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CELLULASE EFFECT ON ANAEROBIC DIGESTION OF MAIZE SILAGE UNDER DISCONTINUOUS OPERATION

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ABSTRACT: This paper studied the effect of adding an enzyme (ellulose) on anaerobic digestion of maize silage. We compared materials at chopping lengths of 8 mm (MSL), 4mm (MSS) and natural size (Ms) under a mesophilic and discontinuous operation (batch process). Hence, we found the process to be significantly influenced by particle size. Moreover, the ellulose addition did not significantly impact biogas production after a 35-day digestion period. Ms and MSS displayed an improved response to all variables when compared with MSL and MSL+C, with significant differences. Studies on the refractory fraction at infinite time (R_0) have demonstrated that the lowest values correspond to Ms and MSS (0.122 and 0.155, respectively). The Kinetic approach and the Ultimate Biodegradability test are useful tools to evaluate the effect of the addition of an enzyme to the anaerobic process.

KEYWORDS: ellulose, silage, biogas, kinetics, biodegradability.

EFEITO DA ADIÇÃO DE ENZIMA (CELULASE) NA DIGESTÃO ANAERÓBIA DE SILAGEM DE MILHO EM OPERAÇÃO DESCONTÍNUA

RESUMO: O efeito da adição de celulase sobre a digestão anaeróbia de silagem de milho em partículas, com ellul de 8 mm de comprimento (MSL), em reatores operados em bateladas sob condição de temperatura mesófila, foi avaliado e comparado com silagem de milho em ellul natural (Ms) e partículas menores de silagem de milho, 4 mm de comprimento (MSS). Constatou-se que o ellul das partículas desempenha um importante papel sobre o desempenho do ellulo de digestão anaeróbia. Para todos os tamanhos de partículas de silagem de milho avaliados, a adição de celulase © não produziu efeitos significativos sobre a produção de biogás após 35 dias de digestão anaeróbia. Ms e MSS responderam melhor em todas as variáveis quando comparadas com MSL e MSL+C, com diferenças significativas. Estudos sobre a fração refratária no tempo infinito (R_0) mostraram que os menores valores corresponderam a Ms e MSS (0,122 e 0,155, respectivamente). Ajuste de modelo cinético e avaliação da biodegradabilidade última foram as ferramentas utilizadas para avaliar o efeito da adição de enzimas sobre ellulos anaeróbios.

PALAVRAS-CHAVE: celulase, silagem, biogás, cinética, biodegradabilidade.

INTRODUCTION

Lignin is a very well-known compound, which makes up approximately 50% (w/w) of agricultural wastes and energy crops. Lignocellulosic substrates are the main feedstock of biogas plants in countries such as Germany and Austria, where favorable rates for feeding renewable

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energy into the power grid have led to an increase in the use of anaerobic technology (NEUREITER et al., 2005). The rate-limiting step during the anaerobic digestion of lignocellulosic material is the hydrolysis of cellulose and ellulose oses. The increase of hydrolysis rate is critical to the improvement of biomass conversion efficiency during anaerobic digestion (ROMANO et al., 2009).

The addition of a wide variety of enzyme products comprising cellulases, hemicellulases, xylanase, pectinase, lipase, lactase and others, either alone or in combination to anaerobic digestion systems, can increase the bioavailability of carbon sources, producing energy by acting directly in both pre-hydrolysis and hydrolysis of not only energy crops and agricultural residues but also sludge and grease wastes. Target feedstocks so far have been maize silage, corncob mix, straw, rye, wheat, and isolated fibers (SUÁREZ-QUIÑONES et al., 2012).

