Pressure Effects on Proliferation of Methane-Producing Bacterias in Mesophylic Anaerobic Fermentation

Carmen MATEESCU

National Institute for Research and Development in Electrical Engineering ICPE-CA,
313 Splaiul Unirii Str.., Bucharest-3, 030138, Romania, Tel: +40 213468297/ext. 115,
Fax: +40 213468299, e-mail: carmen.mateescu@icpe-ca.ro

Abstract

Theoretical studies have assumed that the hydrostatic pressure in anaerobic digesters is likely to have a negatively influence on the effectiveness of the biochemical processes in the bioreactors and consequently on the biogas production. These assumptions made the authors approach this interesting topic and carry on experimental studies aimed to reveal the methanogens response on the hydrostatic pressure in order to allow designers adopt the most effective technical solutions in designing and construction of the biogas reactors.

This paper has as a main scope to investigate the influence of the hydrostatic pressure on the microorganisms producing biogas. For this scope, experimental researches for anaerobic digestion of organic slurry have been carried out in stainless steel pressure vessels which were set for four different conditioning pressures. A comparative study of the methanogens loading for the initial sample and for the samples conditioned under various work pressure has been recorded and analyzed.

Key words: methanogens, biogas digesters, metabolic activity, waste treatment

Introduction

A lot of industrial processes are supported by bacteria that are used as a starter for various fermentative processes in the food industry or for producing bio-fuels (J. B. SILVA & al. [1]). Likewise, bacteria are involved for synthesis of a wide variety of chemicals which have a high economic value, such as vaccines, enzymes, proteins, amino acids, organic acids, solvents, antibiotics, insecticides, vitamins, hormones, etc.

Knowledge of the fundamental processes involved in anaerobic digestion is important for the designing, construction and operation of biogas plants (U. MARCHAIM [2]). The conversion of organic compounds to complex molecules via a multitude of biochemical reactions and finally to biogas within the anaerobic fermentation processes, involves the participation of some different populations of microorganisms among which there are synergistic interactions (F. A. SHAH & al. [3]).

Though in the anaerobic digesters some fungi and protozoa have also been identified, bacteria represent the dominant microorganisms in these fermentative processes. Microbiology of anaerobic decay of organic wastes is a process which involves many different bacterial species, such as hydrolytic, acid forming, acetogenic, and methanogenic bacteria which produce CO_2 and CH_4 as the main products of the digestion processes (F. A. SHAH & al. [3]).

Methane production is carried out by methanogenic groups belonging to *Archaea* species, which are known to have very sensitive growth and activity behaviors concerning different substrates and operation conditions (B. CALLI & al.

[4]). The stable formation of the methanogenic community determines the success or failure in anaerobic digestion systems treating wastewaters including highly concentrated organic and toxic materials (F. A. SHAH & al. [3]). Optimization of the anaerobic processes, their stability over time, resistance to any disturbances and the general processes progress could not be achieved without monitoring of the specific communities of microorganisms living in the organic mass. These microbial communities are very complex and consist of different taxonomic groups of bacteria, depending mainly on the type of the residual substrate.

Many environmental factors affect the biochemical processes that occur in anaerobic digesters to produce biogas. Amongst them, the hydrostatic pressure in the digester has proved an important role in maintaining an optimal biochemical balance. Depending on hydrological conditions, this parameter shows a stimulatory or adverse effect on microbial metabolism (A. BIANCHI & al.[5]).

In anaerobic digestion processes, it has been shown that when the hydrostatic pressure in the environment increases over 4-5 meters of water column, the methane production practically stops but it starts again when the hydrostatic pressure decreases to lower values. This finding is very important to design the digester (V. NIKOLIC & al. [6]).

Very few studies have been performed in laboratories to demonstrate the effect of pressure on bacterial activities. Bianchi et al. compared the microbial activity of surface-water and deep-sea water but concluded that it is difficult to assess a precise depth limit where pressure affects bacterial activity (A. BIANCHI & al. [5]). Bernhardt et al. studied the activity of thermophilic methanogens in high pressure environments and noticed that below 40 MPa, cell proliferation is enhanced while beyond 40 MPa, elongated,

large sized cells are formed. Further increase of pressure finally causes cell lyses (G. BERNHARDT & al. [7]). In another paper, the authors have developed a high-pressure equipment to study whether high pressure might alter the range of viability for methanogens but the results were provided the same for thermophylic species (G. BERNHARDT & al. [8]).

The current paper aims to present several experimental researches that demonstrate how the hydrostatic pressure in the mesophylic anaerobic digesters could influence the proliferation and activity of microorganisms responsible to produce biogas. These experimental results could be very useful to designers of biogas plants, having an important role in optimizing of the anaerobic processes even in the preliminary stage of bioreactor designing.

