Enzymatic and metabolic activities of four anaerobic sludges and their impact on methane production from ensiled sorghum forage

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1. Introduction

Anaerobic digestion is an old and well established biological process used to treat a wide range of substrates (i.e. industrial and municipal waste activated sludges, municipal solid wastes, manures, residues from the agro-processing industry, agricultural residues and energy crops) which contain high concentrations of readily biodegradable organic material in form of carbohydrates, proteins and fats. It involves the degradation and stabilization of organic materials under anaerobic conditions by microbial organisms and it leads to the formation of biogas which consists mainly of CH_4 (55–75%) and CO_2 (25–45%). The ecology of anaerobic digestion is complex, and involves several bacterial groups and up to nine steps of conversion of organic matter. However, it is possible to distinguish four main steps, namely hydrolysis, acidogenesis, acetogenesis, and methanogenesis, and four major bacterial groups: the hydrolytic-fermentative bacteria that hydrolyze complex organic compounds into simple ones; fermentative bacteria that convert the simple organic compounds into volatile fatty acids with the simultaneous production of hydrogen (H_2) and carbon dioxide (CO_2); the acetogenic bacteria that convert the above-mentioned acids into acetic acid and finally the methanogenic bacteria that produce methane, either from acetate or from H_2 and CO_2.

The major challenge in producing methane through anaerobic digestion of lignocellulosic substrates is their structure and composition, which mainly consist of cellulose (40–50%), hemicelluloses (25–35%) and lignin (15–20%), strongly linked to each other. Some compositional and structural features (i.e. the degree of polymerization and crystallinity of the cellulose, the structure of hemicelluloses, the lignin content and composition, the pectins content, the accessible surface area and pore volume) can affect the hydrolysis as well as the accessibility of biodegradable compounds (Monlau et al., 2013).

During the last years, various methods of pretreatment (physical, chemical, biological and combination of them) have been studied to enhance methane production of lignocellulosic substrates

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(Sonakya et al., 2001; Lehtomaki et al., 2004; Frigon et al., 2012; Kafle et al., 2013; Orozco et al., 2013; Sambusiti et al., 2013)

When microorganisms are able to excrete the suitable enzymes, hydrolysis is a relatively fast step (Björnsson et al., 2001). On the contrary, if the substrate is hardly accessible for the enzymes, as in the case of lignocellulosic substrates (i.e. sorghum), hydrolysis becomes the rate-limiting step (Taherzadeh and Karimi, 2008).

During the hydrolysis step, organic polymers (i.e. proteins, lipids and carbohydrates) are hydrolysed into amino acids, long chain fatty acids, and simple sugars, through the action of facultative hydrolytic bacteria, known as primary fermenting bacteria, which hydrolyse the substrate by secreting extracellular enzymes. A wide range of enzymes (i.e. cellulases, hemicellulases, proteases, amylases and lipases) can be produced, through which bacteria can hydrolyze almost all kinds of substrates (Taherzadeh and Karimi, 2008; Bruni, 2010). Polysaccharides (i.e. cellulose, hemicelluloses and starch) are converted into simple sugars; the cellulase enzymatic complex hydrolyses cellulose into glucose through the synergic action of endoglucanase, exoglucanase, and β-glucosidase enzymes; hemicelluloses degradation results in monosaccharides such as xylose, glucose, galactose, arabinose and mannose, while starch is converted into glucose by amylase enzymes (Rajoka and Malik, 1997; Yang et al., 2001).

Enzymes are also naturally secreted by microorganisms existing in the anaerobic digestion inoculum and their activity may influence the methane yields and anaerobic digestion kinetics during BMP tests. However, the characteristic of the inoculum (i.e. microbial consortia and enzymatic activity behaviours) depends mostly on its origin (treatment of industrial, domestic or agricultural residues) and may influence the hydrolysis performance of the substrate.

To date, little information is available about the dynamics of hydrolytic enzymes present in anaerobic digesters fed with ensiled sorghum forage. Only few studies have considered the potential influence of inoculum in anaerobic digestion systems performed both in batch and continuous mode (Marchetti et al., 2009; Keating et al., 2011).

Therefore, the aim of the present research was twofold: (i) to study the basic enzymatic activity naturally displayed by anaerobic microbial consortia when lignocellulosic biomasses become available, i.e. which kind of enzymes and which kind of dynamics; (ii) to evaluate whether the enzymatic activity displayed during anaerobic digestion of lignocellulosic biomasses is correlated with biomethane production. To this aim, batch anaerobic digestion tests were performed using sorghum as substrate and four type of anaerobic sludge samples as inoculum. Methane production was correlated to both metabolic and enzymatic activities. Therefore, the present study constitutes an effort to understand the dynamic of the anaerobic digestion process with respect to the hydrolytic enzyme activities and it is also of interest in view of the proposed application of enzymatic pretreatment of lignocellulosic biomasses to enhance biomass recovery.

