

*Review*

# Transformations, stages and microorganisms that participate in methane fermentation process

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Accepted 26 April, 2016

**Anaerobic decomposition of organic compounds is conducted in close cooperation of specialized bacteria of different types, including mostly hydrolyzing, digestive, acetogenic, homoacetogenic, sulfate-reducing (VI) and methanogenic bacteria. A great interest in the anaerobic digestion process results mainly from its advantages, as compared to aerobic methods. The main advantages of the methane digestion process are: Production of an insignificant amount of biomass and lower energy input, as compared to degradation conducted under aerobic conditions. This paper reviews the transformations, stages and microorganisms that participate in methane fermentation process.**

**Key words:** Biogas, methane fermentation, methanogens, microorganisms, anaerobic digestion.

## INTRODUCTION

The demand of the world economy for electrical and thermal energy in over 88% is covered from non-renewable resources, mainly petroleum and natural gas (IEA, 2006). The extraction, processing and combustion of these raw materials adversely affect the natural environment, besides they are gradually exhausted (Energy, 1997; EC, 2007). According to forecasts, the demand for fuels used for energy production will be further increased.

The European Union's energy policy aims at using more and more unconventional sources of energy. It was assumed that up to 2010 in the European Union the electrical energy produced in the environmentally friendly way would constitute 22.1% of the total amount of produced energy. On the one hand, this is connected with energy safety; on the other hand it contributes to improvement of the environment. Until 2050 the use of modern technologies of energy acquisition should cause 50% reduction of CO<sub>2</sub> emission (IPCC, 2000). The use of 1 million equivalent

hectares for energy crops may provide approximately 65 TWh energy in the primary fuel. The use of biogas, acquired in this way, for production of electrical energy and heat would allow the reduction of the annual emission of CO<sub>2</sub> by about 57 million tons.

The European Parliament Committee on Industry, External Trade, Research and Energy calls on to increase efforts within the research on new technologies of using biogas as a biofuel. Studies are carried on to use biogas not only for the production of electrical energy and heat but also to introduce it in the natural gas supplied network, which would allow to reduce Europe's dependence on the import of natural gas from the third countries. In the European Union in 2007 the production of biogas amounted to over 6 million tons. Since then, a 20% increase annually has been noted in the amount of produced biogas (EuroObserver, 2008; Fachverband, 2009). Biogas production from large quantities of agricultural residues, animal wastes, municipal and industrial wastes (water) appears to have potential as an alternative renewable energy for many African countries if relevant and appropriate research is carried out to adopt the biogas technology to the local conditions in African countries (Mshandete and Parawira, 2009). Biogas is

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presently obtained not only from landfill degasification, anaerobic sludge digestion in wastewater treatment plants, but more and more often also from industrial biogas-plants where the combined processing of organic municipal and industrial wastes as well as sewage sludge is conducted (Mata-Alvarez et al., 2000; Weiland, 2010). Such procedure is connected with drastic reduction of the possibilities of landfilling organic wastes; in 2010 the mass of biodegradable wastes was reduced to 75% as compared to the wastes disposed in 1995.

## PROCESS OF ANAEROBIC DIGESTION

Methane digestion is a complex, reduction process of a number of biochemical reactions occurring under anaerobic conditions. Under symbiotic effects of various anaerobic and relatively anaerobic bacteria, multi molecular organic substances are decomposed into simple, chemically stabilized compounds – mainly of methane and carbon dioxide (Naik et al., 2010). Generally, this process consists of liquefaction and hydrolysis of insoluble compounds and gasification of intermediate products. This is accompanied by a partial or complete mineralization and humification of organic substance (Lyberatos and Skiadas, 1999). An advantage of the process of anaerobic digestion is production of biogas, a high energy fuel which may be used to produce environmentally-friendly energy. It is basically for this reason that scientists and power industry companies have been interested in anaerobic digestion for almost 140 years. Biotechnology of biogas production usually refers to digestion of various types of organic wastes, food industry wastewater, sewage sludge, animal excrements or organic fraction of municipal wastes etc. In some countries subjected to anaerobic digestion are plants deliberately grown for this purpose, e.g. maize (Boone et al., 1993; Mata-Alvarez et al., 2000). Currently in many European countries, the production of biomass as a substrate for the biogas plants has developed. In the most extreme European case, the government of Germany has taken steps in 2011 to reduce even monoculture maize production for energy purposes (Graaf and Fendler, 2010; Weiland, 2010).

Digestion connected with biogas production may play a triple part. First, it is a method of converting the energy contained in biomass into a useful fuel (biogas) which may be stored and transported. Second, it is a method of recycling of organic wastes into stable soil additives, that is, valuable liquid fertilizer and energy. Third, it is a method of wastes treatment aimed at a reduction of their hazardous effects on the environment.

## TRANSFORMATIONS OF METHANE FERMENTATION PROCESS

Methane digestion is used for stabilization of wastes,

such as: Sewage sludge, manure, industrial wastewater and organic fraction of municipal wastes (Classen et al., 1999; Finstein, 2010; Verstraete et al., 2002). In the process of biochemical degradation, the complex organic compounds become decomposed to simple organic and inorganic compounds (Bryant, 1979). During the methane digestion process, microbiological reduction of sulfates (VI) to sulfides and hydrogen sulfide occurs along with anaerobic ammonification and reduction of nitrates (V) to ammonia. Apart from assimilative reduction of nitrates (V), denitrification may occur (Scherer et al., 2000).

Initial stages of anaerobic metabolism are similar to those of aerobic processes. When dissolved oxygen is missing, some chemolithotrophic organisms make use of oxidized mineral compounds (sulfates (VI) and nitrates (V)) as the final acceptor of hydrogen. Oxidation proceeds then, as under aerobic conditions, through the respiratory chain, but the final products are respectively hydrogen or molecular nitrogen and energy (Santosh et al., 2004). The process of digestion releases into the environment the high energy final products, such as alcohol or methane. Methane digestion is a complex process which undergoes four phases: Hydrolysis, acidogenesis – acidification phase, acetogenesis, methanogenesis (Figure 1). Involved in biochemical conversions of  $H_2$  and  $CO_2$  to methane and acetate to methane and  $CO_2$  are various enzymes and prosthetic groups which occur only in methanogenes. The basic structures of these compounds are presented in Figure 2 and comprise: Deazariboflavine derivative  $F_{420}$ , methanopterin, methanofurane, nickel-tetrapyrrol factor  $F_{430}$  and coenzyme M (mercaptan sulfonate). Autotrophic binding of  $CO_2$  by methanogenes occurs without a share of the reaction of ribulose-bisphosphatic cycle. Synthesis of cellular material with  $CO_2$  occurs through the reductive pathway of aceto-CoA with pyruvate (Mashaphu, 2005; Saxena et al., 2009).

