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Evaluation of CH_4 and H_2 Yield with Different Mixtures of Digested and Fresh Buffalo Manure

Filomena di Cristofaro[*a], Claudia Carotenuto[a], Petronia Carillo[b], Pasqualina Woodrow[b], Biagio Morrone[a], Mario Minale[b]

[a]Dipartimento di Ingegneria Industriale e dell'Informazione, Seconda Università degli studi di Napoli, Via Roma 29, 81031 Aversa (Caserta), Italia
[b]Dipartimento di Scienze e Tecnologie Ambientali Biologiche e Farmaceutiche, Seconda Università degli studi di Napoli, Via Vivaldi 43, 81100 Caserta, Italia
filomena.dicristofaro@unina2.it

In this work hydrogen and methane production from buffalo manure has been scrutinized, by means of the anaerobic dark fermentation process and different process conditions.
Animal manure characteristics are largely variable during different periods of the year because of the unavoidable changes in animal food supply and digestion apparatus of buffalo. A very significant variability was observed for water buffaloes manure, collected from the same supplier. This implies also a large variability in biogas production by anaerobic fermentation of such substrates.
To overcome this variability, we mixed fresh with digested manure. This procedure is consolidated as regard methane production. We also investigated a similar strategy to optimize and stabilize H_2 yield.
We were able to find optimal process conditions for hydrogen and methane production with this type of substrate. The experiments have been run in batch mode. The products of fermentation, H_2, CO_2 and CH_4, have been measured with a micro-gas chromatograph.
Moreover the potential production of H_2 has been maximized by thermally pre-treating the substrate and using a process temperature of the fermenter equal to 55 °C and an initial pH value of 5.5, while very high levels of potential production of CH_4 have been obtained at 37 °C and with an initial pH of 7.0.
The effect of different strategies has also been analyzed to highlight the different microbial community present in the substrate using the denaturing gradient gel electrophoresis (DGGE).
This investigation has been carried out with the aim at isolating and selecting of natural bacterial species to use as inoculum to improve and stabilize the fermentation process of buffalo manure. More results concerning the microbial communities present in the substrate and responsible for biogas production are presented in a companion paper. Here we only list the main types of bacteria identified in the manure.

1. Introduction

The growing global environmental concerns related to the use of fossil fuels together with the political instability in major oil exporting countries have prompted the interests for alternative environmental-friendly energy sources (Carotenuto "et al.", 2012): biogas from animal manure is one of them.
In this work hydrogen and methane production from buffalo manure has been analyzed, by using the anaerobic dark fermentation with different process conditions (Gottardo "et al.", 2013). Anaerobic fermentation of animal manure for biogas production is highly variable because of the unavoidable changes in fermenting substrate. These differences imply different yield, process lag time and biogas growth rate. We observed a large variability of water buffaloes (*Bubalus bubalis, subsp. River*) manure parameters, despite collected always from the same farmer using the same protocol. The manure was collected from two different sheds, one hosting lactating buffaloes, the other non-lactating ones.
To overcome this variability and to obtain a nearly constant biogas yield, as required by industrial processes, we mixed fresh with digested manure. This procedure is quite consolidated for methane production and indeed the fermentation lag times shortened from four to one day and the final CH_4

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concentration increased from 55 % to 65 % in the produced biogas. A similar strategy to optimize and stabilize H_2 yields is also under scrutiny. The manure is used here without any addition of nutrients or external microbial communities.
This strategy seemed not to be working properly for H_2 production. We also looked for the correct inoculum by collecting the fermenting manure during different stages of the process. In particular the substrate was collected at the beginning of the process and when the maximum H_2 growth rate was observed.
The anaerobic fermentation experiments have been run in batch mode. The biogas composition is measured by a micro-gas chromatograph. In particular, the process conditions for maximizing H_2 production for the different collected fresh manures and a compulsory thermal pretreatment to inhibit the H_2-consumer bacteria have been determined.
We identified the favorable conditions for hydrogen production as: i) dark anaerobic fermentation; ii) thermal pre-treatment of substrate at 90 °C for six hours; iii) initial pH ~ 5.0 - 5.5; iv) process temperature of 55 °C, and those for methane production as: i) dark anaerobic fermentation; ii) initial pH ~ 6.0 - 7.0; iii) process temperature of 37 °C, iv) no thermal pre-treatment of the substrate.
Because the final goal of this work is the individuation and the selection of natural bacterial species to use as inoculum to improve the fermentation process, this research provides a picture of the dynamics of microbial communities in the buffalo manure during this process. For this reason, the different process conditions have been analyzed also in terms of different microbial communities. The phylogenetic analysis of 16S-rRNA gene sequences resulting from denaturing gradient gel electrophoresis (DGGE) is used to individuate the bacterial community of water buffalo manures as enhanced by the fermentation process (Carillo “et al.”, 2012). Anyway, detailed results pertaining to the bacterial communities are not shown in this paper for the sake of brevity and they are presented in a companion paper devoted to this investigation.

