PRE-TREATMENT EXPERIMENTS FOR THE USE OF *CYNARA CARDUNCULUS L.* AS A SUBSTRATE FOR THE PRODUCTION OF BIOGAS

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SUMMARY:
The purpose of the present study was to explore and evaluate the influence of different pre-treatments on *Cynara* stalks over anaerobic digestion and potential methane production. Different pre-treatments followed by anaerobic digestion batch experiments, were carried-out to *Cynara* stalks feedstock in order to select the most effective combination. After selecting the most suitable inocullum/substrate (*I/S*) ratio, different pre-treatments: mechanical, thermal, thermal chemical and enzymatic were studied to enhance the biogas and methane yield in correlation with volatile solids (VS) reduction. The most promising pre-treatment was submitted to a final experiment, in order to set up optimized operational parameters. The thermal chemical pre-treatment applied to the substrate, in the final assay, doubled the cumulative methane yield in comparison with the trial conducted with the untreated one. The methane yield achieved was 0.59 l/g VS_{added} and 0.31 l/g VS_{added} for the pre-treated and untreated substrate respectively. The enhancement achieved is also shown in terms of VS reduction**.** Enzymatic pre-treatment can contibute to an improvement of untreated substrate hydrolyses and also an increase in methane yield of 18% in comparison with the one without enzymatic addition. Mechanical pre-treatment combined with the addition of enzymes enhance hydrolyses of the substrate improving *inoculums* efficiency however, more experiments are required within higher incubation times.

1. INTRODUCTION

Different studies have shown that, methanogenesis and hydrolysis are the limiting steps of anaerobic digestion and biogas production, however, in terms of using biomass or lignocellulosic substrates it's evident that hydrolyses is the most important and limiting step of the anaerobic digestion process and consequently on the specific biogas yield (Hendriks, 2009; Ward, 2008; Lehtomäki, 2006; Di Berardino, 2006; Mladenovska, 2006; Siegert, 2005; Wyman, 2005;

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Mosier, 2005a; Alvarez, 2000;Palmowski, 2000; Boone, 1993; Mata-Alvarez, 1987; Chynoweth, 1987). Some works referred that the inoculums have a good influence in hydrolyses step increasing its rate when rumen is applied (Sullivan, 2008, 2005; Hu, 2005, 2004; Mosier, 2005b; Gunaseelan, 1997, 1995; Chynoweth, 1987). Others show that a pre-treatment step before feeding the digester also increases hydrolyses rate (Hendriks, 2009; Lehtomäki, 2006; Angelidaki, 2000; Palmowski, 2000; Hartmann, 2000; Chynoweth, 1987). Different pre-treatments have been applied in a lab scale with good results such as: mechanical, hydrothermal, thermal-chemical using acids or alkalis and biological like enzymatic. Although, the works related to biogas production are limited to specific crops and/or the majority of these works are specific for ethanol production. Notwithstanding, it seems that thermal, alkali and mechanical pre-treatments give better biogas and methane yields. Depending on the crop material and the particle size achieved through mechanical pre-treatment, it's expected that hydrolyses yield increase at least 5-25% reducing consequently the digestion time between 23-59 % and increasing the specific biogas yield between 5-25% (Hendriks, 2009; Lehtomäki, 2006; Mshandete, 2005; Angelidaki, 2000; Palmowski, 2000; Hartmann, 2000). In general, particle sizes less than 40 mesh doesn't improve hydrolyses yield, hydrolyses rate and specific methane yield. Thermal pre-treatment with liquid hot water and steam explosion improve herbaceous crop's digestibility increasing the specific methane yield (Bauer, 2009; Hendriks, 2009). Alkali pre-treatments however remove more lignin (Buranov, 2008; Wyman, 2005; Mosier, 2005b) leaving cellulose and hemicelluloses available for the bacteria resulting in an increase of the specific biogas and methane yield (Hendriks, 2009; Ward, 2008; Angelidaki, 2000; Gunaseelan, 1995; Chynoweth, 1987, 1985).

