INVESTIGATION OF ANAEROBIC DIGESTION IN A TWO-STAGE BIOPROCESS PRODUCING HYDROGEN AND METHANE

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Abstract

Hydrogen has received wide attention in the last decades as a clean energy vector. The major advantage of energy generation from hydrogen is the near-zero carbon emissions, since the utilization of hydrogen, either via combustion or via fuel cells, results in pure water. Recently, there has been increasing interest on the biological production of hydrogen gas from renewable biomass such as carbohydrates from agriculture or agro-food industries. This specific anaerobic digestion is called dark fermentation and is involved in the classic anaerobic digestion producing methane. Indeed, in a two-step process, *i.e.* when acido- and aceto-genesis are carried out in a different bioreactor than methanogenesis, it is feasible to generate separated biogas flows containing either H_2 or CH_4 depending on specific operating parameters.
This paper deals with the comparison of the first stage performances whether operated in optimum conditions for H_2 production with a mixed culture or a pure *Clostridium butyricum* strain. Hydrogen yields of about 1.75 and 2.3 mol H_2/mol glucose were achieved respectively. The metabolites, mainly acetate and butyrate, contained in the spent medium were efficiently converted to methane in a second anaerobic digester with a methane yield of about 170 ml/g COD initially fed in the first stage.

Introduction

From different point of view methane is considered as an interesting renewable energy provider. Anaerobic digestion producing methane has been largely investigated and many industrial processes are currently available for the methanogenic fermentation of various carbon sources (Cervantes *et al.* 2006; De Lemos Chernicharo 2007). Moreover, the energy recovery from simple carbohydrates such as molasses is about 46% higher than with alcoholic fermentation (Park *et al.* 2010). Therefore, aiming at the reduction of their dependence on fossil fuels, most of agro-food industries and waste treatment plants are investing in anaerobic digestion of their residual organic matter (Lens *et al.* 2004; Levin *et al.* 2007; Trzcinski and Stuckey 2009; Verstraete *et al.* 2009).

From the microbiological point of view since a large number of microorganisms have different and successive contribution in this bioprocess it has been divided in four steps: hydrolysis, acidogenesis, acetogenesis, and eventually methanogenesis (Levin *et al.* 2007; Ding and Wang 2008). Many literature is available about operating parameters (e.g. C/N ratio, hydraulic retention time, etc.) affecting the performances of the methanogenic microorganisms rather than about the diversity and specific metabolism of the microbial populations involved in the first three anaerobic digestion stages. Recently, a great interest has been shown for H_2, an intermediate biogas produced during acido- and aceto-genesis. Hydrogen is a promising energy vector with some advantages and complementarities compared to methane : 2.4-fold higher energy density (*i.e.* 33 kWh/kg H_2), no carbon dioxide emissions from hydrogen combustion and potential use of hydrogen for high energy conversion in fuel cells. Nevertheless, though the metabolism of the acidogenic bacteria producing H_2 is faster than that of methanogenic Archaea, the hydrogen produced in anaerobic digesters is rapidly converted to methane. Indeed, even a small amount of H_2 (> 0.1%) in the gas phase indicates a malfunctioning process (Cooney *et al.* 2007).

In a two-step process, *i.e.* when acido- and aceto-genesis are carried out in a different bioreactor than methanogenesis, it is feasible to generate separated biogas flows containing either H_2 or CH_4 depending on specific operating parameters such as the pH (Kyazze et al 2007; Antonopoulou *et al.* 2008; Ding and Wang 2008; Xie et al 2008; Hafez et al 2010; Park et al 2010). According to the highest yields reported by these authors, the mean volumetric H_2 and CH_4 potential would be in a stoichiometric ratio of 2:2 to 2:3. Equations 1 to 4 (Mohan *et al.* 2008, Calusinska *et al.* 2010) describe the main metabolic pathways involved in optimised two-stage anaerobic digestion from carbohydrates. Pathways from protein or lipid substrates or with other acid intermediates occur in methanogenic fermentation but with, if any, a low production of the intermediate H_2. Besides the fore-mentioned advantages for producing both H_2 and CH_4 biogas mixtures, decoupling the faster acidogenic fermentation from the methanogenic production is quite interesting for an industrial process with non-regular energy needs, likely in agro-food industries. Indeed, this makes the process more resistant to shock loading (Cooney et al 2008) and would allow satisfying daily peaks of energy demand with rapidly produced hydrogen without the need of a costly and hazardous CH_4 storage.