2. Methods

2.1. Origin of anaerobic sludge inocula and of sorghum

To perform BMP tests, four types of anaerobic digested sludge were used: (1) WW: collected from a digester fed on waste activated sludge; (2) AGR: collected from a digester fed on agro-wastes (cattle manure and corn silage); (3) GR: collected from a UASB reactor treating wastewater from a chemical industry; (4) MIX: a sludge obtained by mixing (50% each on a volatile solids basis) WW and AGR.

Ensiled sorghum forage (*Sorghum Sudanense hybrid*), used as substrate, was collected from a farm near Cremona (Lombardy Region, Italy). After collection, the sorghum sample was oven dried at 60 °C for 2 days to a moisture content of less than 10% and then grounded into 1 mm particles sizes with a kitchen blender. Finally, it was stored into air-tight containers at ambient temperature.

2.2. Analytical determinations

Total solids (TS), volatile solids (VS) were analysed according to APHA methods (APHA, 2005). Chemical oxygen demand (COD) was determined according to the open reflux method (APHA, 2005). Fats and proteins were determined with a NIR System (5000 monochromator, Foss). Cellulose (CEL), hemicelluloses (H-CEL) and klason lignin (K-LIG) were measured using a strong acid hydrolysis method adapted from Effland, 1977. Samples (200 mg) were first hydrolyzed with 12 M H_2SO_4 acid for 2 h at room temperature, then diluted to reach a final acid concentration of 1.5 M and kept at 100 °C for 3 h. The insoluble residue was separated from the supernatant by filtration on fibreglass filter (GF/F, WHATMAN). This insoluble residue was washed with 50 mL of deionized water and then placed in a crucible. The crucible and the paper fibreglass were dried at 100 °C during 24 h to determine by weighting the amount of klason lignin. After centrifugation of the sample in 2 mL Eppendorf® tubes, followed by filtration at 0.2 μm (Nylon membrane, Acrodlsc®), 800 μL of supernatant were transferred to a vial prior to the analysis by high performance liquid chromatography (HPLC). Structural carbohydrates (i.e. glucose, xylose, arabinose, glucuronic and galacturonic acids) were measured by HPLC coupled to refractometric detection (Waters R410). The components were separated by an Aminex column HPX-87H column (300 × 7.8 mm, Bio-Rad) equipped with a protective precolumn (Microguard cation H refill cartbridges, Bio-Rad). The eluting solution corresponded to 0.005 M H_2SO_4, and the flow rate was 0.3 mL min^{-1}. The column temperature was maintained at 50 °C and the refractometric temperature was fixed at 45 °C. A refractive index detector (Waters 2414) was used to quantify the carbohydrates. The system was calibrated with glucose (0–6 g L^{-1}), xylose (0–6 g L^{-1}), arabinose (0–2 g L^{-1}), and uronic acids (0–2 g L^{-1}) (galacturonic and glucuronic) standards (Sigma–Aldrich®). Thereafter, cellulose and hemicelluloses contents were estimated as follows (Eq. (1) and (2)):

$$\text{Cellulose } (\%\text{VS}) = \text{Glucose } (\%\text{VS})/1.11 \quad (1)$$

$$\text{Hemicelluloses } (\%\text{VS}) = [\text{Xylose } (\%\text{VS}) + \text{Arabinose } (\%\text{VS})]/1.13 \quad (2)$$

where: 1.11 is the ratio of the molecular weights of glucose to glucan (180/162) and 1.13 is the ratio of the molecular weights of xylose and arabinose to xylan (150/132).

2.3. Biochemical methane potential (BMP) tests

BMP tests were performed in duplicate using a commercial laboratory instrument (AMTPS, Bioprocess control, Sweden). This is a volumetric device consisting of 15 gas-tight glass bottles (500 mL of working volume) placed in a water bath at 35 ± 0.5 °C. Each bottle was continuously mixed with a rotary stirrer. The biogas produced passes through a NaOH solution (3 M), for CO_2 absorption. Methane flows through a liquid-displacement automated measuring unit with a resolution of 11–13 mL. A data acquisition system allows flow-rate data to be recorded continuously. To perform experimental tests, all four anaerobic digestion sludges were used as inoculum, separately. Before BMP tests, each inoculum was kept under endogenous anaerobic conditions at 35 °C for about 7 days

to reduce non-specific biogas generation. An amount of 2.5 g VS of sorghum (previously oven dried at 60 °C and grounded into 1 mm particles) was then mixed with 2.5 g VS of each inoculum, obtaining a substrate to inoculum ratio around 1 g VS g^{-1} VS, as suggested by Raposo et al. (2011) and Chandra et al. (2012). Finally, to reach 500 mL of working volume, 50 mL of mineral medium of macronutrients (OECD 311, 2006) and deionised water were also added to each bottle. A blank sample was performed by mixing the inoculum, the mineral medium, and the deionised water, without the addition of substrate. The initial pH value was around 7.5 for all samples. The BMP test duration was 32 days for all samples and the methane yield at 32 days (BMP_{32}) was calculated as follows (Eq. (3)):

