

Novel insight on the impact of enzymatic addition on organic loading rate in anaerobic digestion

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Abstract

Addition of enzymes to anaerobic digesters (ADs) has been reported as beneficial to the hydrolytic step of the process. Additional benefits have been described for bioadded reactors such as improved dewatering and lower energy requirements. This work aimed to assess the long-term and unaccounted effects of enzymatic addition on sludge digestion. Enzymes' impacts were tested using different addition modes (bulk or gradual addition) and during operational changes on reactors operated for 295 days. Enzyme added in bulk, generated a 14% increase in biogas production (144 ml/gVS_{added}) compared to control (126 ml/gVS_{added}), whereas the same amount of enzyme added gradually produced a 10% increase (139 ml/gVS_{added}). These values however, were higher when the OLR was increased from 3 to 5.5 kg VS/(m³·d): 257, 212 and 149 ml/gVS_{added} for the enzyme added in bulk, the enzyme added gradually and the control respectively. Specific biogas yields (SBY), higher in bioadded reactors, were significantly different between control reactors and those reactors dosed in bulk. Furthermore, following OLR increase, the mode of enzyme addition resulted in different increases in gas production rate (GPR) when the enzyme was added in one dose compared to control and to a gradual addition, 121%, 32% and 93% respectively. These results offer a new hypothesis on the impact of bioadditions to AD during changing operational conditions, suggesting a potential stabilising effect of the enzymes in continuous systems.

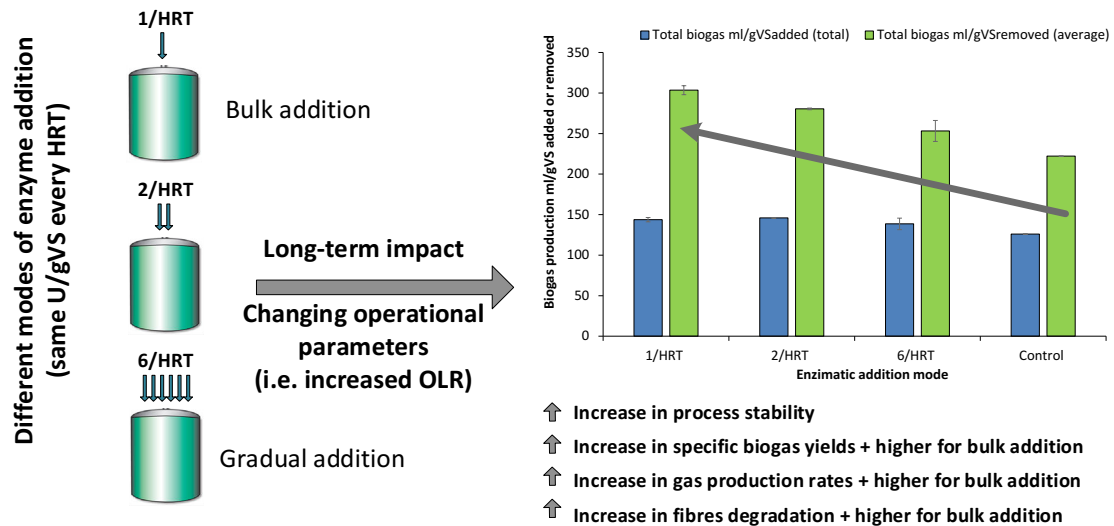
Keywords

Direct addition; biogas specific yields; biogas production rate; addition mode; cellulases; enzyme

Highlights

The benefits of enzyme addition to AD have been identified by many operators at full-scale but not properly elucidated. This is the first paper explaining the long-term impact of enzymatic addition during operational changes and the importance of how they are added to the reactor. The way enzymes were added to the reactor influenced COD and fibers removal rates and hence biogas production yields. The same amount of enzyme added in bulk, as one dose showed higher specific biogas yields and gas production rates compared to a gradual addition and control reactors. Under increasing organic loading rates, bioadded reactors had more stable methane yields, in particular those where enzymes were added in bulk.

Graphical Abstract



1 . Introduction

Anaerobic digestion (AD) or biomethanisation is one of the most frequently used techniques for sludge management and stabilisation [1,2]. The process can yield up to 90% of the energy contained in the organic matter through its conversion into biogas by a series of fermentative and methanogenic interconnected bacterial and archaeal pathways [3]. However, notwithstanding its merits, the process can still pose problems linked to feedstock degradability and process conditions, which can result in slower methanogenesis and lower biogas yields [2]. The polymeric components of the feedstock material cannot be consumed by microorganisms unless they undergo hydrolysis and are converted into soluble compounds able to cross the cell membrane, thus creating a limiting step in the overall process [4]. Physical, chemical and biological feedstock pre-treatments can be used to improve the hydrolysis of the organic matter [5]. Above all, biological treatments involving the use of hydrolytic enzymes have been successfully used to improve AD performances by enhancing the solubilisation step and allowing greater renewable energy yields [6-8]. The range of enzymes, enzyme combinations and their optimal working parameters (e.g. pH, temperature, salinity) are vast depending on the enzyme and conditions used in the process [9]. Furthermore, enzymes have been used in two ways: as pre-treatment, or in bioaugmentation added directly to the anaerobic digester. Both methods aim at increasing feedstock solubility and digestibility and thus improve the efficiency of the overall process. Enzyme direct addition, in particular, has shown to produce higher biogas yields coupled with additional benefits, such as increased solids destruction and digestate dewaterability. Recktenwald et al. [10] conducted a full-scale experiment where glycosidic enzymes were dosed in AD directly through a heat exchange loop. The enzymes not only produced a 20% increase in methane but also improved the digestate dewaterability. This was expressed as a 38% decrease in dewatering polymer requirements for the enzyme treated sludge resulting in financial saving in sludge disposal and

polymer costs. Other full-scale studies, showed that the direct addition of hydrolytic enzymes to 30 full-scale plants treating agricultural residues produced up to 35% increase in terms of biogas yield [11]. Whereas Müller et al. [12] recorded a decrease in both apparent viscosity and consistency factor in the treated sludge after enzymatic addition, which translated into economic benefits from reduced energy demand for mixers and pumps. Other studies have shown enhanced sludge solubilisation but no improved biogas production [8,13]. These contrasting results are likely to be related to the variability of the enzyme added, the type of substrate used, the mode of addition and dosage, the reactor operating conditions, or even the stability of the different enzymes adopted [9]. The lack of understanding of the impact of enzyme dosing has led to non-optimised processes or to enzyme dosing being regarded as not cost effective [14]. It is therefore important to provide a more impartial evaluation of their impact on AD. This work aims to fill this gap and by contributing to a better understanding of the effects of external hydrolytic enzymes in sludge digestion. In particular the work focused on different enzymes addition modes and their impact when changes in process parameters, such as organic loading rate, are applied to the reactor.

