Biomethane production improvement by enzymatic pre-treatments and enhancers of sewage sludge anaerobic digestion.

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Abstract

Enzymatic hydrolysis is recognised as an effective pre-treatment for increasing biodegradability of sludge. In this work, isolated commercial enzymes as well as in-situ enzymes producer bacteria were used respectively as enhancers and pre-treatments of sewage sludge. Biodegradability of sample as well as biomethane potential production were studied. Results showed that depuration efficiencies in terms of CODs (73.5-85.5 %) and TVS (28.5-42.7 %) were more than twice the control value. In addition, pre-treated samples as well as enhanced samples with enzymes generated more biomethane than control. The optimal ones, were those with the isolated proteases (P) and with bacteria (*Bacillus licheniformis*) treatment in-situ (F), producing a total volume of 72.4 ± 2.62 ml CH_4 and 114 ml ± 0.46 CH_4, respectively, increasing the biogas volume in 3.65 and 5.77 times respectively compared with control.

1. Introduction

The sludge line from conventional wastewater treatment plant (WWTP) generates high amount of sludge after decanting solids coming from primary (sedimentation) and secondary (biological) treatments. All the sludge is concentrated by flotation, thickening, centrifugation and dewatering [1]. The variations in quantity and quality of mixed sludge are mostly defined by domestic habits as well as by correct operation of the different treatment units in WWTP.

However, the common composition includes organic and inorganic compounds. Organic compounds are mainly microbial organisms and extracellular polymeric substances from secretion and cell lysis as well as sedimentable organic matter from

wastewater such as cellulose or humic acids [2]. Inorganic matter is normally 20-50% of dry matter [3-4]. Stabilization of sludge by anaerobic digestion is a crucial step to remove pathogens, solids and bad odours, to increase the ammonia content and to enhance the partial mineralization of organic matter. This operation has an extra value due to biomethane potential production and hence energy saving. In this sense AEBIOM estimated a potential of 6 billion Nm^3 of biomethane coming from sewage sludge in 2018 [5].

Different technologies to increase biomethane potential in anaerobic digestion processes are being widely studied. These studies were mainly focused on increasing the biodegradability of sludge by physico-chemical, biological and/or biochemical methods, improving hydrolysis step in overall anaerobic digestion process. All these methods have obtained higher recovery volumes and yields of biomethane even at full-scale level as a consequence of: (i) the disruption of pathogen cellular membranes avoiding competitiveness with anaerobic digestion microbial consortia; (ii) the increase of available compounds such as proteins, sugars, ammoniacal compounds or volatile fatty acids (VFAs) that serve as anaerobic digestion consortia feed [2].

Among different pre-treatments, biological and biochemical treatments have been designed in order to improve hydrolysis step in an eco-friendly way and with no special equipments [6-7]. In this sense, enzymatic hydrolysis is recognised as an effective pre-treatment for increasing biodegradability of sludge. There are different types of enzymes (lipases, glucanases, proteases) and the selection of the optimal treatment depends basically on the origin and the characterization of each sample. Duarte et al. [8] used lipases (glycerol ester hydrolase, E.C. 3.1.1.3) for the hydrolysis of triacylglycerols

in fish industry effluent. Yu et al. [6] studied the effect of application 10% endogenous hydrolases (amylases from *B. subtilis* and proteases from *A. hydrophila*) as pre-treatments to sewage sludge. Results showed that biogas production was increased by 23.1% compared to control after 11 days when a combination of both hydrolases was used. Bonilla et al. [9] used commercial and self-making proteases to enhance the anaerobic digestibility of paper biosludge. In BMP assays results, self-making protease BCE_2078 pre-treatment did not show any improvement in biogas production. However, the maximum improvement (26% after 62 days) happened using commercial protease from *Bacillus licheniformis*. *B. licheniformis* is used at industrial scale to produce hydrolytic enzymes. It is a Gram-positive bacterium commonly found in multiple natural habitats due to its ability of degrade different substrates by secreting hydrolytic enzymes and its versatility and adaptability to multiple environmental conditions. It is known that, *B. licheniformis* is a dominant natural bacterial strain in multiple kinds of wastewaters. It is able to easily metabolize nutrient content, favouring its growth against other bacterial strains in these substrates. This competition is mainly due to proteins degradation efficiency because its production of proteolytic enzymes [10-11].