Acetic acid production	$C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 2CO_2 + 4H_2$	(1)
Butyric acid production	$C_6H_{12}O_6 \rightarrow CH_3CH_2CH_2COOH + 2CO_2 + 2H_2$	(2)
Acetoclastic methanogenesis :	$CH_3COOH \rightarrow 2H_2 + CO_2$	(3)
Hydrogenotrophic methanogenesis :	$4H_2 + CO_2 \rightarrow CH_4 + 2H_2O$	(4)

Many investigations have been carried out in order to improve the H_2 production from organic matter. The results achieved and reviewed by many authors (Bartacek *et al.* 2007; Das and Veziroglu, 2008; Wang and Wan, 2009a, b, c) are useful for improvement of the whole anaerobic digestion process, whether performed in one or two stages. Predominance of spore-forming H_2-producers and an optimum pH around 5.5 are reported for high H_2 production with pure or mixed cultures (Fang *et al.* 2002; Chen *et al.* 2005; Kim *et al.* 2008; Wang and Wan 2009a; Chen *et al.* 2009; Masset *et al.* 2010). They achieved hydrogen production yields of about 1.5 - 2 mol H_2 / mol glucose or 200 ml H_2 / g glucose and production rates of about 10 mmol H_2 / h or 120 ml H_2 / h.l of culture medium.

The purpose of the researches reported here is to comprehensively compare the yields achieved in the first stage operated in optimum conditions for H_2 production with a mixed culture (this study) or a pure *Clostridium butyricum* strain (results reported in Masset *et al.* 2010). The mixed culture was collected from an anaerobic digester and was thermally pre-treated to enrich the acidogenic microflora with spore-forming H_2 producers. By comparison to other studies of the two-stage anaerobic digestion (Kyazze *et al.* 2007; Xie *et al.* 2008; Hafez *et al.* 2010; Ljungren and Zacchi 2010), we promoted the simple technology of sequenced-batch reactor for both stages instead of a more complex setup e.g. using a UASB digester. This research also studied the feasibility of the second stage converting the metabolites of the first stage to methane.

Materials and methods

Experimental setup

Acidogenic fermentation (1st stage) was carried out in a 2.3 l bench-scale batch bioreactor as described elsewhere (Masset *et al.* 2010). The bioreactor containing 2.3 l of the MDT medium was inoculated with 200 ml of a sludge collected from an UASB anaerobic digester treating organic wastewater from sugar-beet and bioethanol industries. In order to enrich the hydrogen-producing microflora, the sludge was heat treated at 80 °C for 30 min. The MDT medium contained, per litre of deionised water : glucose monohydrate (5g), casein pepton (5g), yeast extract (0.5g), Na_2HPO_4 (5.1g), KH_2PO_4 (1.2g), $MgSO_4.7H_2O$ (0.5g).
Needles placed through the septum were used to control the pH level (METTLER TOLEDO combined probe) by automatic addition of sterile 1.5 N sodium hydroxide (maximum range of 0.15 pH unit around the pH level). During fermentation the bioreactor was maintained at 30 ° C and stirred at 60 rpm.
Methanogenic fermentation (2nd stage) was carried out in a 20 liters stirred anaerobic digester used in CWBI for biochemical methane potential assays of different agro-food organic wastes. This lab-scale digester is similar to the 2.3 L bioreactor and has been inoculated two years ago with a sludge collected from a full-scale anaerobic digester treating the activated sludge from a municipal wastewater treatment plant. The 20 l bioreactor was completely filled, stirred at 60 rpm and maintained at 55 °C and at a pH level around 7.3. The spent medium used for the experiments of anaerobic digestion was collected from a 20 l bioreactor that was inoculated with the pure *Clostridium butyricum CWBI1009* strain and performed according to the SBR mode. It contained mainly acetate and butyrate and the *Clostridium* biomass. It was kept at 4°C without significant evolution regarding metabolite concentrations. Before addition in the digester the acids were neutralized at pH 7.3 with KOH 9N.