2. Materials and Methods

The biological matrix used in the experiments consists of buffalo manure, collected from a farm, located in the municipality of Villa Literno, in Campania region. It is the region with the largest number of buffalo breeding, both in Italy and Europe. The manure samples for experiments are collected through a sterile plastic scoop in the morning, while cleaning the sheds, during different periods of two years. They are placed in sterile containers and immediately stored in the refrigerator at 4 °C.
The material used in the experiment has different origins: buffalo manure deriving both from lactating and from non-lactating heads. Anyway, results are presented only for the lactating ones, for sake of brevity. The feed of the two groups is based primarily on corn, cereals, grass hay and alfalfa. The difference in the feeding of two groups consists in the intake of forage and protein content. Lactating heads are provided with a daily quantity of high content proteins fodder, to facilitate the milk production.
Before starting the fermentation process, a chemical and physical characterization of the substrate is performed. Not all substrates have the same performance in terms of produced biogas. In fact, the amount of biogas is related to the degradation of the organic substance, influenced by several factors, such as: the quality of the biodegradable fraction of substrate, temperature, pH, total and volatile solids (TS and VS), C/N ratio, etc. These differences involve different yield, process lag time and growth rate. The characterization has been performed by the determination of total solids and elementary chemical composition in terms of carbon, nitrogen and C/N ratio. TS are the residue after evaporation of the sample taken in an oven at 105 °C overnight and they are representative of the organic content estimate.
Several experiments of anaerobic fermentation have been carried out in batch mode at different conditions. The fresh material was mixed by hand with distilled water, in order to achieve a manure/water ratio of 30/70. Then the mixture was pre-treated in different ways. The pH was corrected by the addition of hydrochloric acid 1 M, with the aim at achieving the desired pH values to start the fermentation process. The typical initial pH value of the original water/manure mixture is usually 7.0 - 8.0; after the correction, it is 5.0 - 5.5 for H_2-oriented fermentation and 6.0 - 7.0 for CH_4. The sample is split in batch bottles of borosilicate glass of 280 mL. For each typology of prepared sample, three bottles are filled with 80 g of material in order to have three replicates. Each bottle is sealed with a butyl rubber cap. Each cap is coated with shellac to ensure sealing. Since the fermentation is anaerobic, nitrogen is blown in each bottle before starting the process. Two fermentation temperatures are used: 55 °C and 37 °C for H_2 and CH_4-oriented fermentation, respectively.
To investigate the capacity of selecting a bacterial community for H_2 potential production maximization, the thermal pre-treatment on some fresh samples is carried out, with the aim of eliminating the methanogenic bacteria, which are responsible for hydrogen consumption during fermentation processes. In particular, the

samples are placed in an oven at 90 °C for 3 – 6 h (Gilroyed “et al.”, 2008). Then, the material is prepared according to the same procedure described above.
The biogas composition is measured using the micro-gas chromatograph Agilent 3000A (S.R.A. Instruments, France). It is equipped with two capillary columns, crossed by two distinct carrier gases: argon is used for the column MolSieve 5A at 110 °C, while helium is used for the column Poraplot U at 85 °C.
On each type of sample an extraction of genetic material has been performed to identify the different bacterial communities in the buffalo manure. The phylogenetic structure of the microbial community in fermentation samples was studied using denaturing gradient gel electrophoresis (DGGE) to generate fingerprints of 16S rRNA genes. The analysis of gene sequences provided a good estimate of eubacterial community and evidenced the presence of very active H_2-producing bacteria (Baker “et al.”, 2003).

