BIOLOGICAL POST HYDROLYSIS OF DIGESTATE ENHANCES THE BIOGAS PRODUCTION IN ANAEROBIC DIGESTION OF AGRO-WASTE

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ABSTRACT

This work simulates a post-thermal treatment to the recirculated fraction of digestate, still rich in recalcitrant organic materials. By this way, the digestate can be further exploited for methane production, increasing the substrates exploitation's grade. The influence of the digestate post treatment has been studied by the conduction of a thermal treatment on a mesophilic digestate, considered exhausted. Three different duration of the thermal treatment have been considered. Then the digestate has been processed once at mesophilic condition AD. It was found that HRT of 3 days of the thermal treatment assures an increasing of the 25% and 65% of the soluble organic matter and VFA concentrations, respectively. As consequence biogas production increased of the 30% respect to the case without thermal post treatment.

Keywords: Anaerobic digestion, biogas, thermophilic, post-hydrolysis, thermal treatment

1. Introduction

The worldwide production of solid waste has reached the alarming level of 17 billion tons and it has been estimated it will be around of 27 billion tons within 2050 as consequence of the human population growth, especially in Africa and Asia continents [1]. The increasing awareness of the problem is leading the Western countries' governments to legislate in favor of more sustainable waste management based on the circular economy concept [2, 3]. Circular economy considers waste as raw materials for the production of new environmental friendly and biofuels or for the recovery of high added value molecules and nutrients. Consequentially waste disposal on soil or landfill must be avoided.

Organic residual from farm and agricultural activities are among the most abundant wastes in Europe with more than 1,500 million tons/year and 250 million tons/year, respectively [4, 5]. Circular economy model encouraged the exploitation of these wastes streams close to farms where they are produced to reduce the energetic and economic costs for transport [6]. Anaerobic digestion (AD) is the most adopted technology for the management of great amount of agricultural wastes. Some of the benefits of this technology are the production of biogas rich in methane to be used for heat and electricity cogeneration or as automotive fuels. Moreover, AD reduces the odor, the amount of the organic wastes stabilizing the wastes, contributes to the reduction of the Greenhouse Gases (GHG) emissions and allows the production of a high value fertilizer to be commercialized or applied directly in the farm [7]. One of the major disadvantages of AD from agricultural wastes consist in the recalcitrant nature (essentially lignin and crystalline cellulose) of these substrates. When feeding as manure or straw to anaerobic digesters, the inherent resistance of these lignocellulosic fraction in the raw material limit the convertibility of the materials. As a result only between 40% and 50% of the feed stock will be converted to biogas and the rest leaves the reactor unused, reducing the profitability of the AD system [8]. Consequentially, the output digestate is still rich in not stabilized organic matter and can be not applied as soil improvers [9].

The pretreatment stage becomes fundamental to increase the lignocellulosic materials degradation, to increase the porosity and consequentially the specific surface available to microorganisms involved in the AD. Pretreatments allow also the optimization of the biogas production and the stabilization of the organic substrates at the end of the AD. The combination of physical and chemical pretreatments are the most adopted for substrates with high content of lignin and cellulose. The wet explosion (WEx) pretreatment showed a high efficiency in the solubilization of cellulose in hexose sugars favoring their following conversion in methane by AD [8]. WEx contemplates the use of oxygen as oxidizing agent to destroy the cellulose polymers in glucose and to degrade the lignin in lower molecular weight aliphatic acids and phenols. Steam explosion is an alternative pretreatment where cellulose and lignin solubilization is performed by the substrates soaking in an acid solution, usually sulfuric acid, at high temperature (150-200°C) and high pressure (10-15 bar) for some minutes (5-15 minutes) [10]. Good performances have been also obtained by the steam explosion in an alkali solution (NaOH), which was able to hydrolyze 69 and 38% of the cellulose and hemicellulose, respectively, and favour the partial solubilization of the 75% of the lignin [11]. An alternative approach is the ammonia fiber expansion (AFEX) pretreatment. Biomass enters in contact with concentrated ammonia at temperatures of 70-180 °C and pressure ranges between 200 and 1000 psi. After a brief residence time, the pressure is explosively released, effectively disrupting the structure of the biomass. AFEX decrystallizes cellulose, partially hydrolyzes hemicellulose, and depolymerizes lignin [12], inducing a better methane production.

All these pretreatments are characterized by high use of reagents and energy. Thermal pretreatments can be used as valid processes to avoid the use of reagents resulting is particularly well suited to locations where there is a supply of waste

heat, for example from a nearby factory or power plant [13]. The thermal treatments require heating the substrates between 130-200°C for about 30 minutes and under high pressure. This action results in a better degradation of the proteins and of the fibers, and in general improves the volatile solids removal by 20% of the conventional AD process. In addition, thermal treatment contributes to reduce the reaction medium viscosity improving the heat and mass transfer [14]. By this way, thermal pretreatments is able to impact the AD kinetics reducing the hydraulic retention time (HRT) and the methane production [15]. Recently a new process has been tested at laboratory/pilot scale, the Intermediate Thermal Hydrolysis Process (ITHP) in which thermal hydrolysis is not a pretreatment but an intermediate process [16]. In this process, the substrates are treated by AD before the ITHP. The resulting digestate from ITHP has still adopted for a final stage of AD step. This process improved organic matter removal by 15% as compared to the conventional thermal treatment with a consequent methane increasing of about 10%. It was demonstrated that post-treatment of digestate improved volumetric methane yields by 7% and the COD-reduction increased from 68% to 74% in a mesophilic (37 °C) semi-continuous system [17]. Moreover, the HRT was compared to a conventional system with pre-treatment of feed substrates at 70 °C [18].