Monitoring and analytical methods

The composition of biogas was measured using a gas chromatographer fitted with a thermal conductivity detector as described elsewhere (Hamilton *et al.* 2010). Nitrogen was used as carrier gas for H_2 measurement and Helium for N_2, O_2, CH_4 and CO_2 measurements.
Culture samples were centrifuged at 13000 g for 10 min and the supernatants were filtered through a 0.2 µm cellulose acetate membrane (Sartorius Minisart). The glucose, ethanol, formate, acetate, propionate, butyrate, lactate and succinate were analyzed using a HPLC equipped with a differential refraction index detector as described formerly (Masset *et al.* 2010).

The data for the glucose and metabolite concentrations were used to calculate the mass balance (MB) of the glucose conversion using the equation:

$$MB = \frac{\sum N_i \cdot \Delta C_i}{N_G \cdot \Delta C_G} \quad (5)$$

where N_i is the number of carbon atoms in a molecule of metabolite i; ΔC_i is the concentration of metabolite i effectively produced (*i.e.* the difference between the final and initial concentrations for a given culture sequence); N_G is the number of carbon atoms in the glucose molecule (*i.e.* 6) and ΔC_G is the concentration of the glucose consumed during the culture sequence (Eq. 5)
Water or a KOH solution (9N in water) was used in replacement equipment (*i.e.* an inverted 5 l glass vessel for the 1st stage and a 55 l polycarbonate column for the 2nd stage, both with an inside diameter of about 18 cm) to monitor the biogas production of the batch and sequenced batch bioreactors.

Results and discussion

Investigation of H_2 production from glucose in sequenced batch reactor mode

The fermentative H_2 production by mixed culture was investigated in a 2.3 l bioreactor equipped with pH control and liquid replacement equipment to monitor the biogas production (as described in Materials and Methods). After running a culture in batch, a sequenced-batch reactor (SBR) mode was performed in the same bioreactor for 33 successive sequences according to the procedure used in former works to investigate H_2 production by a pure *Clostridium butyricum* strain (Masset *et al.*, 2010). The SBR mode enables to easily assess the substrate conversion yields and the limitations due to metabolites-inhibiting concentrations. Each sequence lasted for 24 hours, or 72 h when indicated, and was carried out with removal-addition of 40 % (900 ml) of the culture medium and addition of glucose up to the standard initial concentration *i.e.* 5 g/l glucose monohydrate (as described in Materials and Methods). Tests were conducted at two different pH levels *i.e.* 5.2 and 5.5 while keeping other operating conditions constant (stirring, temperature, pressure, etc.). A first series of experiments at pH 5.2±0.15 involved 16 sequences including the batch culture (1st sequence). A second series of 5 sequences was conducted at pH 5.5±0.15 to study the effect of pH on H_2 production yields and the metabolism of mixed cultures. Both sequences were carried out with water replacement equipment to monitor biogas production and H_2 content was measured by gas chromatography. Finally a third series of experiments was carried out with pH maintained at 5.2 and in sequences 28 to 31 with a 9N KOH - solution used in the replacement equipment. This allowed to directly monitor the biohydrogen production and to compare the results with the performances of a pure *Clostridium butyricum* strain (Masset *et al.* 2010). It is to mention that the sequences 2, 7, 12, 17, 22, 27 and 32 lasted for 72 h (*i.e.* during the week-end) with pH control but no temperature control. Moreover, no results were collected during seq. 7.

Results of the first series of 16 sequences at pH 5.2

A biogas production of 2.5 l was achieved during the batch culture. It increased progressively in the 3 following sequences up to about 4 l of biogas and reasonably maintained at this level for the 11 following sequences. The H_2 production yields (1.34±0.2 mol H_2 / mol glucose) indicated in Figure 1 were calculated taking into account the measured H_2 content and the glucose effective