Materials and Methods

The experiments of anaerobic digestion under hydrostatic pressure conditions were performed in stainless steel vessels having a capacity of 5 liters. The substrate used as biomass to be fermented in pressurized tight containers is an organic mixture consisting of cattle manure, unfiltered whey and water dilution.

Temperature control in anaerobic digestion experiments was achieved by incubating the pressured vessels in a climate test chamber type Vötsch Industrietechnik VC 4018, with digital control unit display of temperature and humidity.

The methanogens loading of the fermented slurry was determined initially by sampling fresh organic slurry which was prepared to be used in anaerobic fermentation experiments but also finally, at the end of experiments by sampling slurry which was conditioned at pressures of 0, 2, 4 and 6 bar respectively.

These samples were subjected to microbiological analysis, by using decimal dilutions and the Most Probable Number technique. The principle for this technique

is dilution and incubation of replicated cultures across several serial dilution steps. This technique does not rely on quantitative assessment of individual cells but on specific qualitative attributes of the microorganism being counted.

The total number of methanogenic microorganisms has been determined for the initial and final samples in order to assess their metabolic activity and consequently the influence of the hydrostatic pressure on microbial proliferation.

For methanogens enumeration, a selective growth medium, as well as decimal dilutions of the samples, was prepared. For growth medium preparation, the components were dissolved in distilled water, afterwards the solution pH was adjusted with a solution of KOH 0.1 N, so that after sterilization the pH to be maintained at the value 7 ± 0.2 at 25^0C.

The stabilized broth was distributed in glass tubes of 20 ml capacity, fitted with screw cap, after which the tubes were sterilized in autoclave at a temperature of 121^0C for 15-20 minutes. In scope to have the oxygen released, prior to use the glass tubes were heated in boiling water for 15 minutes, afterwards were rapidly cooled to the temperature of incubation.

A selective broth proposed by Zeikus has been used for the enumeration and isolation of methanogens. This culture medium allows the growth of a wide variety of species of methanogens. The addition of some nutrients such as sodium formiate or methanol (in volumetric ratio of 0.5 - 1%) to the basal medium was done in scope of enhancing the growth of methanogens, ensuring them the basic nutrient requirements. According to Zeikus, the basal medium for methanogens growth was prepared by using the following minerals and nutritive solutions: 0.75 g KH_2PO_4, 1.45 g K_2HPO_4 x $3H_2O$, 0,9 g NH_4Cl, 0,2 g $MgCl_2$ x H_2O, 0,5 g Na_2S x $9H_2O$, 9 ml mineral solution, 5 ml vitamin solution, 1 ml resazurin 0,2%, 1000 ml distilled water. The natrium sulphide solution was added after the broth sterilization. The mineral solution contained, in grams per liter of distilled water, the followings: nitrilotriacetic acid, 4.5; $FeCl_2 \cdot 4H_2O$, 0.4; $MnCl_2 \cdot 4H_2O$, 0.1; $CoCl_2 \cdot 6H_2O$, 0.17; $ZnCl_2$, 0.1; $CaCl_2$, 0.02; H_3BO_3, 0.019; and sodium molibdate, 0.01 (J. G. ZEIKUS [9]).

The basal medium was completed with 2% agar Difco, purified. Afterwards the prepared mixture was sterilized using an autoclave Systec V-150. The sulphide solution $Na_2S \cdot 9H_2O$ was added after sterilization.

For the microbiological analysis of the organic slurry to be anaerobically fermented in the pressure vessels, a representative sample was extracted and decimal dilutions were prepared according to the standard SR EN ISO 6887-1:2002 [10].

A number of three samples were prepared for each decimal dilution, by adding of 1 ml raw, undiluted sample to a correspondent solvent volume multiplied by 9 times undiluted sample. As a solvent, a buffer solution of phosphate salts including disodium phosphate, monopotassium phosphate but also natrium chloride has been used.

The enriched medium was dispensed into sterile glass tubes, after which the sample to be analyzed, which was likely to contain methanogens, was inoculated into the medium. A special care was shown to this inoculation process since the sample was intended to touch the bottom of the tube sample. The inoculated sample volume was in ratio of 1/10 of the broth volume.

Preparing of the decimal dilutions as well as inoculation of the nutritive broth has been done in strictly aseptic conditions, in a biologic cabinet type Astec Microflow ABS 1000 fitted with UV lamp.

