groupspecific quantification of aceticlastic methanogens in anaerobic processes: population dynamics and community structures. Biotechnol. Bioeng., 93:424-430.