About enzyme addition, there have been many studies aimed at enhancing biogas production in feedstock preservation techniques such as silage, mainly because it can lead both to energy losses during storage and specific methane yields. NEUREITER et al. (2005) tested different additives for silage preparation, their effect on the composition of whole crop maize silage and their potential to improve methane production. Treatments included ensiling without additives, inoculation with silage starters and the addition of amylase. Additionally, the effects of improper silage preparation such as the insufficient compression of the material and the addition of spoilage organism *Clostridium tyrobutyricum,* were evaluated. They found that although *C. tyrobutyricum* caused the highest storage losses, this was compensated by the increased formation of methane. It remains to be investigated whether storage losses in laboratory experiments are significant on a technical scale. The results demonstrate that there is a trade-off between desirable storage properties and maximum methane yields. Further studies developed by PLÖCHL et al. (2009) investigated alfalfa, grass and maize silages without additives, with chemical and with biological additives and used these both to compare to fresh material and in batch anaerobic digestion tests. In an economic assessment, the costs of the silage additives were compared to the additional proceeds that could be achieved from improving digestibility and preventing silage losses. In their study, it is significant that the question of whether or not silage additives can produce improved digestibility is not yet answered, although there are hints that the increased concentrations of organic acids does increase specific methane yields. It is therefore necessary to continue with basic research studies on the effects of ensiling on the methane yield of crops and to further develop selective silage additives for the ensiling of biogas feedstock.

Regarding the direct addition of an enzyme to the anaerobic digestion of feedstock, there are studies with both positive and negative results in targeting the enhancement of methane yield. ROMANO et al. (2009) found no biogas production improvement when adding ellulose, hemicellulase and β-glucosidase to a thermophilic anaerobic digestion of wheat grass. Three digestion configurations were simulated and investigated. Results showed that even though the enzyme products were capable of solubilizing wheat grass when used directly, when they were added with the anaerobic digester ellulose, the biogas yield, methane yield and the reduction of solids were not enhanced. The direct application of enzyme to batch digesters was also attempted by SCHIMPF & VALBUENA (2009) and HÄBLER et al. (2010). Enzyme preparations of ellulose, pectinase and lactase, either alone or in combination, were applied to various energy crops at different chopping lengths. Corn and rye silages were the analyzed substrates and the authors found both increasing and decreasing effects. Similar behavior was found by DIAK et al. (2012), who used batch reactors and continuous flow reactors, which were designed and operated as septic tanks, in order to evaluate whether enzymatic treatment would increase the hydrolysis and digestion rates in primary sludge. Overall, no significant improvement was observed in enzyme-treated reactors compared with control reactors.

Recent studies have focused on improving the solubilization of sludge (YU et al., 2013), focusing on enzyme mechanisms in terms of solubilization of extracellular polymeric substances. DONOSO-BRAVO & FDZ-POLANCO (2013) used a first-order model to obtain some kinetic parameters and therefore the preliminary basic design of a continuous process, by stimulating

methane production through the addition of lipase to anaerobic treatments of sewage sludge and grease trap.

A lack of reports addressing the kinetic approach and aiding a better understanding of changes to biodegradability when adding enzymes is the primary motive for this paper, which evaluates the effects of the addition of ellulose to maize silage of different chopping lengths.

MATERIAL AND METHODS

Enzyme preparation

The enzyme used was industrially produced ellulose, with an Acremonium sp. (mold) microorganism. The culture filtrate was stored at pH 4 and with an optimum temperature of 50 °C. The major components were carboxymethyl cellulose [1309 ± 103 U g^{-1} (dilution of enzyme 1:50/60/70; mean: 1:60)], arabinogalactan [1641 ± 117 U g^{-1} (dilution of enzyme 1:80/90/100; mean: 1:90)], glucomannan [1454 ± 84 U g^{-1} (dilution of enzyme 1:50/60/70; mean: 1:60)], arabinoxylan [1522 ± 95 U g^{-1} (dilution of enzyme 1:80/90/100; mean: 1:90)], pectin [9283 ± 582 U g^{-1} (dilution of enzyme 1:80/90/100; mean: 1:90)] and filter paper [105 ± 5 U g^{-1} (dilution of enzyme 1:4,9/5,1; mean: 1:5)]. The effect of adding the enzyme was measured under biogas reactor conditions at pH 7.5 and at 38 °C (100.4 °F).