At the first stage of the process,  $CO_2$  is bound by methanofurane (MFR) which is then reduced to methenyl, methylene, methyl and at the final stage – methane, which is bound in turn by coenzymes: Tetrahydromethanopterin, 2-methylthioethanesulfonic acid and 2-mercaptoethanesulfonic acid (Medigan et al., 2000). Hydrogenase accounts for assimilation of  $H_2$ . As a result of activation of hydrogen by hydrogenases which react with factor  $F_{420}$ , a reducing force is provided. Most methanogenes use  $H_2$  as a source of electrons, which is connected with the occurrence of hydrogenases.

Methanopterin accounts for the stage of the reduction of  $CO_2$  to methyl groups of pyruvate. Methyl groups in the carbonylation process are converted into carbonyl groups with a share of carbon monoxide dehydrogenase enzyme (Mashaphu, 2005; Saxena et al., 2009). Involved in methanogenesis pathway are many coenzymes which do not have any flavinic or quinonic groups (Figure 2). Metabolism of methanogenesis is unique, because it runs along the pathway which requires coenzymes which do

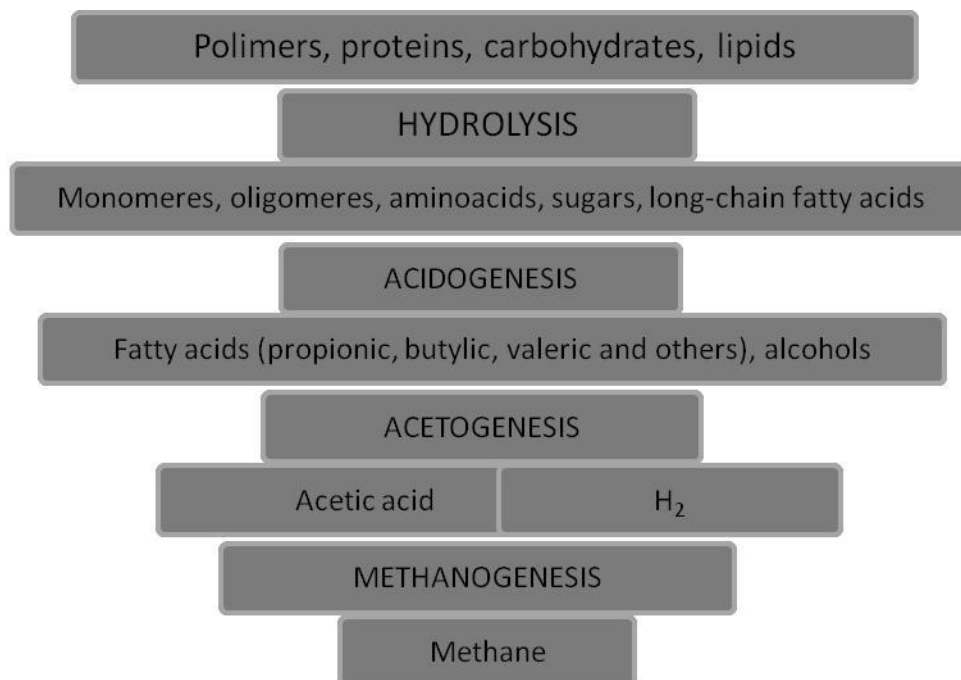


Figure 1. Stages of methane fermentation process

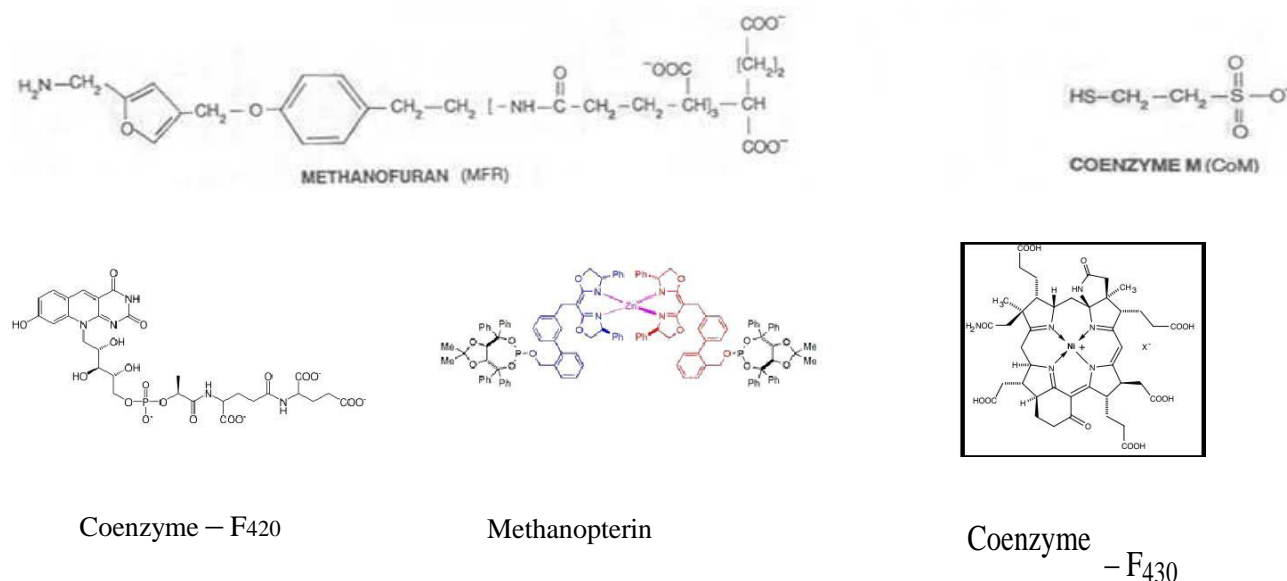


Figure 2. Coenzymes and prosthetic groups of methanogenes

not occur in any live organisms, except for methanogenes (Smith, 1966; Zeikus, 1977).

Methanogenes C1 participated in the metabolic pathway of methanofurane, methanopterin and coenzyme M, whereas coenzymes F<sub>420</sub> and B act as electron donors. C1 compounds do not contain any carbon-carbon bonds. They contain monocarbon compounds, such as methane (CH<sub>4</sub>), methanol (CH<sub>3</sub>OH), dimethyl carbonate (CH<sub>3</sub>OCOOCH<sub>3</sub>) and other

monocarbon compounds. These compounds appear in the environment as a result of the digestion and decay of products of vegetable and animal origin and also pesticides. Methane is produced by methanogenic archaeons using carbon dioxide as electron acceptor (Medigan et al., 2000; Mashaphu, 2005).

