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Highlights

Extrusion and enzymatic hydrolysis treatments enhanced methane produced from corn cob.

Sequential alkali extrusion and enzymatic hydrolysis achieved the major improvement.

Fungal extract was as suitable as Ultraflo® L for the corn cob enzymatic treatment.

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Extrusion and enzymatic hydrolysis as pretreatments on corn cob for biogas production

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Abstract

Although agricultural wastes such as corn cob could be employed as substrates for biogas production, their lignocellulosic nature entails difficulties for their use. For this reason, a pretreatment of the biomass is recommended. Considering that there is limited information about the effects of extrusion as pretreatment on biogas production from biomass, this pretreatment alone or in combination with alkali and/or with the enzymatic hydrolysis was evaluated to improve the production of methane by anaerobic digestion of corn cob. The lignocellulosic composition, methane yields and biogas composition of the corn cob before and after pretreatments were compared. Among all the pretreatments studied, the sequential alkali extrusion and enzymatic hydrolysis (A+FE+H) pretreatment accomplished the major improvement with an increase of the methane volume produced of 22.3% in regards to the anaerobic digestion of the raw corn cob.

Keywords: methane, biogas, pretreatment, extrusion, enzymatic hydrolysis, corncob.

Abbreviations: AD, anaerobic digestion; A+FE, alkali and fast extrusion; A+FE+H, alkali and fast extrusion followed by enzymatic hydrolysis; BMP, biochemical methane potential; FE, fast extrusion; FE+H, fast extrusion followed by enzymatic hydrolysis; H, enzymatic hydrolysis; NDS, neutral detergent soluble compounds; SE, slow extrusion; SE+H, slow extrusion followed by enzymatic hydrolysis; VS, volatile solids.

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1 Introduction

Biogas, a mixture formed mainly by methane and carbon dioxide [1], is a renewable and cleaner form of energy that could partially substitute the conventional petroleum-based sources of energy [2].

Anaerobic digestion of lignocellulosic agricultural residues represent a good alternative for biogas generation because of the large availability of this kind of biomass, the lack of competition with food or feed chains, and the conversion of these wastes into feedstocks taking advantage of its composition while mitigating the problem of the management of these wastes [3]. Despite the many advantages offered by the use of agricultural wastes, its degradation is complex due to the inherent properties and structure of lignocellulosic materials which limit their digestibility [4,5]. To overcome those limitations, pretreatment of materials is recommended in order to make them more amendable for anaerobic digestion [5–7]. In this regard, de-polymerization of lignocellulose into monomers accomplished by an enzymatic hydrolysis can help to promote biogas production [8–10].

However, previous pretreatments, to disturb and change the highly ordered structure of native lignocellulose are positive to increase the accessibility of enzymes [11]. Among the various pretreatment processes available for lignocellulosic materials, extrusion is simple, cheap, and has proven to be viable. Moreover, this technology is applicable to a wide range of biomass and has great flexibility and adaptability with respect to scale-up and process modifications [12]. Extrusion is a process where uniformly moistened biomass material is passed through a extruder barrel applying pressure with a screw [7]. Extruded lignocellulosic materials result in larger specific areas [11], a lower bulk density and a higher water-holding capacity which favors the

access for microorganisms and enzymes [11,13]. This technology has been reported as an effective physical method for biomass-size-reduction that enhance anaerobic digestion of lignocellulosic materials, and consequently the biogas production [11,14].

Although extrusion can effectively enhance the anaerobic digestion performance, its effect is still limited [13]. To improve the extrusion process, chemical solutions such as alkalis can be incorporated to increase the severity of the pretreatment to the lignocellulosic biomass [7,11]. Alkalis (e.g. sodium hydroxide) are delignification agents [11] with a marked role in de-esterification of plant cell walls [15], and in solubilization of some hemicelluloses and lignin [11]. Moreover, the combination of alkali and extrusion processes produces more pores [11].

Most researches have focused on the extrusion effects on bioethanol production. However, there is limited information about the effects of extrusion as pretreatment on biogas production from biomass [12]. For this reason, the objective of this study was to analyze the effect of extrusion, the combination of extrusion with alkali, and the sequential of extrusion or extrusion combined with alkali and enzymatic hydrolysis, as pretreatments of corn cob for the anaerobic production of methane.