Analytical methods

Total solids (TS) content and volatile solids (VS) percentage were analyzed according to standard methods (APHA et al., 2005) and pH measurements were conducted with a WTW PMX3000 pH-meter. Volatile fatty acids and Total organic carbon ratio (VFAs/TOC) were conducted with FOS/TAC 2000 equipment. The composition of biogas (CH_4, CO_2, O_2 and H_2S) was determined using a Multitec 540 Gas Analyzer.

Experimental procedure

Experiments were carried out based on VDI 4630 guidelines for the "Fermentation of organic materials" methodology (VDI, 2006). The tests were performed in 500 mL reactors containing 300 g of the ellulose sludge, with a concentration of 64% VS. The batch tests were conducted in triplicates at 38 ºC (100.4 °F) for a time period of 35 days using Eudiometer methods (DIN 38414-8, VDI 4630). Inoculum from a full-scale biogas plant (KTG Biogas AG, Germany), fed with maize silage, was used for the batch experiments. The seeding sludge was filtered to eliminate particles larger than 4mm. The TS was 3% and the VS accounted for 64%. The substrate/ ellulose ratio based on the initial VS was 0.4. The cumulative volume of produced biogas from each eudiometer was measured daily as the equivalent volume of acidified water displaced. Results are presented at standard temperature and pressure (STP).

Maize silage (Ms) was the main substrate, which was chopped into 8mm (MSL) and 4mm (MSS) lengths. Tests were performed adding ellulose © only to MSL. In Table 1, the main characteristics of the maize silage and ellulose are given.

TABLE 1. Characteristics of the ellulose and each substrate used in the tests (mean values from triplicate samples).

Sample		Inoculum	Ms	MSS	MSL	MSL+ C
Mass substrate	(g)	120.00	6.22	5.81	6.34	6.56
TS substrate	(%)	0.03	0.37	0.39	0.36	0.35
VS substrate	(%)	0.64	0.96	0.96	0.96	0.96
Inoculum	(g)	300.00	300.37	300.10	301.17	300.03
Substrate fed	(g)		6.22	5.81	6.34	6.56
Enzyme fed	(mL)					5.0
Total weight	(g)	300.00	306.59	305.91	307.50	311.56
VS ratio		0.00	0.40	0.40	0.40	0.40
VS Substrate	(g)	0.00	2.205	2.205	2.205	2.205

VS – volatile solids; TS – total solids; Ms – maize silage; MSS – maize silage chopped into 4 mm length; MSL – maize silage chopped into 8 mm length; MSL + C – maize silage added with ellulose.

Kinetics

The kinetics were analyzed according to the following First-order kinetic model:

$$y(t) = y_{max} \times \left(1 - e^{-k \times t}\right) \quad (1)$$

where,

y(t) is the cumulative yield (m^3 CH_4 kg^{-1} VS),

y_{max} is the potential maximum yield (m^3 CH_4 kg^{-1} VS),

k is the kinetic constant (d^{-1}), and

t is the time (d).

Methane productivity ($r_{s(t)}$) was calculated using a modified Hill model according to eq. (2):

$$r_{s(t)} = y'_{(t)} = y_{max} \times \frac{b \times c^b \times t^{b-1}}{(c^b + t^b)^2} \quad (2)$$

where,

$r_{s(t)}$ is the specific methane rate at the time *t* (m^3 CH_4 kg^{-1} VS d^{-1}),

$y'_{(t)}$ is the derivation of the methane cumulative curve (m^3 CH_4 kg^{-1} VS),

y_{max} is obtained from equation 1, and

b and *c* are the coefficients of the curve fitting.

In order to make clear that the biomethane potential test is close to finishing, it is necessary to calculate the time in which the difference in methane production between two consecutive points is less than 1% (PAGÉS-DÍAZ et al., 2012). This is possible by estimating t_{Grenz} according to eq. (3):

$$t_{Grenz} = -\frac{1}{b} \times \ln\left(\frac{1}{100\,b\,c + 1}\right) \quad (3)$$

Biodegradability

Ultimate biodegradability (UB) was determined by graphical statistical analysis. This method consisted of a linear regression of the remaining total volatile solids portion (TVS_e) of the initial total volatile solids mass (TVS_0) at the time *t*, as the duration of the time (or hydraulic retention time, HRT) of the test tends to infinity.