Cynara cardunculus L. is a perennial crop that grows wild in the Mediterranean region and in other parts of the world with an annual growth cycle, sprouting every autumn (more than 15 cycles have been reported) (Fernández and Curt 2005). In scientific trials the average production is 20 tons/ha.year of dry material, with 40% stalks, 25% leaves, and 35% capitula. The biomass produced in the successive phases of the growth cycle has different potential uses such: biofuels (pellets, biodiesel), paper and pulp production, pharmacologic active compounds, green fodder, rennet for cheese-maker. (Gominho et al 2001, Férnandez et al, 2006).

Recently *Cynara* stalks were tested for ethanol production (Ballesteros, 2008) coupling a low-dose sulfuric acid (0.1-0.2% w/v) thermal-chemical pre-treatments and enzymatic hydrolyses. The achieved ethanol yields were relatively low for industrial process application, requiring further improvements. The purpose of the present study was to investigate and evaluate the influence of different pre-treatments on *Cynara* stalks over anaerobic digestion and potential methane production. Different pre-treatments followed by anaerobic digestion batch experiments, were carried-out to *Cynara* stalks feedstock in order to select the most effective combination. After these experiments the best pre-treatment was submitted to a final experiment, in order to set up optimized operational parameters.

2. MATERIAL AND METHODS

2.1. Substrate and inoculums

The *Cynara cardunculus* L. used in this work was collected from the pedagogic field (BioEnergISA) at ISA campus (Instituto Superior de Agronomia, Lisboa) in October 2008. The leaves and the capitula were removed and only the stalks were used. The stalks were grounded using a knife mill and sieving in different fractions: 10 mm, 2mm, 40 mesh, 40-60 mesh, 60-80 mesh and 80 mesh. This material is described as substrate (*S*) in the anaerobic digestion assays.

As *inoculums* (*I*) was used digested sewage sludge from a Waste Water Treatment Plant (WWTP, ETAR de Chelas, Lisboa). The sewage sludge was freshly collected during the start-up of each experiment.

2.2. Batch reactors

Two batch systems with different working volumes were used: 300 ml for the experiments assays and 2000 ml for the final assay. Both were operated using a mesophilic temperature of 37 ºC. In the *experimental assays* (B_n) the reactors were placed in heated thermostatic bath, maintained at 37ºC temperature. These reactors were manually agitated everyday for 1-2 min. In the final assay the temperature of each reactor was maintained using a water-heated jacket vessel, and mixed everyday for 10 min by a mechanical stirrer.

2.3. Experimental procedure

The anaerobic digestion experiments were conducted at different incubation times; the experimentals operated at 11-12 days incubation time, and final assay was extended to 30-32 days.

2.3.1. Experimental assays

In each experiment twelve reactors (500 ml) were used, six reactors for biogas accumulation measurement (X_1, X_2, X_3, X_4, X_5 and X_6) and the remaining six reactors (Y_1, Y_2, Y_3, Y_4, Y_5 and Y_6) for biogas analyses. In each experiment the reactors X_6 and Y_6 were used as a control, containing just *inoculums*. In the *experimental assays* there was also a preparation of six samples with 300 ml containing the same material content present in each reactor.

I/S ratio determination: Five different *I/S* ratios were used for each reactor respectively, 349.8 (X_1/ Y_1), 174.2 (X_2/ Y_2), 116.1 (X_3/ Y_3), 87.5 (X_4/ Y_4) and 69.7 (X_5/ Y_5) and the best *I/S ratio* was selected to run all the following trials.

2.3.2. Mechanical pre-treatment

In order to select the best granulometry for the following experiments, different particle sizes were used: 2 mm (reactors X_1/Y_1), 40 mesh (reactors X_2/Y_2), 40-60 mesh (reactors X_3/Y_3), 60-80 mesh (reactors X_4/Y_4) and 80 mesh (reactors X_5/Y_5).

2.3.3. Thermal pre-treatments

Thermal batch pre-treatment can differ in terms of temperature, reaction time, solids concentration and solubilizing agent with *I/S ratio* selected. The thermal pre-treatment applied was conducted at fixed temperature (160ºC), solids (12.5 %) andsolid/solubilizing agent (ratio of 1/8). Different reaction times: 10 min (X_1/Y_1), 15 min (X_2/Y_2), 20 min (X_3/Y_3), 30 min (X_4/Y_4) and 0 min (X_5/Y_5) were tested.