This research has the aim to simulate a 70°C thermal treatment to the recirculated fraction of digestate, derived from a full-scale digester, treating manure and straw. After the thermal post-treatment, the digestate is mixed with new fresh organic matter for a mesophilic AD process (Figure 1). By this way, the digestate, still rich in recalcitrant lignocellulosic materials, can be further hydrolyzed for methane production, increasing the substrates exploitation's grade. The influence of the digestate post treatment has been studied by the conduction of a mild thermal treatment on a mesophilic digestate, considered exhausted. Three different duration of the thermal treatment have been considered. Then the digestate has been processed once at mesophilic condition AD. The influence of thermal treatment on the microbial community composition has been also studied through PCR-DGGE analysis carried out on the bioreactor under thermophilic conditions.

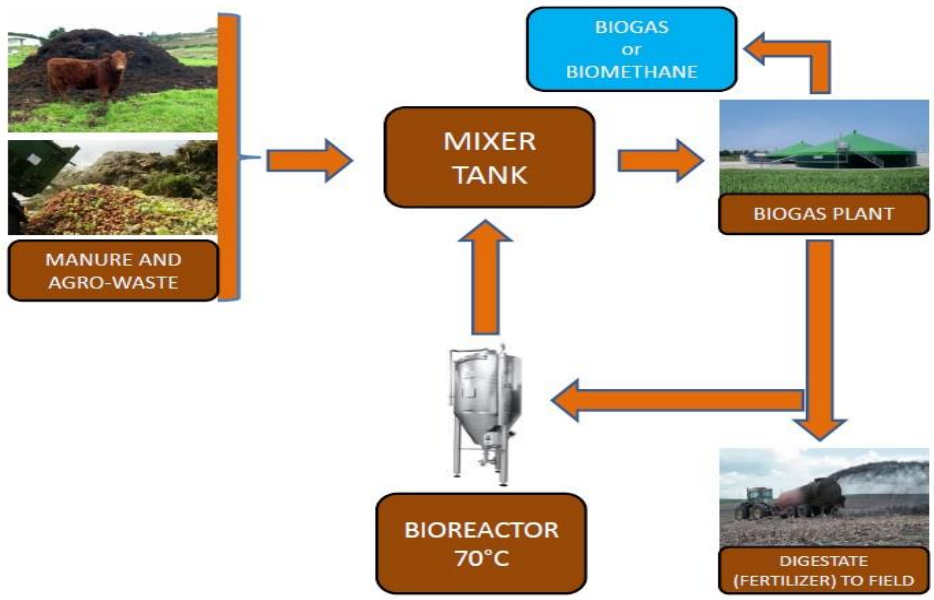

Figure 1. Concept of the thermal treatment to the recirculation fraction of digestate.

2. Materials and Methods

The research activity is composed by two experimental parts: i) the Biochemical Methane Potential (BMP) tests conducted in batch and ii) the semi-continuous tests.

2.1 Characterization of the digestates

The two experimental parts were conducted adopted two digestates: the first digestate is represented by a mesophilic digestate from a full scale reactor located at Isola della Scala (Italy) working at 37°C and used to simulate a typical mesophilic AD process. The second is constituted by a thermophilic digestate from a full scale reactor located at Treviso working at 65°C. Table 1 shows the chemical characterization of the both the digestates.

	Mesophilic Inoculum	**Thermophilic inoculum**
TS (% *w/w*)	67.18 ± 0.52	62.40 ± 1.27
TVS (% *w/w*)	43.48 ± 0.37	44.40 ± 0.61

pH	8.36 ± 0.02	5.30 ± 0.01
Alkalinity (g $CaCO_3$/L)	1.49 ± 0.26	2.94 ± 0.17
COD (mg O_2/gTS)	758.93 ± 2.60	706.56 ± 5.54
sCOD (mg O_2/L)	7,110.39 ± 34.91	14,131.00 ± 42.31
VFA (mg O_2/L)	675.48 ± 4.73	6,889.00 ± 10.45
TKN (mg/gTS)	21.91 ± 1.67	33.49 ± 1.57
NH3-N (mg N/L)	3,190.00 ± 19.23	662.00 ± 12.48
NH3 (mg N/L)	367.39 ± 4.95	0.96 ± 0.04
P (mg/gTS)	16.41 ± 0.91	14.32 ± 0.60

Table 1. Chemical characterization of the digestates used for the tests

2.2 BMP tests

BMP tests have been conducted to determine the methane production from two agricultural digestates, representing the output of poultry and bovine manure and rice straw, treated by two different anaerobic full scale digesters in Italy. In particular, the first digestate is represented by a mesophilic digestate from a full scale reactor located at Isola della Scala (Italy) working at 37°C and used to simulate a typical mesophilic AD process. The second is constituted by a thermophilic digestate from a full scale reactor located at Treviso working at 65°C.

BMP tests were based on the procedure described in Angelidaki et al. [19] and Holliger et al. [20]. Two typologies of BMP tests were performed: the first to simulate a mesophilic AD process to use as control test, the second to simulate the thermophilic post treatment on a mesophilic digestate (PTMD). The difference in methane production has been used to evaluate the performance of thermal treatment.