In this work, pre-treatments by applying directly the microorganisms and comparing with commercial isolated enzymes were investigated. To date there is no studies about previous controlled fermentation only with adapted *B. licheniformis* bacteria at exponential growth phase as a pre-treatment. In this sense, it was registered their effects in biomethane potential production during subsequent AD process.

2. Materials and methods

101

102 *2.1. Inoculum*

103 The inoculum was obtained from 5L single-phase dry-mesophilic anaerobic digester
104 operating at HRT = 20 d. The raw sludge characterization includes: pH = 7.4; total
105 chemical oxygen demand (CODt) = 21.3 g/L; soluble chemical oxygen demand (CODs)
106 = 1.2 g/L; total solids (TS) = 14.5 g/L and total volatile solids (TVS) = 8.58 g/L; fixed
107 total solids (FTS) = 5.92 g/L.

108

109 *2.2. Substrate*

110 The raw sewage sludge as substrate was obtained from an experimental aerobic digester
111 from Center for New Water Technologies (CENTA) in Carrión de los Céspedes
112 (Seville, Spain). It was kept at 4ºC during 4 months. The initial composition is can be
113 observed in Table 1.

114

Table 1. Physico-chemical characterization of sludge used as substrate.

Physico-chemicals parameters	Values (%)	Microelements	Values (mg/g)
pH*	6.55	Si	78.86
Total Solids (TS)	4.91	Ca	56.97
Total Volatile Solids (TVS)	2.78	Al	26.97
Fixed Total Solids (FTS)	2.13	Fe	12.61
Total Carbon (TC) **	29.11	P	18.86
Total Nitrogen (TN)**	4.48	S	9.87
Proteins**	29.14	Mg	8.43

**pH units; **from dry matter*

115

116 *2.3. Pre-treatments and enhancers*

117 Hydrolysis of initial substrates was promoted by two methods: (i) biological pre-
118 treatment and (ii) enzymatic enhancers; as it is shown in Table 2. The crude sludge was

119 autoclaved (30 min 121 ºc) before biological pre-treatment (fermentation) in order to
120 remove residual microorganisms that could compete with *B. licheniformis.*
121 Fermentation was carried out by inoculating an exponential *B. licheniformis* ATCC
122 21415 culture kept under LB medium. Fermentation conditions: T =37 ºC, Agitation
123 rate =150 rpm, Time = 12 d.

Table 2. Applied pre-treatments and enhancers before BMP

Samples	Pre-treatments and enhancers
WP	Without pre-treatment
G	Addition Glucanase
C	Addition Cellulase
P	Addition Protease
F	Fermentation
1F:1S	Fermented sludge and crude sludge mixture 1:1
1F:9S	Fermented sludge and crude sludge mixture 1:9

124

125 Enzymatic additions were carried out using 0.3% (v/v) of enzymes from BIOCON
126 company directly in the digester. The characterization of enzymes is shown in Table
127 A.1 in Supplementary information file. Biocellulase enzymes comprise a mixture
128 among biocellulases with betaglucanase, xilanase and hemicellulase activities very used
129 in food processing and textile finishing. Betaglucanase showed 1.3 (4) Betaglucanase,
130 cellulase, xilanase and arabinoxilanase activities and it is also commonly used in food
131 industry above all in brewing factories. Bioprotease showed proteolytic optimal activity
132 between pH 7-11.

133 *2.4. Experimental set-up procedures*

134 BMPs were used in order to determine the methane potential of different samples. The
135 anaerobic digestion of different pre-treated and enzyme-rich samples were studied in
136 250 ml serum bottles with effective volume of 120 ml. The digesters were initially
137 loaded with a mixture of crude sludge (the inoculum) and different substrates (Table 2)

in a final concentration of 40% v/v of inoculum, which is considered optimum for biogas production and substrates acclimatize [12]. Control reactors (sample WP) were also incubated to determine the background gas production. All the anaerobic digestion experiments were carried out until all the available carbonic content was converted to biogas (23 days) or in other words, there was no more biogas production detected and pH was stable. All reactors were run in duplicates and average values of results were calculated. At the beginning and at the end of each experiment, the samples were characterized in order to evaluate their biodegradability. During the experiment, the volume and the composition of biogas produced were registered.

2.5. Analytical methods

Controlling AD reaction is made by measuring different parameters involved in the process. The main parameters measured were: pH, TS, TVS, alkalinity, VFAs, CODt, CODs, biogas volume and composition. In addition, at the beginning of the experiment total carbon (TC) and total nitrogen (TN) were measured for characterization.