consumption, *i.e.* about 53±2 % and 95 % respectively for these 12 sequences. The Figure 1 also shows the concentration of residual glucose and soluble metabolites measured at the end of each sequence. The major metabolites are butyrate (52.7±11,1 mM), formate (10.7±4.9 mM) and acetate (15.8±7.1 mM). Similar results for both H_2 yields and butyrate and acetate concentration in the culture medium were mentioned in the literature for experiments in small scale bioreactors with pure or mixed cultures containing spore-forming H_2-producing bacteria (Fang *et al.* 2002; Chen *et al.* 2005; Wang and Wan 2009a; Chen *et al.* 2009; Masset *et al.* 2010). Ethanol (2.2±1 mM) and sometimes propionate (about 0.7 mM) were released at lower amounts than the other metabolites. Ethanol is usually produced either by facultative anaerobic enterobacteriaceae, some of them being H_2 producers, or by Clostridium population during the stationary growth phase at pH levels below 6 (Hawkes *et al.* 2002; Kapdan and Kargi 2006; Hamilton *et al.* 2010). In our experiments, the enterobacteriaceae population would be in quite minority since no lactate, a frequent metabolite of these microorganisms (Oh *et al.* 2008), was detected during this series of experiments.

The pattern of metabolite concentrations measured in seq. 1 to 11 and seq. 12 to 16 seems relatively different. This suggests that the metabolism or the microbial populations have changed after a relative stabilization in seq. 8 to 11 while the H_2 yields were not significantly affected. Indeed, as shown in Table 1 and also observed in former SBR experimentations with a pure *Clostridium* strain (Masset *et al.* 2010), the glucose conversion into formate decreases from a maximum (9% of the initial glucose carbon content) achieved during the batch culture to a few percents after some culture sequences. By contrast, in the experiments reported here, after an acetate production peak (15 %) observed in seq. 5 to 6 simultaneously to the H_2 production peak, the amount of glucose converted to acetate declined too whereas it increased with the pure strain as for butyrate.

Results of the sequences 17 to 21 at pH 5.5

The results (Figure 1) achieved in similar conditions at pH 5.2 and 5.5 highlight an obvious decrease of about 40 % of the H_2 production yield when increasing the pH to 5.5. This trend is consistent to the results of other authors (Fang and Liu 2002; Chen *et al.* 2009). However, regarding the metabolites yields, no global trend may be evidenced. Indeed, in our experiments (seq 17 to 19), glucose conversion to both formate and lactate increases when

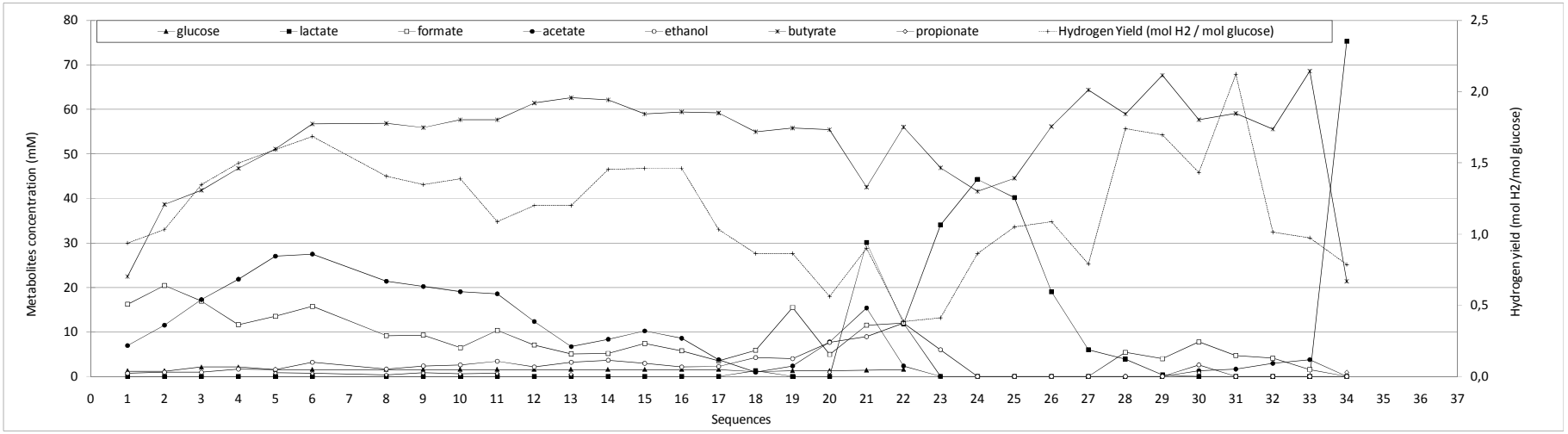

Table 1: Metabolites yields expressed as carbon converted (%) from glucose during different sequences for fermentative hydrogen production in a 2.3l sequenced batch bioreactor.