$$BMP_{32} = (V_{CH4,s} - V_{CH4,blank})/VS_s \quad (3)$$

where VCH4,s (NmLCH4) is the volume of methane produced from the substrate and measured at the end of the test; VCH4,blank (NmLCH4) is the volume of methane produced from the inoculum and measured at the end of the test; (VCH4,s−VCH4,blank) (NmLCH4) is the net volume of methane production measured at the end of the test; VSs (gVS) is the mass of volatile solids of the added substrate. All gaseous volumes hereafter reported are referred to STP conditions.

2.4. Theoretical biochemical methane potential and anaerobic biodegradability

The experimental BMP_{32} was used to calculate the anaerobic biodegradability under the tested conditions by comparing it with the theoretical value (BMP_{th}), as follows (Eq. (4)):

$$BD(\%) = (BMP_{32}/BMP_{th}) \cdot 100 \quad (4)$$

As generally accepted, the theoretical biochemical methane potential of the substrate BMP_{th} ($mL_{CH4}\ g^{-1}$VS) can be calculated by considering the theoretical methane yield of each degradable compound during anaerobic digestion process as 415 $mLCH_4\ g^{-1}$ cellulose $(C_6H_{10}O_5)_n$, 424 $mLCH_4\ g^{-1}$ xylan $(C_5H_8O_4)n$, 420 $mLCH_4\ g^{-1}$ proteins $(C_{14}H_{12}O_7N_2)_n$, 1014 $mLCH_4\ g^{-1}$ lipids $(C_{57}H_{104}O_6)$, 350 $mLCH_4\ g^{-1}$ soluble sugars (Symons and Bushwell, 1933).

2.5. Kinetic study

The anaerobic digestion process was assumed to follow a first order kinetic model, as it is the case of lignocellulosic substrates for which hydrolysis is the limiting steps (Angelidaki et al., 2009). The first order kinetic constants were calculated by using the least-squares fit of methane production data from BMP tests during time (t), according to the following Eq. (5):

$$BMP\ (t) = BMP_{t\to\infty} \cdot (1 - \exp(-k_h \cdot t)) \quad (5)$$

where: BMP (t) is the cumulative methane yield ($NmLCH_4\ g^{-1}$VS) at the time t (d), $BMP_{t\to\infty}$ is the ultimate methane yield ($NmLCH_4\ g^{-1}$VS) of the substrate, k_h (d^{-1}) is the first order kinetic constant and t (d) is the digestion time. $BMP_{t\to\infty}$ and k_h were determined by the mean square error method.

2.6. Determination of enzymatic activities

In order to characterise the sludge inocula in terms of their enzymatic behaviour, a set of bottles were prepared in the same way as those used in BMP tests. Then, at appropriate intervals, samples were taken from each BMP bottle and centrifuged. Enzymatic activities, expressed as IU $g^{-1}VS_{inoculum}$, were determined on supernatants.

Endoglucanase (CMCase) activity was determined by measuring the amount of glucose released from carboxymethylcellulose (CMC, Sigma) using the Somogyi–Nelson method with glucose as standard (Somogyi, 1952) (detection limit 5 mg L^{-1}). For this purpose, an aliquot of diluted sample (0.5 mL) was mixed with 0.5 mL of a CMC suspension (1% w v^{-1}) in citrate buffer (0.05 mol L^{-1}, pH 5). Reaction mixtures were left at 55 °C for 30 min, and then boiled to stop the enzymatic activity. Sugars release was then determined with glucose as standard (Somogyi, 1952). One unit of enzyme (IU) was defined as the amount of enzyme which hydrolyzes 1 μmol of reducing sugars, expressed as glucose, in 1 min.

Exoglucanase (avicelase) activity was determined according to Desrochers et al. (1981). An aliquot of diluted sample (1 mL) was mixed with 1 mL of microcrystalline cellulose Avicel® suspension (Serva, 2% w v^{-1}) in acetate buffer (0.1 mol L^{-1}, pH 5). Samples were then incubated at 30 °C for 24 h, and then boiled to stop the enzymatic activity. The amount of glucose released from cellulose Avicel®, was measured according to Somogyi–Nelson method with glucose as standard (Somogyi, 1952) (detection limit 5 mg L^{-1}). One unit of enzyme (IU) was defined as the amount (μmol) of glucose released from 1 mL of sample, in 1 min.