2 Materials and methods

2.1 Substrate

Corn cob is the elongated woody core of maize resulting from the removal of embedded grains, and it is considered a lignocellulosic agricultural waste. Corn cob was collected from Mondariz (Pontevedra, Spain). Corn cob was dried at room temperature, grinded in an electric shredder MTD 220E (Saarbrücken, Germany), then milled in an

77 IKA® Werke universal mill model M 20 (Staufen, Germany) and sieved to get a particle
78 size below 2 mm.

79 2.2 *Van Soest fiber analysis*

80 Total solids (TS) and volatile solids (VS) of the substrate and the inoculum were
81 determined gravimetrically following standard methods.

82 The Van Soest fiber analysis allows the organic matter fractionation into soluble
83 components, hemicellulose, cellulose and lignin by sequential extraction with neutral
84 and acid detergents, followed by strong acid extraction [16]. Therefore, substrates
85 composition was determined with the help of a Gerhardt Fibrebag system (Les Essart Le
86 Roi, France). Changes in fiber composition induced by enzymatic hydrolysis, extrusion,
87 alkaline extrusion and combined pretreatments were investigated by analyzing the
88 separated solid fraction as described Pérez-Rodríguez et al. [17].

89 2.3 *Pretreatments*

90 2.3.1 *Extrusion pretreatment of corn cob*

91 A twin-screw extruder (HAAKE Rheomex™ PTW OS, Fisher, France) was
92 employed to pretreat the milled corn cob.

93 Two different extrusion conditions were assayed: a slow feed rate extrusion
94 using 200 g corn cob/h and 179 g distilled water/h for 55 s (SE), and a faster feed rate
95 extrusion using 400 g corn cob/h and 360 g distilled water/h for 35 s (FE).

96 Additionally, the best condition (FE) was also assayed in combination with an
97 alkali (sodium hydroxide) as pretreatment (A+FE) by including a solution of 0.4%
98 NaOH (w/v) to the extruder using a volumetric pump.

Extrusion was carried out mixing equal masses of corn cob and distilled water or sodium hydroxide solution (1:1 (w/w)). After alkali extrusion pretreatment, samples were washed until neutrality.

Extrusion was carried out at room temperature. As consequence to the friction between the screw and the biomass during the extrusion operation a slightly elevation in temperature was detected but no extra heating was applied.

2.3.2 Enzymatic hydrolysis

Raw or pretreated samples were submitted to enzymatic hydrolysis using the commercial enzymatic preparation Ultraflo® L or *Aspergillus* enzymatic extract produced by solid-state fermentation of corn cob.

Ultraflo® L, an enzymatic cocktail with endo-1,3(4)- β -glucanase, and collateral xylanase, cellobiase, cellulase, and feruloyl esterase activities among others, was graciously provided by Novozymes (Bagsvaerd, Denmark).

Obtainment of *Aspergillus* enzymatic extract used in this work, including fungal production and enzyme extraction, was described in Pérez-Rodríguez et al. [18]. Briefly, solid state fermentation (SSF) was carried out in 250 mL Erlenmeyer flasks containing 5 g (dry weight) of corn cob moistened (1:3.6 (w/v)) with a concentrated nutrients salts solution. After being sterilized, each flask was inoculated with one milliliter of 1×10^6 spores of *Aspergillus terreus* CECT 2808/g dry corn cob. Cultures were incubated statically at 30°C for 7 days. Crude enzymatic extracts were obtained by adding distilled water (10 mL/g dry corn cob) to each flask and incubated for 1 h, 200 rpm at 30 °C. After incubation, the flaks content was filtered, and the permeate obtained was centrifuged at 2755×g for 15 min. The supernatant was recovered and constitute the hereinafter denominated *Aspergillus* enzymatic extract.

The feruloyl esterase, xylanase, cellulose and cellobiase activities of both Ultraflo® L and *Aspergillus* enzymatic extracts were determined in a previously work [18].

Enzymatic hydrolyses were performed as it was previously described by Pérez-Rodríguez et al. [17] by the addition of a volume of Ultraflo® L or of the *Aspergillus* enzymatic extract, which mean 0.2 U of feruloyl esterase per gram of dry substrate. After hydrolysis, samples were boiled (100°C for 10 min) for thermal inactivation of enzymes, and centrifuged in a Sorvall Legend X1 centrifuge from Fisher Scientific at 11250 x g for 15 min.