Deazariboflavine – derivative F<sub>420</sub> is a coenzyme of electron transfer used by many enzymes, such as hydrogenase, formate dehydrogenase, methylene

dehydrogenase of tetrahydromethanopterin ( $H_4MPT$ ), methylene reductase  $H_4MPT$  and heterodihydrogen sulfide reductase. As earlier mentioned, MFR participated in the stage of methanogenesis initiation only when  $CO_2$  is bound with furane. In successive stages, it is reduced to the level of formyl and transformed to the next coenzyme of tetrahydromethanopterin. There are four types of tetrahydromethanopterin which may occur in three different degrees of oxidation (Mashaphu, 2005).

## BIOGAS

Biogas is a digester gas arising from the activity of methanogenic anaerobic bacteria which decompose organic matter. Its composition depends on the type of raw material subjected to the digestion process and on the method of conducting this process and is as follows: Methane  $CH_4$  (50–75%), carbon dioxide  $CO_2$  (25–45%), hydrogen sulfide  $H_2S$  (0–1%), hydrogen  $H_2$  (0–1%), carbon monoxide  $CO$  (0–2%), nitrogen  $N_2$  (0–2%), ammonia  $NH_3$  (0–1%), oxygen  $O_2$  (0–2%) and water –  $H_2O$  (2–7%) (Graaf and Fendler, 2010). The obtained biogas may be used in various fields of economy (Classen et al., 1999; Naik et al., 2010; Verstraete et al., 2002), mainly in technological processes and for power engineering purposes, including the following:

1. Production of thermal energy in gas boilers and production of thermal and electrical energy in associated units – (from  $1\ m^3$  of biogas – in associated production of energy 2.1 kWh of electrical energy and 2.9 kWh of heat is obtained);
2. Production of electrical energy in spark - ignition or turbine engines;
3. Using the obtained gas as a fuel in motor-car engines;
4. Using the obtained gas in various technological processes, e.g. in the production of methanol.

The average efficiency of methane digestion reaches approximately  $0.24\ m^3$  of methane from 1 kg of dry organic matter.  $1\ m^3$  of biogas of  $26\ MJ\ m^{-3}$  calorific value may replace  $0.77\ m^3$  of natural gas of  $33.5\ MJ$  calorific value, 1.1 kg of hard coal of  $23.4\ MJ$  calorific value or 2 kg of firewood of  $13.3\ MJ$  calorific value (Arbon, 2002).

## STAGES OF ANAEROBIC DEGRADATION OF ORGANIC WASTES

Microbiology of anaerobic transformation of organic wastes is a process which involves many different groups of bacteria, such as hydrolysing, acidifying, acetogenic and methanogenic bacteria which in the final stage produce  $CO_2$  and  $CH_4$ , that is, the main products of the digestion process (Demirel and Scherer, 2008; Nealson, 1997). A specific characteristic of methane digestion is its

phasing. Each of them accounts for degradation of a different type of compounds.

**Hydrolysis:** During hydrolysis of the polymerized, mostly insoluble organic compounds, that is, carbohydrates, proteins, fats are decomposed to soluble monomers and dimers, that is, monosugars, amino acids and fatty acids. This stage of the methane digestion process passes through extracellular enzymes from the group of hydrolases (amylases, proteases, lipases) produced by appropriate strains of hydrolyzing bacteria. Hydrolysis of hardly decomposable polymers, that is, cellulose and cellucottons is considered to be a stage which limits the rate of wastes digestion. During solid wastes digestion, only 50% of organic compounds undergo biodegradation. The remaining part of the compounds remains in their primary state because of the lack of enzymes participating in their degradation (Conrad, 1999; Parawira et al., 2008).

The rate of hydrolysis process depends on such parameters as: Size of particles, pH, production of enzymes, diffusion and adsorption of enzymes on the particles of wastes subjected to the digestion process. Hydrolysis is carried out by bacteria from the group of relative anaerobes of genera: *Streptococcus*, *Enterobacterium* (Bryant, 1979; Smith, 1966).

**Acidogenesis (acidification phase):** During this stage, the acidifying bacteria convert water-soluble chemical substances, including hydrolysis products to short-chain organic acids (formic, acetic, propionic, butyric, pentanoic), alcohols (methanol, ethanol), aldehydes, carbon dioxide and hydrogen. From decomposition of proteins, amino acids and peptides arise, which may be a source of energy for anaerobic microorganisms. Acidogenesis may be two-directional due to the effects of various populations of microorganisms. This process may be divided into two types: Hydrogenation and dehydrogenation. The basic pathway of transformations passes through acetates,  $CO_2$  and  $H_2$ , whereas other acidogenesis products play an insignificant role. As a result of these transformations, methanogenes may directly use the new products as substrates and energy source. Accumulation of electrons by compounds such as lactate, ethanol, propionate, butyrate, higher volatile fatty acids is the bacteria's response to an increase in hydrogen concentration in the solution. The new products may not be used directly by methanogenic bacteria and must be converted by obligatory bacteria producing hydrogen in the process called acetogenesis. Among the products of acidogenesis, ammonia and hydrogen sulfide which give an intense unpleasant smell to this phase of the process should also be mentioned (Ntaikou et al., 2010; Classen et al, 1999; Conrad, 1999). The acid phase bacteria belonging to facultative anaerobes use oxygen accidentally introduced into the process, creating favourable conditions for the development of obligatory

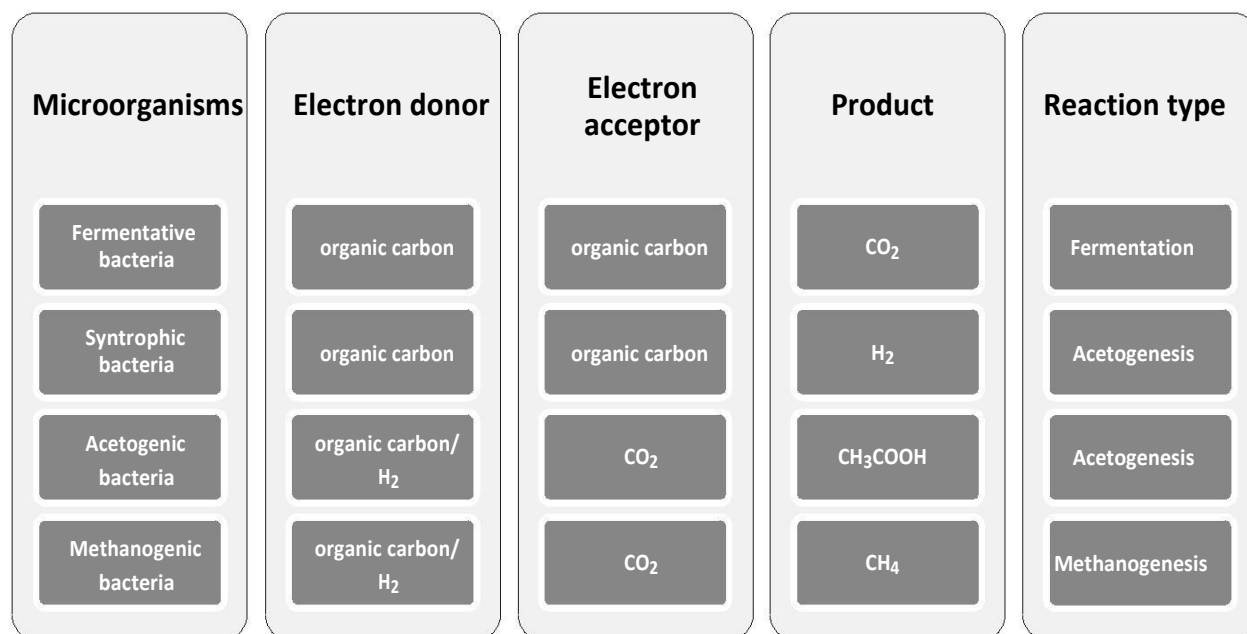


Figure 3. Microorganisms cooperation in organic matter degradation.