TC and TN of sewage sludge samples were determined by a LECO Elemental Analyzer, model CHNS 932. Protein content was calculated as %N * 6.5. The rest of the microelements were analysed by inductively coupled plasma atomic emission spectrometry (ICP-AES) using a Fisons-ARL 3410 multielement sequential instrument, equipped with a data acquisition and control system. The standard operating conditions for this instrument are summarized below: argon as carrier, cooling and plasma gas, used at 80 psi pressure, being carrier gas flow of 0.8 L min^{-1}, refrigerant gas of 7.5 L min^{-1}, plasma gas of 0.8 L min^{-1}, and the integration time of 1 second. A mini-flame consumes argon gas at a radio-frequency power of 650 W.

pH, solids, CODt, CODs and alkalinity were determined using standard methods [13]. pH determination was taken by pHmeter type CRISON MICROPH 2001 with a temperature probe. For TS, TVS and FTS, samples were weighed in ceramic boats in a laboratory balance Cobos type and drying in oven type ELF14 de CARBOLITE. After drying, they were transferred to the desiccator. For alkalinity determination, samples were previously filtered and diluted in Milli RO water in 1:25 proportion. Titration was automatic using a titrator type Compact Tritator S+ from CRISON and sulphuric acid (0.2 N) from MERCK. Thermoreactor used in COD determination was also from MERCK. The measurement of the sample was taken in a spectrometer type HEλIOS α TERMO from ELECTRON CORPORATION.

Volatile acidity was measured by determination of different VFAs (Table A.2 in Supplementary information file). For determination, samples were previously washed out with distilled water at 3000 rpm 1 min and filtered with a diameter pore filter 0.22 µm. The result was mixed with a solution of ortophosphoric acid and phenol in 1:1 proportion. VFA were determined using a gas chromatograph (Shimadzu GC-2010) according to Montañés et al., [12]. Table A.2 shows the goodness of fit (R^2) of answering factor and retention time of each VFA determined. The system measured the peaks and they were converted to mg VFA/L automatically. Total acidity can be also calculated by weighted sum using molecular weights of VFAs and expressed as mg AcH/L.

Biogas production was determined indirectly, by measuring the cumulative pressure inside the bottles via pressure transducers. Biogas composition was measured by gas chromatograph (SHIMADZU GC-2010) according to Zahedi et al., [14] Commercial

187 mixtures of H_2, CH_4, CO_2, O_2, N_2 and H_2S from Abelló Linde S.A. were used to
188 calibrate the system.
189
190 **3. Results and Discussion**
191
192 *3.1. Pre-treatments and effect in sludge*
193 It can be observed the final biodegradability parameters in terms of CODs, TVS, VFAs
194 and alkalinity after pre-treatments in Table 3. As it can be observed, all the pre-
195 treatments result in an increase of solubility in terms of CODs and TVS. Among
196 different pre-treatments, pre-treatment F showed the highest value of CODs ~ 13.5 g
197 O_2/L; 7 times higher than experiment without pre-treatments (sample WP). So, *B.*
198 *licheniformis* fermentation achieved the maximum solubilization of organic matter in
199 terms of CODs after 12 days of pre-treatment.

Table 3. Values ofCODs, TVS, VFAs and alkalinity before pre-treatments (WP) and after different pre-treatments

Samples name	CODs (g CODs/L)	TVS (g TVS_f/L)	VFA (mg AcH/L)	$Alkalinity_f$ (mg CaO_3/L)
WP	1.88±0.35	20.45±0.18	18.5±6.92	4697
G	2.90±0.39	21.68±0.17	48.3±17.8	5755
C	3.14±0.30	21.13±0.56	143±63.7	5522
P	3.29±0.68	21.05±0.36	263±15.0	6040
F	13.48±0.68	23.99±0.30	554±2.78	6787
1F:1S	6.23±0.24	22.38±0.28	83.5±0.02	5720
1F:9S	3.58±0.41	20.85±0.32	44.2±29.3	3437

200
201 The second best result was obtained after 1F:1S pre-treatment with a final CODs = 6.23
202 g O_2/L, increasing 3 times the CODs value regarding control experiment. The rest of
203 pre-treatments (G, C, P and 1F:9S) reached similar values of CODs = 3.22 ± 0.29 g

O_2/L only 1.7 times superior than WP. Regarding sample 1F:9S, the proportion of fermented sludge was too low for producing a considerable change in CODs of raw sludge. While samples G, C and P comprised the mixture of sludge with enzymes glucanases, cellulases and proteases respectively without reaching optimal conditions for enzymes in order to avoid their reaction before anaerobic reaction process. By this procedure, it was ensured the use of these enzymes as enhancers during anaerobic reaction process instead of as pre-treatments. This fact also explains the great difference in terms of CODs between using *B. licheniformis* in a fermentation unit (F) and using only the *B. licheniformis* isolated proteases (P).