3. Results and Discussion

The biogas concentration as a function of time can be usually very well described by the Gompertz equation Eq(1) (Khanal “et al.”, 2004):

$$H = P\ exp\left\{-exp\left[\frac{R_m\, e}{P}(\lambda - t) + 1\right]\right\} \quad (1)$$

where H is the percent instantaneous biogas (CH_4 or H_2) concentration, P the potential biogas concentration, R_m the maximum production rate, λ the lag time and t the incubation process time.
Figure 1 shows the evolution of the methane volumetric percent concentration as a function of the time during the fermentation process, for different experimental campaigns, process temperature of 37 °C and an initial pH value of 6.0. The different samples (A, B, C and D) shown in Figure 1 pertain to manure collected in different moments spanning two years. Each point is reported with error bar to represent the measurement uncertainty. For samples A, B and C after a period of about 50 h, the substrate starts to produce CH_4, following a sigmoidal trend, very well described by the Gompertz equation, Eq(1). The values of the fitting parameters are reported in Table 1, together with the corresponding regression coefficient R^2. In addition, the parameters have been estimated with a 95 % Confidence Interval. Usually, the highest CH_4 concentration in the produced biogas is about 55 %. For sample D very low methane production equal to only 10 % is obtained and the Gompertz regression equation is not satisfactory.
To improve the methane production with sample D, it has been mixed with digested sample C, acting as an “inoculum”. The new sample is indicated as WI (With Inoculum) and it presents a zero lag time and a higher growth rate (Figure 1). The CH_4 final concentration in biogas is increased up to about 60 %. The use of the inoculum has been proven to improve methane production. This procedure was performed with different concentrations of inoculum (20 – 33 - 50 % wt in the digested manure) obtaining the same results in all cases (not shown in Figure 1).
In Figure 1 we observed also a very significant variability of CH_4 production, despite the manure was collected from the same farmer and all the samples were prepared with the same protocol. This situation is well evidenced by evaluating TS and C/N ratio of the substrate. In fact, the average total solid (TS) content was about 23.3 % ± 5.4 and the average C/N ratio for the same samples was about 30.6 % ± 6.7. The large Standard Deviation for both the quantities is due to the biological variability of the substrate. This large variability is also documented in terms of pH values. In Table 2, in fact, in the first column the initial pH values of fresh manure are reported, while in the second the final pH values of digested manure for the same samples are shown. We can see that the samples with a high CH_4 production, attain a final pH value of about 7.0 even if the fermentation was started with a pH value of 6.

Table 1: Fermentation process parameters of Gompertz equation, Eq(1), for the samples A, B and C.

Parameters	*A*	*B*	*C*
P [%]	56.92	53.52	55.20
λ [h]	41.90	69.10	138.70
Rm [%/h]	0.20	0.14	0.29
R^2	0.99	0.99	0.99

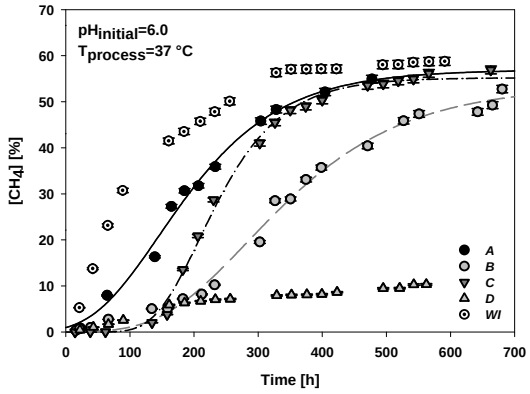

Figure 1: Methane concentration during the fermentation process for different samples. Process conditions: T = 37 °C and initial pH = 6.0. Lines represent the Gompertz equation, Eq(1). WI=With Inoculum (50 % wt).

Table 2: CH_4 Production - pH values. pH_i=Initial pH, pH_f=Final pH.