anaerobes of the following genera: *Pseudomonas*, *Bacillus*, *Clostridium*, *Micrococcus* or *Flavobacterium*.

**Acetogenesis:** In this process, the acetate bacteria including those of the genera of *Syntrophomonas* and *Syntrophobacter* convert the acid phase products into acetates and hydrogen which may be used by methanogenic bacteria (Schink, 1997). Bacteria

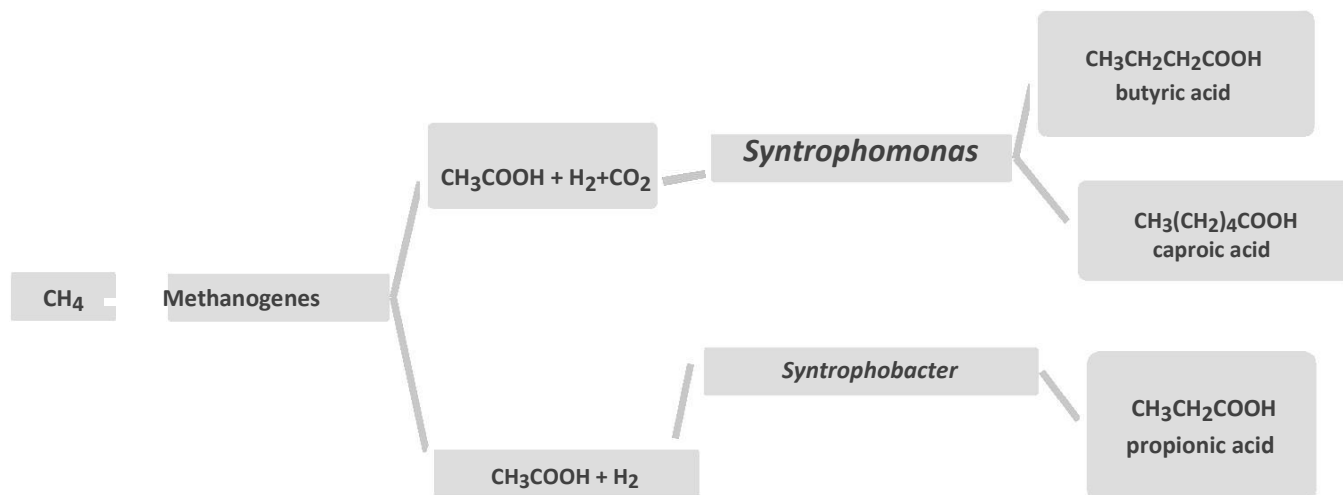
*Methanobacterium suboxydans* account for decomposition of pentanoic acid to propionic acid, whereas *Methanobacterium propionicum* accounts for decomposition of propionic acid to acetic acid. As a result of acetogenesis, hydrogen is released, which exhibits toxic effects on the microorganisms which carry out this process. Therefore, a symbiosis is necessary for acetogenic bacteria with autotrophic methane bacteria using hydrogen, hereinafter referred to as syntrophy (Schink, 1997; de Bok et al., 2005). Acetogenesis is a phase which depicts the efficiency of biogas production, because approximately 70% of methane arises in the process of acetates reduction. Consequently, acetates are a key intermediate product of the process of methane digestion. In acetogenesis phase approximately 25% of acetates are formed and approximately 11% of hydrogen, produced in the wastes degradation process.

**Methanogenesis:** This phase consists in the production of methane by methanogenic bacteria. Methane in this phase of the process is produced from substrates which are the products of previous phases, that is, acetic acid, H<sub>2</sub>, CO<sub>2</sub> and formate and methanol, methylamine or dimethyl sulfide. Despite the fact that only few bacteria

are capable to produce methane from acetic acid, a vast majority of CH<sub>4</sub> arising in the methane digestion process results from acetic acid conversions by heterotrophic methane bacteria (Demirel and Scherer, 2008). Only 30% of methane produced in this process comes from CO<sub>2</sub> reduction carried out by autotrophic methane bacteria. During this process H<sub>2</sub> is used up, which creates good conditions for the development of acid bacteria which give rise to short-chain organic acids in acidification phase and consequently – too low production of H<sub>2</sub> in acetogenic phase. A consequence of such conversions may be gas rich in CO<sub>2</sub>, because only its insignificant part will be converted into methane (Griffin et al., 2000; Karakashev et al., 2005).

#### COOPERATION OF MICROORGANISMS OF METHANE FERMENTATION PROCESS

Conversions of complex organic compounds to CH<sub>4</sub> and CO<sub>2</sub> are possible owing to the cooperation of four different groups of microorganisms (Figure 3). These microorganisms may be counted among: primary fermentation bacteria, secondary fermentation bacteria (syntrophic and acetogenic bacteria) and two types of methanogenes belonging to domain *Archaea*. These microorganisms occur in natural environment and fulfill various roles during the process of anaerobic degradation of wastes (Conrad, 1999; Mashaphu, 2005). Syntrophy is a form of symbiosis of two metabolically different groups of bacteria, which enables a degradation of various substrates (de Bok et al., 2005; Demirel and Scherer,



**Figure 4.** Metabolism of fatty acids during syntrophy.

2008). Cooperation of the population of microorganisms enables a synthesis of certain products which are then used by another group of bacteria. The bacteria belonging to domain *Archaea*, which are involved in the production of methane exhibit synergistic relationships with other populations of microorganisms. They may develop only when hydrogen is used by hydrogenotrophs. Such cooperation between microorganisms producing hydrogen and using hydrogen was defined as interspecific transfer of hydrogen (Conrad, 1999; Mashaphu, 2005). Syntrophy between microorganisms producing and using hydrogen allows for the growth and activity of these microorganisms.