2 Material and methods

2.1 Sludge characterisation

Primary and waste activated sludge (feedstock) and digested sewage sludge (seed) were collected from a full-scale UK WWTP serving a 2.5 million population equivalent, with a mesophilic AD plant, treating mixed primary sludge and waste activated sludge (WAS). The plant operates using a varying ratio of primary and WAS, maintained in the range 40:60 - 60:40. Total solids (TS) and volatile solids (VS, as % of TS) were measured using standard protocols [15] and are reported in Table 1.

Table 1. Material characterisation, sludge feed and seed (post AD). All the organic components are reported as % on dry weight. Composition not available for feedstock HRT 3-24).

		TS (%)	VS (% of TS)	pH	Fibres (%)	Lipid (%)	Proteins (%)	Carbs (%)
Batch	Feedstock	5.4	78.9	6.10	32.3	7.7	23.6	45.6
	Seed	3.4	65.1	7.51	5.9	9.1	20.5	38.6
Continuous (Long-term)	Feedstock (HRT 1-2)	5.1	78.6	6.15	33.8	10.3	22.3	50.5
	Seed	2.9	64.1	7.60	5.3	8.9	24.2	39.5
	Feedstock (HRT 3-5)	5.1	80.3	6.05				
	Feedstock (HRT 5-8)	5.5	80.1	6.05				
	Feedstock (HRT 9-13)	5.3	78.6	6.35				
	Feedstock (HRT 14-18)	5.2	79.0	6.09				
	Feedstock (HRT 19-24)	5.3	79.0	6.12				

Protein, lipid and fibre content were quantified using the ASTM D5176-08 method using a Shimadzu Total nitrogen analyzer (ASTM, 2015), a DIONEX Accelerated Solvent Extractor (ASE) 200 with acetone as an extraction solvent and the AOAC method 973.18 [16], respectively. The amount of 'others + sugars' was calculated by difference from the others on a TS basis. The AOAC method 973.18 allows to quantify acid detergent fibre (ADF) and lignin (ADL). This fraction contains cellulose, lignin, and insoluble ash (including silica) 'Others + sugars' should therefore include hemicellulose and soluble sugars (monomers and small polymers).

Comparison of sludge pre- and post- digestion suggested that the most recalcitrant fractions for sludge were lipids (25% degradation) and the fibres. Data reported in supplementary materials (Table 1S).

2.2 Batch anaerobic digestion trials

Impact of different enzymatic doses was tested using batch anaerobic digesters where selected enzymes were directly added to the reactors at the start of the experiment. Batch tests were carried out in triplicates at 37°C in 1 l reactors using the AMPTS II automated system (Bioprocess control, Lund, Sweden). The working volume of the reactors was 700ml with a 2:1 seed:substrate ratio. TS and VS of the material used are reported in Table 1. Off-gas -methane concentration was measured using SERVOPRO1400 CH₄ gas analyser (Servomex, UK). The digestion process was terminated when the increase in daily production of biogas/methane was below 1% for 3 consecutive days.

Table 2. Enzyme characteristics and doses applied. Both enzymes were dissolved in distilled water at a concentration of 2.5 g/l to minimise the amount of water added to the system. Doses are reported as U/gTS to allow comparison with other studies.

Enzyme	pH activity range	Activity (U/g)	Dose suggested by the supplier	Batch test Direct addition		Continuous reactors	
				Feed stock ²	Digestate ²	Feed stock ²	Digestate ²
Cellulase CEL200	5.5-7	2E 06	50-300 mg/kg material (fresh weight)	2100 U/gTS 36 mg/kg ³	3290 U/gTS 58 mg/kg	2100 U/gTS 36mg/kg	4200 U/gTS ¹ 71 mg/kg
NCE 8L	4.0-8.0	8E 04	1.0-10 g/kg material (fresh weight)				4200 U/gTS 1822 mg/kg 83 U/gTS 36 mg/kg

¹Different batch of post-digestion composition. ²Doses of enzymes calculated using sludge composition before (feedstock) or after digestion (digestate). ³Amount of enzyme added.

Samples were collected before and after digestion experiments and analysed for TS, VS, carbohydrates, total fibres, lipids and protein content. The results reported are expressed as average of triplicates. Batch digesters were dosed with two different enzyme amounts (Cellulase CEL200), selected according to

manufacturers' recommendation identified using the feedstock composition (pre-digestion) or the composition of the inoculum (post-digestion), resulting in doses of 2100 U/g·TS and 3290 U/g·TS respectively. A summary of the enzymes characteristics and their doses is reported in table 2.

2.3 Continuous anaerobic digestion trials

Continuous digestion of sludge was carried out in 1 l reactors with a 700 ml working volume, using modified bottles of the AMPTS II automated system (Bioprocess control, Lund, Sweden) to allow anaerobic input and output of material. TS and VS of the material used are reported in Table 1. The reactors, maintained at 37°C, were operated for 295 days and fed every two days at an initial organic loading rate (OLR) of 2.5 kg·VS/(m³·day), with an equivalent 16-day hydraulic retention time (HRT). The HRT matched that of the full-scale plant where the sludge was collected. All reactors were seeded with digested sludge from a full-scale sewage treatment digester and fed with a mix of primary and secondary sludge. Off-gas methane content of the biogas was measured using a SERVOPRO1400 CH₄ gas analyser (Servomex, UK).