The tests were conducted at mesophilic temperature (37 ± 1°C). 1000 mL glass bottles were used as reactors. The control tests bottle were filled with the mesophilic digestate till to reach the working volume of 500 mL. The PTMD tests were performed according to an inoculum to substrate ratio of 2.5:1 on a VS-basis, where the term inoculum is referred to the digestate from thermophilic AD process, while substrate is represented by the digestate from mesophilic process. Finally, the bottles were flushed with N2 to guarantee anaerobic conditions, closed and incubated in a temperature-controlled chamber. A SMC pressure Switch manometer (1 bar, 5% accuracy) was used to measure biogas production in the headspace of the bottles, up to the depletion of the biogas production. Accumulated volumetric biogas production was calculated considering the pressure increase in the headspace volume. Periodically, gas samples were taken from the reactors to analyse biogas composition by the portable biogas analyzer BIOGAS5000 (Geotech, United Kingdom). Assays were carried out in triplicate, and the results were expressed as the biogas volume produced per gram of volatile solids under standard ambient temperature conditions (L biogas or methane kg VS-1 at T=298 K, P=1.0133 bar).
Kinetic parameters were calculated using the modified Gompertz model described in Eq. 1 [21].

$$P_{net}(t) = Pmax \exp\{-\exp[\frac{Rmax*e}{Pmax}(\lambda - 1) + 1]\} \quad \text{Eq. 1}$$

Where $P_{net}(t)$ is the net accumulated methane production (NL_{CH4}/kg_{TVS}) at time t, P_{max} is the methane potential production (NL_{CH4}/kg_{TVS}), R_{max} is the maximum methane production rate (NL_{CH4}/kg_{TVS} d), and λ is the lag phase (d).

2.3 Semi-continuous tests

The performances of the PTMD were evaluated in semi-continuous mode too and compared with a semi-continuous control test (SCC). Figure 2 summarizes the SCC and PTMD processes. In the PTMD test the mesophilic digestate was digested at 70°C and then digested for a second time at mesophilic temperature (37°C). On the contrary SCC was simply digested a second time at 37°C.

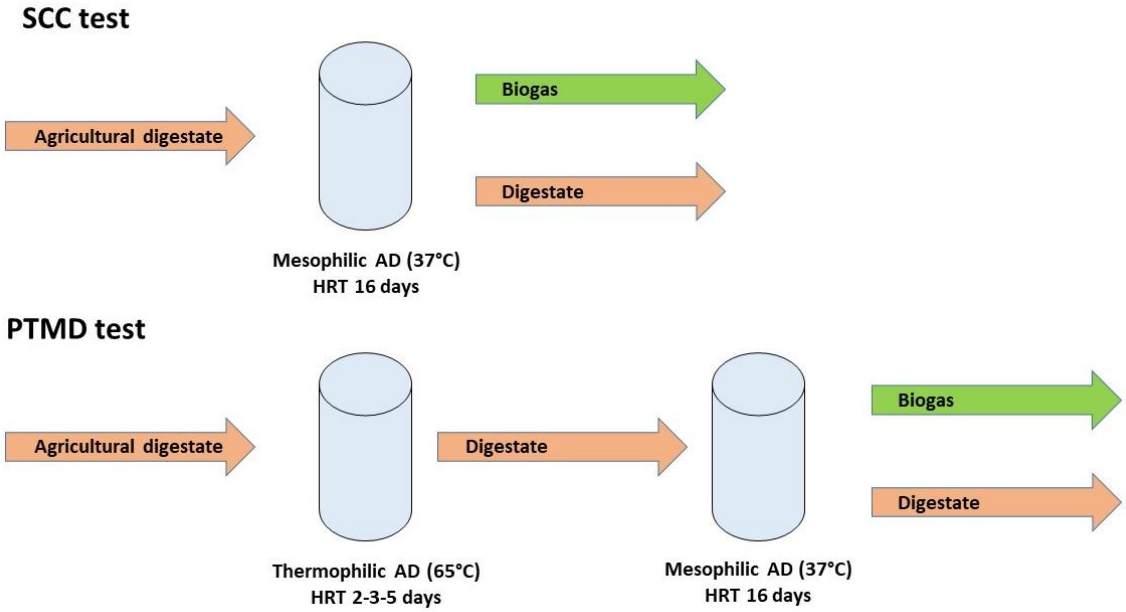

Figure 2. Schemes of SCC and PTMD tests.

SCC and PTMD tests were conducted at lab scale reactors, with a total volume and a working volume of 4.5 L and 4.0 L, respectively. The PTMD experimental campaign included a 14 days start-up phase where the digestates have been gradually added until to reach the working volume and three phases corresponding to three different Hydraulic Retention Time (HRT) of the thermophilic reactor set up at 2.0; 3.0 and 5.0 days. The HRT of the mesophilic reactor was keep constant at 16 day; the duration of the mesophilic AD was established at 56 days corresponding to 3.5 HRT to assure the reaching of steady state methane production. Every day SCC and PTMD were fed according the Organic Load Rate (OLR) reported in Table 2. It is possible to observe that the OLR depends on the HRT of the reactors. Consequentially, SCC reactor and mesophilic phase of PTDM process were characterized by constant OLR along the duration of the semi-continuous tests, while the thermophilic phase of PTMD test' OLR decreased with the HRT augmentation. The working volumes of the reactors were kept constant by the daily discharged of the same digestate amounts.