2.3.4. Thermal-chemical pre-treatments

A thermal chemical pre-treatment was applied using the reaction time and *I/S ratio* previous selected. Different chemical solutions were used: NaOH (14%), $NH_{3(aq)}$ (1.5%), HNO_3 (1%) and CH_3COOH (1%). After the pre-treatment step each pre-treated substrate was placed in each reactor: X_1/Y_1 (NaOH), X_2/Y_2 ($NH_{3(aq)}$), X_3/Y_3 (HNO_3), X_4/Y_4 (CH_3COOH) and X_5/Y_5 (untreated substrate).

2.3.5. Enzimatic pre-treatment

To analyze the effect of enzymatic pre-treatment two different types of enzymes were used: *Celluclast* composed of exo-1, 4-β-D-glucanase and endo-1, 4- β-D-glucanase, and *Novozymes 188* composed of 1, 4-β-D-glucosidase. The substrate was placed in a flask adding acetate buffer 0.2M (diluted 10 times) to maintain the pH at 4.8. The enzyme loading concentrations were: 25 FPU (g dry substrate)$^{-1}$ for cellulases and 25 IU (g dry substrate)$^{-1}$ for β-Glucosidase. The pre-treatment was conducted at 50ºC with a rotary shaker for 60h. Enzymes activities were 90 FPU ml^{-1} and 450 IU ml^{-1} for cellulases and Novozyme 188 respectively. The substrate was enzymatic pre-treated for 40 h and 60 h, and during that time (24h, 40h and 60h) samples were taken to determine the sugar concentration in HPLC. After the enzimatictreatment step each substrate was placed in each reactor: X_1/Y_1 (pre-treated substrate at 40 h), X_2/Y_2 (pre-treated substrate at 60h), X_3/Y_3 (untreated substrate and both enzymes) and X_5/Y_5 (untreated substrate).

2.3.6. Final Assay

At the end of *experimental assays* the best pre-treatment was selected and applied again on the *Cynara* stalks with a particle size of 10 mm. In this *Final Assay* three reactors were used: B_0 (containing just inoculums), B_1 (containing inoculums + pre-treated substrate) and B_2 (containing inoculums + untreated substrate).

2.4. Analytical methods

The chemical composition of the whole stalks, pith and depicted material was determined using Tappi standard methods in relation to ash, moisture content, extractives, lignin and polysaccharides as described in (Gominho, 2001). Cynara stalks were also characterized in terms of total solids, volatile solids, total organic carbon (TOC) and total nitrogen (N_{total}) according to Standard Methods. Pre-treated were analyzed in terms of holocelulose and α- celulose according to the method in Handbook of Wood Chemistry and Wood Composites (Rowell et. al, 2005). During the enzymatic pre-treatment HPLC analysis were made at 24h, 40h and 60h in terms of sugar content (glucose, xylose).

The analyses of pH, total and volatile solids (TS; VS) and ammonia were carried out according to Standard Methods. The chemical oxygen demand (CQO) was determined using a Hanna Instruments Series C99 multi-parameter photometer. Total carbon content was evaluated as suggested by Zucconi and Bertoldi (1987). The homogeneized sample was digested in the presence of dichromate at 150ºC for 2 hours in a Hanna C9800 reactor. Volatile Fatty Acids (VFA) were determined by gas chromatograph with flame-ionization detector using helium as the carrier gas at 99.9995 % according to standard methods. The injector, column and detector temperatures were 175ºC, 170ºC and 250ºC respectively. VFA were also determined at the same time as Alkalinity using the 5 pH point titration method. Daily biogas production was measured using 500 ml graduated reversible gas- liquid displacement holders during experimental a*ssays* and 5l bottles and 2l graduated cylinders in the *Final Assay*. The methane content was determined at 10th day in the experimental a*ssays* and at weekly intervals during the f*inal assay* using a gas chromatograph with a thermal conductivity detector with helium as the carrier gas at 99.9995 %. The injector, column and detector temperatures were 60ºC, 50ºC and 150ºC respectively.