Then, the amount of carbohydrate reducing ends (Dinitrosalicylic acid method described by [19]) of the supernatant (liquid fraction) was determined.

Finally, supernatants were separated and the solid residues were used as substrate for anaerobic digestion to produce biogas.

2.4 Anaerobic digestion of corn cob samples

Raw or pretreated corn cob (1 g dry substrate) were anaerobically digested by a mesophilic anaerobic sludge in 575 mL serum bottles with a working volume of 400 mL as it was previously described by Pérez-Rodríguez et al. [17].

Two control tests were done under the same conditions. Controls without substrate were done to determine the endogen gas production of inoculum and make the appropriate correction of results. Other controls with ethanol as substrate were done to verify the aptitude of inoculum for biomass degradation.

2.5 Biochemical Methane Potential (BMP) test

Bottles were periodically submitted to Biochemical Methane Potential (BMP) test. The BMP value can be used as an index of the anaerobic biodegradation potential [20], measuring the methane or biogas produced by a known amount of substrate in batch anaerobic conditions.

Biogas volume produced by the anaerobic digestion of corn cob samples was measured volumetrically using the displacement method with acidified water as solution. The biogas was introduced into the liquid column directly from the bottle and the biogas volume produced was calculated from the measurement of change in barrier solution height. All volumes were expressed under normal conditions (273 K temperature and pressure 1013 mbar).

The gas composition (CH_4 , CO_2 , O_2 , N_2 and H_2S) was analyzed employing a Varian CP 4900 micro-gas chromatograph (Middelburg, Netherlands) under the conditions and using the method previously described by Pérez-Rodríguez et al. [17].

The methane produced by a known mass of volatile solids of corn con sample ($\text{VS}_{\text{substrate}}$) was calculated as the difference between the volume of methane produced from the corn cob sample ($V \text{CH}_{4\text{substrate}}$) minus the volume of methane produced by the control without substrate ($V \text{CH}_{4\text{blank}}$):

$$L \text{CH}_4/\text{kg VS} = (L \text{CH}_{4\text{substrate}} - L \text{CH}_{4\text{blank}})/\text{VS}_{\text{substrate}}$$

The methane yield was expressed as the volumetric methane production (liter) under standard conditions per unit of substrate fed expressed as volatile solids (VS).

Results are presented as the average of at least two replicas along with their standard deviation.

3 Results & Discussion

3.1 *Effect of slow or fast extrusion, alkali extrusion and enzymatic hydrolysis on corn cob composition*

Untreated corn cob is composed by $42.0 \pm 0.15\%$ of cellulose, $45.9 \pm 0.90\%$ of hemicellulose, $2.8 \pm 0.20\%$ of lignin, and $9.3 \pm 0.95\%$ of neutral detergent soluble compounds (NDS).

The use of pretreatments involved modifications in the main lignocellulosic fractions (cellulose, hemicellulose, lignin and NDS) of corn cob in comparison with untreated material as it can be seen in [Figure 1](#) and [Figure 2](#).

The pretreatments of extrusion (slow (SE) or fast (FE) extrusion and the alkali fast extrusion (A+FE)) involved similar variations in the composition of corn cob ([Figure 1](#)). These modifications were characterized by a decrease in the percentage of hemicellulose (from $45.9 \pm 0.90\%$ in the raw corn cob to $38.9 \pm 0.52\%$ (SE), $40.6 \pm 0.19\%$ (FE), and $39.4 \pm 1.20\%$ (A+FE)) as well as in the percentage of cellulose (from $42.0 \pm 0.15\%$ in the raw corn cob to $34.8 \pm 0.23\%$ (SE), $34.8 \pm 1.24\%$ (FE), $37.6 \pm 1.22\%$ (A+FE)). The changes in these fractions were accompanied by an increment in the NDS whose percentages doubled the value quantified in the raw corn cob (from $9.3 \pm 0.95\%$ in the raw corn cob to $19.0 \pm 0.60\%$ (SE), $18.7 \pm 1.39\%$ (FE), and $17.1 \pm 0.48\%$ (A+FE)). Thus, extrusion pretreatments caused depolymerization of cellulose, hemicellulose, and lignin of corn cob which is in accordance with the analysis reported by Panepinto [21].

