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Research Article

Increased Biomethane Production from Endoglucanase-Pretreated Feedstock

Giuliano Degrassi[1]*, Rodrigo Hoshino[2], Milan Koijc[3], Mariela Catone[4], Vibha Bhardwaj[1] and Mario A. Rosato[5]

[1]Industrial Biotechnology Group, ICGEB, International Centre for Genetic Engineering and Biotechnology, Parque Tecnológico Miguelete, Av. General Paz N° 5445, Buenos Aires, Argentina

[2]Agronomy Department, Londrina State University, Rodovia Celso Garcia Cid, PR, Brazil

[3]Institute of Molecular Genetics and Genetic Engineering, IMGGE, Beograd, Serbia

[4]Instituto Nacional de Tecnología Industrial (INTI), Centro De Biotecnología Industrial, Buenos Aires, Argentina

[5]Sustainable Technologies srl, Via S. Biagio 4, 30025 Fossalta di Portoguaro (VE), Italy

*E-mail: degrassi@icgeb.org

Abstract

A *Bacillus subtilis* endoglucanase gene was amplified and cloned in *Escherichia coli*, the enzyme was expressed and purified to homogeneity by affinity chromatography and conditions for its optimal activity and stability were determined. The *E. coli* M15 (pREP4) heterologous expression system was grown and induced under controlled fermentation conditions and the purified enzyme was used for enzymatic pre-treatment of corn silage and digestate from a corn silage-fed anaerobic digestor. The biochemical methane potential of enzyme pre-treated and non-pre-treated corn silage and digestate was estimated, showing that in both cases there is a statistically significant increase of BMP of more than 30% due to the enzymatic pre-treatment. This result suggests that enzymatic pre-treatment of feedstocks could be a technology to be further explored as a tool for increasing the efficiency of biogas production process, also from the economic sustainability point of view.

Keywords: Biomethane•Endoglucanase-Pretreated• Bioenergy

Introduction

Polysaccharides such as cellulose, hemicellulose and pectin are complex polymers with high energy content and potential for being used in bioenergy production. In the last decades, research has focused on the identification and production of enzymes for depolymerization of these molecules to transform them into products/forms suitable for use in bioenergy production processes such as the second-generation biofuels [1,2]. Biogas production is one of the processes for obtaining bioenergy that could benefit from the depolymerization of plant cell wall components [3]. The average yield of methane from this process is relatively low compared to the energy contained in the feedstock used for the biogas generation by methanogenic bacteria, mainly due to the poor hydrolysis of the plant cell wall components in the anaerobic digestor [4]. Cellulose is one of the most abundant polysaccharides produced on earth and its degradation requires time and the combined participation of many microorganisms, the so-called "microbial consortia" [5], mainly because of its complexity. Furthermore, cellulose is embedded in an even more complex structure that includes both hemicellulose and lignin, linked to each other, therefore, its degradation requires the cooperation of cellulases with hemicellulolytic and ligninolytic enzymes [6].

Depolymerization of cellulose can be achieved mainly by the action of three types of enzymes: (i) endocellulase or β-1, 4-endoglucanase hydrolyzes the chain internally providing oligosaccharides; (ii) exocellulase or cellobiosidase removes disaccharides or cellobiose from the ends of the chain; and (iii) β-glucosidase hydrolyzes cellobiose into glucose. These enzymes are broadly studied for their biotechnology potential application [7].

Fungi are the most studied microorganisms able to degrade cellulose [8] however, also bacteria can attack this substrate although they are much more efficient when they operate in consortia [9,10]. Bacteria with cellulolytic activity can be found in soil [11], but also among the bacterial plant pathogens [12]. Soil is a highly competitive environment where bacteria fight for food. Among the organic matter present in the soil cellulose is abundant, therefore the degrading and utilizing abilities confers a competitive advantage. *Bacillus* species are predominant in the soil and reported as cellulose well-users [13]. On the other hand, many phytopat hogenic bacteria can use or attack cellulose, mainly due to the need for weakening the cell wall to invade the host [14]. There is a broad range of bacteria that can be used for the production of cellulolytic enzymes. However, the production of such enzymes directly from cellulolytic bacteria has some limitations mainly due to the regulatory and catabolic repression mechanisms [15,16]. Therefore, a possible alternative is the expression of genes encoding for enzymes to be used in the degradation of cellulose through heterologous expression/overexpression systems. In our study, we focused on two soil bacteria and one plant pathogen to develop such systems for the production under controlled conditions of cellulolytic enzymes. *Bacillus subtilis*, *Bacillus pumilus* and *Xanthomonas axonopodis* were chosen as donors of the endocellulase, β-glucosidase and cellobiosidase genes, respectively.