3. RESULTS AND DISCUSSION

3.1. I/S ratio effect (B_1 assay)

In this experiment the aim was to evaluate inoculums activity and select a suitable I/S ratio for the all experiments in terms of biogas and methane yield. Figure 1 shows that the lowest I/S ratio

(69.7 ml/g VS) biogas production increases achieving a higher specific methane yield, due to a lesser amount of microrganisms and, consequently, to a less respiration rate, being the adopted I/S ratio, for the following B_n *assays.*

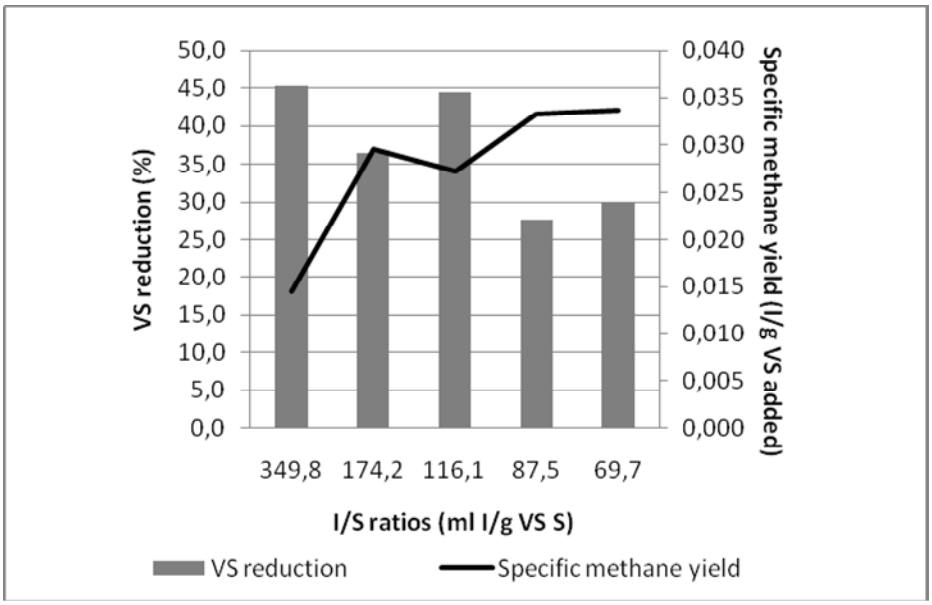

Figure 1. I/S ratio effect on the VS reduction and specific methane yield (*B_1 assay*)

3.2. Improving Biogas production using mechanical pre-treatment (B_2 assay)

The mechanical pre-treatment of *Cynara* stalks shows that the biogas production and methane yield improves when the particle sizes are decreased (Figure 2). There is a strong increase in methane yield between *Cynara* stalks particle sizes of 2 mm and 40 mesh and a moderate evolution between particle sizes of 40 and 80 meshes.