Regarding the samples submitted to the enzymatic hydrolysis (H) as sole pretreatment or after the extrusion pretreatments (SE+H, FE+ H and A+FE+H) ([Figure](#)

2), the reduction of the percentage of cellulose was remarkable comprising between 43.3 (H) to 57.5% (SE+H). Moreover, the NDS percentages of the samples enzymatically hydrolyzed ($41.9 \pm 0.73\%$ (H), $41.7 \pm 0.86\%$ (SE+H), $42.9 \pm 0.84\%$ (FE+H), and $43.5 \pm 0.04\%$ (A+FE+H)) were 4.5 times higher than the percentage represented by the same fraction in the raw corn cob composition ($9.3 \pm 0.95\%$). Due to the term NDS includes soluble carbohydrates, proteins, lipids, minerals and vitamins [16], which are easily assimilable compounds for methanogenic microorganisms, higher NDS availability in samples should increase the methane production rate. In summary, the results shown a de-polymerization of the samples as consequence of the pretreatments applied and it could be assumed that their anaerobic digestion would lead to higher methane productions.

Additionally, the carbohydrate reducing ends were also analyzed in the liquid phase from enzymatic hydrolyses, obtaining the following amounts: 8.39 ± 0.05 g carbohydrate reducing ends/L (H), 8.86 ± 0.04 g carbohydrate reducing ends/L (SE+H), 8.95 ± 0.06 g carbohydrate reducing ends/L (FE+H) and 9.34 ± 0.02 g carbohydrate reducing ends/L (A+FE+H). According to [22] an increment in sugar availability due to extrusion was one of the causes to explain the increment observed in methane production. Therefore, higher sugars concentration can be related to higher volumes of methane produced. Consequently, in our case, the highest methane level could be expected to be achieved by the anaerobic digestion of alkali fast-extruded corn cob (A+FE+H).

3.2 Impact of the extrusion pretreatments on the production of methane

The evolution of methane production from raw or extrusion pretreated corn cob during 32 days of anaerobic digestion is shown in Figure 3.

214 All the extrusion pretreatments enhanced the volume and rate of methane
 215 production from the anaerobic digestion of corn cob (Figure 3a). However the extrusion
 216 feed rate hardly influenced the process, since a methane yield of 269.6 ± 1.31 L CH₄/kg
 217 VS ($6.54 \pm 0.03\%$ more than the raw corn cob) was obtained during the lower speed rate
 218 (SE) and 270.6 ± 3.11 L CH₄/kg VS ($6.94 \pm 0.08\%$ more than the raw corn cob) during
 219 the faster speed rate (FE). There is a correspondence between the production of methane
 220 and the composition of the extruded samples since no compositional differences
 221 appeared between fast or lower speed rate pretreatments (Figure 1). Moreover, our
 222 results are in agreement with Khor et al. (2015), which lend support to the claim that
 223 different extrusion speeds do not cause important variations in the volume of methane
 224 released, since these authors did not observe notable differences in the percentage of
 225 improvement between fast and slow extrusion neither for maize straw ($36.4 \pm 6.9\%$
 226 from fast extruded and $36.3 \pm 4.7\%$ from slow extruded) nor for sprout stem ($29.6 \pm$
 227 3.2% from fast extruded and $26.2 \pm 5.9\%$ from slow extruded). However, from an
 228 energy point of view fast extrusion is more favorable as biomass pretreatment [23].
 229 Thus, the higher feed rate extrusion (FE) was selected for the assay of extrusion
 230 combined with sodium hydroxide solution (A+FE). The anaerobic digestion of the
 231 A+FE corn cob resulted in 275.6 ± 1.14 L CH₄/kg VS, which implicated an additional
 232 surplus in the methane produced versus the FE of 1.28 time. This enhancement in
 233 methane respect to FE is analogous to those obtained from other alkali pretreated and
 234 extruded biomass such as maize straw (1.20 times), grass (1.15 times) and sprout stem
 235 (1.08 times) [23].