In sample P, on the one hand; the conditions for an optimal enzymatic activity were not reached: the physical contact time was reduced, the temperature was different from the optimal (60ºC) and the concentration was low in comparison with extracellular enzymes produced by *B. licheniformis. B. licheniformis* is a bacterium extensively used for large-scale industrial production because it can secrete large quantities of external enzymes up to 20–25 g/l [10].

On the other hand, regarding sample F, the use of the submerged culture is advantageous because of the ease of sterilization and the self-control of the operation conditions such as pH and/or temperature. In addition, the participation of other kinds of enzymes produced by *B. licheniformis* could enhance the biodegradability of the substrate. Other authors such as Sun et al. [15] suggested that the co-existence of accessory enzymes boosted the action of cellulases depending on the substrates at different degrees. As it has been observed in this work, glucanase and cellulases increase the CODs. So, it is proposed a synergic effect among all the pool of enzymes produced by *B. licheniformis*, not only the proteases but also other hydrolytic enzymes. However, Yu et al. [6] concluded that using a combination of protease and amylase did

not imply a significant improvement in biomethane production efficiency in comparison with using only amylase. So, more investigations must be conducted to determine the synergic effect of combination of different *B. licheniformis* enzymes in the sewage sludge.

The same explanation that CODs can be used for explaining VFA behaviour. Normally, the more organic matter hydrolized (reflected in CODs) the more VFA content. Due to in the enhancer samples (G, C and P) the optimal enzymatic activity conditions were not reached, the VFA values were increased in low proportion (2.6, 7.7 and 14.2 times respectively) in comparison with sample F (30 times) respect to the control WP (Table 3). However, pre-treatments 1F:1S and 1F:9S only increased the VFA content in 4.5 and 2.4, respectively; so, the majority of soluble compounds in these cases were distinct of VFA structures. Furthermore, the protein content of these samples were hydrolized delivering ammonia leading to an increase in alkalinity. In spite of that, in all the cases the calculated proportions VFA/ alkalinity were in the desirable range (0-0.4) for a correct anaerobic digestion process [16]. The ratio VFA/alkalinity is important to be maintained at this level in order to control pH balance between acids generated (VFAs) from acidogenic bacteria and basic compounds contained in digestate (HCO_3-alkalinity) and generated (CO_2) during methanogenic step in AD [17].

In the case of TVS, all of them had similar final values. There was a slight increase in the case of pre-treatments 1F:1S and F. TVS is an analytical parameter that includes both organic solids: suspended and dissolved. One of the main desired effect of pre-treatments is to transform particulate solids to dissolved solids but the total must be the same. The slight increase can be due to better homogeneity of these samples that

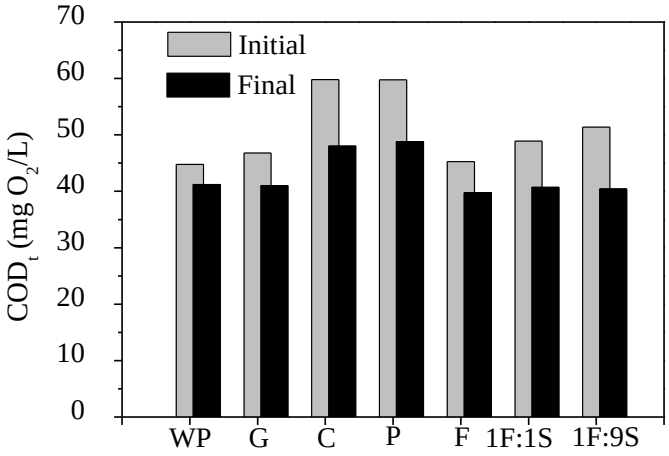
implies more accurate TVS determination.