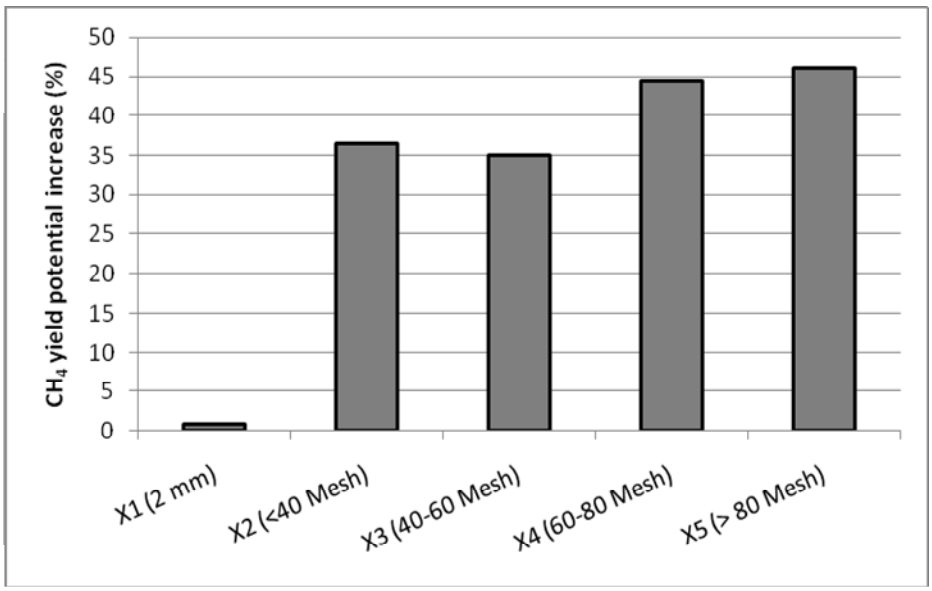

Figure 2. Effect of the *Cynara* stalks particle size on the methane yield (%) in B_2 assay

3.3. Thermal reaction time effect (B_3 assay)

Thermal pre-treatment by mean of heating of the substrate at referred temperature and related pressure also improves *Cynara* stalks digestibility due to carbon solubilization, according to the results shown in Figure 3. Increasing thermal reaction time from 10 to 20 min improves significantly the biogas yield, when compared to the degradation of the untreated substrate. Figure 3 and Figure 4 show also that at longer thermal reaction time (30 min), despite of achieving higher carbon solubilization, the biogas yield is lower than the obtained at 20 min retention time pre-treatment. During the thermal pre-treatment a change on C/N ratio was observed according to reaction time, achieving a better ratio for the 20 min thermal pre-treated substrate. Based on these results 20 min of reaction time was the suitable operational option to carry-out the ***B_3 assay***.

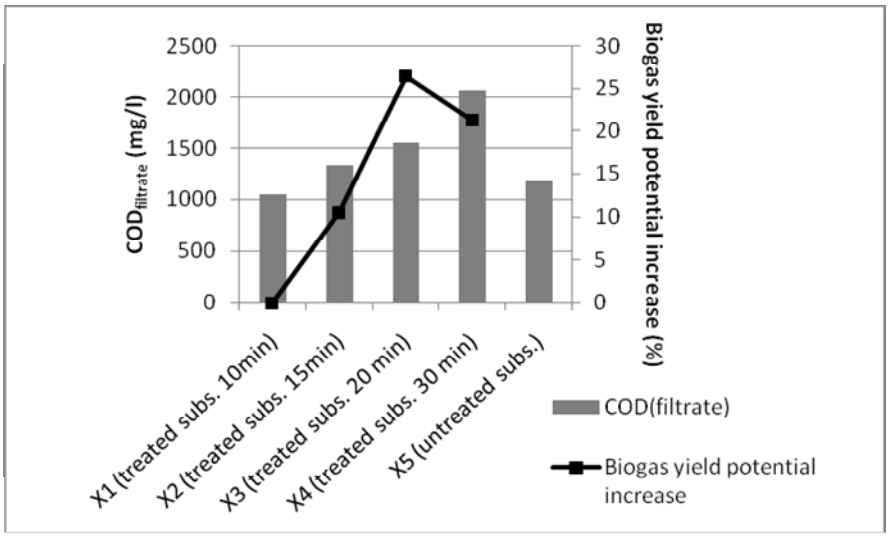

Figure 3. Thermal reaction time effect on carbon solubilization and specific biogas yield (B_3 assay)