Cellulase CEL200 and Cellulase NCE8L (Sinobios, China) were used for the continuous systems, with the doses reported in Table 2. NCE8L, a more neutral cellulase, was introduced at day 123 (at the beginning of HRT no 9) until the end of the experiment (day 295).

The long-term impact of enzymatic additions was assessed in different ways:

(1) dosing two different enzymes doses using the feedstock (pre-digestion) composition or the digestate (post-digestion) composition, resulting in 2100 U/g·TS and 4200 U/g·TS respectively, as reported in Figure 1 and Table 2.

(2) dosing the same enzyme amount but with different addition modes i.e. 4200 U/g·TS as one addition every HRT (1/HRT), 4200 U/g·TS in two doses during the HRT (2/HRT) and 4200 U/g·TS in 6 doses, mixed with the feedstock at every feed (6/HRT).

(3) dosing the enzymes as in (2) and increasing the OLR of the reactors. To avoid changing the composition of the feedstock which would have introduced another variable, the HRT was decreased to mimic changes in OLR. A summary of the conditions tested is provided in Figure 1.

The following reaction condition were applied (also displayed in Figure 2).

Day 0 - 81: OLR 2.5 kg/(m³·day) and HRT 16 days. Enzyme CEL200 added at a concentration of 4200 U/g TS from day 19 till day 81.

Day 82 - 165: OLR 3 kg/(m³·day) and HRT 14 days. Enzyme CEL200 added from day 82 to 123 and NCE8L from day 124 to 165 at a concentration of 4200 U/g TS.

Day 166 - 234: OLR 3 kg/(m³·day) and HRT 14 days. Enzyme NCE8L concentration was decreased to 83U/g TS.

Day 235 - 278: OLR 4.5 kg/(m³·d) and HRT 9 days. Enzyme NCE8L concentration of 83U/g TS.

Day 279 - 295: OLR 5.5 kg/(m³·d) and HRT 7 days. Enzyme NCE8L concentration of 83U/g TS.

Biogas and methane production were expressed as:

-Biogas yield (By) or methane yield (My): the volume of biogas produced per g of

VS in the treated wastes (ml/gVS added)

- Specific biogas (sBy) or specific methane yield (sMy): the volume of biogas produced per g of VS degraded from wastes (ml/gVS removed).

Number of hydraulic retention time (HRT)																							
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
Days 1-81						Days 82-123			Days 124-165			Days 166-235			Days 236-280					Days 281-295			
OLR: 2.5 kg/(m ³ ·d) HRT: 16 days Feeding volume 87.5 ml						OLR: 3 kg/(m ³ ·d) HRT: 14 days Feeding volume 100 ml			OLR: 3 kg/(m ³ ·d) HRT: 14 days Feeding volume 100 ml			OLR: 3 kg/(m ³ ·d) HRT: 14 days Feeding volume 100 ml			OLR: 4.5 kg/(m ³ ·d) HRT: 9 days Feeding volume 100 ml					OLR: 5.5 kg/(m ³ ·d) HRT: 7 days Feeding volume 100 ml			
CEL200: 4200 U/(g·TS·HRT) 50 mg						CEL200: 4200 U/(g·TS·HRT) 50 mg			NCE8L: 4200 U/(g·TS·HRT) 1275 mg			NCE8L: 83 U/(g·TS·HRT) 25 mg			NCE8L: 83 U/(g·TS·HRT) 25 mg					NCE8L: 83 U/(g·TS·HRT) 25 mg			

Figure 1. Summary of the conditions applied to the long-term experiment: OLR and mode of enzymatic addition.

2.4 Enzyme doses

Two types of enzymes CEL200 and NCE8L (Sinobios, China) were used for direct addition tests in batch and continuous trials. CEL200 is a concentrated cellulase preparations made from several strains, whereas NCE8L is from *Trichoderma reesei* sp. One unit of cellulase is equal to the amount of enzyme which is able to hydrolyse 1µmol of reducing sugar (in glucose) from CMC-Na in 1 minute at 50°C and pH 4.8. Both enzymes were added to the reactors from a concentrated solution prepared at 2.5. g/l. The delivery of 2100 and 4200 U/g·TS corresponded to 100 and 200 µl of the solution respectively. Manufacturer suggested working rate, reaction optimum pH and temperature and doses used in the study are reported in Table 2.

3 Results and discussion

3.1 Batch anaerobic digesters: effect of different enzyme doses

Enzymes were added directly to batch reactors using doses calculated from the feedstock composition (before digestion) or doses calculated from the digestate composition (after digestion), as summarised in Table 2. The digestate doses aimed at tackling the most recalcitrant organic components of the feedstock to digest, e.g. those not easily degraded in standard conditions. Both enzymatic additions produced an increase in biogas and methane content above the untreated control (Table 3).

Table 3. Cumulative biogas production and methane content of batch trials using the material composition before digestion and after digestion to determine the enzyme dose.

Cellulase	Dose (U/gTS)	Biogas (ml/gVS added)	Methane (%)
CEL 200	2100 (before digestion)	250 ± 8.5 (+ 1.2% over control)	64.3 ± 0.6 (average over time of triplicates)
CEL 200	3290 (after digestion)	286 ± 4.3 (+ 4.0% over control)	70.9 ± 0.3 (average over time of triplicates)

As expected, an increase in enzyme/substrate ratio led to an increase in reaction yields as more enzyme was available to interact with the substrate. Methane content, in particular, was higher in both bioadded systems compared to control reactors. Microbial extracellular hydrolytic enzymes (EHE) production varies in response to environmental signals of resource availability (e.g. substrates, products, metabolic intermediates and co-factors), as well as chemical signals [17]. Allison and Vitousek [18] also highlighted that environmental factors such as pH and temperature influence the hydrolytic activity of enzymes. In addition, the rate of hydrolysis, which is a function of enzyme activity, is directly linked to the chemical composition of the substrates. For example, the presence of simple sugars, have shown to have an effect on lipase activity and lipid degradation rate [19]. It is therefore possible that the presence of free sugars, released from cellulose degradation, could have impacted on other enzymatic activities and hence enhanced methane content or that the cellulase itself could have acted as a chemical signal. In addition, it has been proved that enzyme-driven reactions can reach a saturation point after which, a higher amount of enzyme will not necessarily produce an increase in reaction yields, as the enzyme concentration will no longer be the limiting factor in the reaction. This was also demonstrated by other authors [20] which tested different doses of enzymes and showed that higher doses do not necessarily translate in higher biogas yields, like in our case. Therefore, an enzyme optimal concentration value exists for each reaction, which can be identified using batch digestion trials. A comprehensive testing of concentrations should be done for each enzyme to assess this saturation point to identify the optimal combination of concentration vs solubilisation and avoid overdosing or inefficient use of enzymes.