Xylanase activity was determined employing a procedure adapted from Shewale and Sadana (1978), by mixing 0.5 mL of sample with the same volume of a xylan solution (Sigma, 1% w v^{-1}) in citrate buffer (0.025 M, pH 5). A blank sample with 0.5 mL of deionized water was also prepared. Samples were incubated at 50 °C for 30 min and then, boiled to stop the enzymatic activity. Reducing sugars were determined again through the Somogyi procedure, employing xylose as standard (detection limit 5 mg L^{-1}). One unit of enzyme (IU) was defined as the amount which releases 1 mol of reducing sugar equivalents per minute under the conditions specified above.

Laccase activity was determined according to Li et al. (2008) by mixing 0.5 mL of sample with 0.5 mL of 2,2′-azino-di-3-ethyl benzothiazoline-6-sulphonic acid (ABTS, Sigma, $_{420}$ = 36,000 $M^{-1}\ cm^{-1}$) and 0.5 mL of acetate buffer (0.1 M, pH 5). A blank sample with 0.5 mL of deionized water was also prepared. Samples were incubated at 37 °C for 10 min and laccase activity determined by using a spectrophotometer (OD 420 nm) (6705 UV/Vis Spectrophotometer, Jenway, UK). One unit of enzyme (IU) was defined as the amount of enzyme which oxidizes 1 μmol of ABTS in 1 min (detection limit OD 0.020).

2.7. Determination of metabolic activities

Metabolic activity tests were performed on the four inocula by measuring the methane production rate after addition of specific substrates, according to the BMP protocol.

According to Angelidaki et al. (2009), acetic (1 g L^{-1}), propionic (1 g L^{-1}) and butyric (1 g L^{-1}) acids were dosed to assess acetoclastic, methanogenic and acetogenic activities, respectively. For the determination of hydrolytic and acidogenic activities, 1 g L^{-1} of carboxymethylcellulose and glucose were used as substrate, respectively.

Those substrates were dosed to each inoculum and the cumulated methane production was measured as in BMP tests. Each specific metabolic activity was assessed by linear regression of the cumulated methane production curve. The standard error of the slope estimate was used to quantify the reliability of each specific activity value.

3. Results and discussion

3.1. Inoculum and sorghum characteristics

Table 1 summarizes total solids (TS) and volatile solids (VS) concentration of each inoculum used. TS concentration was

different for the four inocula. Nevertheless, similar VS/TS ratios were found for all sludge samples.

As for ensiled sorghum forage, TS content was 93 ± 4 gTS 100 g^{-1} wet weight, while VS was 86.6 ± 0.4 gVS 100 g^{-1}TS. Sorghum sample had an average COD/VS value of almost 1.2, which is close to the typical value for carbohydrates (1.19 and 1.21 gCOD g^{-1} for cellulose and hemicelluloses, respectively). Proteins and fats contents were 9 ± 3 g 100 g^{-1} VS and 1.8 ± 0.3 g 100 g^{-1} VS, respectively. Cellulose, hemicelluloses, Klason lignin contents of sorghum were: 32.2 ± 1.1 g 100 g^{-1} VS, 16 ± 0.6 g 100 g^{-1} VS, and 25.7 ± 0.2 g 100 g^{-1} VS, respectively. Soluble sugars content of sorghum was estimated as [VS (gVS 100 g^{-1}TS) − (Cellulose + Hemicelluloses + Klason lignin + Proteins + Fats) (g 100 g^{-1}TS)]/[VS (gVS 100 g^{-1}TS)] and it was 15.3 g 100 g^{-1} VS.

Despite the high variability of substrates composition, varying according to plant type and variety, results can be considered in accordance with literature values (Li et al., 2010; Manzanares et al., 2012). Typical compositional values of forage sorghum ranged between 32% and 36%TS for cellulose, between 20% and 23%TS for hemicelluloses and 18–26%TS for Klason lignin. Soluble sugars content was in accordance with typical values of sorghum samples, ranging between 10 and 30%VS (Monlau et al., 2012).

3.2. Enzymatic activities

The enzymatic breakdown of carbohydrates polymers (i.e. cellulose, hemicelluloses and lignin) is considered as the dominant mechanism in the hydrolysis of complex substrate (i.e. sorghum). Enzymes are naturally secreted by microorganisms existing in anaerobic digesters, but their activity can vary during the course of the biochemical methane potential (BMP) tests, as a consequence of the composition of the organic substrate to be degraded.

Therefore, tests were performed to study the extent and variation of relevant enzymatic activities during the course of a BMP test.