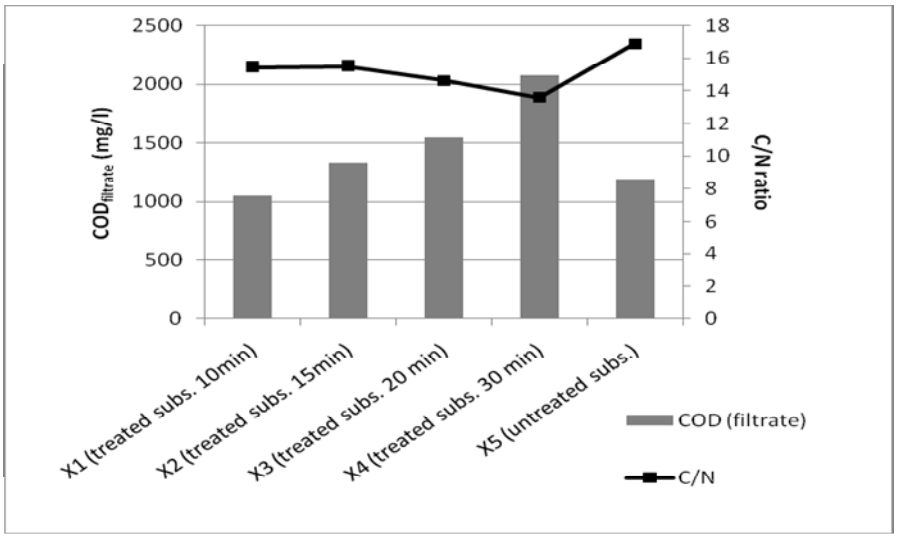

Figure 4. Thermal reaction time effect on the carbon solubilization and C/N ratio (**B_3 assay**)

3.4. Thermal chemical effect (B_4 assay)

Thermal chemical pre-treatment as it was expected showed a great effectiveness on carbon

solubilization. Figure 5 shows the methane yield potential increased and solubilized carbon for different solubilizing agents in comparison with the untreated substrate. NaOH and CH_3COOH were the only solubilizing agents that led to an increase in biogas production and CH_4 yield in comparison with the untreated substrate. Table 1 shows that HNO_3 and $NH_{3(aq)}$ had favored hydrolyses and fermentation due to the higher concentration of VFA. On the other hand the $NH_{3(aq)}$ concentration used led also to an increase of $N\text{-}NH_4^+$ (1321-1488 mg $N\text{-}NH_4^+$/l) which possibly contributed to methanogenic bacteria inhibition (Alvarez, 2000; Di Berardino, 2006).

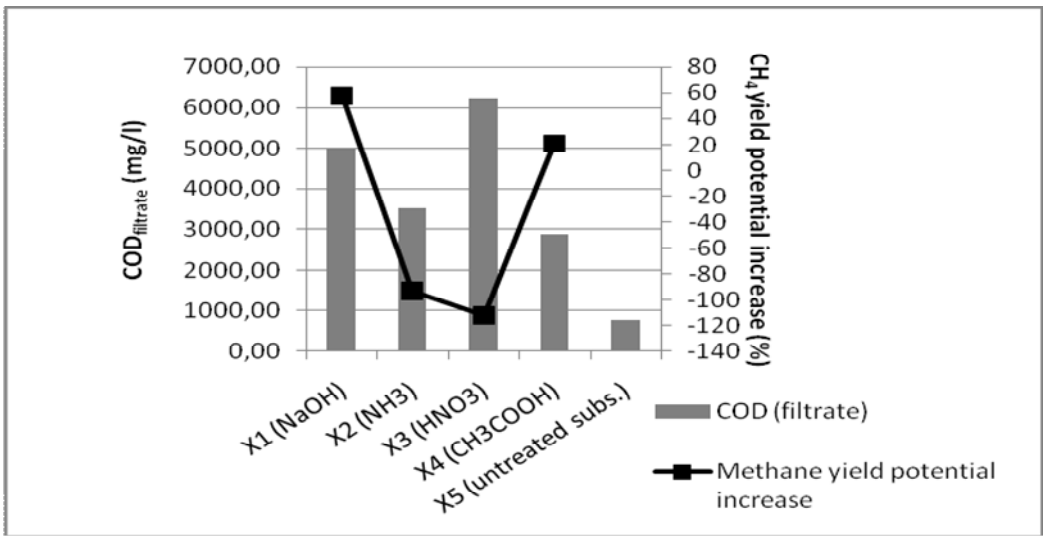

Figure 5. Thermal Chemical pre-treatment effect on carbon solubilization (mg COD/l)) and biogas production as potential increase in methane yield (%) for different chemical solubilizing agents in comparison with untreated substrate in B_4 assay

Table 1. VFA and Alkalinity concentration at the end of B_4 assay ($X_1|Y_1$- NaOH pre-treated substrate; $X_2|Y_2$- $NH_{3\ (aq)}$ pre-treated substrate; $X_3|Y_3$- HNO_3 pre-treated substrate; $X_4|Y_4$- CH_3COOH pre-treated substrate; $X_5|Y_5$- untreated substrate)