3.2 BMP results

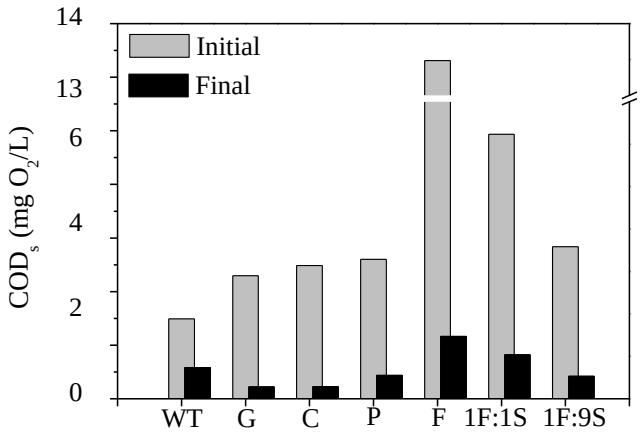
3.2.1. Biodegradability parameters

COD, solids and VFAs degradation are the main factors that determine the biodegradability of the samples. In figure 1 it is shown the initial and final values of CODt and CODs after BMP experiments. Regarding CODt removal using different substrates (Figure 1(a)), in order of decreasing: 1F:9S (27.7%)> P (25.6%)> C (19.7%) > 1F:1S (16.7%) > F and WP (12.1%) > G (3.99%). In general, CODt removal efficiency is in the range 10-20%. However, CODs removal percentages were very similar and more than twice higher (73.4-85.5%) than control G (38%); common CODs removal value in sewage sludge anaerobic digestion at mesophilic range.

As it can be observed the CODt removal is low in comparison with CODs. This is because CODs from sewage sludge does not include microorganisms. But, CODs comprises mainly low molecular weight particles such as proteins, monosaccharides and VFAs which are available for microorganisms to be degraded easily, leading to high CODs removal percentages. A part of this available organic matter, became part of microorganisms which are included in CODt analysis, resulting in low removal CODt percentages [18-19]. For this reason, CODs removal has been usually considered as the key indicator for evaluating the hydrolysis efficiency of pre-treatment, assuming that, biomethane yield is solely related to CODs concentration. However anaerobic digestion is not only related to CODs concentration but also composition; because some recalcitrant soluble structures (high-molecular polymers, long-chain volatile fatty acids, ammonia nitrogen etc) can be formed as a consequence of pre-treatments [18].



(a)



(b)

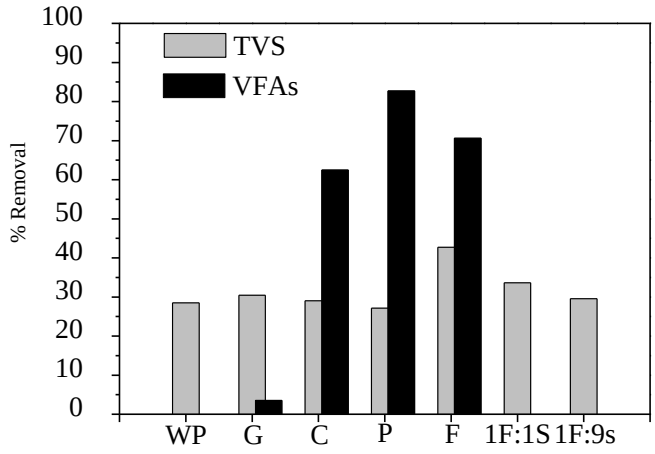
Figure 1. Initial and final CODt (a) and CODs (b) values after biodegradability tests using different pre-treated and enzyme-rich substrates and without pre-treatments (WP).

279 In this case, the results indicate that the majority of available organic matter is degraded.
280 So, although final total organic matter (CODt) was high, the soluble part (which can be
281 utilized to acidogenesis) was low. In this sense, the amount of CODs compared to the
282 CODt can be used as an index of solubilisation. In this case WP and enzymatic
283 enhancers (P, G and C) had 4-6.2% of CODs/CODt whereas pre-treatments had 7.0%;

12.7% and 29.8% for 1F:9S; 1F:1S and F respectively similar than other treatments used in bibliography for increasing solubility [20].

It is important to remark that addition of glucanase (G treatment) (3.99%) is worse than without treatment (12.1%) in terms of CODt. The possible causes can derive from breakage of biofilms formed by the anaerobic consortium. Biofilms are assemblages of microorganisms because of extracellular polymeric substances (EPS) matrix. This matrix is composed basically by polysaccharides such as ß-glucans. The addition of glucanase produce the disaggregation of this cooperative structure reducing the efficiency of the whole process [21].