Figs. 1A and B report xylanase enzymatic activity (IU g^{-1} $VS_{inoculum}$) trends detected in sludge samples during biochemical methane production (BMP) tests. In absence of sorghum (Fig. 1A), xylanase activity was detected in all inocula since the beginning of the test, although at different levels. The higher activity was observed in the sludge acclimated to agrowastes (AGR) which is one order of magnitude higher that that one measured on sludges collected from digesters treating industrial wastes (GR) and waste activated sludge (WW). During the course of the following endogenous methane production, xylanase activity significantly increased during the first 17 days for the AGR sludge (max. around 7 IU $g^{-1}VS_{inoculum}$), to decrease later on. As for the GR sludge, no dynamic evolution was observed. These data suggest that this enzymatic activity is physiologically present in all sludge inocula. Upon sorghum addition, a similar dynamic trend was observed for all tested inocula (Fig. 1B). As expected, the presence of sorghum increased xylanase activity in all samples suggesting that enzyme production is induced by the substrate, i.e. by hemicelluloses concentration. This behaviour was also confirmed by Kumar et al. (2008). The net enzymatic activity has been also computed as the difference between activity values measured in presence and absence of sorghum (Fig. 1C) that quantifies the enzymatic activity that is induced by putting in contact each inoculum with sorghum. It appears that the net enzymatic activity values had similar trends for WW, AGR and MIX, suggesting that sorghum induced the production of similar amount of hydrolytic enzymes in those sludge samples. On the contrary, a much lower values were observed for the granular sludge suggesting that this inoculum was unable to express the same level of xylanase activity.

Exoglucanase, that is active on crystalline cellulose, was found in lower (two order of magnitude) levels than the xylanase one (Figs. 1D and E). Under endogenous condition (Fig. 1D), an increasing followed by a decreasing trend was again observed. In presence of sorghum, exoglucanase activity was found to increase in all samples (Fig. 1E), even if levels remained modest, with a maximum of 0.08 IU $g^{-1}VS_{inoculum}$. Again, the time-trend of net enzymatic activity values (Fig. 1F) was very similar for WW, AGR and MIX inocula; again, lower values were observed for GR.

No significant endoglucanase activity, whose substrate is amorphous cellulose, was observed for AGR, GR and MIX sludges. Only at the beginning of incubation (5–7 days), WW sludge showed an activity up to 1.24 and 1.96 IU $g^{-1}VS_{inoculum}$ in absence and in presence of sorghum, respectively. The fact that endoglucanase activity has been detected only at the beginning of the test may suggest that its presence was induced by residual organic matter in the inoculum.

Laccase activity, an oxidative enzyme as well as lignin and manganese peroxidases, was always found in traces (max. 2.5×10^{-4} IU $g^{-1}VS_{inoculum}$ at 17 days for AGR inoculum and in presence of sorghum), presumably because anaerobic BMP condition does not favour its activity.

The agricultural (AGR) inoculum showed always the highest enzymatic activities, while granular sludge (GR) showed the lowest values, with municipal (WW) sludge in-between. However, when the net enzymatic activity values are considered, AGR and WW showed more similar values and trends indicating a similar induction capacity of sorghum. The net values calculated for the mixed sludge (MIX) are similar to those computed by averaging values from AGR and WW suggesting that enzyme expression is additive.

On the overall, xylanase was the prevailing enzymatic activity for all inocula and it was furthermore seen that the maximum activity of xylanase and exoglucanase enzymes occurred at different times during the tested period. Xylanase enzyme exhibited its maximum activity earlier than exoglucanase. These results suggest that a correlation exists between sorghum structure and the enzymatic activities found. In the first 15 days of BMP trials, xylanase acts on hemicelluloses that are mainly located outside and in-between cellulose fibres. Then, when cellulose becomes more accessible, exoglucanase enzymatic activity increases; this activity was found to increase until the end of BMP trials, presumably because cellulose hydrolysis is a slow process and the cellulose availability never decrease during the course of the test. This hypothesis implies the presence of residual undigested cellulose at the end of the test.

The enzymatic dynamics observed have been also confirmed by previous authors (Perez et al., 2002; Cohen et al., 2005). Perez et al., 2002 reported that high reduction of hemicelluloses led to a highest chance for cellulolytic microbes to degrade cellulose, because hemicelluloses bound a layer of cellulose forming a microfibril to increase the stability of cell wall. Cohen et al. (2005) found that the crystalline form is highly resistant to microbial and enzymatic degradation, while amorphous cellulose is hydrolysed much faster. So, the rate of the enzymatic hydrolysis of cellulose is greatly affected by its degree of crystallinity.

However, quantitatively, the comparison of the enzymatic activities reported here was not performed because no data for

Table 1
TS, VS and pH values of the four sludge inocula (AGR, WW, MIX and GR). Values correspond to mean ± standard deviation of measurement performed in duplicate.