For ensuring anaerobic conditions, the medium was covered with a 1 cm depth layer of sterile paraffin oil after inoculation. The tubes containing the culture medium, the inoculated sample and sealed with the paraffin oil were incubated at a temperature of 37^0C for several days by using an incubator with temperature control and indicator of the incubation time.

The specimens were investigated every 24 hours for identifying any changes to their original aspect, as for example the presence of gas bubbles with or without fragmentation of the culture medium.

The methodology for this investigation technique was dilution and incubation of replicated cultures across several serial dilution steps. Firstly, it was assumed that microorganisms in the initial and all subsequent dilutions are randomly distributed. Secondly, it was assumed that one or more organisms contained within an inoculant volume are capable of producing a positive result.

For the determination of methanogens loading, a procedure with seven decimal dilution steps and three units per dilution level has been used. The assessment of the most probable number was done based on the statistic principle by using the indexes shown in the McCrady tables, reported to the sample dilution and the volume unit (1 ml), according to the procedure described in the standard SR ISO 7218:2000 [10].

The procedure for preparation of decimal dilutions, inoculation of the broth, the samples incubation as well as the enumeration of methanogenic microorganisms have been performed both for the initial biomass sample and for the fermented samples conditioned for various hydrostatic pressures: 0 bar, 2 bar 4 bar and 6 bar respectively.

Results and Discussions

The quantitative assessment of the most probable number of microorganisms present in the mixture of fermentative biomass was done based on the Most Probable Number index obtained from the combination of positive and negative tubes.

The positive tubes indicating the presence of metanogens and consequently of the methane gas are those which show bubbles randomly distributed in the whole broth volume and sometimes the fragmentation of the broth. The negative tubes do not show any visible changes in the aspect of broth.

The results are calculated and expressed in terms of the number of positive tubes for the highest dilution which shows a positive reaction. In the Table 1, combinations of positive and negative tubes are presented. Based on the index selected from McCrady table, the total number of methanogens which is most likely to exist in 1 ml sample has been determined for the slurry consisting of cattle manure and unfiltered whey.

In the pictures from the figure 1 the positive tubes corresponding to the four working pressure 0 bar, 2 bar 4 bar and 6 bar respectively are shown.
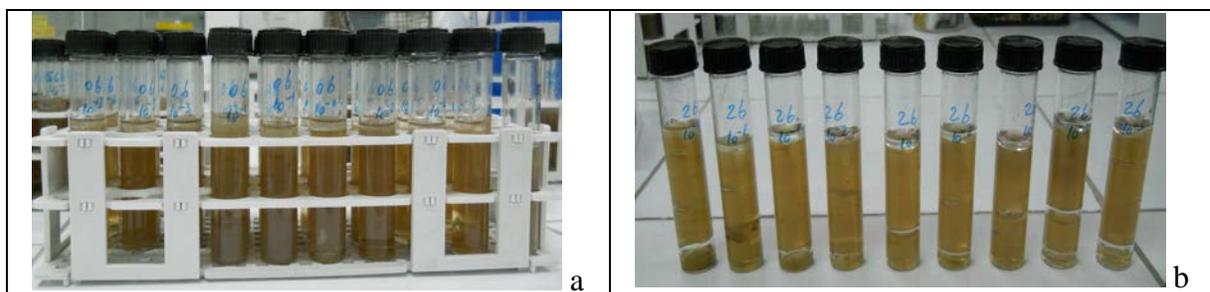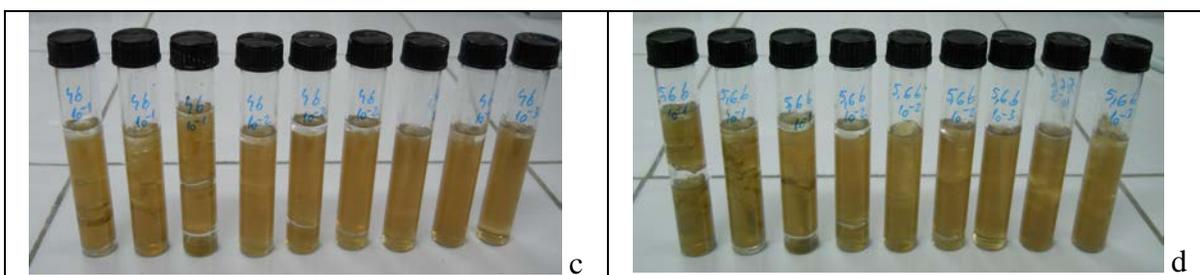
Fig. 1 Positive tubes indicating the presence of methanogens after conditioning at 0 bar (Figure 1a), 2 bar (Figure 1b), 4 bar (Figure 1c) and 6 bar (Figure 1d)

To record the measured values, a data sheet that assigned either a positive or a negative value for each experimental unit was prepared. The combination of positive and negative tubes for the microbial inoculation of equal volumes of sample, three for each dilution, as well as the Most Probable Number index showing the estimated number of microorganisms present in 1 ml of fermented slurry that is suspected of containing methanogens, are presented in the table 1 for all the work pressures used in the experimental.