The phenomenon of interspecific transfer of hydrogen enables the growth of the so called syntrophic bacteria (*Syntrophomonas*, *Syntrophospora*, *Syntrophobacter*) which oxidise such compounds as propionate and butyrate and thereby obligatorily use hydrogen as electron acceptor. The reaction of butyrate oxidation, carried out by these microorganisms, may occur only when low pressure of  $H_2$  is maintained (Demirel and Scherer, 2008; McMahon et al., 2004). Syntrophic bacteria cannot grow in the form of pure cultures, but only when accompanied by microorganisms using hydrogen produced by them, e.g. methanogenic archaeons. So syntrophy is a process in which the compound's decomposition occurs, with participation of two or more microorganisms and none of them can use this compound separately. Syntrophic bacteria produce adenosine triphosphate (ATP) as a result of substrate phosphorylation or oxidation, used in their metabolism of not only fatty acids but also alcohols produced by other microorganisms as a result of digestion. These compounds are converted to acetate and hydrogen which are used by methanogenic archaeons (de Bok et al., 2005). Bacteria of *Syntrophomonas* genus oxidise butyric

acid and caproic acid to  $CH_3COOH$  and  $H_2$ . Species belonging to this genus oxidise also pentanoic and enanthic acids to  $CH_3COOH$ ,  $CO_2$  and  $H_2$ . Oxidation of propionate is also an important stage of methanogenesis process in which syntrophic consortia of acetogenic bacteria and methanogenic archaeons obligatorily participated (Demirel and Scherer, 2008). Most of the propionate-oxidising syntrophic bacteria, so far isolated from different environments, belonged to

*Syntrophobacter* genus from  $\delta$ -*proteobacteria* group.  $CH_3COOH$  and  $H_2$  produced by the mentioned bacteria are used by methanogenic archaeons to produce methane (Nealson, 1997). Species of *Syntrophobacter* genus are capable to use sulfates as electron acceptor in the process of propionate oxidation. Syntrophic oxidation of propionate is not limited to gram-negative bacteria but also to gram-positive bacteria in which a similar phenomenon was observed. Two thermophilic, gram-positive species of syntrophic bacteria oxidising propionate have been described: *Desulfotomaculum thermobenzoicum* subsp. *thermosyntrophicum* and *Desulfovibrio* (de Bok et al., 2005, Mashaphu, 2005). *Desulfotomaculum*, similarly to *Syntrophobacter* may use sulfates as electron acceptors. *Desulfovibrio* may participate, along with *Metanobacterium* genus, in forming  $CH_3COOH$  and  $H_2$  in the process of anaerobic respiration and digestion, using sulfate and lactate to make these products. Metabolism of fatty acid during syntrophy was presented in figure 4.

#### **METHANOGENES – KEY MICROORGANISMS OF THE METHANE FERMENTATION PROCESS**

Methanogenes are strict anaerobes; the presence of oxygen is lethal for them. These microorganisms contain

neither catalase nor superoxide dismutase. Due to extraordinary sensitivity of these microorganisms to oxygen, their biochemistry, physiology and ecology are less known.

Methanogenes as absolutely anaerobic microorganisms inhabit anaerobic environment ecosystems, such as tundras, marshlands, rice fields, bottom deposit, swamps, sandy lagoons, tanks where wastewater is decomposed, sewage sludge, solid wastes landfills, ruminants' stomach (Bryant, 1979; Smith, 1966; Zeikus, 1977). These microorganisms are particularly sensitive to changes in temperature and pH, their development being inhibited by a high level of volatile fatty acids and other compounds, that is, hydrogen, ammonia, sulphur hydrogen in the environment (Zeikus, 1977).

Among methanogenic microorganisms, we can distinguish psychro-, meso- and thermophilic microorganisms. Mesophilic and thermophilic bacteria described in literature exhibit high activity within temperature, respectively 28 to 42°C and 55 to 72°C. So far, no anaerobic psychrophilic bacterium has been found which would exhibit activity at a temperature lower than 25°C. Temperature is very important for methanogenic bacteria, due to a limited temperature resistance of their enzymatic structures.

Methanogenic bacteria usually develop in inert conditions, with environmental pH from 6.8 to 7.2. This, however, does not mean that methanogenesis does not occur in environments of acid or alkaline reaction. Methanogenes which decompose acetates (*Methanosarcinabarkeri* and *Methanosarcina* sp.) were isolated from environments of approximately pH 5, while methylotrophic and hydrogen-oxidising methanogenes were found in strongly alkaline ecosystems (Smith, 1966). Methanogenic bacteria belong to chemolithotrophs, because they are capable to use CO<sub>2</sub> as a source of carbon (Smith, 1966; Zeikus, 1977).

Methanogenes are an important group of microorganisms for several reasons, the most important being their ability to process organic matter to methane. Methanogenic bacteria are used in anaerobic decomposition of wastewater, as a part of the wastes treatment system. Sedimentation processes are also used in stabilization of primary and secondary sludge arising in the process of aerobic wastewater treatment. These microorganisms arouse also some interest in pharmaceutical industry, because they may constitute a source of vitamin B<sub>12</sub> (Yanga et al., 2004).

### Taxonomy and morphology of methanogenes

All alive organisms, based on analysis and comparison of conservative phylogenetic features, through analysis of 16S and 18S rRNA, were classified to three main taxonomic units of the living world. Three phylogenetic domains may be distinguished: *Archaea*, *Bacteria* and *Eukarya*. According to analysis of sequence 16S rRNA,

methanogenic bacteria were classified to domain *Archaea*. Among the microorganisms within domain *Archaea*, four groups are distinguished, the most visible being *Crenarchaeota* and *Euryarchaeota*. In taxonomic and phenotypic respect, methanogenes belong to *Euryarchaeota* group (Vignais et al., 2001). Methanogenic bacteria are divided into 4 classes, 5 orders, 9 families and 26 genera (Demirel and Scherer, 2008). Phylogenetic classification of methanogenes is presented in Figure 5.

As earlier mentioned, according to assumed classification of live organisms, methanogenes are archeons. Unlike bacteria, methanogenes do not have a typical peptidoglycan (mureinic) skeleton and are characterized by a different metabolism. Besides, methanogenes' cytoplasmatic membrane consists of lipids composed of isoprenoid hydrocarbons glycerol lipids. Methanogene ribosomes exhibit a similar size to that of eubacteria ribosome, but the sequence of principles in ribosome RNA, especially 16S rRNA, is completely different (Watanabe et al., 2004).

Methanogenes are largely differentiated morphologically. Methanogenes exhibit almost all shapes occurring in bacteria: Cocci (*Methanococcus*), rods (*Methanobacterium*), short rods (*Methanobrevibacter*), Spirillaceae (*Methanospirillum*), sarcina (*Methanosarcina*), filiforms (*Methanothrix*). The size of these microorganisms ranges from 0.3 to 7.4 µm (Karakashev et al., 2005). The properties of selected methanogenes are presented in Table 1. Typical reactions carried out by methanogenes during anaerobic processes are shown in Table 2.