Sequence number	1	2	3	4	5	6	9	10	11	12	13	14	15	16
Lactate	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0
Formate	9,0	6,2	3,0	1,2	3,8	4,5	2,3	0,7	3,7	0,7	0,6	1,3	2,5	0,9
Acetate	7,7	8,3	12,2	13,5	16,0	13,6	9,0	8,4	8,7	2,1	-0,2	5,1	6,0	3,2
Ethanol	0,8	0,7	0,5	1,3	0,6	2,6	1,6	1,4	2,1	0,2	2,2	2,1	0,9	0,5
propionate	0,0	0,0	0,0	0,0	1,3	0,4	0,9	0,2	0,6	-0,6	0,8	-0,5	0,0	0,0
Butyrate	50,2	56,9	45,1	51,6	54,0	61,8	52,7	57,8	55,6	63,7	61,8	59,5	53,0	58,0

Sequence number	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34
Lactate	0,0	2,1	-1,2	0,0	49,4	-8,2	42,8	40,5	24,7	-5,5	-7,5	0,9	-2,9	-0,4	0,0	0,0	0,0	121,0
Formate	0,2	2,1	6,7	-2,1	4,8	3,1	-3,5	0,0	0,0	0,0	0,0	2,9	0,5	3,0	0,2	0,8	-0,4	-0,5
Acetate	-1,1	-1,2	2,0	7,0	12,1	-6,8	-1,4	0,0	0,0	0,0	0,0	0,0	0,0	1,4	1,0	2,1	2,3	-2,3
Ethanol	1,2	3,2	1,7	5,8	5,1	7,7	-0,7	-3,6	0,0	0,0	0,0	0,0	0,0	2,8	-1,6	0,0	0,0	0,0
propionate	0,0	0,0	0,0	1,6	-0,9	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	1,4	-0,8	0,0	0,0	1,6
Butyrate	56,7	47,1	54,7	52,7	25,1	70,8	32,4	32,8	45,4	67,0	70,5	49,1	74,2	42,4	57,4	48,2	80,2	-36,4
Sum	57,0	53,3	64,0	65,2	95,6	66,6	69,5	69,7	70,1	61,5	63,0	52,9	71,9	50,6	56,2	51,1	82,2	83,4

pH raises from 5.2 to 5.5 whereas butyrate and acetate production decreases (Table 1). By contrast Chen *et al.* (2009) and Fang and Liu (2002) observed respectively either an increase of butyrate and acetate concentrations or a decrease of butyrate and an increase of acetate.
It is to mention that seq. 20 was not carried out successively to seq. 19 but after a delay of two weeks. It seems that it did not affect the H_2 yields. However significant differences were recorded for the metabolites concentrations measured at the end of the following sequences (seq. 21 to 25). Indeed, lactate production increased drastically up to concentrations higher than 30 mM. Wang *et al.* (2008) also reported such high concentrations of lactate with a pure *Clostridium butyricum* strain producing hydrogen at a yield of 0.6 mol H_2/mol glucose.

