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DYNAMICS OF METHANOGENIC ARCHAEAL COMMUNITIES BASED ON THE RRNA ANALYSIS AND THEIR RELATION TO METHANOGENIC ACTIVITY

Changes in phylogenetic groups of methanogenic microorganisms during methane fermentation have been studied. Phylogenetic groups of methanogens were quantified and visualized by hybridization of oligonucleotide probes complementary to rRNA of the major phylogenetic groups. At the beginning of fermentation, *Eubacteria* are the main group and the count of Archaea constitutes only an insignificant percentage of the population of microorganisms. During the process of fermentation, a very significant increase in methanogenic microorganisms was recorded after 70 days of the process in progress. It is reflected in the quantity and the composition of released biogas.

1. INTRODUCTION

Biogas plants may play important roles in providing society with a sustainable mix of energy. Such plants convert biomass and biological waste into the so-called biogas, i.e. a mix of CH_4, CO_2 as well as H_2O containing in addition traces of N_2, H_2S. Methane is later converted into energy [1–3].

The understanding of the microbiology of such continuously operated industrial plants is still very rudimentary. The microbial 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, including such physiological groups as hydrolytic bacteria, fermentative acidogenic bacteria, acetogenic syntrophic bacteria, hydrogenotrophic and acetotrophic methanogens, and sulphate reducing bacteria. For better understanding and control of this diverse community, the groups must be analysed quantitatively. Because of difficulties with cultivation of the methanogenic microorganisms, for their quantification fluorescence *in situ* hybridization (FISH) was used with rRNA-

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targeted fluorescent oligonucleotide probes [4, 5]. 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 enable one not only to detect bacteria from specific groups, but also to determine their abundances in samples [6]. 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 [7].

The purpose of this study was to determine the dynamics of methanogenic archaeal communities and their relation to methanogenic activity in a reactor.

2. MATERIALS AND METHODS

Object of study. Methane fermentation was conducted in five laboratory fermenters of the capacity of 10 dm^3. Maize silage from an experimental cultivation intended as biomass for methane fermentation constituted the raw material for fermentation. The chemical composition of silage maize biomass is presented in Table 1.

Table 1

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%

For T0 sample, the composition of fermentation feedstock was as follows: maize silage biomass – 1800 g; water 6 dm^3; inoculum (post-fermentation liquid from previous processes of methane fermentation) – 0.5 dm^3. 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.

Collection of samples and microbiological analyses. Collection of samples depended on parameters of biogas released during fermentation. 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 dependent on the bacteria count and was determined individually for each sample. Filters with the biomass retained on their surface were subject to the following microbiological analyses:

Determination of the total number of microorganisms. The total number of microorganisms was determined by the method of direct counting under an epifluorescence microscope (Nicon Eclipce T300). For the determination purpose, microorganisms retained on the filter surface were being stained with the pigment DAPI.

Determination of the number of living and dead microorganisms. In order to perform this determination, microorganisms retained on the filter surface were subject to LIVE/DEAD staining. In the procedure of staining and identification, the diagnostic set LIVE/DEAD (Invitrogen) was applied [8].

Determination of the number of methanogenic microorganisms. Determination was performed with the FISH method [9, 10]. In the process of hybridization, oligonucleotide probes were applied, complementary to rRNA of the major phylogenetic groups of methanogenic microorganisms (Table 2). Also Eubacteria and Archaea were included in the phylogenetic analysis. Termini 5′ of oligonucleotides were marked with the fluorescent pigment Cy3. Conditions of the hybridization process for particular phylogenetic groups are presented in Table 3.

Table 2

Sequences of oligonucleotides applied in the FISH process

Group of microorganisms	Sequens (5′ ⟶ 3′)	References
Archaea	GTGCTCCCCCGCCAATTCCT	[10]
Methanosarcinales	GGCTCGCTTCACGGCTTCCCT	[10]
Methanomicrobiales	CGGATAATTCGGGGCATGCTG	[11]
Methanobacteriales	ACCTTGTCTCAGGTTCCATCTCC	[10]
Methanococcales	GCAACATAGGGCACGGGTCT	[10]
Eubacteria	GCTGCCTCCCGTAGGAGT	[4]

Microscopic analysis. In all microscopic analyses, the epifluorescence microscope (Nicon Eclipse T300) with digital registration of images was used. During the analyses, 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. All results were statistically analysed with the analysis of variance applying the software Statistica 6.0.

3. RESULTS AND DISCUSSION

Processes leading to methane production in the anaerobic environment require co-operation between many species of microorganisms and result from interactions between organisms of various metabolisms [12]. During the fermentation, an increase in the total number of microorganisms was observed. However, between sample T2 and T3, i.e. between the 42nd and the 70th day of fermentation, reduction in the total number of microorganisms was observed from 44.5×10^8 to 39.2×10^8. At the same time, the increased contribution of dead cells was recorded with respect to living ones (Table 3). However, in the subsequent measurements, both the total number of cells, as well as the contribution of living cells increased significantly.