Sample	pH_i	pH_f	$[CH_4]$ [%]
A	8.8	7.0	54.9
B	8.8	7.0	52.7
C	8.6	7.2	57.9
D	7.3	6.7	10.3
WI	7.2	7.0	58.8

Since the samples that have provided a higher CH_4 concentration, attained a final pH value of 7.0, other experiments were conducted using this condition as starting point. We aimed at finding the process conditions to maximize CH_4 production. In particular, the favorable conditions which have been determined for CH_4 production are: i) initial pH=7.0; ii) process temperature of 37 °C; iii) no thermal pre-treatment.
The evolution of $[CH_4]$ (for three replicates shown with letter E), under these favourable conditions, is reported in Figure 2. The fitting curve is obtained by interpolating the whole set of data. It can be observed that the maximum CH_4 concentration is greater ($[CH_4]$ E = 65.0 %) than that of the WI sample (Figure 1), but the lag time phase is larger. The final pH value is 7.3. The values of Gompertz parameters are reported in Table 3. The biogas produced during 640 h of the experimental campaign is 87.7 mL_N/g_{TS}, while the corresponding methane production is 59.0 mL_N/g_{TS}. It is worth noticing that these values do not indicate the maximum production obtainable from the substrate since the fermentation process was voluntarily interrupted when a constant value of $[CH_4]$ is attained. In fact, these experimental tests were primarily devoted to the individuation of the correct protocol to maximize $[CH_4]$ and to accelerate the fermentation.
The strategy to use the digested manure as inoculum, which has been shown valid for methane production, presents some problems as regards the hydrogen production. The inoculum has been thermally pretreated the same way as the substrate used for hydrogen production.

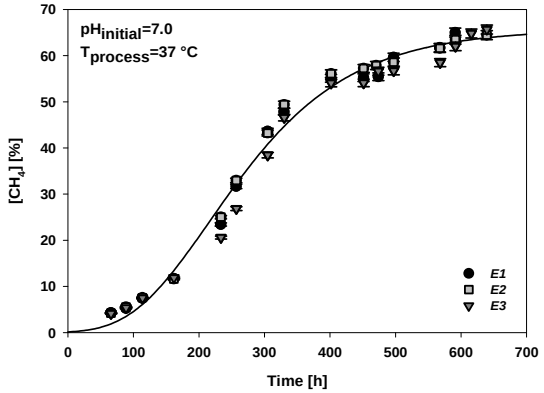

Figure 2: Methane concentration during the fermentation process for different samples E. Process conditions: T = 37 °C and initial pH = 7.0. Line represents the Gompertz equation Eq(1).

Table 3: Fermentation process parameters of Gompertz equation, Eq(1), for the samples E.

Parameters	*E*
P [%]	65.73
λ [h]	97.80
Rm [%/h]	0.20
R^2	0.99

Figure 3 shows the evolution of the hydrogen concentration during the fermentation process, for different experimental campaigns, at process temperature of 55 °C and with an initial pH value of 5.0. For the sample F after a period of about 50 h, the [H_2] reached a value of 6 % in the biogas and after 350 h the maximum [H_2] was equal to 13 %. Instead, for the G sample after a period of about 50 h, the [H_2] reached a value of 2 % and after 350 h the [H_2] maximum was 6 %. For this reason, the digested sample F was mixed with fresh sample G, at the same process conditions. Sample G manure was thermally pre-treated at 90°C for 3 h. Different concentrations of inoculum equal to 20, 33 and 50 % wt were used. In Figure 3 the sample WI (With Inoculum) has a similar behaviour as the sample G, so the inoculum does not give satisfactory results. For data reported in Figure 2 it was not possible to find an equivalent Gompertz fitting curve, because of a different kinetic pathway followed by the substrate.
Consequently, we have been looking for the correct inoculum by collecting the fermenting manure at different stages of the fermentation process. In particular, we extracted the fermented material when the maximum H_2 growth speed is observed and at maximum H_2 concentration value. These substrates, then, would be used as inoculum with fresh manure.