3.2 Continuous anaerobic digesters: long-term effects of enzymatic additions at increasing organic loading rate and different addition modes

Long-term effects of enzymes on sludge digestion were evaluated using three enzyme addition modes (1/HRT, 2/HRT and 6/HRT) at increasing organic loading rates. The raw data is available in supplementary Figures S1 and S2.

3.3.1 Effect of enzymes on biogas and methane production

Biogas and methane production reported as ml/gVS added was averaged over each HRT as the length of these varied between 16 days (2.5 kgVS/(m³·day)) and 7 days (5.5 kgVS/(m³·day)). The results are reported in Figure 2.

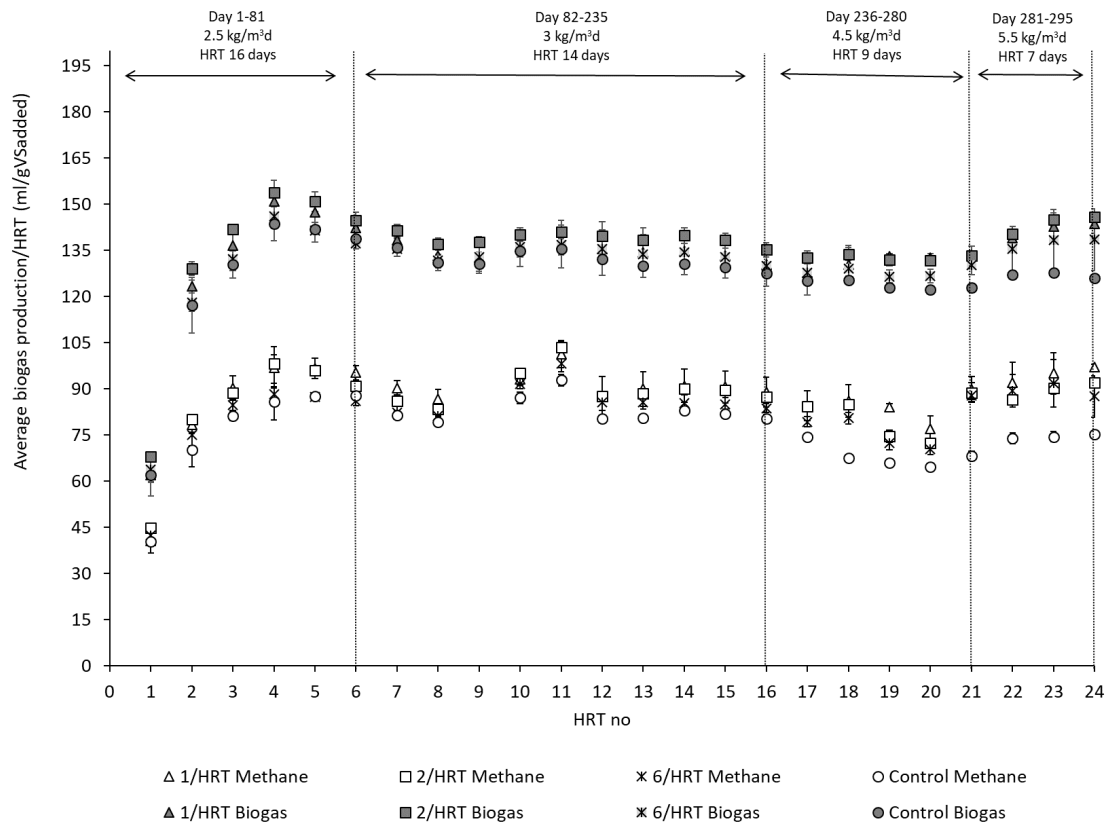


Figure 2. Cumulative biogas production of continuous trials at increasing OLRs. The values have been averaged over each HRT. The same enzyme dose was added to each bioadded-reactor using different addition modes: once every HRT (1/HRT), twice every HRT (2/HRT) and 6 times every HRT (6/HRT). The error bar represents the standard deviation of the data across each HRT.

Up to HRT 16 (234 days of operation) biogas production and methane content of the test digesters were greater than for control units, although the three systems did not show any significant difference. From day 235 (HRT 17), both biogas and methane content decreased in all reactors when OLR was increased to 4.5 from 3 kgVS/(m³·day). The control reactors however, did not recover as promptly and the difference in methane and biogas increased between the bioadded reactors and the control ones after that (Figure 2). The decrease in biogas and methane at HRT 8-9 (Figure 2) was due to a change in the sludge used as feedstock which required a change in enzyme, from CEL200, with a pH optimum of 5.5-7, to NCE8L with a pH range of 4.0-8.0 and optimum of 7.0.

Similarly to what was found in batch reactors, the bioadded reactors behaved in

a different way compared to the control ones. In particular, the bioadded reactors showed an increase in biogas production when the OLR changed (day 235, HRT 17). On average, the CH₄ content (%) of the different systems varied between the three different addition modes 64.9 ± 0.4 %, 62.4 ± 0.2 %, 62.1 ± 0.2 % for 1/HRT, 2/HRT, 6/HRT respectively and the control, 60.1 ± 0.1 %.

The methane percentages presented in the Box and Whisker graph in Figure 3 show the distribution of the values obtained over the 295 days at the different OLRs (2.5, 3, 4.5 and 5.5 kg VS/(m³·d)) between the control reactors (Figure 3a) and the reactors 1/HRT, where the enzyme was added in bulk (Figure 3b). The data illustrates how enzymatic additions have influenced the methane yields when the OLR was increased over time. The bioadded reactors showed a more stable production compared to control units when operational conditions were changed.