TVS and VFAs degradation are shown in Figure 2 in terms of percentages. Regarding TVS%removal, in general, all the experiments achieved depuration efficiencies around 30%; with the exception of the F case, where the values were higher than 40%. It can be concluded that the behaviour was similar in all experiments and in the common range (30-50%) of TVS degradation at mesophilic range (even in the control experiment) [17]. According to VFA degradation, 1F:1S, 1F:9S, and WP treatments showed more VFA content at the end of BMP experiment. Accumulation of VFA in one-phase digesters are due to a disequilibrium between production and consumption leading to inhibition of the process. This can be explained due to the low initial content of VFA enhancing more hydrolysis and acidogenesis activity instead of methanogenesis and then more production of VFAs. Anyway, in this work VFA did not produce the inhibition of the process due to



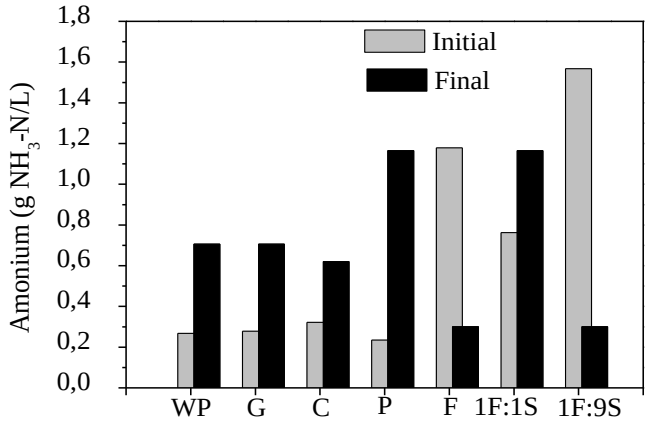
307

308 **Figure 2**. Depuration efficiency in terms of %Removal of TVS and VFAs.

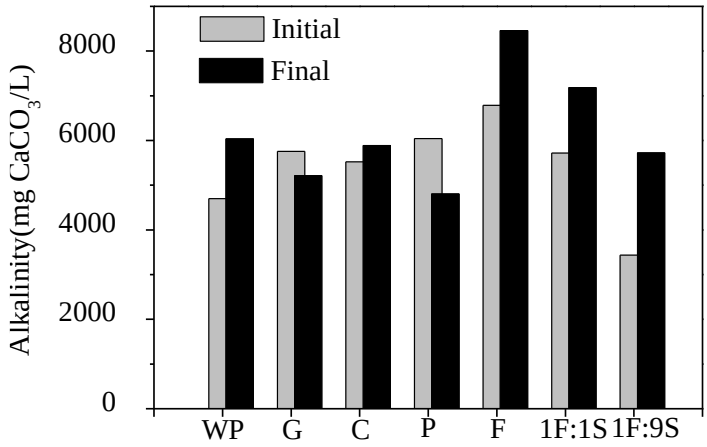
309 initial VFA values were below VFAs inhibiting threshold previously reported [22]. In
310 the case of experiment C, P and F the elimination of VFAs were optimal and in the
311 range of 63-83% typical from sewage anaerobic digestion process. In the case of
312 addition of glucanase (G sample), the removal of VFA was reduced (about 3.5%) due to
313 the inefficient substrate biodegradation by using betaglucanase as it was explained in
314 previous paragraph.

315 In Figure 3(a) and (b) it is shown the initial and final ammonium and alkalinity values
316 in each BMP experiment. As it was explained in section 3.1 hidrolysis implies ammonia
317 release leading to alkalinity increase. In all the experiments, after anaerobic digestion
318 alkalinity was higher (Figure 3(b)), starting from values 3500-6800 to 4800-8200
319 mgCO_3Ca/L (with the exception of samples G and P). Ammonium behaviour before and
320 after biodegradability tests were shown in Figure 3(a). It is known that desirable
321 ammoniacal nitrogen content for anaerobic digestion is around 0.2 g NH_3-N/L [23]. In
322 this sense the fermentation pre-treatment of crude and mixed substrates obtained high
323 values of ammoniacal nitrogen with values of 0.762, 1.57 and 1.17 g NH_3-N/L

respectively for pre-treatments 1F:1S-9S and F. This fact can be explained because protein degradation efficiency during fermentation pre-treatments. It is important to remarck the high content of ammonia of sample P after BMP digestion.