	AGR	WW	MIX	GR
TS (g L^{-1})	54 ± 3.6	18 ± 0.3	27 ± 3.8	32 ± 0.2
VS (g L^{-1})	35 ± 2.3	12 ± 0.1	18 ± 2.5	21 ± 0.1
VS/TS (%)	65	67	67	65
pH	7.9	7.8	7.8	7.7

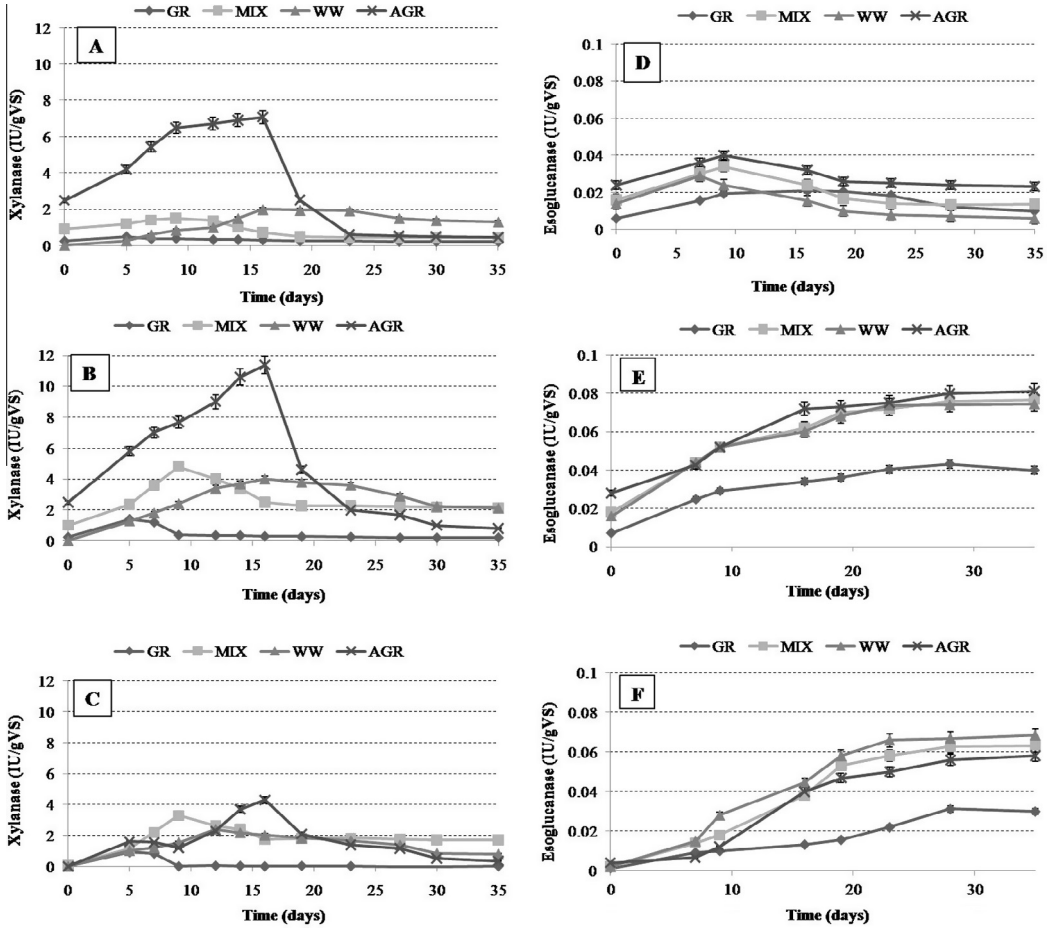

Fig. 1. Time course of xylanase and exoglucanase enzymatic activities in BMP samples, employing four different digested sludges in absence (A, D) and in presence (B, E) of sorghum. Graphs C and F show the net curves (values obtained in presence of sorghum minus values obtained in absence sorghum).

enzymatic activities during anaerobic digestion of ensiled sorghum forage have yet been found in literature.

3.3. Metabolic activity

In order to define the metabolic activities of the four sludge inocula, tests were performed by measuring the methane production rate after the addition of specific substrates. According to Angelidaki et al. (2009), acetic (1 g L^{-1}), propionic (1 g L^{-1}) and butyric (1 g L^{-1}) acids were dosed to assess acetoclastic–methanogenic (acetic acid) and acetogenic (propionic and butyric acids) activities. For the determination of hydrolytic and acidogenic activities, 1 g L^{-1} of carboxymethylcellulose (CMC) and glucose were used as substrate, respectively. Metabolic activities measured on the sludge inocula are represented in Fig. 2.