	OLR of the different phase of PTDM test (kg TVS / m3 d)		
Reactor	**HRT (2 days)**	**HRT (3 days)**	**HRT (5 days)**
SCC process	3.64 ± 0.24	3.25 ± 0.30	3.54 ± 0.11
Thermophilic reactor of PTDM	24.78 ± 0.84	17.33 ± 1.60	11.34 ± 0.34
Mesophilic reactor of PTDM	3.49 ± 0.28	3.20 ± 0.18	3.18 ± 0.14

Table 2. OLR values for the different HRT of the thermophilic reactor in PTMD process.

2.4 Evaluation of the tests' performances and analytical methods

BMP tests were evaluated simply considering the biogas production and the methane content, according the methods previously described.

The performances of the semi-continuous tests were evaluated considering the difference of: i) the methane productions between SCC test and the mesophilic reactor of PTMD tests; ii) soluble Chemical Oxygen Demand (sCOD) and Volatile Fatty Acids concentrations between SCC and PTMD tests. These latter parameters are considered as indicators of the hydrolysis efficacy of the thermal treatment.

Several parameters were measured using samples taken at the outlet stream from SCC and PTMD processes: pH, alkalinity, Volatile Fatty Acids (VFAs), total Chemical Oxygen Demand (COD), soluble COD (sCOD), Total Solids (TS), Total Volatile Solids (TVS), and the concentrations of total nitrogen compounds (TKN), of ammonium (NH_3-N) and free ammonia (NH_3) and phosphorus (P). They were determined using the standard methods described in the scientific literature [22]. In addition, the biogas produced was measured both quantitatively and qualitatively, using a TG1PP gas meter (RITTER, Germany) and a portable biogas analyzer, the BIOGAS5000 (Geotech, United Kingdom).

2.5 Total DNA extraction, PCR amplification, and DGGE analysis

DNA extractions and PCR setups were performed under microbiological safety cabinet (SafeFAST Elite class II, Carlo Erba). Total DNA was extracted using the FastDNA® SPIN Kit for soil (MP Biomedicals, USA) according to the manufacturer's instructions. Samples collected from the hyperthermophilic reactor were extracted in duplicate, starting from 0.5 g of biomass for each extraction. PCR comprised about 50 ng of template DNA, 0.8 µM of each primer, 0.4 mM of the four dNTPs, 1 unit of GoTaq™ DNA Polymerase and 5 µl of 5X PCR buffer. Eubacterial 16S rRNA genes were amplified through primers fD1 and rp2 [23]. The PCR reaction was performed with the following program: initial denaturation at 94 °C for 5 min; 30 cycles of denaturation at 94 °C for 30 s, annealing at 45 °C for 30 s, extension at 72 °C for 2 min; and final extension at 72 °C for 5 min. The following nested PCR was performed on the hypervariable V3 region using primers p2/p3 [24], with a GC-clamp. The nested PCR was as follows: initial denaturation at 94 °C for 5 min; 35 cycles of denaturation at 94 °C for 30 s, annealing at 57 °C for 30 s, extension at 72 °C for 45 s; and final extension at 72 °C for 5 min. Archaeal 16S rRNA genes were amplified using primers A109-f [25] and 1510-r [26]. Afterward, a nested PCR was performed on the hypervariable V2-V3 region using primers A109(T)-f and 515-GCr with a GC clamp. The first archaeal PCR reaction was performed with the following thermocycle program: initial denaturation at 94 °C for 5 min; 38 cycles of denaturation at 94 °C for 30 s, annealing at 52 °C for 30 s, extension at 72 °C for 1 min; and final extension at 72 °C for 5 min. The nested PCR was as follows: initial denaturation at 94 °C for 5 min; 35 cycles of denaturation at 94 °C for 30 s, annealing at 52 °C for 30 s, extension at 72 °C for 45 s; and final extension at 72 °C for 5 min. All primers were purchased from Sigma-Genosys (Milan, Italy). The PCR products were quantified using Low DNA MassTM Ladder (Euroclone, Italy) in a 2.0 % agarose gel. DGGE analyses were performed on amplicons obtained for V3 regions for Eubacteria and V2-V3 region for Archea as previously reported [27, 28]. The gel (8% acrylamide/bisacrylamide 19:1, BioRad) was cast using a denaturing gradient of 30–60%, with 100% denaturant defined as 7 M urea and 20% (v/v) formamide.

2.6 Cloning, sequencing, and taxonomical analysis

Major bands in the DGGE-gel were cut off. Twenty-five µl of sterilized water was added to the excised DGGE bands and incubated on a rotary shaker (200 rpm) at 37°C for 4 h. Afterwards, 5 µl eluate was used as DNA template for re-amplification. PCR amplification was carried out as described before, except for the use of non-GC-clamped primers. PCR products were transformed in *Escherichia coli* Xl1blue using the pGEM-T vector system according to the manufacturer's instructions (Promega, Italy), sequenced on both strands (GATC Biotech; Cologne, Germany), and finally searched for identity using the NCBI [29] and EzBioCloud [30] databases.

3. Results and discussions

3.1 BMP tests

As reported above, two BMP tests have been carried out to show the methane production's difference from a mesophilic AD process with and without a thermal process. By this way, the performances a thermal post treatment and the recirculation of the digestate from a mesophilic AD process, may be predicted.