### Substrates used by methanogenic bacteria

Methanogenes process a limited quantity of simple organic substrates, the most important of which are: CH<sub>3</sub>COOH and H<sub>2</sub>-CO<sub>2</sub> (Conrad, 1999). Most methanogenic bacteria are capable to use H<sub>2</sub> and CO<sub>2</sub> for their growth (Table 2), although certain species process CH<sub>3</sub>COOH, CH<sub>3</sub>NH<sub>2</sub> and HCOOH (Classen et al., 1999).

Owing to used substrates, methanogenes are divided into two groups (Demirel and Scherer, 2008):

1. Hydrogenotrophic – which use only H<sub>2</sub> and CO<sub>2</sub>. Partial pressure of hydrogen is an important parameter which defines stability and disturbances in the anaerobic digestion process. Therefore the activity of hydrogenotrophic methanogenes is essential for stability and efficiency of the digestion process. Efficiency and activity of hydrogenotrophic methanogenes are important both in anaerobic processing of simple, soluble types of substrates (such as acetate, ethanol, dextrose, propionate), and various types of wastes (e.g. oil). The role of this group of microorganisms in anaerobic processing of biomass of complex organic compounds

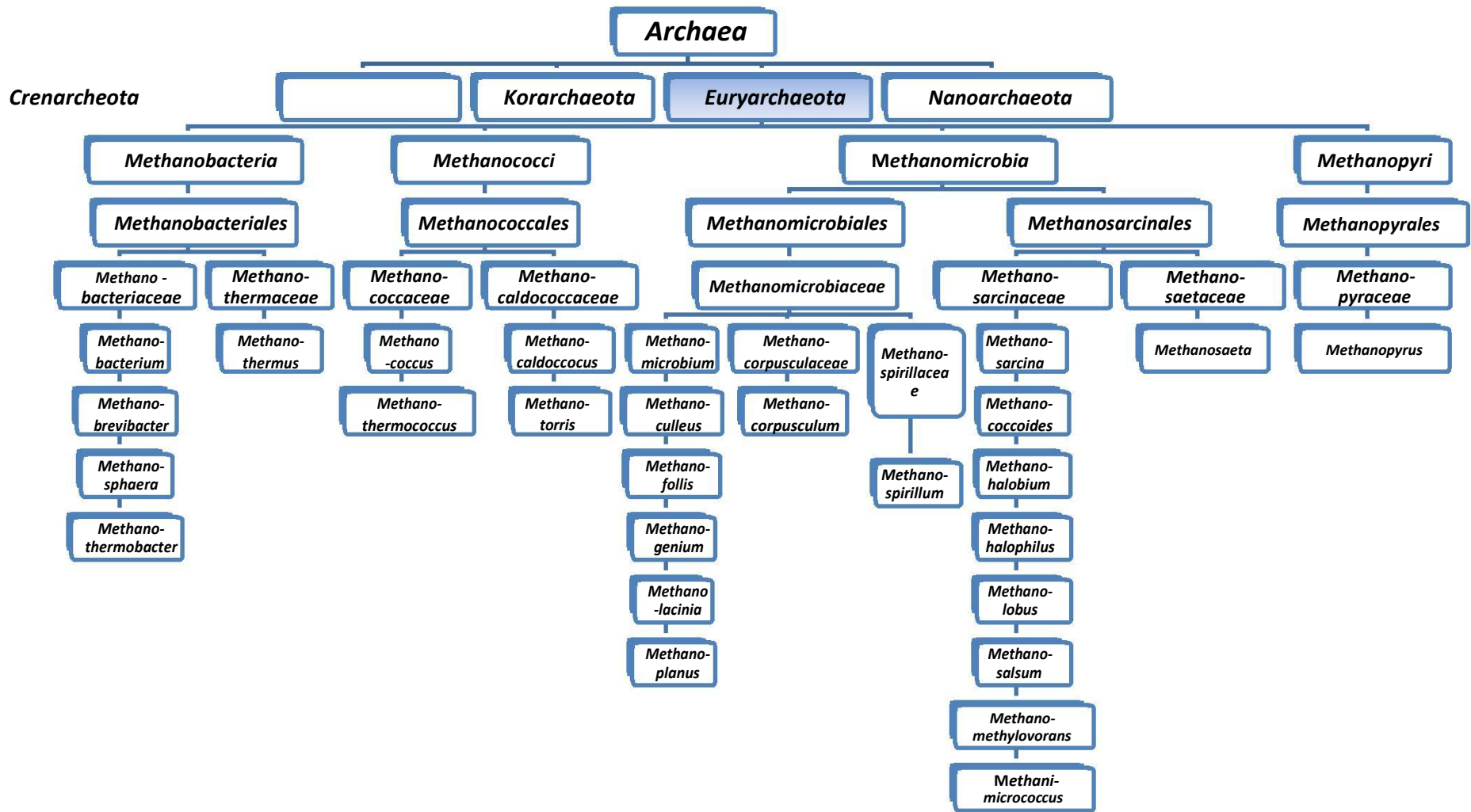


Figure 5. Phylogenetic hierarchy of methanogenes. Source: Demirel and Scherer 2008.

is not well known yet.

2. Acetotrophic – reduce methyl groups, such as: Species of *Methanosarcinales* genus which use

simple compounds for their growth; that is, acetate. Acetate is one of the most important substrates for methanogenic bacteria, because over 70% of biomethane comes from processing

of acetic acid. Acetotrophic methanogenes are obligatory anaerobes which process acetate to methane and carbon dioxide. The activity and efficiency of this group of microorganisms are