(a)



(b)

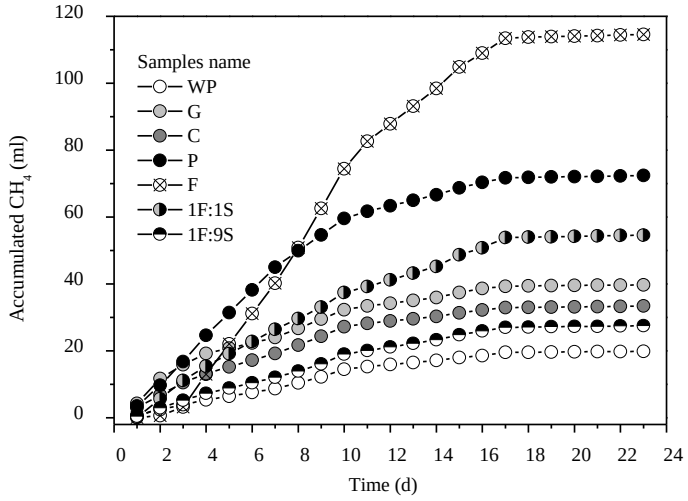
Figure 3. (a) Amoniacal nitrogen content (g NH_3-N/L) ; (b) alkalinity values (mgCO_3Ca/L) at the beginning and at the end of the BMP

This can be explained because the greatly enhanced hydrolysis step or because the effect of protease in other proteins such as other exo-enzymes coming from microbiota

329 [24]. Regarding pH conditions, the pH values were kept constant (data not shown) in
330 the optimal range near 7.5 as it is determined for mesophilic range with a slightly
331 reduction.

332 *3.2.2. Biomethane potential*

333 Figure 4 shows the daily biogas production for each experiment during 23 days. As it
334 can be observed, in general since 15-17 days biomethane production is less than 1%.
335 Maximum values of biomethane production were obtained using substrates F and P with
336 generation of 114 y 72 mL CH_4 as it was expected due to VFAs removal percentages.
337 On the other hand, C experiment only produce 33.2 mL CH_4 biogas, probably due to
338 lower values of VFAs and alkalinity. The rest of experiments also increased biomethane
339 production generating values between 30-40 mLCH_4 in 20 days. Regarding that, control
340 sample (WP) produced only 20 mLCH_4. So, it can be concluded that any of the tested
341 pre-treatments or enhancers improved biomethane generation.



342

343 **Figure 4**. Accumulated biomethane production through the time for different substrates.

344 Table 4 shows the total biomethane production in each experiment. In order of
345 decreasing CH_4 production: F (115) > P (72) > 1F:1S (55) > 1F:9S ≈ G ≈ C (34) > WP

346 (20) mL CH_4. F and P registered the highest CH_4 volume and CH_4 productivity in base
347 of initial and consumed TVS and CODs. In this sense P showed 3 times more
348 productivity than those from pre-treatment F in base of initial and consumed CODs.
349 This fact could be explained because, by using the bacterial treatment (F), it was
350 obtained more quantity of biodegradable compounds reflected in more CODs (4 times
351 higher than P enhancer) after pre-treatment (Table 1) but also more ammoniacal
352 nitrogen content at the beginning of the experiment that cause a period of adaptation of
353 3 days (Figure 4) before starting to produce biomethane

354 It is known that the biomethane production process is easily inhibited at thermophilic
355 temperatures than at mesophilic ones. However, pH also has an important effect and at
356 the beginning of the experiment at pH = 8, increasing free ammonia concentration could
357 be highly increased [17, 23, 25].

Table 4. Parameters of biodegradability: (V) total CH_4 volume collected, CH_4 production yield (Y) based on the initial CODs and initial TVS and on the consumed CODs and consumed TVS.