Results suggest that, as for both the acetoclastic and methanogenic activity and the acidogenic activity, the granular (GR) and agricultural (AGR) inocula showed similar values, while the inocula grown on waste sludge showed higher values. As for glucose degradation (acidogenic activity), the methane production rate observed for the WW inoculum is higher than the acetoclastic–methanogenic activity, suggesting that hydrogenotrophic methanogenesis was contributing to the observed methane production rate upon glucose dosage. This is not the case of the GR and AGR sludge, due to a different microbial consortia present in the different inocula, which depend mostly on their origin (treatment of industrial, domestic or agricultural residues). The AGR sludge showed a balanced and high acetogenic activity; a higher capacity

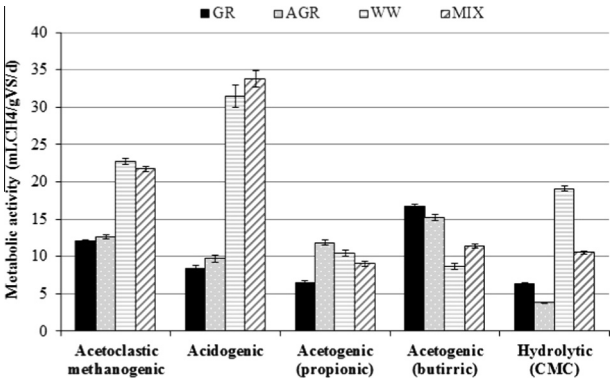

Fig. 2. Results of the metabolic (acetoclastic–methanogenic, acidogenic, acetogenic and hydrolytic) activities, measured on the four sludge inocula.

for degrading butyric than propionic acid was observed for the GR sludge, while an opposite behaviour was found for the WW sludge which showed the most balanced acidogenic activities.

The hydrolytic activity measured by using CMC as substrate was found to be higher (from three to four times) for the WW than for the other inocula. Surprisingly, the agricultural inoculum was found to have the poorest hydrolytic capacity.

The inoculum obtained by mixing AGR and WW did not show the expected intermediate behaviour showing synergistic (on acetate and glucose) or slightly antagonistic (on propionic acid) effects. A coherent intermediate behaviour between WW and AGR

Table 2
BMP_{32}, k_h and R^2 values, using ensiled sorghum forage as substrate and four anaerobic sludges as inocula. Values correspond to mean ± standard deviation of measurement performed in duplicate.

	BMP_{32} ($NmLCH_4\ g^{-1}VS$)	k_h (d^{-1})	R^2
AGR	274 ± 18	0.049	0.95
WW	248 ± 20	0.146	0.98
MIX	265 ± 12	0.080	0.94
GR	246 ± 15	0.093	0.99

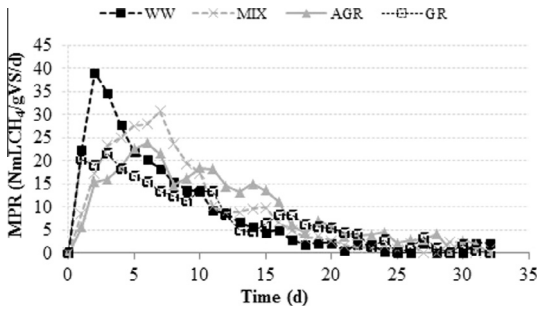

Fig. 3. Methane production rates (MPR) at normal temperature and pressure conditions, using ensiled sorghum forage as substrate and four anaerobic sludges as inoculum. Values correspond to mean ± standard deviation of measurement performed in duplicate.

inocula was found only upon dosage of CMC, in agreement with the addictive response already observed for the enzymatic activities.

3.4. BMP test

Methane yields (BMP_{32}, $NmLCH_4\ g^{-1}VS$) and methane production rate trends (MPR, $NmLCH_4\ g^{-1}VS\ d^{-1}$), of ensiled sorghum forage were monitored by performing batch tests to compare the behaviour of the four anaerobic inocula (Table 2 and Fig. 3, data refer to normal temperature and pressure conditions).

After 32 days of anaerobic digestion, no significant differences were obtained in terms of methane yields (Table 2). The average BMP_{32} was 258 ± 14 $NmLCH_4\ g^{-1}VS$ and it is equal to the value (269 ± 22 $NmLCH_4\ g^{-1}VS$) obtained on a similar sorghum sample by Sambusiti et al., 2012, and well within the range (260–390 $NmLCH_4\ g^{-1}VS$) indicated by Chynoweth et al. (1993) for sorghum.

However, a slight higher mean value of methane yield (274 ± 18 $NmLCH_4\ g^{-1}VS$) was obtained from the agricultural sludge (AGR) than from the other inocula, probably due to the adaptation of the bacterial consortium to similar agricultural wastes and to the highest increase in xylanase and exoglucanase enzymatic activities, measured both in present and in absence of sorghum, during the course of the BMP test. By knowing the chemical composition of the ensiled sorghum forage and by considering the theoretical methane yield of each degradable compound (Symons and Bushwell, 1933), a theoretical methane potential (BMPth) of 311 $mLCH_4\ g^{-1}VS$ would be expected. Thus, the anaerobic biodegradability of this sorghum sample was 83%. The lower experimental methane yield value obtained compared to the theoretical one could confirm the presence of residual undigested cellulose at the end of the test.