**Table 1.** Characteristics of selected methanogenic bacteria

Species	Morphology	Width/length of cell (µm)	Substrate	Optimum temperature (°C)	Optimum pH
<i>Methanobacterium Brytanii</i>	Longrods	0.5-1.0/1.5	H <sub>2</sub> /CO <sub>2</sub>	37	6.9-7.2
<i>Methanobacterium formicicum</i>	Longrods	0.4-0.8/2-15	H <sub>2</sub> /CO <sub>2</sub> . formate	37-45	6.6-7.8
<i>Methanobacterium thermoalcaliphilum</i>	Rods	0.3-0.4/3-4	H <sub>2</sub> /CO <sub>2</sub>	58-62	8.0-8.5
<i>Methanothermobacter thermoautotrophicum</i>	Longrods	0.3-0.6/2-7	H <sub>2</sub> /CO <sub>2</sub>	65-70	7.0-8.0
<i>Methanothermobacter wolfii</i>	Rods	0.4/2.4-2.7	H <sub>2</sub> /CO <sub>2</sub>	55-65	7.0-7.5
<i>Methanobrevibacter smithii</i>	Shortrods and chains	0.6-0.7/1.0-1.5	H <sub>2</sub> /CO <sub>2</sub> . formate	37-39	-
<i>Methanobrevibacter ruminantium</i>	Shortrods and chains	0.7/0.8-1.7	H <sub>2</sub> /CO <sub>2</sub> . formate	37-39	-
<i>Methanothermobacter fervidus</i>	Shortrods	0.3-0.4/1.3	H <sub>2</sub> /CO <sub>2</sub> . formate	83	<7.0
<i>Methanothermococcus thermolithotrophicus</i>	Cocci	-	H <sub>2</sub> /CO <sub>2</sub> . formate	65	-
<i>Methanococcus voltaei</i>	Cocci	1.5 (diameter)	H <sub>2</sub> /CO <sub>2</sub> . formate	35-40	6.0-7.0
<i>Methanococcus vannielii</i>	Cocci	1.3 (diameter)	H <sub>2</sub> /CO <sub>2</sub> . formate	65	7-9
<i>Methanomicrobium mobile</i>	Shortrods	0.7/1.5-2.0	H <sub>2</sub> /CO <sub>2</sub> . formate	40	6.1-6.9
<i>Methanolacinia paynteri</i>	Shortrods	0.6/1.5-2.5	H <sub>2</sub> /CO <sub>2</sub>	40	7.0
<i>Methanospirillum hungatei</i>	Shortrods	0.5/7.4	H <sub>2</sub> /CO <sub>2</sub> . formate	30-40	-
<i>Methanosarcinaacetivorans</i>	Irregularcocci	-	Methanol. acetate	35-40	6.5
<i>Methanosarcina barkeri</i>	Irregularcocci, irregularpackets	-	H <sub>2</sub> /CO <sub>2</sub> . methanol. acetate	35-40	5-7
<i>Methanosarcina mazeii</i>	Irregularcocci, irregularpackets	-	Methanol. acetate	30-40	6-7
<i>Methanosarcina thermophila</i>	Irregularcocci	-	H <sub>2</sub> /CO <sub>2</sub> . methanol. acetate	50	6-7
<i>Methanococcoides methylutens</i>	Irregularcocci	0.8-1.2 (diameter)	Methanol	42	7.0-7.5
<i>Methanosaeta Consilii</i>	Rods	0.8x2.5-6.0 (dimensions)	Acetate	35-40	7.0-7.5
<i>Methanosaeta thermophila</i>	Rods	0.8-1.3x6.0 (dimensions)	Acetate	55-60	7

Source: Demirel and Scherer (2008).

important parameters in the process of anaerobic conversion of acetate.

In the studies of hydrogenotrophic, methanogenes participated in degradation of organic fraction of municipal wastes. The studies were conducted under thermophilic and hyperther-

mophilic conditions (up to 70°C) (Scherer et al., 2000). Hydrogenotrophic methanogenes were also found under the conditions of digestion of sewage sludge, manure and compost (Jackel et al., 2005). The studies on the effects of temperature on populations of microorganisms in

anaerobic manure processing indicate that hydrogenotrophic methanogenes are a group which maintains a high specific methanogenic activity (SMA) and is characterized by invariable number at 65°C. The activity of other methanogenes at a high temperature was considerably reduced

**Tabela 2.** Typical reactions carried out by methanogenes during anaerobic process

Reaction carried out by methanogenes	
Hydrogen:	$4 \text{ H}_2 + \text{CO}_2 \longrightarrow \text{CH}_4 + 2 \text{ H}_2\text{O}$
Acetate :	$\text{CH}_3\text{COOH} \longrightarrow \text{CH}_4 + \text{CO}_2$
Formate:	$4\text{HCOOH} \longrightarrow \text{CH}_4 + \text{CO}_2 + 2 \text{ H}_2\text{O}$
Methanol	$4 \text{ CH}_3\text{OH} \longrightarrow 3 \text{ CH}_4 + \text{CO}_2 + 2 \text{ H}_2\text{O}$
Carbon monoxide:	$4 \text{ CO} + 2 \text{ H}_2\text{O} \longrightarrow \text{CH}_4 + 3 \text{ H}_2\text{CO}_3$
Trimethylamine:	$4(\text{CH}_3)_3\text{N} + 6 \text{ H}_2\text{O} \longrightarrow 9 \text{ CH}_4 + 3 \text{ CO}_2 + 4 \text{ NH}_3$
Dimethylamine:	$2(\text{CH}_3)_2\text{NH} + 2 \text{ H}_2\text{O} \longrightarrow 3 \text{ CH}_4 + \text{CO}_2 + 2 \text{ NH}_3$
Methylamine:	$4(\text{CH}_3)\text{NH}_2 + 2 \text{ H}_2\text{O} \longrightarrow 3 \text{ CH}_4 + \text{CO}_2 + 4 \text{ NH}_3$
Methylmercaptans:	$2(\text{CH}_3)_2\text{S} + 3 \text{ H}_2\text{O} \longrightarrow 3 \text{ CH}_4 + \text{CO}_2 + \text{H}_2\text{S}$
Metals:	$4\text{Me}^0 + 8 \text{ H}^+ + \text{CO}_2 \longrightarrow 4 \text{ Me}^{++} + \text{CH}_4 + 2 \text{ H}_2\text{O}$

Source: Demirel and Scherer (2008).

(Ahring et al., 2001). Methanogenic bacteria binding hydrogen were found to belong to family *Methanobacteriaceae* (Boone et al., 1993).

Manure is a complex type of substrate, composed of hydrocarbons, proteins and acids. Characteristics of the population of bacteria and archaeans in anaerobic thermophilic processing of manure indicated a dominance of two species: *Methanoculleus thermophilicus* (hydrogenotrophic) and *Methanosarcina thermophila* (acetotrophic). The main hydrogenotrophic microorganisms, participating in anaerobic processing of fruit and vegetable wastes comprise *Methanosphaerastadtmanii* and *Methanobrevibacter wolini* (Bouallagui et al., 2004).

Counted among acetotrophic methanogenes should be the species belonging to genus *Methanosarcina*. It was found out that during anaerobic processing of sewage sludge and manure, the number of microorganisms of *Methanosaeta* genus decreased with increasing acetate in the environment, with simultaneous intensive growth of the bacteria belonging to *Methanosarcina* genus which are acetotrophic methanogenes (Griffin et al., 2000). The studies on the dynamics of the population of anaerobic microorganisms participating in degradation of municipal wastes and sewage sludge indicated that *Methanosaeta concillii* was a dominant species among acetotrophic methanogenes (McMahon et al., 2004).

The rate of the development of digestive bacteria depends on the type of applied substrate. If the substrates are carbohydrates, the rate of bacteria generation in acid phase amounts to 5 h, whereas in the case when fats are the substrate, the time is prolonged to approximately 72 h. The generation time, in acetogenesis phase, for bacteria using propionic and fatty acids reaches respectively 84 and 131 h. In the methanogenesis phase, the generation time ranges between 15 and 85 h (Ilyin et al., 2005; Santosh et al., 2004).