Results of the third series of 13 sequences at pH 5.2

The third series of experiments was carried out again at pH 5.2 and in the sequences 28 to 31 a KOH solution was used in the replacement equipment to absorb carbon dioxide from the biogas produced and assess directly the hydrogen production. The consequences of the metabolic pathway shift (stated in section 3.1.2.) to high lactate production are noticed until the end of seq. 27 : lactate and butyrate are the sole metabolites produced in seq. 23 to 25 from the total initial amount of glucose (Table 1). In the two following sequences lactate is even consumed with a possible contribution to hydrogen production (Li and Fang 2007). After that period, a similar pattern of metabolite concentrations than in seq. 13 to 16 is recovered. However, the acetate concentration is quite lower.
During the experiments with direct measurement of the H_2 cumulative production yields of 1.75±0.3 mol H_2/mol glucose were achieved. They are at the same level or a few higher than the highest yields obtained in the first series of experiments at pH 5.2. This suggests that the H_2 content measured by gas chromatography would be sharply underestimated, probably due to the operating conditions that are not quite identical for calibration and measurement of sampled gas (*i.e.* the 500 µl calibration gas are sampled from a 150 ml serum bottle regularly filled at atmospheric pressure with gas mixture from a 50 l pressurized container (B50 size from Air Liquide) and biogas is sampled directly from the bioreactor headspace through the septum). However, the H_2 yields achieved in the experiments reported here are lower than those (2.3 mol H_2/mol glucose) obtained in the same SBR operating conditions with the pure *Clostridium butyricum CWBI1009* strain (Masset *et al.* 2010). However, with this strain a significant amount (about 18 %) of the substrate initial carbon content was converted in acetate that is involved in the *Clostridium* metabolic pathway to the highest hydrogen production (Eq. 1). Moreover, about 70 % of the carbon mass balance was recovered in the soluble metabolites whereas in our experiments the sum varies between 50 and 72 % as mentioned in Table 1 for seq. 28 to 31. The rest of carbon would be converted in carbon dioxide and biomass. It should be noted that the H_2 yields achieved in the seq. 32 and 33 are similar to those of seq. 24 and 25 (*i.e.* before the series with KOH replacement equipment). Nevertheless, the hydrogen production yields of the first series of experiments were not attained in the third series. Furthermore, high lactate production are recorded again in seq. 34 probably indicating a kind of unstability in the microbial ecology.

Investigation of CH_4 production from the soluble metabolites of H_2-producing dark fermentation

This section presents the results of the experiments carried out in a 20 l anaerobic digester in order to assess the feasibility of converting the metabolites from a bioreactor producing H_2 into methane. Aiming for relevant investigations, the culture medium from a stable dark fermentation (as described in section 2.2.) was preferred to the spent medium from the former SBR producing hydrogen in which the metabolite concentrations were relatively unstable (as stated in section 3.1.). A first series of 3 sequences was carried out with removal-addition of 1.5, 2 and 2 liters of metabolites mixture. As shown in Figure 2, they allowed producing 2.4, 2.5 and 3.0 liters of biogas respectively. The duration of the active period when biogas production was effective decreased progressively from seq. 1 to seq. 3. Therefore the production rate increased up to a maximum of 2.7 l/d (Figure 2).

A second series of 14 sequences was carried out with removal-addition of 4 liters of spent medium from the first anaerobic digestion stage. Different period of time were considered for the successive sequences in order to assess the resistance of the methanogenic microflora to loading shocks. The Figure 2 shows the cumulative biogas production for this series that

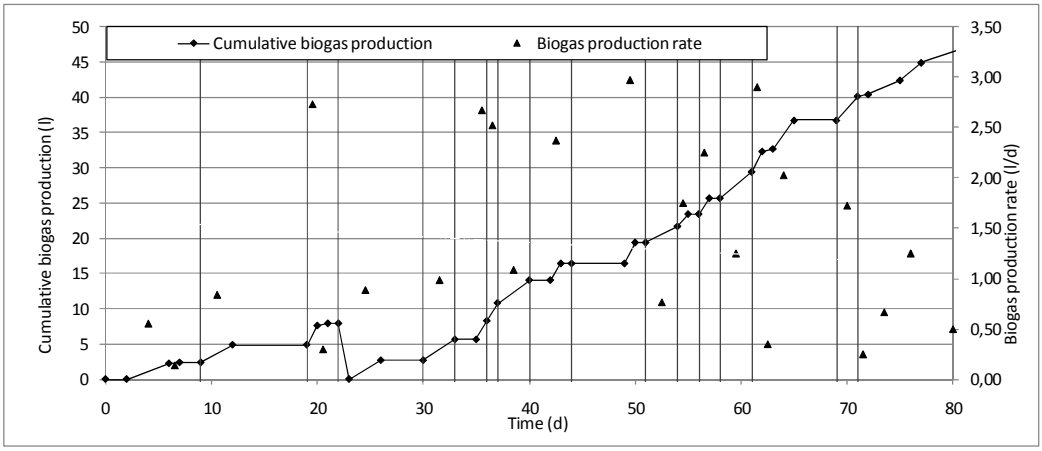

Figure 2: **Cumulative biogas production (l) and biogas production rate (l/d) in a 20 l anaerobic digester fed with spent medium from fermentative hydrogen production. The vertical lines indicate the removal-addition of spent medium (starting a new sequence)**