As reported in Fig. 3, the MPR trends suggest that during the first 5 days of digestion, the fastest methanization occurred when using the urban sludge (WW), while the slowest was obtained from the granular sludge (GR). Later on, a higher MPR was measured with the AGR inoculum. The MIX inoculum generally showed an intermediate behaviour between WW and AGR. Finally, the overall kinetic constants (kh, d^{-1}) of the first order kinetic model, described by Eq. (5), were computed (Table 2).

Despite the high variability of crop methane yields, depending mainly on plant variety, and harvesting time, methane yields obtained are in agreement with literature values (270–420 $mLCH_4\ g^{-1}VS$), as reported by Jerger and Tsao (1987), Chynoweth et al. (1993), Bauer et al. (2009); Herrmann et al. (2011). As observed, the inoculum type may influence methane production rates during BMP tests, suggesting that the origin of the inoculum is another key parameter, with the substrate/inoculum ratio, which has to be taken into account, performing BMP tests. Despite, only few studies on the effect of different inocula on BMP of organic substrates were found in literature (Marchetti and R., 2009; Keating et al., 2011), they are in accordance with those of this study.

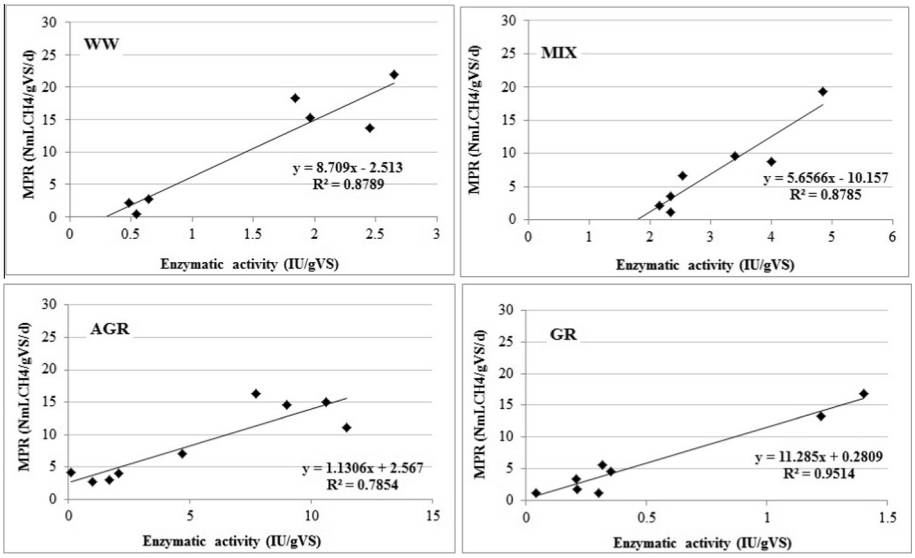

Fig. 4. Correlation between MPR and total enzymatic activities for all sludge inocula.

3.5. Correlations between enzymatic and metabolic activities and anaerobic digestion performances

Interestingly, in this study (Fig. 4) positive correlations between the overall enzymatic activities (EA, IU $g^{-1}VS^{-1}$) detected in each sludge sample and methane production rates (MPR, $NmLCH_4\ g^{-1}VS^{-1}\ d^{-1}$) were observed for all sludges, showing that a high enzymatic activity may favour the hydrolysis of complex substrate and accelerate the methanization process of sorghum.

Results about the first order kinetic constants are in agreement with the observed hydrolytic metabolic activities that were highest for the WW inoculum, lowest for the AGR sludge, with MIX sludge in-between. Indeed, a positive correlation was found between the first order kinetic constants (k_h, d^{-1}) and the hydrolytic metabolic activities measured by adding CMC as substrate (HA $NmLCH_4\ g^{-1}VS^{-1}\ d^{-1}$) in presence of all sludge inocula ($k_h = 0.0055$ HA + 0.00369, $R^2 = 0.84$). No other correlation was observed between metabolic activities and the value of k_h.

Apparently, when degrading complex lignocellulosic substrates, the only relevant metabolic activity that is worth measuring is the hydrolytic one, while acidogenic, acetogenic and methanogenic ones are less relevant to the overall anaerobic degradation process.

4. Conclusion

In this study, four anaerobic digested sludges were characterised in terms of both enzymatic and metabolic activities and data were correlated with BMP results of sorghum. Xylanase resulted the prevailing enzymatic activity for all inocula, reflecting their capability in degrading hemicelluloses. Moreover, it exhibited its maximum activity earlier than exoglucanase, which is active on crystalline cellulose. Furthermore, results of this study show that, although the enzymatic and metabolic activities of the inoculum are not as relevant to the final BMP value, they are indeed relevant to the methane production rate, as confirmed by positive correlations founded.

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