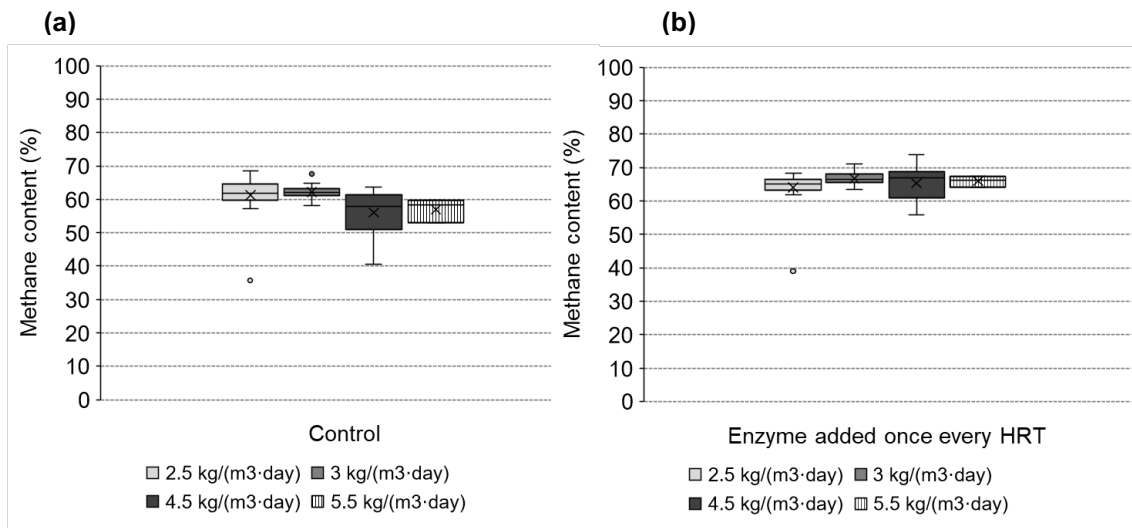


Figure 3. Box and Whiskers plots of the methane content in biogas of continuous trials at increasing OLRs for control (a) and 1/HRT reactors (b), where enzyme was added all in once every HRT.

When analysing performance parameters such as Specific Biogas Yield (SBY) (Figure 4a) and Gas Production Rates (Figure 4b) for the different reactors and each OLR interval, it is clear that enzymatic additions allowed an increase in both values at higher OLRs. SBY did not change in the control reactors when OLR was increased from 3 to 5.5 kg VS/(m³·d), with values of 201 ± 6.14 ml/gVS_{removed} and 268 ± 4.13 ml/gVS_{removed} recorded, whereas it increased from 244 ± 16.09 ml/gVS_{removed} to 697 ± 6.61 ml/gVS_{removed} with the 1/HRT addition mode. Similarly, the GPR increased from 0.26 ± 0.02 ml/(ml_{reactor}·day) to 0.47 ± 0.04 ml/(ml_{reactor}·day) for the control digesters but from 0.26 ± 0.02 ml/(ml_{reactor}·day) to 0.81 ± 0.01 ml/(ml_{reactor}·day) for the reactors where the enzyme was added once every HRT.

Reactors where enzymes were added once every HRT, had a 41% increase in GPR at OLR 4.5 kg VS/(m³·d) and 72% increase at OLR 5.5 kg VS/(m³·d)

compared to control digesters. GPR at OLR 5.5 kg VS/(m³·d) was slightly higher for the reactors where the enzyme was added twice every HRT (2/HRT) at 0.89 ± 0.04 ml/(ml_{reactor}·day). These GPR values are in the range of those reported in literature for similar values of OLR [1].

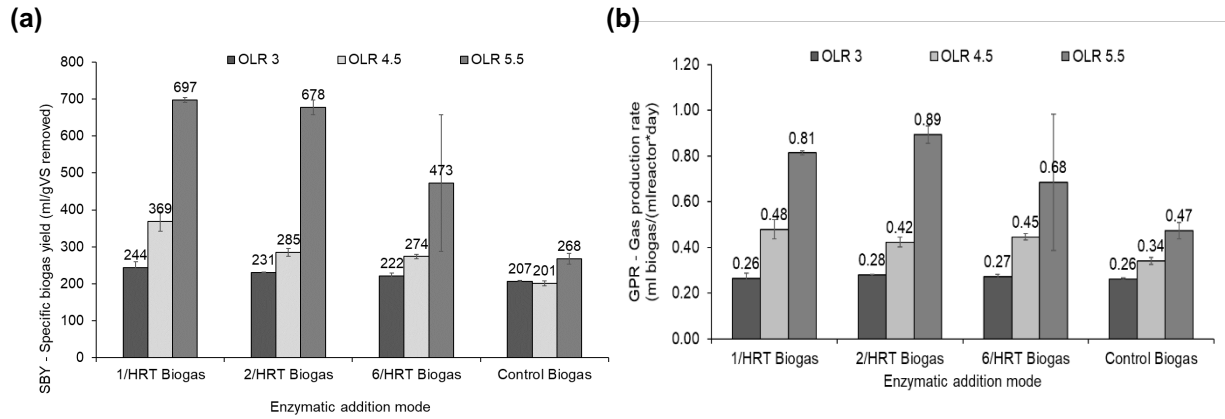


Figure 4. Specific Biogas Yield (SBY) (a) and Gas Production Rates (b) of the different reactors conditions for each OLR interval. OLR 2.5 kg VS/(m³·d) was not reported for ease of reading, as results were similar to OLR 3 kg VS/(m³·d).

A similar trend was reported by Asquer et al. [21], who applied enzymes to hemp digestion. Following an increase in OLR (from 2.8 to 3.8 kg VS/(m³·d)) the bioadded reactors increased their biogas production rate and resulted in a 117 % improvement in biogas formation. The same authors reported that the OLR increase also produced higher energy yields, with increases of SBY for both biogas and methane, 77% and 129% respectively. An enhancement of both of GPR and SBY related to input mass was also reported by Quiñones et al. [22]. These authors reported that the application of enzymes was able to enhance biogas production by 10-15% and increase the methane content of biogas by an increment of 5-10% for farm waste and cattle manure.