The total volatile solids remaining at infinity is assumed to be the refractory fraction of the substrate (R_0), defined as the non-biodegradable portion of the initial substrate TVS_0 mass. Then an extrapolation of the linear plot of TVS_e/TVS_0 versus 1/HRT for the y-axis showed the refractory fraction as the value of the y-intercept. The remaining portion of the TVS_e/TVS_0 at any time was calculated by the biogas produced during each interval. The ultimate biodegradability of a substrate was estimated as UB = 1 - R_0 (KANG & WEILAND, 1993; CONTRERAS et al., 2012).

Statistical analysis

Statistical analysis of the results was developed using the Statgraphics Centurion XV software pack (Version 15.2.05). One-way ANOVA (Analysis of Variance) was used to analyze the data and to test for significant treatment effects (particle size and enzyme addition) for the response variables (k, y_{max}, $r_{(s)\ max}$ and UB). The experimental data was tested for significant differences using the method of 95% Least Significant Difference (LSD).

RESULTS AND DISCUSSION

In the cumulative methane production curves (Figure 1) we can observe that higher biogas production was reached when Ms was used as a single substrate. As raw maize was not used as a control, it is not a definitive conclusion that ensiling improves methane yield. MSS reached higher yields than MSL, with significances differences (p-value <0.05) indicating that size reduction has a positive effect. Evidently, particle size had a major role in the digestion process, as was expected.

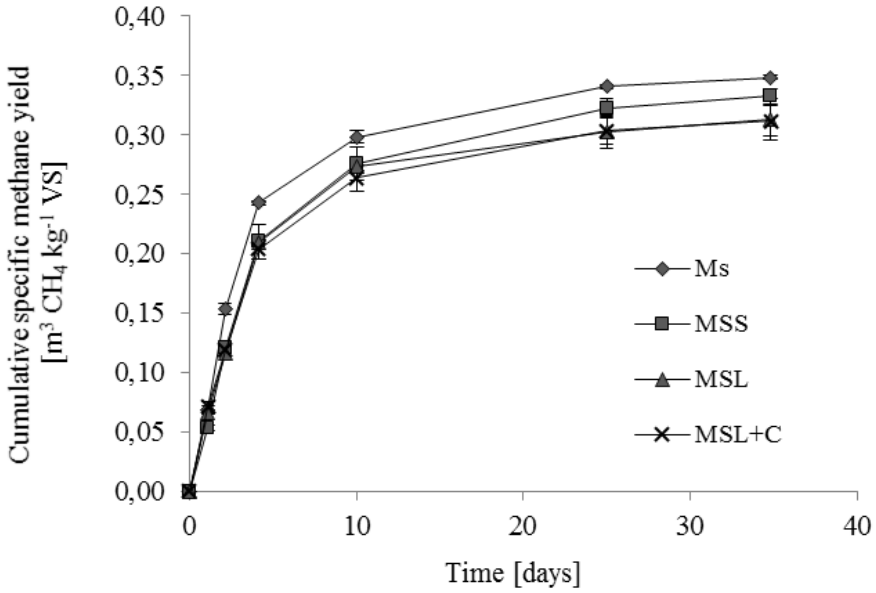

FIGURE 1. Cumulative specific methane yield for the substrates analyzed during the anaerobic process.

Subtitle: M_s – maize silage; MSS – maize silage chopped into 4 mm length; MSL – maize silage chopped into 8 mm length; MSL + C – maize silage added with cellulase.

Kinetics and biodegradability

In general, kinetic behavior was proper. The exponential curves show that ensiling at least does not lag the anaerobic process, as expected. It seems that volatile fatty acids are promoted by ensiling, taking into account that the substrates used in this investigation were lignocellulosic materials. In Table 2 the main results obtained for kinetics and biodegradability are summarized.

TABLE 2. Anaerobic conversion of maize silage in terms of kinetics and biodegradability (mean values and standard deviations from triplicate samples).