Samples Name	V (mL_{CH4})	Y_{CODS0} ($mL_{CH4}/gCODs_0$)	Y_{TVS0} ($mL_{CH4}/gTVS_0$)	Y_{CODSC} ($mL_{CH4}/gCODsc$)	Y_{TVSC} ($mL_{CH4}/gTVSc$)
WP	19.8 ±0.40	88.5	8.15	236±41.4	28.6±0.08
G	39.7 ±0.14	115	15.5	136±5.33	50.8±1.32
C	33.4 ±0.17	94.9	14.1	111±4.87	48.8±3.11
P	72.4 ±2.62	212	33.1	289±19.9	122±1.89
F	114 ±0.46	72.3	40.6	87.4±0.63	95.1±0.66
1F:1S	54.6 ±0.82	74.2	20.7	101±6.97	61.4±1.76
1F:9S	27.5 ±3.46	68.8	11.8	89.9±1.32	40.0±0.81

358

359 Sludge protein content was around 30% (6.5 times %TN). In this sense, it can be
360 concluded that the protease showed high efficiency for sludge proteolysis not only used
361 as a purified enzyme but also as a part of degradation machinery of *B. Licheniformis*

bacterium.

However, 1F:9S, G and C showed the lowest biomethane production. In the G and C cases the low amount of initial organic load (CODs) and nitrogen (ammonia) could cause the bacterial washout by nitrogen deficiency limiting the biogas production [26]. On the other hand, 1F:9S caused also inhibition by ammonia content but because excess of that. This effect could also have happened in the 1F:1S pre-treated sample but, here, the organic load content was higher, increasing the C/N ratio (and thus the biogas yield). If the C/N is expressed as available COD (mainly CODs) divided between available N (ammonium) then F (11.3) > 1F:1S (8.3) > 1F:9S (2.25). For this reason, the productivity of methane in base of TVS showed the same order in values F (40.6) > 1F:1S (20.7) > 1F:9S (11.8) ml CH_4/ g TVS_0. The productivity increase of different enhancers and pre-treatments studied can be compared with others previously reported [6,27-29]. In this sense the enhancer P and pre-treatment F obtained the best results in %biomethane enhancement (306% and 398% respectively) even in comparison with the best previously reported by Yin et al. [27] (236% biomethane enhancement) which used rich enzyme fungal mash (mainly carbohydrases) during 24h at 60ºC as pre-treatment. Other authors also have used proteases as pre-treatments [6] and enhancers [28-29] but the %biomethane enhancements obtained were only 23.1%; 37 and 155%, respectively.

4. Conclusions

Biochemical treatments tested for sewage sludge, previously to anaerobic digestion, result in higher depuration efficiency in terms of CODs (73-85%), CODt (16-28%) and TVS (30-42%) in comparison with control experiment: CODs (38%), CODt (12%) and TVS (28%) enhancing the stabilization and biodegradability of sludge. This fact is

reflected in biomethane potential production. All the pre-treated and enzyme-rich sludge generated more biomethane than control one. The optimal pre-treatments are due to protein degradation using proteases from *B. licheniformis* purified (72.4 ml CH_4) or by treatment with the bacteria population in situ (114 ml CH_4). Both treatments increase the biogas volume in 3.65 and 5.77 times respectively compared with control. The selection of optimal pre-treatment must take into account the final C/N ratio. In this way, the combination of several pre-treatments could be beneficial. Apparently all these methods have extra costs derived from different additional operations. However, all of them have a net positive benefit as a results of higher levels of biogas production, or in other words, more energetic self-sufficiency.

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527 *Samples names:*

WP: without pre-treatment;

G: addition of glucanase enhancer;

C: addition of cellulase enhancer;

P: addition of protease enhancer,

F: fermentative pre-treatment with *B. licheniformis*;

1F:1S: mixture 1:1 of fermented sludge and raw sludge;

1F:9S: mixture 1:9 of fermented sludge and raw sludge

Figures 1 and 3

 Values of indicated parameters measured before starting BMP experiment

 Values of indicated parameters measured after concluding BMP experiment

Indicated parameters:

Figure 1(a): COD_t

Figure 1(b): COD_s

Figure 3(a): Ammoniacal nitrogen (g NH_3-N/L)

Figure 3(b): Alkalinity (mgCO_3Ca/L)

Figure 2

 Total Volatile Solids removal percentage

 Volatile fatty acids removal percentage

Figure 4 *Samples names:*

 , WP: without pre-treatment;

 , G: addition of glucanase enhancer;

 C: addition of cellulase enhancer;

 P: addition of protease enhancer,

 F: fermentative pre-treatment with *B. licheniformis*;

 1F:1S: mixture 1:1 of fermented sludge and raw sludge;

 1F:9S: mixture 1:9 of fermented sludge and raw sludge