Table 4. Final digester analysis.

	Methane [%]	H ₂ S [ppm]	sCOD [mg/l]	NH ₄ ⁺ [mg/l]	pH
1/HRT	67.5 ± 2.3	45.0 ± 11.4	2028.3 ± 57.5	12.0 ± 1.4	7.65 ± 0.05
2/HRT	63.0 ± 3.5	69.0 ± 13.7	2275.0 ± 108.9	19.4 ± 3.7	7.63 ± 0.04
6/HRT	63.2 ± 2.6	17.0 ± 16.9	2262.5 ± 115.1	14.4 ± 4.7	7.65 ± 0.05
Control	60.2 ± 0.2	160.0 ± 36.4	2557.5 ± 36.9	17.0 ± 3.5	7.69 ± 0.05

The final analysis of the different digesters (digestate), reported in Table 4, also showed higher level of H₂S in the biogas in the control reactors, suggesting that enzyme addition had a stabilising action in the process when operation parameters such as OLR were changed. Tian et al. [23], have reported lower concentrations of H₂S when the C/N increases as protein percentage in the feed

is reduced. As ammonia levels were higher in the control reactors, it is possible that the enzymatic addition has had an impact on the C/N ratio of the reactor and limited the activity of the sulphate reducing bacteria (SRB).

3.3.2 Effect of enzyme dosing on total solids, volatile solids and digestate composition

The data reported in Figure 5a show the TS input and output over the 24 HRT (295 days), whereas Figure 5b includes the VS removed over time for control and for enzyme added reactors. TS removal was not different for each of the conditions, showing a similar trend for all the reactors. Significant changes in VS removal only happened when reactors were subjected to a change in conditions such as an increase in OLR e.g. from HRT 10 onwards and for just bioadded reactors, but in particular with bulk enzyme dosing modes (1/HRT and 2/HRT).

Other authors have reported very little difference in TS removal between bioadded systems and controls [7,22]. A smaller VS removal with increase in SBY was recorded also by Noyola and Tinajero [6] using an enzyme additive containing lipase, protease and cellulase. Similarly, cellulase applied at full-scale cow slurry, corn silage and triticale showed a 12% decrease in feedstock required to maintain biogas output (with a 12% increase in biogas yield) [24].

The bulk addition has triggered a different response compared to the gradual one (both 1/HRT and 2/HRT). As the production of microbial extracellular hydrolytic enzymes (EHE), responsible for the hydrolytic step, can vary in response to environmental signals of resource availability (e.g. substrates, products, metabolic intermediates and co-factors), as well as chemical signals [17], it is possible that a more gradual addition has had an impact on the endogenous microbial response.

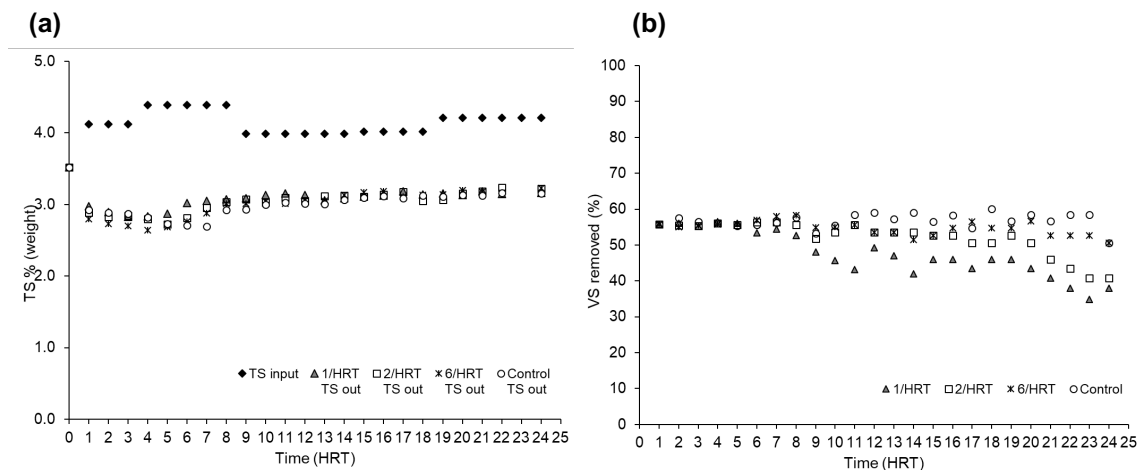


Figure 5. TS input and output over the 24 HRT (295 days) (a) and VS removed over time for control and for enzyme added reactors (b).

A detailed analysis of the organic matter was done on the initial feedstock and the digestate at day 122, at HRT 8, when OLR was 3 kg/(m³·day) and are

reported in figure 6. The values of fibre content in all bioadded reactors, 21.3% for 6/HRT, 20.8% for 2/HRT and 19% for 1/HRT, were lower than in the control units with values of 27.4%. The dose of enzyme added to the system was the same in all three settings (6/HRT, 2/HRT and 1/HRT), so the effect on the fibre component is linked to the mode of addition. The protein content in the digestate, measured as total nitrogen, comprise also the protein component of the soluble microbial products (SMP) [25]. All bioadded reactors showed a smaller amount of proteins compared to controls, suggesting a decrease of SMP and a potential impact on viscosity. Lipids also seem more effectively degraded when enzymes are added in bulk (1/HRT).

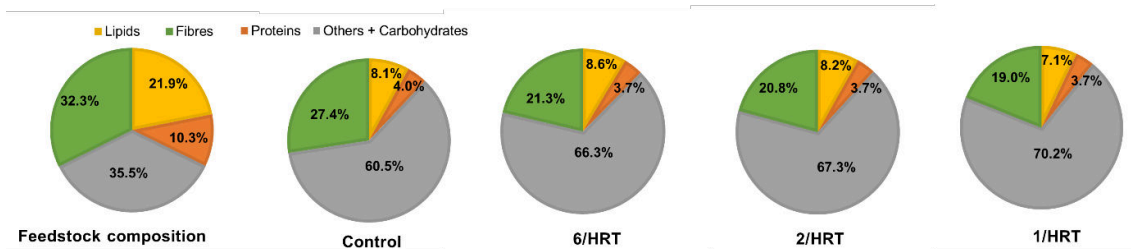


Figure 6. Composition of feedstock and the reactors (digestate) at 122 and 0 days for control and bioadded units after 8HRT and at OLR of 3 kg/(m³·day).