Figure 3 shows the Gompertz curves of the BMP tests. The first 15 days of the tests have been characterized by a very similar trend of methane production: the two tests had almost the same exponential phase growth. The lag phases of both the BMP tests was inferior to 24 hours, demonstrating the agricultural digestate are still rich in edible organic matter. After the 20th day, the kinetics of the BMP tests decreased and methane production recorded smaller and smaller daily rates. The mesophilic AD with and without thermal pretreatment BMP tests reached the definitive methane production after about two and three months, respectively.

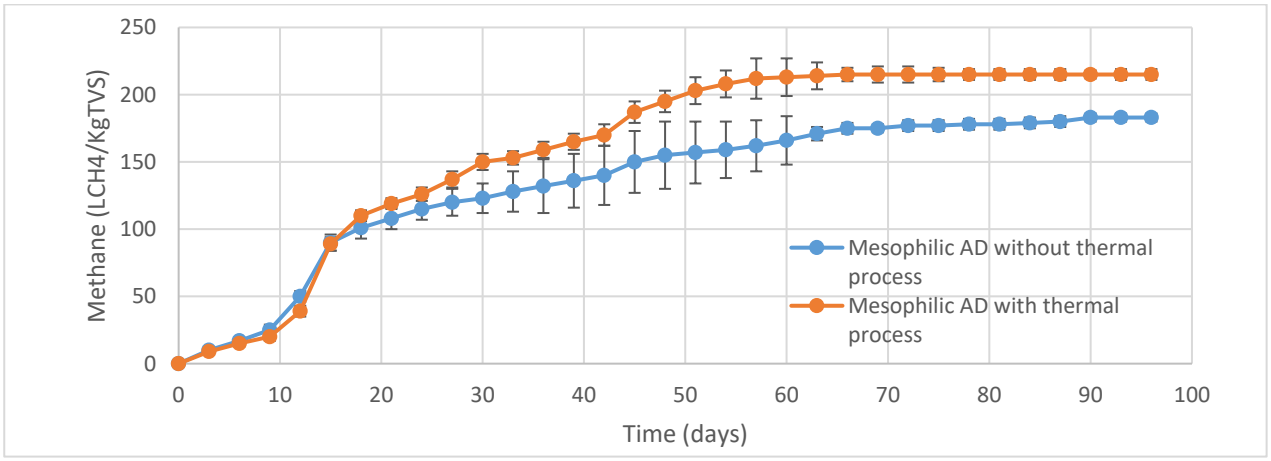

Figure 3. The Gompertz curve of the BMP tests

The trend of the BMP tests, having similar exponential growth in the first 15 days, demonstrate that thermal treatment seems to be efficacy on the degradation of more recalcitrant and slowly degradable organic compounds. The kinetic of residual simple compounds (sugars and carbohydrates) was not affected by thermal treatment. Instead, it improved the solubilization of more complex molecules, such as lipids and proteins [31], present in high concentration in digestate from manure AD [9]. These compounds, and in particular proteins, are thermos-labile structures whose hydrogen and covalent blonds can be definitively compromise by heat, for temperatures higher to 60-65°C [32]. Moreover, previous studies on thermal treatment at 70°C before AD showed that heat is able to hydrolyze the lignocellulosic materials. In particular, the reduction rates of cellulose were 15.7%, 23.5%, 24.9%, and 44.3% and those of hemicellulose were 1.8%, 13.2%, 19.0%, and 32.5% for a duration of the thermal process of 1 d, 2 d, 3 d, and 4 d, respectively [33]. This effect is helpful in both alleviating the binding force and facilitating solubilization allowing a higher biogas production [34].

BMP tests confirmed the beneficial effect of thermal treatment on digestate in the increasing of the more complex organic matter solubilization, with a consequent optimization of the methane production, which passed from 183 LCH_4/kg_{TVS} for mesophilic AD without thermal treatment of digestate to 215 LCH_4/kg_{TVS} for the mesophilic AD with thermal treatment of digestate. It corresponded to a methane increment of about 15%, which encouraged the conduction of new tests at bigger scale and semi-continuous mode.

3.2 Semi continuous tests

Table 3 summarizes the chemical parameters of the digestates of the semi-continuous SCC and of the PTDM at the three different thermophilic stage's HRTs.

SCC test showed high residual concentration of TS and TVS, respectively at the 75.80 and 53.20% w/w. The COD content, parameter usually used to measure the organic matter concentration, is almost of the 800 mg O2/gTS, while the sCOD is lower than all the other semi-continuous tests (6.53 gO2/L), where the thermal process were applied. It demonstrated that agricultural digestate is still rich in recalcitrant organic matter, not degradable by a mesophilic AD even at long HRT [35]. Consequentially, digestate cannot be considered stabilized and adapted for fertilizer application. These criticisms justify the need of a mild thermal treatment.