## IDENTIFICATION OF ANAEROBIC ENVIRONMENTAL MICROORGANISMS

The studies on microorganisms' ecology require identification of microorganisms based on universal system of classification which reflects microorganisms' evolutionary relationship (Hofman-Bang et al., 2003). Three basic aims have to be achieved in all studies on microorganisms' ecology:

1. Identification and classification of microorganisms;
2. Determination of the quantity of microorganisms;
3. Determination of microorganisms' activity.

### Traditional methods of culture and identification

Traditional methods of investigating the microorganisms' quantity and identification are based on microorganisms' morphology and phenotypic features (Smith, 1966; Zeikus, 1977). The studies of methanogenic microorganisms carried out by Grothenius et al. (1991) were based on microscopic techniques and consisted in identification of acetotrophic methanogenes based on their morphology. Hydrogenotrophic methanogenes were demonstrated by autofluorescence, at the wavelength of 420 nm.

The culture of methanogenes is difficult because of a low rate of the growth of those microorganisms, specific nutritional requirements and restrictive environmental conditions.

Phylogenetic analysis allows the identification of microorganisms according to molecular techniques. This diagnostic method eliminates the need for the culture of these microorganisms. This means that sequences of nucleic acids may be isolated from environmental samples, sequenced and compared to the known sequences appropriate for identified related microorganisms

(Hofman-Bang et al., 2003).

In the past, many procedures were published, which enabled calculation and isolation of methanogenic bacteria (Ferry et al., 1974; Smith, 1966; Zeikus, 1977). One of the possible methods of isolation and culture of methanogenes is the use of LPBM substratum of the following composition:

$\text{KH}_2\text{PO}_4$  – 0.75 g,  $\text{KH}_2\text{PO}_4 \cdot 3 \text{H}_2\text{O}$  – 1.45 g,  $\text{NH}_4\text{Cl}$  – 0.9 g,  $\text{MgCl}_2 \cdot \text{H}_2\text{O}$  – 0.2 g,  $\text{Na}_2\text{S} \cdot 9 \text{H}_2\text{O}$  – 0.5 g, solution of trace elements Fe, Mn, Co, Zn, Ca, B – 9 ml, solution of vitamins – 5 ml, distilled water – 1000 ml.

The substratum is prepared in anaerobic atmosphere with 95% share of nitrogen and 5% share of carbon dioxide (Zeikus, 1977). In conventional studies, the identification of methanogenes is mainly based on analysis of morphological forms. We can distinguish four basic morphological forms of methanogenes, that is, sarcina, rods, spirocheta and spherical forms. The mentioned forms may create various types of aggregates and clusters, or they may occur in the form of single cells in the environment. Their shapes may be more or less regular, depending on the species, rods may be shorter or longer, straight or curved (Zeikus, 1977).

### Molecular techniques in ecology of anaerobic microorganisms

Contemporary studies on the diversity and function of microorganisms are more and more often based on the techniques of molecular biology. This is connected with the fact that only a part of microorganisms can be cultured *in vitro*. Molecular techniques enable the description of environmental microorganisms' populations according to analysis of appropriate molecules, e.g. rRNA. Isolation of the sequence of nucleic acids occurs without the culture of microorganisms and isolation of pure cultures (Nealson, 1997).

In recent years, the research on molecular diversity of microorganisms developed extensively. Therefore various techniques are being developed, based on extraction of nucleic acids and adapted to environmental studies. These techniques comprise among others: DNA separation, hybridization of DNA-DNA and mRNA-DNA, cloning of DNA, sequencing and other techniques based on the polymerase chain reaction (PCR), such as Denaturing gradient gel electrophoresis (DGGE), temperature gradient gel electrophoresis (TGGE), fluorescent *in situ* hybridization (FISH) (Iqbal et al., 2009; Keyser et al., 2006).

Typical studies on methanogenes using molecular biology techniques comprise:

1. Extraction of fragments of DNA, rRNA from the environmental sample;
2. Amplification of respective fragments of genes, using the PCR;

3. Cloning the products of reaction;

4. Electrophoretic separation using the techniques of DGGE, TGGE or T-RFLP;

5. Sequencing the products or clones (Hofman-Bang et al., 2003).

Another technique used in the research on methanogenes population is fluorescent *in situ* hybridization (FISH). Hybridization occurs at the cellular level and is conducted *in situ*. It enables determination of the distribution of microorganisms in the environment. For labelling of oligonucleotide probes, the fluorescently labelled probes are used. This method is based on stabilization and permeabilization, hybridization, removal of non specifically bounded probes and microscopic analysis of dyed cells in fluorescent or scanning electron microscope or flow cytometer (Demirel and Scherer, 2008; Tabatabaei et al., 2009). The FISH method was used in identification of dominant methanogenic archeons participating in anaerobic degradation of manure and sludge (Karakashev et al., 2005).

The use of advanced techniques of molecular biology is essential in the studies aimed at the comprehension and systematization of complex reactions going on during the digestion process (Demirel and Scherer, 2008).

### CONCLUSION

A great interest in the anaerobic digestion process results mainly from its advantages, as compared to aerobic methods. The main advantages of the methane digestion process are: Production of an insignificant amount of biomass and lower energy input, as compared to degradation conducted under aerobic conditions. Another advantage is the production of biogas which may be used as a source of energy. Furthermore, the anaerobic process does not need energy for aeration, which results in high energy saving. It is characterized by a lower demand for nutritive substances and higher resistance, as compared to aerobic processes, with high organic loads. Disadvantages of the process are difficulties connected with maintaining appropriate concentration of microorganisms in bioreactor, higher sensitivity to changes in reaction and temperature. Besides, methane digestion process do not always causes a complete degradation of organic impurities. Besides, it requires heating of wastes to carry out the digestion under mesophilic or thermophilic conditions. In addition, odour nuisance is observed in the case of ineffective hermetization of reactors and containers designed for storage of digested substrates.

In accordance with the assumption of the European Union development of fuel III deficiency has gradually complement conventional fuels. These fuels also reduced  $\text{CO}_2$  emissions to the atmosphere. One of the main research topics is the use of methane fermentation, not

only for the disposal of sewage sludge or liquid agro wastes but also of plant residues. Methane fermentation process and biogas production has many advantages compared to other types of renewable fuels. Methane fermentation process not only provides fuel (biogas), but also a valuable fertilizer.

## ACKNOWLEDGEMENTS

This paper was partially supported by The National Centre for Research and Development (NCBiR) in Poland – LIDER PROGRAMME. This paper was also partially supported by Operational Programme of Innovative Economy in Poland.

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