Parameters	Ms	MSS	MSL	MSL+ C
k_0 (d^{-1})	0.27 ± 0.007	0.219 ± 0.013	0.247 ± 0.021	0.245 ± 0.016
$y_{max.}$ (m^3 CH_4 kg^{-1} VS)	0.337 ± 0.002	0.327 ± 0.008	0.305 ± 0.014	0.306 ± 0.013
$r_{(s)max.}$ (m^3 CH_4 kg^{-1} VS d^{-1})	0.0245 ± 0.0006	0.0188 ± 0.005	0.0191 ± 0.005	0.0154 ± 0.0014
t_{Grenz} (d)	11.3	13.3	12.2	13.7
UB	0.878	0.845	0.776	0.787

Ms – maize silage; MSS – maize silage chopped into 4 mm length; MSL – maize silage chopped into 8 mm length; MSL+C – maize silage added with cellulose; VS – volatile solids;

As can be observed, in all cases Ms showed the best results in comparing to the effect of chopping length. Moreover, MSS had similar values when compared to Ms, this indicates that further efforts could be justified for the mechanical treatment of maize silage.

The effect of the addition of cellulase on the kinetics and biodegradability of MSL was neither positive nor negative. According to the statistical analysis with 95% confidence, results for MSL and MSL + C had no significant differences (p-value >0.05). The specific methane yields obtained

in this study ranged between 305 and 337 L kg^{-1} VS, which are in all cases comparable with other reports in literature (AMON et al., 2007; NEUREITER et al., 2005; OSLAJ et al., 2010). Estimated values of t_{Grenz} demonstrated that Ms reached stability first, compared with the other substrates.

In Figures 2 and 3, we graphically show the methane rate behavior and the refractory fraction remaining in the maize silage during anaerobic treatment. It is interesting to observe how the enzyme role is not effective in the remaining refractory fraction of MSL + C. Only Ms and MSS have ultimate biodegradability higher than 84%. In the case of MSL and MSL + C, there is no possibility that they, under the conditions studied, could express biodegradability higher than 80%.

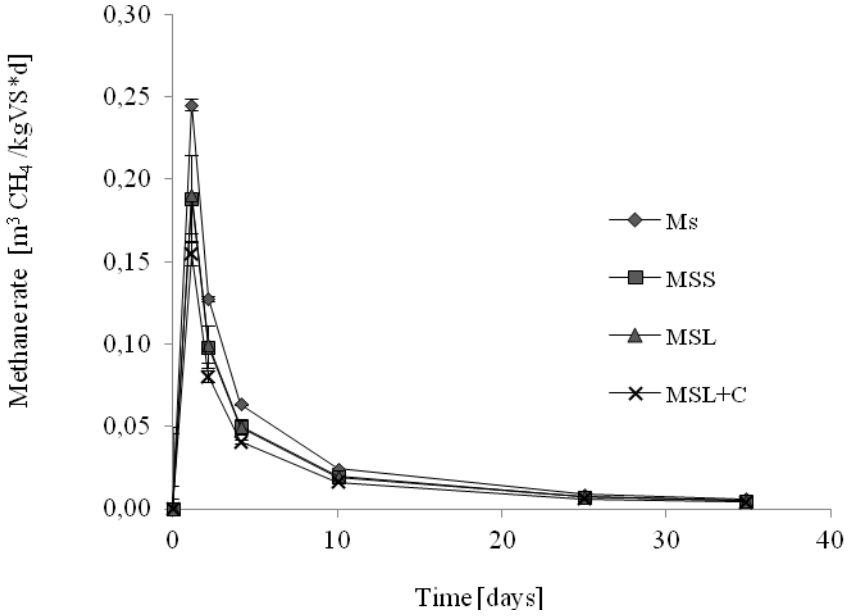

FIGURE 2. Methane rate of maize silage during anaerobic treatment.
Subtitle: M_s – maize silage; MSS – maize silage chopped into 4 mm length; MSL – maize silage chopped into 8 mm length; MSL + C – maize silage added with cellulase.