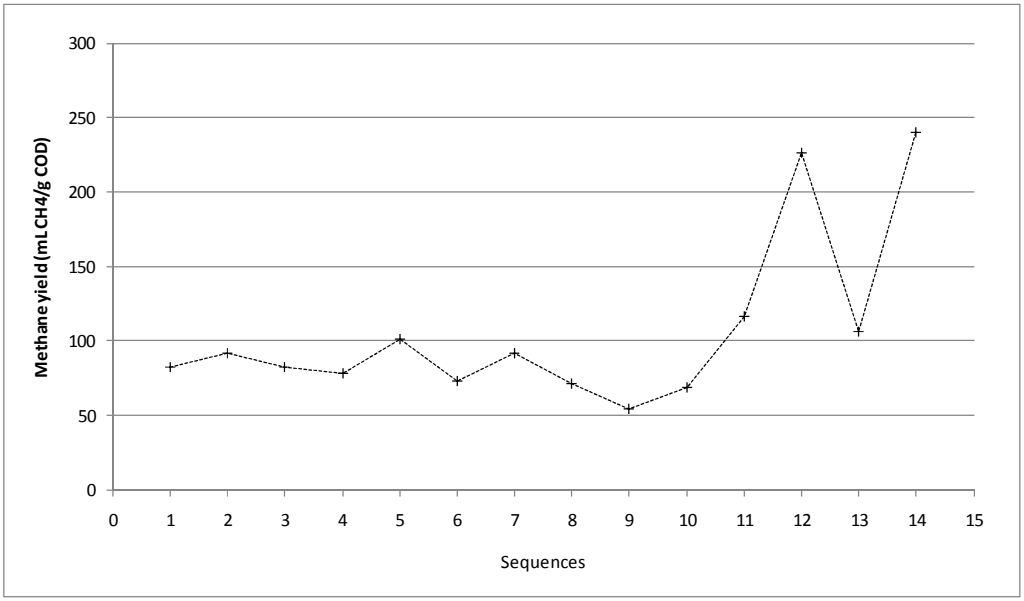

Figure 3: Methane production yields (ml CH_4 / g COD initially loaded in the first stage) for different sequences in a 20 l anaerobic digester fed with 4 liters of spent medium from fermentative hydrogen production (2nd series of experiments).

started after the first series (22 days duration). The profile is relatively linear suggesting a mean biogas production rate of about 0.83 l/d. By comparison, the production rate during the active period of each sequence varied between 1 and 3 l/d. A mean methane content of about 60 % was measured in the biogas. Therefore the methane yields were calculated for each sequence (Figure 3). They are relatively similar for the first 10 sequences at about 100 ml CH_4/g COD (initially fed in the first stage) and increase in the following sequences to a mean 170 ml CH_4/g COD. These results are promising even if they are lower than those reported in the literature (Hafez *et al.* 2010; Xie *et al.* 2010) and if large yields variations are recorded probably due to non regular loading.

Conclusions

In this paper the performances of the first stage of an anaerobic digestion process operated in optimum conditions for H_2 production were compared using a mixed culture or a pure *Clostridium butyricum* strain.

1. The hydrogen yields achieved in a 2.3 l sequenced-batch bioreactor were of about 1.75 and 2.3 mol H_2/mol glucose respectively.
2. A decrease of about 40 % of the H_2 production yields was recorded when increasing the pH from 5.2 to 5.5. These results are consistent to other works.
3. The major metabolites of glucose fermentation were in the decreasing order of concentration in culture medium butyrate, acetate and formate at a lower concentration. This suggests a predominance of *Clostridium* strains in the microflora.
4. A relative instability of the population or metabolism of the mixed culture was evidenced which affects severely the H_2 yields. Therefore, an efficient control of the different operating parameters would be required when using mixed cultures in the first stage of anaerobic digestion.

The investigations carried out in a second 20 l digester focused on the performances of the second stage of an anaerobic digestion process.

5. The results confirm the feasibility to produce methane from the soluble metabolites produced in the first stage with a methane yield of about 170 ml/g COD initially fed in the first stage.

Globally the initial COD contained in glucose was converted to produce from 200 to 260 ml H_2 /g COD and 170 ml CH_4/g COD.

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