Reactors	VFA (mg/l)	Alkalinity (mg $CaCO_3$/l)	Methane content (CH_4 %)
$X_1\|Y_1$	105.5	3844.5	70.6
$X_2\|Y_2$	4200.8	236.0	45.2
$X_3\|Y_3$	2465.0	0.5	45.2
$X_4\|Y_4$	0.0	1416.9	64.6
$X_5\|Y_5$	0.0	1445.5	60.3

3.5. Improving Hydrolyses – adding hydrolytic enzymes (B_5 assays)

According to Table 2 and Figure 6 the enzymatic pre-treatment enhanced strongly the biodegradability of the feedstock due to a quick acidification, resulting in a high level of volatile acids after 40 hours of degradation time (reactor X_1). In the following 24 hours of time the methanogenic activity increased and reduced substantially the organic acids concentration in the solution (reactor X_2). In these reactors the biogas composition showed a relatively low methane concentration, as consequence of the acidification reactions and the slow response of methanogenic population during the trial assay. At the end of experiment (reactor X_3) methanogenic activity consumed most of available acids controlling completely the acidification

and the test run under methanogenic controlled phase. Methane concentration is relatively high (63%) and low volatile acids concentrations were detected at the end of the test.Comparig the results obtained with the the untreated substrate, as shown in Figure 3, the enzymatic attack at the beginning of the experiment increases the biogas yield of 18.4%,improving also the methane content. This beahaviour seems very effective for releasing high quantity of biodegradables compounds in the early stage of the enzymatic attack (40 hours).

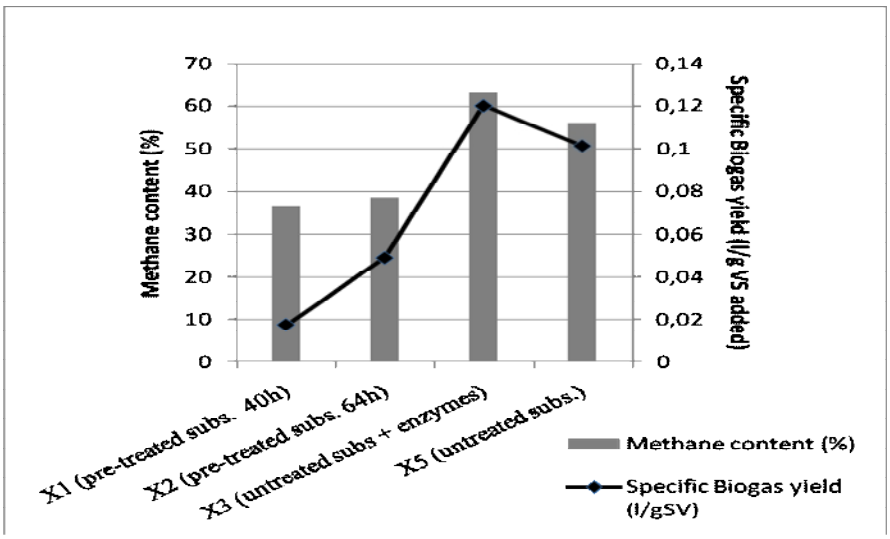

Figure 6- Methane content and Specific biogas yield obtained after 12 days of incubation time (**B_5 assay**)

Table 2. VFA and Alkalinity at the end of experiment in *B_5 assay*

Reactors	VFA (mg/l)	Alkalinity (mg $CaCO_3$/l)
X_1	4439.4	896.2
X_2	2789.0	2495.9
X_3	218.4	1610.6
X_5	122.9	1364.1

3.6. Final Experiment (*Final Assay)*

After the analysis of the data obtained during ***B_n assays*** and combinig the best results of each trial were selected a thermal chemical pre-treatment with NaOH within 20 min of thermal reaction time. In this final experiment a 10 mm of *Cynara* stalks particle size was used. Figure 7 shows the results after the anaerobic digestion experiment in terms of specific and cumulative methane yield.