236 Higher percentages of amelioration were described for the methane production
 237 from other extruded biomass involving 72.2% for rice straw [24], 70 % for maize straw,
 238 62% for grass [14], between 26–49% for grass, maize straw and sprout stem [23], or

between 12–29% for wheat straw [22]). Similarly, the combination of extrusion and alkali pretreatment resulted in an overall increase of 54.0% for rice straw (Zhang et al., 2015), 53.8% for grass, 47.6% for maize straw and 38.6% for sprout stem [23]. Therefore, despite being positive, the extrusion pretreatments of corn cob did not reach a high percentage of improvement in regards to those reported by other authors.

3.3 Impact of the enzymatic hydrolysis of raw or pretreated corn cob on the production of methane

The influence of enzymatic hydrolysis was studied independently or in addition to the previous extrusion pretreatments. Figure 3b summarizes the results obtained by anaerobic digestion after 32 days.

Raw corn cob was enzymatically hydrolyzed by Ultraflo® L. The amount of methane generated from the hydrolyzed corn cob was 289.8 ± 0.79 L CH₄/kg VS, proving a modest enhancement in regard to the anaerobic digestion of the fast extruded (5.2%) and to the raw (14.0%) corn cob. Consequently, the enzymatic hydrolysis accomplished a higher volume of methane than the extrusion.

The samples submitted to the extrusion pretreatments (SE, FE and A + FE) were also hydrolyzed by Ultraflo® L. The initial methane production rate from the extruded and hydrolyzed samples were higher than from the hydrolyzed samples. However, similar levels of methane were attained from the samples hydrolyzed or extruded and hydrolyzed (series H, SE+H and FE+H) in the long term of the anaerobic digestion.

The volume of methane generated only increased appreciably in the A+FE+H samples where 309.4 ± 5.11 L CH₄/kg VS were achieved. Nevertheless, although the anaerobic digestion of the corn cob pretreated with the combination A+FE+H exceed

the volume of methane obtained by the fermentation of the other pretreated samples, some researchers point that the additional methane produced as a result of the sum of pretreatments could be insufficient to offset the additional costs [25]. Therefore, economic studies should be assess to evaluate the convenience of submitting corn cob to the combination of pretreatments or maybe to just one of the types of pretreatment (extrusion or enzymatic hydrolysis).

3.4 Comparison of Ultraflo® L and *Aspergillus* extracts for the enzymatic hydrolysis of raw corn cob on the production of methane

From an economic point of view, reducing the price of the enzymes used in the enzymatic processes is a key research priority [1]. In this way, Singhania et al.[26] and Thomas et al. [27] support that production of enzymes by solid-state fermentation using low-cost agro-industrial residues as substrates, offers potential benefits for bioprocessing because of the high production of the enzymes generated as well as for the reduction in the costs derived from the sources employed. Thereby, solid-state fermentation of corn cob was performed using the strain *Aspergillus terreus* CECT 2808 [18] and it was compared the anaerobic digestion after hydrolysis using the *Aspergillus* enzymatic extract or the commercial Ultraflo® L (Figure 3c).

The volume of methane produced from the corn cob hydrolyzed by *Aspergillus* enzymatic extract was below that produced from the raw corn cob until day 9th of anaerobic digestion. Then, the volume of methane produced from the sample hydrolyzed by the *Aspergillus* extract gradually increased reaching the methane levels obtained from the anaerobic digestion of the sample hydrolyzed by Ultraflo® L. Therefore, despite of the corn cob hydrolyzed by Ultraflo® L resulted in an initial faster methane production rate than that attained by the *Aspergillus* enzymatic extract, the

methane accumulated during 25-26 days was the same (264.85 ± 7.70 L CH₄/kg VS (Ultraflo® L) and 262.9 ± 8.50 L CH₄/kg VS (*Aspergillus* enzymatic extract)), showing the potential of this extract to be used as an alternative to the commercial enzymatic cocktail.

3.5 Methane yield in biogas produced

Biogas comprises primarily methane and carbon dioxide, and other minor components (water, hydrogen sulfide, nitrogen, ammonia and oxygen). However, the substrate employed and the digestion conditions influence strongly on the composition of the biogas [28].

Generally, the main final use of the biogas is for energy [29]. For this application, the methane is considered to be useful, while carbon dioxide and the minor components are not [29]. In this sense, the presence of carbon dioxide reduces considerably the calorific value of the biogas, and the minor components may lead to critical operational problems [28,29]. Moreover, the avoidance of the transport of the carbon dioxide reduce the volume and the density of the biogas, thus reducing transport costs [29]. Therefore, a widespread practice is the biogas upgrading to a higher quality combustible gas, the so-called biomethane, requires removing most of that carbon dioxide and of the minor components [28].