		HRT 2 days		HRT 3 days		HRT 5 days	
	SCC	**Thermophilic reactor of PTDM**	**Mesophilic reactor of PTDM**	**Thermophilic reactor of PTDM**	**Mesophilic reactor of PTDM**	**Thermophilic reactor of PTDM**	**Mesophilic reactor of PTDM**
TS (% *w/w*)	75.80 ± 2.06	67.54 ± 3.13	65.04 ± 3.05	62.80 ± 1.94	53.74 ± 1.64	62.62 ± 3.37	54.20 ± 2.52
TVS (% *w/w*)	53.20 ± 2.51	46.40 ± 2,58	44.62 ± 2.37	42,02 ± 1.93	35.17 ± 2.96	40.24 ± 2.25	36.59 ± 1.52
pH	8.08 ± 0.13	8.46 ± 0.15	8.23 ± 0.10	8.34 ± 0.08	8.20 ± 0.05	8.45 ± 0.15	8.22 ± 0.17
COD (mg O2/gTS)	790.91 ± 29.34	847.84 ± 29.91	843.54 ± 30.36	850.38 ± 33.91	803.51 ± 28.40	795.85 ± 27.34	772.20 ± 44.70
sCOD (g O2/L)	6.53 ± 1.05	7.83 ± 2.27	7.21 ± 1.98	8.67 ± 0.78	7.51 ± 0.26	8.75 ± 0.68	7.39 ± 0.81
VFA (mg O2/L)	129.02 ± 30.92	1500 ± 50	409.05 ± 5.52	1888 ± 270	196.07 ± 31.20	1890 ± 160	129.20 ± 30.57
P (mg/gTS)	13.50 ± 1.10	13.20 ± 0.91	13.00 ± 0.43	13.66 ± 1.57	13.71 ± 0.89	13.68 ± 0.55	13.72 ± 0.08
TKN (mg/gTS)	18.04 ± 3.47	19.90 ± 1.20	23.98 ± 4.05	19.73 ± 2.11	19.38 ± 1.07	20.36 ± 3.20	20.06 ± 2.14

Table 3. Chemical characterization of the digestate at the end of the semi-continuous tests

The thermal treatment was tested with three different HRT of 2, 3 and 5 days. In all the cases, it was always efficacy in the degradation of the organic matter, as showed in Figure 4 where the thermal treatments' effects were illustrated in terms of sCOD and VFAs increasing.

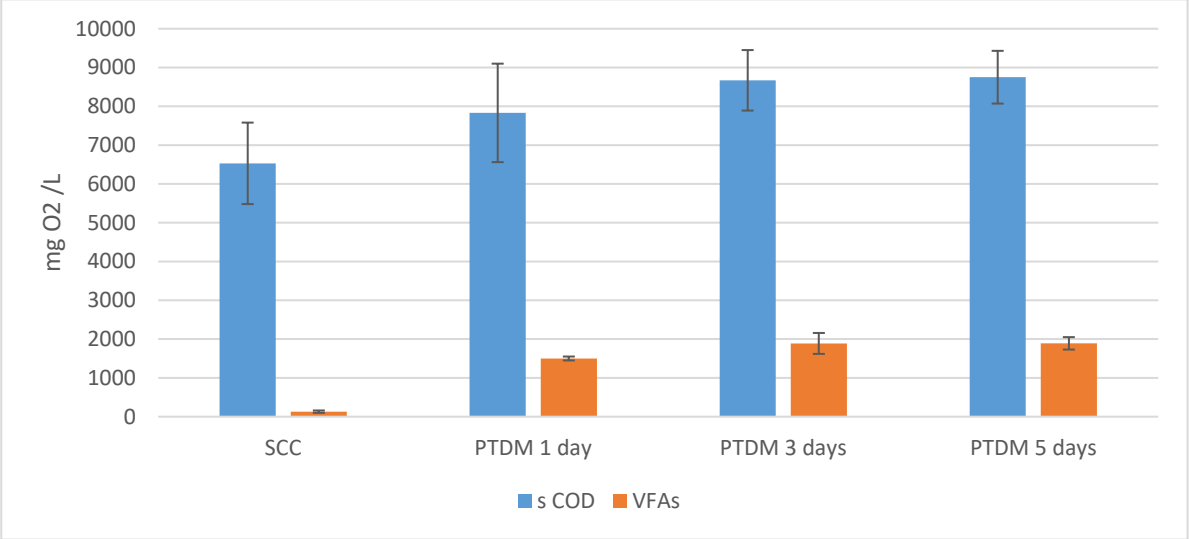

Figure 4. sCOD and VFA concentration of the semi-continuous tests.

The concentration of sCOD passed from 653 mgO2/L for the SCC test to 783, 867 and 875 mgO2/L for the thermal treatment conducted with HRT at 2, 3 and 5 days, respectively. On the contrary, the TS concentrations decreased from 75% w/w of the SCC to about 50-55% of the PTDM tests having the HRT at 3 and 5 days, respectively. Similarly, the TVS dropped from about 55% w/w of the SCC tests to about 35% w/w of the PTDM tests with HRT at 3 and 5 days, respectively (Table 3). These results confirmed the efficacy of the heat application in the degradation of thermos-labile organic structures, as proteins and -lipids, which start to lose their natural conformation for temperature higher than 55-60°C [36]. Thermal treatment has also recognized for its ability to solubilize long polymers chains, such as cellulose hemicellulose and partially of the lignin [37].