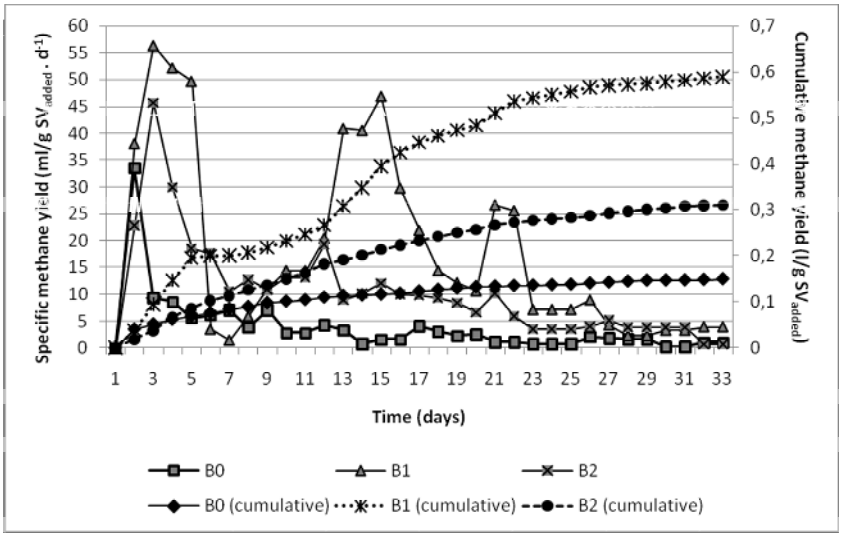

Figure 3. Specific and cumulative methane yield during the ***Final assay*** (***B₀***-reactor containing inoculums, control; ***B₁***- reactor containing pre-treated substrate + inoculums; ***B₂***- reactor containing untreated substrate)

The thermal chemical pre-treatment applied to reactor B_1 doubled the cumulative methane yield in comparison with the trial conducted in reactor B_2. In this way was achieved 0.59 l/g VS_{added} and 0.31 l/g VS_{added} for the pre-treated and untreated substrate respectively. The enhancement achieved is also shown in terms of VS reduction, in reactor B_1 the VS reduction was 44 % and in B_2 the VS reduction was 21%. Despite the high cumulative methane yields achieved there is still an amount of carbon in terms of volatile solids in the substrate that wasn't degraded. *Cynara* stalks are composed of approximately 18% of lignin (%VS). Assuming that lignin isn't degraded in anaerobic conditions there is still approximately 34% and 56% of VS in the pre-treated substrate and untreated substrate respectively that remains to futher degradation.

5. CONCLUSIONS

This work showed that it is possible to increased biogas production using cardoon stalks as substract. Due to the lab scale experiments and imposed incubation time it wasn't possible to test a full range of particle sizes for longer incubation times. Notwithstanding, the results achieved shown that there is a large increase of biogas and methane yield of more than 30% between 2mm and 0.42 mm (40 mesh) particle sizes**.** Thermal and thermal-chemical pre-treatment showed that both led to a better carbon solubilization. However, thermal pre-treatment with H_2O within 20 min of reaction time and thermal chemical pre-treatment with NaOH and CH_3COOH were the ones that led to an increase in biogas and methane yield.

Pre-treatment with NaOH was the one that showed the best results in terms of carbon solubilization, VS reduction, biogas yield and methane yield increment. In this way for the *Final assay* was decided to use a thermal chemical pre-treatment with NaOH. The results achieved in this *Final assay* show, clearly, that thermal pre-treatment with NaOH is very effective even when *Cynara* stalks with a larger particle size are used. *Cynara cardunculus* L. is a good crop for biogas and methane production in Mediterranean countries. Of the several pre-treatments used, the thermal alkali pre-treatment with NaOH resulted in the most promising one due to the improvement of substrate greatly increased biodegradability, which results in an increment of biogas and better methane yields achieved. Mechanical pre-treatment and the addition of enzymes (exo-1,4-β-D-glucanase and endo-1,4- β-D-glucanase and 1,4-β-D-glucosidase)

enhance hydrolyses of the substrate improving *inoculums* efficiency however more experiments are required within higher incubation time. Besides the results achieved in the *Final assay* in terms of cumulative methane yield, there is still work to be done in terms of choosing the most suitable pre-treatment for *Cynara cardunculus* L. in Mediterranean countries.

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