Considering that, the higher percentage of methane present in biogas involves a closer composition of the biogas to biomethane target. Consequently, in addition to the volume of methane generated by the anaerobic digestion, it is important to measure the composition of biogas. The main fractions of biogas, including methane and carbon dioxide, were described in Table 1. Comparing to the biogas produced by the digestion

of raw corn cob, all the pretreatments assayed had a positive effect rising the percentage of methane in biogas produced (Table 1).

4 Conclusions

Although corn cob proved to be an adequate substrate to produce biogas by anaerobic digestion under the studied conditions, the pretreatments assayed demonstrated to be effective in order to increase the methane production. There were not considerable differences between the extrusion feed rate but the addition of an alkali solution during extrusion involved an improvement in the methane yield. In regards to the enzymatic hydrolysis, although both the commercial Ultraflo® L and the *Aspergillus* enzymatic extract were efficient pretreatments increasing the volume of methane obtained from the corn cob compared with the raw material, Ultraflo® L achieved the highest methane production rate. Despite the combination of extrusion and hydrolysis reached similar levels of methane production than the extruded samples, the combination of pretreatments (sequential alkali extrusion and enzymatic hydrolysis, A+FE+H) accomplished the major improvement in both production rate and final volume of methane with an overall increase of 22.3% regarding the anaerobic digestion of raw corn cob.

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Table 1: Main components of biogas produced by the anaerobic digestion of raw and pretreated corn cob.

Corn cob samples/main components of biogas	CH ₄ (%)	CO ₂ (%)
Raw	57.5±0.04	42.4±0.03
Slow extrusion (SE)	60.3±0.37	39.9±0.24
Fast extrusion (FE)	60.6±0.18	39.6±0.19
Alkali+fast extrusion (A + FE)	60.5±0.46	39.5±0.28
Ultraflo L hydrolysis	60.2±0.26	40.2±0.38
<i>Aspergillus</i> extract hydrolysis	61.4±0.44	38.5±0.42
Slow extrusion+hydrolysis (SE + H)	65.6±0.94	37.9±0.26
Fast extrusion+hydrolysis (FE + H)	65.4±0.84	36.5±0.11
Alkali+fast extrusion+hydrolysis (A+ FE + H)	65.4±0.41	37.8±0.66

Figure Legends

Figure 1: Comparison between contents of neutral detergent soluble compounds (dotted), hemicellulose (dark grey), cellulose (light grey), and lignin (striped) of untreated corn cob (raw) or submitted to different extrusion pretreatments: slow extrusion (SE), fast extrusion (FE), and alkali and fast extrusion (A+FE).

Figure 2: Comparison between contents of neutral detergent soluble compounds (dotted), hemicellulose (dark grey), cellulose (light grey), and lignin (striped) of untreated corncob (raw) or submitted to hydrolysis (hydrolyzed) or to the different extrusion pretreatments followed by enzymatic hydrolysis: hydrolysis of the slow extruded corn cob (SE+H), hydrolysis of fast extruded corn cob (FE+H), and hydrolysis of the alkali and fast extruded corn cob (A+FE+H).

Figure 3: Cumulative methane productions as a function of anaerobic digestion time obtained from raw corn cob (*), (a) after the different extrusion pretreatments (slow extrusion (SE) (○), fast extrusion (FE) (△), alkali and fast extrusion (A+FE) (□)); (b) after the hydrolysis by Ultraflo® L (◆), after the combination of extrusion and hydrolysis by Ultraflo® L (slow extrusion followed by enzymatic hydrolysis (SE+H) (●); fast extrusion followed by enzymatic hydrolysis (FE+H) (▲); alkali and fast extrusion followed by enzymatic hydrolysis (A+FE+H) (■)), and (c) after the hydrolysis by Ultraflo® L (◆) or by the *Aspergillus* enzymatic extract (▪). In some cases, the error bars in that scale are smaller than the symbols and cannot be seen.

Figure 1

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Figure 2

495 a)

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497 b)

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499 c)

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Figure 3