The parameter which was more affected by the thermal treatment has been the VFA concentration. PTMD tests showed very high VFA concentration after the thermophilic stage: 1,500 mgO2/L and almost 1,900 mgO2/L for the tests having HRT 2 and 3-5 days, respectively. The control SCC tests, where thermophilic treatment was not applied had lower VFAs concentrations of about 670 mgO2/L and 129 mgO2/L at the beginning and at the end of the mesophilic AD, respectively. It was demonstrated the improving of thermal application on VFA concentrations, testing different operative temperatures. They showed how mild temperature (60-100°C) contributed to the higher VFA production derived from the degradation of proteins, carbohydrates and cellulose. At these temperatures, the formation of inhibiting compounds, which occurs for higher values (150-200°C), can be avoided [37].

Another interesting consideration regards the optimal HRT for the mild thermal treatment. Figure 2 shows how the 1 day - HRT contributed to an important optimization of sCOD and VFA concentrations compared to the SCC test, with an increasing of more than the 15% and 55%, respectively. Anyway, longer HRT of 3 days brought to further sCOD and VFA productions' increasing at 25% and 65%, respectively. Instead, a HRT of 5 days did not lead to better performances. Consequentially, 3 days seemed to be the ideal HRT for thermal treatment conducted at mild temperatures. This result is coherent with previous studies, which observed the best VFA productions for thermal treatment having a duration of 3 days [38, 39]. In particular, Zhang et al. [39] explained that if carbohydrates acidification needs of some hours, the solubilization of proteins and cellulose reach the maximum yield after 3 days. Proteins contribution in the VFA increasing is particularly important in agriculture digestate, containing high percentage of animal manure.

The methane production from the AD follows the trend of the sCOD and VFA concentrations. Figure 5 shows the daily biogas production for the SCC control test and for the PTDM tests at the different HRT values. As for the sCOD and VFA, thermal treatment allowed an optimization of biogas production from a daily production of 0.5 NL/d of the SCC test to about 0.9 NL/d of the PTDM tests with HRT of 3 and 5 days when the steady state was reached (Figure 5).

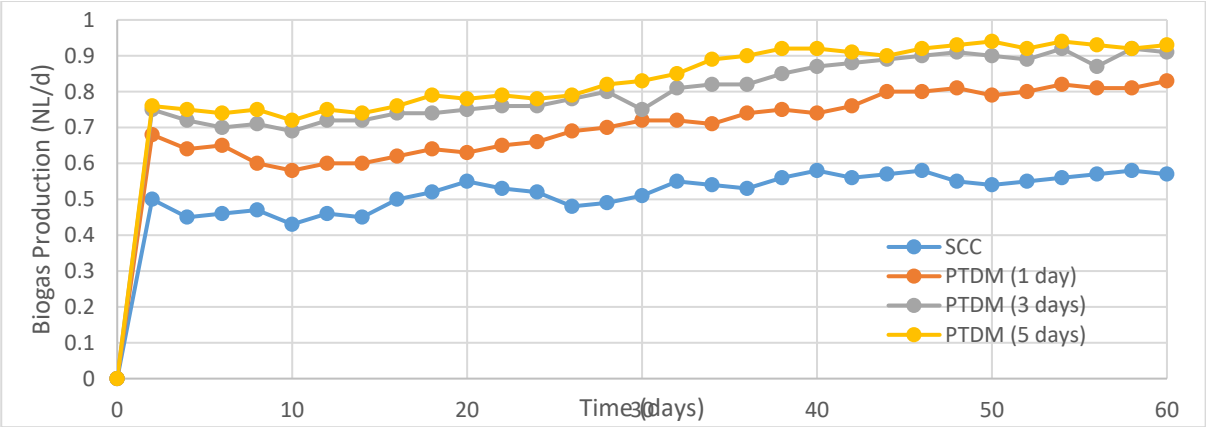

Figure 5. The daily biogas production for the semi-continuous tests.

Lastly, Figure 6 shows the specific methane production for the semi-continuous tests: SCC reached a methane production of 38.90 LCH4/kgTVS, while PTDM at 1, 3, 5 days HRT recorded a methane production of 47.98, 58.65 and 62.92 LCH4/kgTVS, respectively. It means that a post-thermal treatment of digestate with a duration of 1, 3 and 5 days followed by a new mesophilic AD process, could allow to an increasing of methane production of about 20, 33 and 38%.

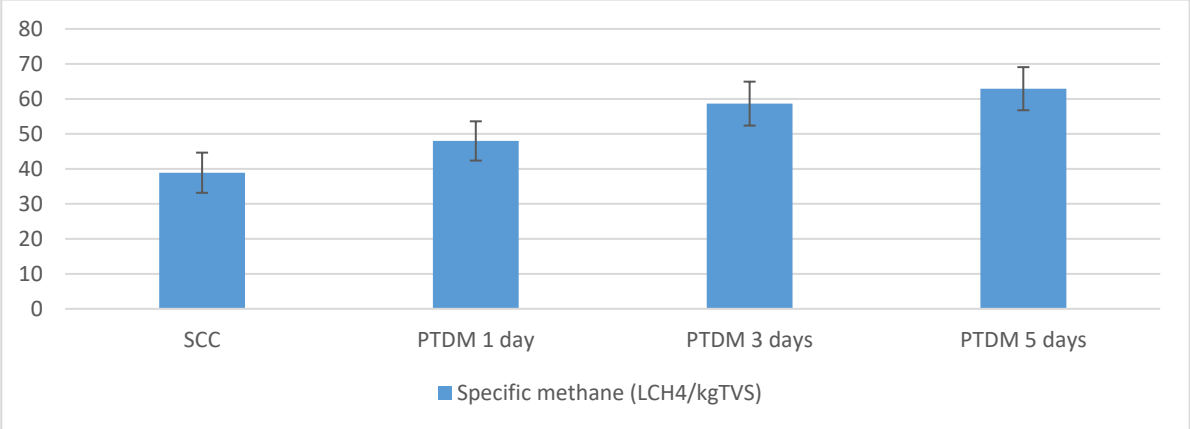

Figure 6. The specific methane production of the semi-continuous tests.