Likewise, sCOD was higher in control digesters (2.6 g/l) than in bioadded reactors (between 2.0 and 2.3 g/l) (Table 4). This has been reported in other studies. For example, using diluted (2% TS) and undiluted (4% TS) sludge with and without enzymes Diak et al. [7] found that soluble COD in the digestate of the control reactor was higher than the soluble COD of any of the enzyme treated reactors.

3.3.3 Effect of enzymes on organic acids and alkalinity concentrations

The process stability parameters, including pH, trends of total volatile acids (VFA) and Ripley ratio (intermediate alkalinity/partial alkalinity) are reported in Figure 7 and Figure 3S. The pH (Figure 3S) ranged between 7.1 and 7.9 and varied in a similar way over time across the different reactors (bioadded and controls alike). Similarly, the VFA content (Figure 7a) had the same trend for all reactors, with overall levels below 1 g/L until OLR was increased to 4.5 kg/(m³·day) (day 245). The only exception to this trend was a peak of VFAs recorded around day 130, which matched a small drop in biogas yield and was linked to a change in sludge composition, but all reactors recovered fast after that.

The Ripley ratio (RR), calculated as ration between intermediate and partial alkalinity, showed similar values for all reactors, and ranged between 0.2 and 0.5 for most of the experiment (Figure 7b). After day 236, when OLR was raised to 4.5 kg/(m³·day) all values increased: 1/HRT to 0.6, 2/HRT and 6/HRT to 1.0, whereas the control reached RR of 1.5. Although higher than those reported in literature, sludge digestion with increasing OLR showed RR values of effective sludge digestion around 0.5 [26]. A second increase in RR was registered after day 260, in parallel to an increase in VFA in all reactors (Figure 7a), with RR values up to 1.8 for control, 2/HRT and 6/HRT reactors and of 1.5 for 1/HRT. All reactors returned to values around 0.6 by day 270. The final increase in OLR to

a throughput of $5.5 \text{ kg}/(\text{m}^3 \cdot \text{day})$ at day 280 caused another increase in the ratio, with a peak at 1.3 for 2/HRT, 1.3 for control and 0.9 for 1/HRT. These values however, did not impact on the biogas production rates (Figure 2 and Figure 1S).

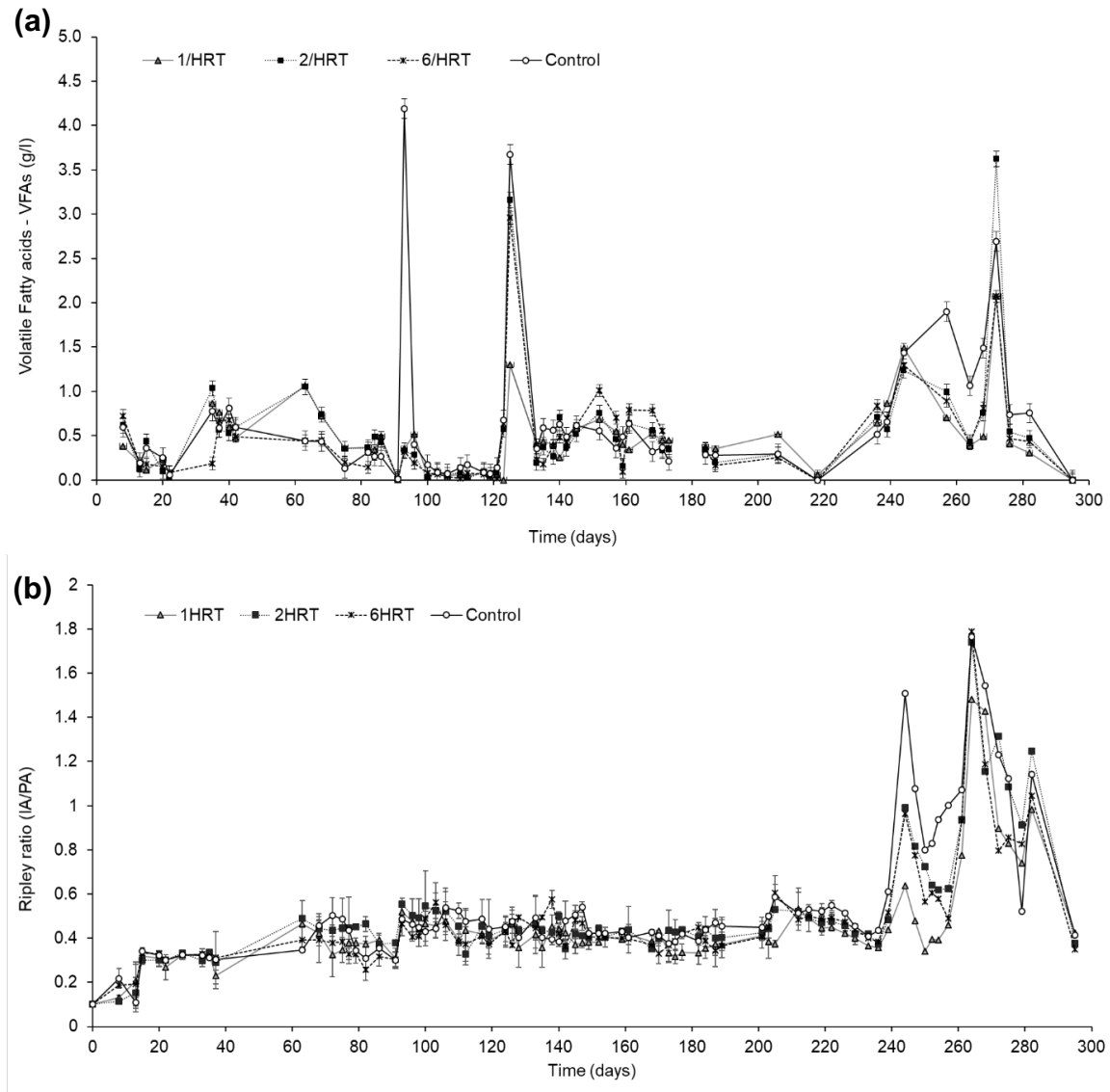


Figure 7. VFA profiles (a) and Ripley ratio (b) over 295 days for all reactors.