Table 1 Combination of positive and negative tubes and the quantitative assessment of methanogens by using the Most Probable Number technique for the initial and conditioned samples

Sample code	Dilution	Result for				
		Raw sample	0 bar	2 bar	4 bar	6 bar
1.1	1 (1ml sample + 9 ml serum)	+	+	+	+	+
1.1	1	-	+	+	+	+
1.1	1	-	+	+	+	+
1.2	2 (1 ml dilution grade 1 + 9 ml serum)	-	+	+	+	+
1.2	2	-	+	+	+	-
1.2	2	-	+	+	+	-
1.3	3 (1 ml dilution grade 2 + 9 ml serum)	-	+	+	-	+
1.3	3	-	+	-	-	-
1.3	3	-	+	-	-	-
1.4	4 (1 ml dilution grade 3 + 9 ml serum)	-	+	-	-	-
1.4	4	-	+	-	-	-
1.4	4	-	-	-	-	-
1.5	5 (1 ml dilution grade 4 + 9 ml serum)	-	-	-	-	-
1.5	5	-	-	-	-	-
1.5	5	-	-	-	-	-
1.6	6 (1 ml dilution grade 5 + 9 ml serum)	-	-	-	-	-
1.6	6	-	-	-	-	-

1.6	6	-	-	-	-	-
1.7	7 (1 ml dilution grade 6 + 9 ml serum)		-	-	-	-
1.7	7		-	-	-	-
1.7	7		-	-	-	-
Most Probable Number Index		300	320	310	300	311
Estimated number of metanogens (cells / ml sample)		$2,3 \times 10^1$ = 23 cel./ml sample	$9,3 \times 10^3$ cel./ml sample	$4,3 \times 10^2$ cel./ml sample	$2,3 \times 10^2$ cel./ml sample	$7,5 \times 10$ cel./ml sample

Analyzing the data in Table 1, it can be noticed that the number of methanogenic microorganisms which are present in samples of fermented slurry is significantly higher for the sample conditioned under atmospheric pressure, which is 9.3×10^3 cells / ml sample.

For the samples conditioned under higher pressures, the number of methanogenic microorganisms present in sludge samples is progressively reduced for each of the three working pressures: 2 bar, 4 bar and 6 bar.

The experimental results confirm the theoretical data presented in the literature. According to both theoretical data and experimental results, it can be underlined that the hydrostatic pressure has a negative influence on growth and metabolic activity of methanogens and consequently on methane content in biogas.

Conclusions

In anaerobic digesters, biogas production is possible due to the different groups of microorganisms, namely, fermentative, syntrophic, acetogenic, and methanogenic bacteria. Methanogens are the vital microbes and have decisive role for biomethane production. Methanogenesis depends on the various factors such as inoculum sludge, the organic substrate but also the process conditions (F. A. SHAH & al. [3]).

From theoretical calculation, it has been founded that in classical cylinder-shaped digesters, fitted with vertical stirrer, the biogas is generated exclusively in the upper layer of the bioreactor, up to a maximum depth of 4 m. The remaining volume of the digester is not biologically active and can not produce biogas. Within this layer, methanogens are living passively; they are fairly dormant and become activated by mixing. The homogenization of the substrate could bring the methanogens to the lower pressure areas. Therefore, this will help bacteria to recover their optimal metabolic activity and continue to decay the organic substrate in proper environment conditions.

The experiments of anaerobic fermentation carried on for an organic mixture consisted of cattle manure and unfiltered whey, performed in stainless steel vessels under four different hydrostatic pressures, have led to the conclusion that the number of methanogenic microorganisms present in fermented slurry is definitely higher for biomass conditioned at atmospheric pressure. For the rest of biomass samples conditioned at various hydrostatic pressures, the number of methanogenic microorganisms present in sludge samples is progressively reduced for each of the three working pressure.

These preliminary data fully confirm the theoretical calculation presented in the literature, according to which the hydrostatic pressure has a negative influence on the growth and the metabolic activity of methane-producing bacteria. Consequently, the biomass samples conditioned at pressures of 2 or 4

to 6 bar produced a much lower quality biogas, which strengthen the theory that the hydrostatic pressure adversely affects the methanogenesis.

Acknowledgments

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