3.3 PCR-DGGE analysis on Eubacteria and Archea communities

The composition of eubacterial and archaeal communities enriched in the hyperthermophilic reactor was analyzed by means of PCR-DGGE molecular technique. DGGE patterns showed a well defined bacterial community for either eubacterial and archeal populations (Fig. 7).

Major bands in DGGE gels were excised, cloned, and sequenced (Tab. 4). Sequencing analyses revealed that the main phyla for Eubacteria were Firmicutes, Bacteroidetes, Actinobacteria and Thermotogae. This findings is in accordance with previous studies which evidenced Firmicutes and Thermotogae as the major phyla in thermophilic digesters [40]. Moreover, members of the Bacteroidales, Clostridiales, and Thermotogales orders were reported to dominate the microbial community of biogas reactor once temperature increased from 55 to 75 °C [41]. In fact, it is worth noting that sequences we retrieved from our reactor have been already described as phylotypes in microbial communities of thermophilic reactor for anaerobic digestion of agricultural wastes. Among Firmicutes phylotypes, we found both Clostridilaes and Hydrogenispora families, whereas among Bacteroidetes clones we identified members of Rikenellaceae and Porphyrimonadaceae family. Of particular interest is the band 5 in the Eubacteria DGGE pattern which corresponds to *Thermotoga neapolitana* species. *Thermotoga* is a genus of (hyper)thermophilic bacteria of the phylum*Thermotogae* [42], with optimum growth temperatures up to 80 °C. Members of the genus *Thermotoga* are anaerobic, rod-shaped bacteria encapsulated by a unique 'toga'- like outer membrane. Members of this genus are able to use wide range of carbon sources (hexoses, pentoses, disaccharides, glucans, xylans, glucomannan, galactomannan, pectin, chitin and amorphouwe found Metrhanosarcina cellulose).

Regarding Archeal community, we found members of *Methanosarcina*, *Methanobacterium* and *Methanothermobacter* genera (Tab. 4). Also in the case of Archea, these results are in accordance with previous studies which showed an increase of *Methanosarcina* and *Methanothermobacter* genera under thermophilic conditions in anaerobic digesters [41].

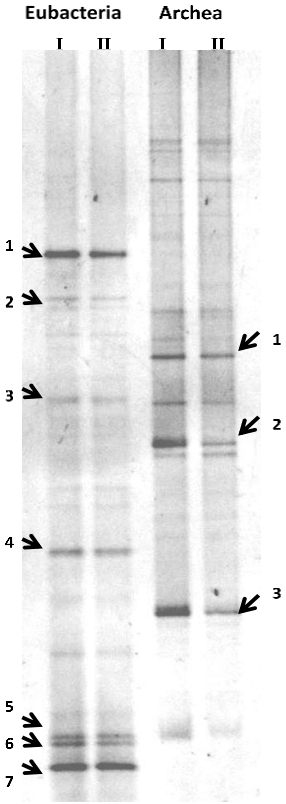

Figure 7. DGGE profiles of eubacterial and archeal communities in the iperthermophilic reactor. Arrows and letters in the gel indicate bands that have been excised, cloned, and sequenced.

Band	Taxon	Accession number	Percentage of identity (%)	Phylogenetic group
EU-1	Uncultured Bacteroidales	FN436068	99,47	Bacteroidales f
EU-2	Uncultured Hydrogenispora	DQ887962	100	Firmcutes, Hydrohgenispora f
EU-3	Uncultured Porphyromonadaceae	FN436026	98,94	Bacteroidetes, Porphyromonadaceae f
EU-4	Uncultured *Catonella*	DQ394697	98,35	Firmicutes, Clostridiales
EU-5	*Thermotoga neapolitana*	DSM 4359	98,96	Thermotogaceae;
EU-6	*Cellulosilyticum lentocellum*	DSM 5427	97,04	Firmicutes, Clostridiales
EU-7	*Bifidobacterium pseudolongum*	DSM 20092	100	Actinobacteria, Bifidobacteriaceae
ARC-1	*Methanosarcina flavescens*	E03.2	99,43	Methanosarcinales
ARC-2	*Methanobacterium kanagiense*	169	97,42	Methanobacteriales
ARC-3	*Methanothermobacter wolfeii*	DSM 2970	99,72	Methanobacteriales

Table 4. Taxonomic characterization of the major bands cloned and sequenced from the DGGE profiles

Conclusions

The efficacy of the post treatment on an agricultural digestate was demonstrated. In particular, a considered exhausted digestate is still rich in recalcitrant organic matter, potentially exploitable for methane production after an adequate hydrolysis step. A thermophilic treatment conducted at 70°C with a HRT of 3 days assured an increasing of the soluble organic matter and VFA concentrations of 25% and 65%, respectively. As consequence, the following AD, conducted in mesophilic condition and in semi-continues mode, resulted in a specific methane production of about 60-65 LCH_4/Kg_{VS}, corresponding to a 30% more than the case without thermal treatment.

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