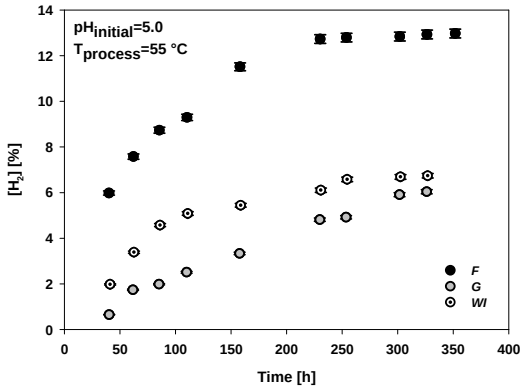

Figure 3: Hydrogen concentration during the fermentation process in different samples. Process T = 55 °C, initial pH = 5.0, thermal pre-treatment T = 90 °C, t = 3 h. WI=With Inoculum (50 % wt).

Because the sample F at the end of fermentation process has a pH value of about 5.5, several experiments were run starting with this pH initial condition. In particular, the favorable conditions for hydrogen production are: i) thermal pre-treatment of substrate at 90°C for 6 h; ii) initial pH = 5.5; iii) process temperature of 55 °C.
The evolution of [H_2] for the three replicates indicated with letter H is reported in Figure 4, where the fitting curve is obtained by interpolating the whole set of data H. After a period of about 20 h, [H_2] reaches 5 %. The maximum H_2 concentration is about 20 % reached after about 160 h. In correspondence of this concentration value the total H_2 produced is 12.3 mL_N/g_{TS}. The final pH value of this sample is 5.5, which is exactly equal to the initial value. The values of fitting Gompertz parameters are reported in Table 4.
The phylogenetic structure of the microbial community in fermentation samples was studied using denaturing gradient gel electrophoresis (DGGE) to generate fingerprints of 16S rRNA genes. The sequences analysis revealed abundance of the phylum Firmicutes, and in particular of the class Clostridia, for the samples that produced hydrogen. We have also identified bacteria of the domain Archaea and class Methanobacteria in the samples that produced methane. In this work the detailed results were not shown for the sake of brevity.

Table 4: Fermentation process parameters of Gompertz equation, Eq(1), for the samples H.

Parameters	*H*
P [%]	21.48
λ [h]	7.90
Rm [%/h]	0.22
R^2	0.95

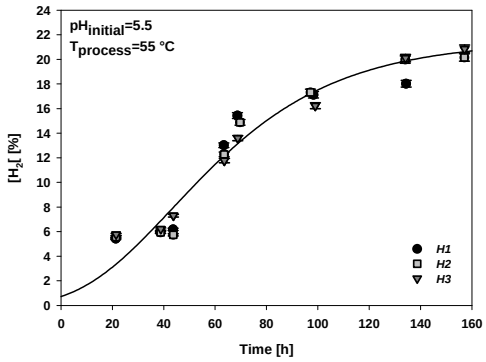

Figure 4: Hydrogen concentration during the fermentation process for different samples H. Process T = 55 °C, initial pH = 5.5, thermal pre-treatment T = 90 °C, t = 6 h. Line represents the Gompertz equation.

4. Conclusions

We have shown that water buffalo manure can be used to produce H_2 and CH_4 by the dark anaerobic fermentation process. Because this material presents a large variability, the use of an inoculum to obtain a constant biogas yield has been investigated.

This procedure is consolidated as regard methane production. In the present experiments we observed that the fermentation lag time was shortened and the final CH_4 concentration was increased by using as inoculum digested material. We also investigated a similar protocol to optimize and stabilize hydrogen yield. Anyway, we did not observe considerable improvement in hydrogen production nor reduction of lag time.

Besides, we determined that the potential hydrogen concentration ($[H_2]$=20 %) is maximized by thermally pre-treating the substrate and keeping the fermenter at 55 °C and with an initial pH equal to 5.5, while the methane concentration is very high ($[CH_4]$=65 %) at 37 °C and with initial pH equal to 7.0.

The final goal of this research is the individuation and selection of natural bacterial species to use as inoculum for the improvement of the fermentation process. The sequences analysis revealed abundance of the phylum Firmicutes, and in particular of the class Clostridia, in the samples that produced hydrogen. While in the methane producing samples, we identified bacteria of the domain Archaea and class Methanobacteria. These results are presented in a companion paper.

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