Ultimate biodegradability (UB) values were in accordance with the anaerobic process evolution. The UB is an important factor to take into account because it establishes the bioconversion boundaries for proper performance in the evaluation of an anaerobic process (CONTRERAS et al., 2012). As can be observed from Figure 2, the refractory coefficient (R_0) (the total volatile solids remaining at infinity) was determined. The lowest values of R_0 correspond to Ms and MSS (0.122, 0.155). LEQUERICA et al., (1984) reported values of R_0=0.365 under mesophilic conditions for the anaerobic treatment of rice straw. CONTRERAS et al., (2012) utilized this analysis to assess the effect of temperature on the bioavailability of rice residues and found significant differences between mesophilic and thermophilic operations. The comparison of such studies will probably lead to false conclusions, principally because of the variations in the composition of the residues. Ultimate biodegradability is a useful tool to provide more elements for the expression of bioavailability under anaerobic conditions.

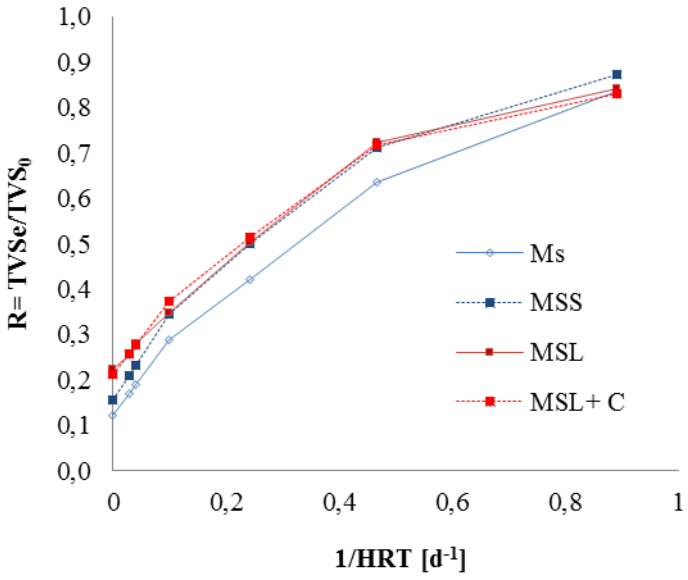

FIGURE 3. Refractory fraction remaining in the maize silage during anaerobic treatment.
TVS_e – remaining total volatile solids portion; TVS_0 – initial total volatile solids
Subtitle: M_s – maize silage; MSS – maize silage chopped into 4 mm length; MSL – maize silage chopped into 8 mm length; MSL + C – maize silage added with cellulase.

As in the studies of ROMANO et al., (2009) and DIAK et al., (2012), results from batch experiments showed that enzymatic treatment does not increase hydrolysis and digestion rates in maize silage chopped at different lengths and with the addition of cellulase. No significant improvements (p-value>0.05) were observed in all variables for MSL and MSL + C compared with Ms and MSS. Overall, the results of this study indicate that the addition of cellulase is not likely to increase digestion rates in the anaerobic digestion of maize silage, but particle size after ensiling.

These results can be attributed to the fact that the optimal conditions for cellulase expression are pH4 and temperature of 50 °C, as commented in the section Materials and Methods, which are not found under the anaerobic conditions studied. Another reason is that the inoculums were already used for maize silage as it came from a full-scale biogas plant fed with this type of substrate. Further studies in microbiology would produce better conclusions about a possible interaction between enzymes and microbiology.

CONCLUSIONS

The addition of cellulase to the anaerobic digestion and the various chopping lengths of maize silage did not produce significant effects on biogas production after a 35-day digestion period. Ms and MSS had a better response for all variables in comparison with MSL and MSL + C, with significant differences. This was generally attributed to inoculum origins and suboptimal conditions for cellulase expression. More emphasis must be placed on direct interaction of feedstock and enzymes in order to understand further enhancing and inhibiting effects. The Kinetic approach and the Ultimate Biodegradability test could be advantageously used in these studies.

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