Table 3

Total number (*TN*) of microorganisms in the fermentation liquid and the contribution of living and dead cells

Sample	$TN \pm SD$ [No. of cells·10^{-8}·cm^{-3}]	Living cells [%]	Dead cells [%]
T0	38.7±5.4	86	14
T1	44.5±3.2	89	11
T2	39.2±4.9	79	21
T3	68.0±5.9	95	5

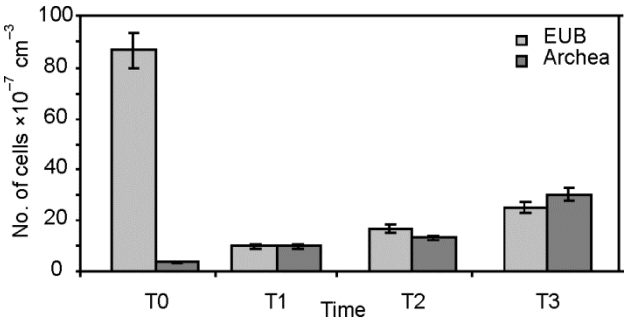

Fig. 1. The count of Eubacteria and Archaea during methane fermentation

Changes observed in Fig. 1 are characteristic of transformations during methane fermentation and are similar to those described by Montero et al. [13]. At the beginning of fermentation, Eubacteria are the main group and the count, Archaea constituting only an insignificant percentage of the population of microorganisms. The count of Archaea increases during the experiment, however the count of Eubacteria basically always remains higher [13].

During the conducted methane fermentation, the count of individual groups of methanogens differed considerably in particular phases of the process (Table 4,

Fig. 2). During the process of fermentation, a very significant increase in methanogenic microorganisms was recorded after 70 days of the process in progress. It is reflected in the quantity and the composition of released biogas (Table 5).

Table 4

Conditions of the hybridization process FISH

Parameter	*Methano-microbiales*	*Methano-bacteriales*	*Methano-sarcinales*	*Methano-coccales*	*Eubacteria* and Archaea
Hybridization buffer					
Formamide concentration	20	30	45	45	35
NaCl 5 M [µl]	360	360	360	360	360
Tris HCl 1 M pH 7.4 [µl]	40	40	40	40	40
Formamide [µl]	400	600	900	900	700
H_2O [ml]	1198	998	698	698	700
SDS 10% [µl]	2	2	2	2	2
Temperature [°C]	46	46	46	46	46
Time [min]	120	120	120	120	120
Washing buffer					
Formamide concentration	20	30	45	45	35
NaCl 5 M [µl]	2150	1020	300	300	800
Tris HCl 1 M pH 7.4 [µl]	1000	1000	1000	1000	1000
EDTA 0.5M pH 8.0 [µl]	500	500	500	500	500
H_2O [ml]	46.3	47.43	48.15	48.15	47.65
SDS 10% [µl]	50	50	50	50	50
Temperature [°C]	48	48	48	48	48
Time [min]	15	15	15	15	15

The volume of the released biogas was significantly increasing till the 15th day and then once again from about the 60th day of the process, and the percentage contribution of methane in the gas increased only after 50 days of the process. The 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. Also the absence of such compounds as H_2S or NH_3 in the nascent biogas indicate quite untypical course of the process.

Among the analysed phylogenetic groups, *Methanosarcinales* are the only ones who use up acetate in the production of methane [14]. Their count increases if the concentration of acetate increases in a reactor; the latter one being a product of de-

composition of complex chemical compounds [15, 16]. 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 further fermentation phases, the count of this group of methanogens is generally different and depends on the availability of substrate.

Table 5

Characteristics of released biogas and composition of populations of methanogenic microorganisms

Sample/ day of fermentation	Volume of biogas [cm^3/d]	Contents				Number of methanogenic microorganisms $\times 10^{-7}$ cm^{-3}			
		CH_4 [%]	H_2S [ppm]	H_2 [ppm]	NH_3 [ppm]	*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

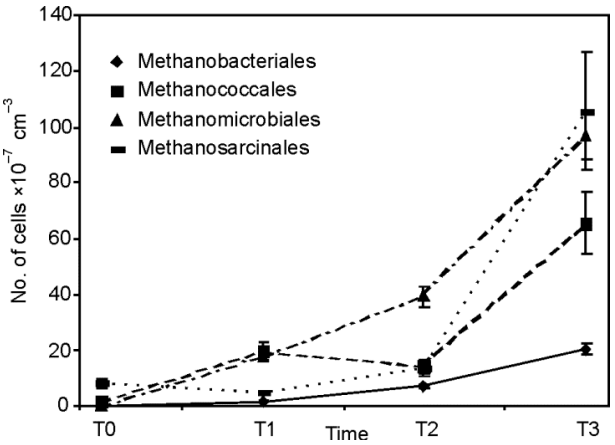

Fig. 2. Dynamics of populations of methanogenic microorganisms during the methane fermentation

From the data presented in this paper, it appears that the count of *Methanosarcinales* in the initial phase of fermentation decreased (Table 5, Fig. 2). Therefore one can assume that the inoculum contained significant quantities of microorganisms from this group, and then their number was decreasing 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, what might result 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 examined. According to Ahring [17] 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 observations by Padmasiri et al. [14] and Lee et al. [16], who state that during the periods when the count of *Methanosarcinales* decreases, the count of autotrophic methanogens increases and the other way around. 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 (Fig. 2). 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 as thoroughly as the bacteria using acetate [18]. In the experiment performed by Padmasiri et al. [14] concerning the fermentation of pig faeces, it was observed that *Methanobacteriales* were the most abundant group at the beginning of the process, the number of which dropped in subsequent stages of the process, and *Methanomicrobiales* dominated among autotrophic methanogens. The domination of this group was also observed during the fermentation of sewage sludge [16, 19, 20]. Similar results were obtained in this paper, where *Methanomicrobiales* after the 40th day of the process were the most abundant group of autotrophic methanogens (Table 5, Fig. 2).

Montero et al. [18] and Lee et al. [16] 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 then 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. [15] 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 concentra-

tions 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, e.g. homoacetogenic bacteria or bacteria reducing sulphates, therefore microorganisms compete for this substrate. One should assume 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 [18].

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.

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