3.3 Enzyme addition mode and its impact on anaerobic digestion

The results reported in this study regarding direct addition of enzymes to digesters and their impact on the increase of specific biogas yield and methane yields are similar to those reported in other studies. However, there are, as far as the authors are aware, no previous publications on the effect of mode of enzyme dosing in anaerobic digestion, with the most relevant literature available being a

couple of studies on cellulase use for bioethanol production. Battista et al. [27] evaluated the glucose production from lignocellulosic material and suggested that compared to a system with zero enzymes a gradual addition of cellulase produced a slower increase in glucose concentration and higher overall glucose yield at the end of the test. Xu, et al. [28] instead assessed different enzymatic modes of addition (additives all at once in the initial stage or added according to the ration of substrate feeding) to optimise the enzymatic hydrolysis of sugarcane bagasse. The results indicated that glucose yield was about 3% higher when enzymes were added at once in the initial stage of enzymatic hydrolysis than using a proportional addition. Both works are in agreement with what was found in this study where the staggered addition of cellulase (6/HRT) increased the amount of fibre degradation (11.5%) compared to controls (4.9%) but the bulk enzyme addition (1/HRT) produced a further increase in fibre degradation (2.3%) with an overall 13.4% degradation. Like for batch systems, it is possible that a higher amount of sugars released from cellulose degradation, might have impacted on the activity of other enzymes such as lipase [19]. This could have then been translated in an improvement of biogas quality while biogas volumes were maintained stable.

The differences between the four systems (control, 1/HRT, 2/HRT and 6/HRT) became more pronounced when the OLR increased to 4.5 kg/(m³·day) and 5.5 kg/(m³·day), i.e. 80% and 120% more organic matter compared to the initial conditions at 2.5 kg/(m³·day). Bioadded systems showed lower fibre and protein content in the digestate than control units. This suggests an activity enhancement in the degradation of the feedstock more recalcitrant fractions when cellulase was added. This was hence translated in greater treatment capacity without apparent changes in the monitored process parameters (e.g. VFAs, pH, alkalinity, Ripley ration), which remained comparable for the different systems. It is possible that changes in physical characteristics, such as viscosity might have contributed to the improvements in specific biogas yields and biogas quality as found in other studies. Changes in viscosity in AD has been also linked to the production of extracellular polymeric substances (EPS) and soluble microbial products (SMP), which can also effect mixing, foaming and dewaterability [29]. According to Neyens et al [30], increase dewaterability and the requirement for a lower amount of flocculation polymer indicate a change in sludge surface structure and EPS production. Similarly, Recktenwald et al. [10] showed increased dewaterability in enzyme dosed reactors, due to enhanced degradation of EPS, in particular of the carbonaceous matter. Ayol et al. [31] reported that this enhanced degradation of the EPS matrix was able to improve sludge solids solubilisation and hence the formation of enzyme-substrate complexes. Muller et al. [29] when dosing enzymes at full-scale recorded an influence on rheological parameters with a significantly decreased consistency factor (K) and hence viscosity compared to the control conditions, with possible economic effects on reduced energy demand for mixers and pumps. In this work, the presence of externally added enzymes, in particular in the mode 1/HRT and 2/HRT, have provided additional degradative power.

Changes in OLR in sludge digestion have been reported to cause an impact on microbial EPS, viscosity and dewatering capability [25]. Some authors [25] noted that though attractive from a methane production perspective, the combination of

a high OLR and low HRT could result in effluents with high content of SMP and potential impact on viscosity and sludge bulking. This would therefore suggest an additional potential beneficial effect of hydrolytic enzymes when applying changes in OLR, like those recorded in this study.

Finally, in addition to organic solubilisation and increased hydrolytic activity, direct additions have also proved to increase process stability and methanogenic activity [32]. This was also shown in this work, where SBY and GPR increased when OLR was doubled, but only in bioadded systems.

These action mechanisms observed when dosing the enzymes directly in the AD are different from what is achieved through enzymatic pre-treatment, where an enhanced hydrolysis due to direct solubilisation of the organic matter is expected. The benefits of enzyme addition are however not fully clear and more investigation is needed on the impact of the type and dose of enzymatic products, the substrate variability, the reactor operating conditions and the mode of addition.

4 . Conclusions

The different mode of addition produced different increases in biogas production rate (GPR) in bioadded reactors compared to control units, higher (41%) when the enzyme was added in bulk. The bulk addition mode (1HRT) produced higher specific biogas yields compared to control and gradual addition (6/HRT). The bulk mode (1/HRT) also generated an increase in fibre degradation compared to control reactors and 6/HRT. Greater fibre degradation, 8.4% and 2% for 1/HRT and 6/HRT respectively, was not accompanied by a parallel decrease in TS removal. Furthermore, enzymes improved biogas production in all bioadded reactors compared to control and for all addition modes (45% for 1/HRT, 42% for 2/HRT and 35% for 6/HRT) when OLR was increased from 3 to 4.5 kg/(m³·day). Specific biogas yields were always higher in bioadded reactors at all OLRs, but significantly different when OLR was increased to 4.5 and 5.5 kg/(m³·day) between control reactors and those reactors dosed in bulk (1/HRT and 2/HRT).

Enzymatic treatment in the AD sector is being underused mainly due the perceived high cost of the enzymes itself and poor understanding of the enzyme action and their long-term impact on the process. The outcome of this study suggests that there could be additional advantages to simple increases of hydrolysis, including increased process stability and methanogenic activity . The results presented in this study and the introduction on the market of 'low-purity, low-cost' enzymes can therefore offer increased application opportunities in the AD sector.

Declaration of Competing Interest

The authors declare no conflict of personal or financial interests that could have influenced the work reported in this paper.

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