The long-term goal of this work is to develop a technology suitable for pre-treatment of cellulose-containing organic matter for improvement of methane yield in processes of biogas production. Feedstock enzymatic pre-treatment using heterologously expressed enzymes for the degradation of plant cell wall components, such as cellulose, that are particularly difficult to be degraded by methanogenic bacteria, could increase the biogas yield in the anaerobic biomethane production process, since cellulose hydrolysis is a limiting step in biogas production, as suggested by the composition of digestate, the residue generated by the biogas digestion process [17,18].

In this study, the construction of heterologous expression systems, enzyme production and purification, as well as trial application in a feedstock pre-treatment process, are reported and discussed.

Material and Methods

Bacterial strains and growth conditions

The bacterial strains used in this study for amplification of β-1,4-endoglucanase (EGC), β-glucosidase (BGL) and cellobiosidase (CBS) genes were *Bacillus subtilis* 152 [19], *Bacillus pumilus* PS213 [20] and *Xanthomonas axonopodis* pv *glycines* NCPPB3658 (*Xag*) [21], respectively, from the ICGEB bacterial collection. *B. subtilis* and *B. pumilus* were grown in Nutrient Broth (NB) liquid or agar medium at 30°C; *Xag* was grown at 28°C on peptone-yeast extract (PY) liquid medium or peptone-sucrose agar (PSA). *Escherichia coli* M15 (pREP4) was used for heterologous expression of 6xHis tagged proteins following the instruction of the supplier (Qiagen).

Recombinant DNA techniques

Digestion with restriction enzymes, agarose gel electrophoresis, purification of DNA fragments, ligation with T4 DNA ligase, end filling with Klenow fragment of DNA polymerase, and transformation of *E. coli* were performed as described [22].

Plasmids were purified using the JetStar plasmid purification kit (Genomed, GmbH); genomic DNA was isolated by Wizard Genomic DNA purification kit (Promega). Nucleotide sequencing was performed by Macrogen sequencing service (www.macrogen.com).

Heterologous expression of enzymes in *E. coli* and purification

The β-1, 4-endoglucanase (EGC) gene from *B. subtilis*, the β-glucosidase (BGL) gene from *B. pumilus* and the cellobiosidase (CBS) gene of *X. axonopodis* pv. *glycines* were amplified by PCR using GoTaq DNA polymerase (Promega), on genomic DNA as template with the following oligonucleotide primers: EGC-Fw-BamHI 5´- CGC CGG GAT CCG CAG CAG GGA C - 3´ and EGC-Rv-HindIII 5´- GCC TAA AGC TTA ACT AAT GGG G - 3´; CBS-Fw-BamHI 5´- GCG GGA TCC CAT GTC GAC AAT CCG - 3´ and CBS-Rv-HindIII 5´- CAG AAG CTT CCA TCC ATT TCC GG - 3´; BGL-Fw-SacI 5´- CAG GCA GAG CTC GCT TGG AAT GTT GAT GG- 3´ and BGL-Rv-SalI 5´- CGT CGA CAT CAC CTT TTC ATG AAT GCG G- 3´ (restriction sites in oligonucleotides are underlined). Primers were designed according to the annotated genome sequences of *B. subtilis* strain Bs-916 (GenBank Accession Number: CP009611.1), *B. pumilus* strain C4 (GenBank accession number: CP011109.1) and *X. citri* pv *glycines* strain 12-2 (GenBank accession number: CP015972.1) which possess endoglucanase, β-glucosidase and cellobiosidase encoding genes highly homologue to the endoglucanase gene of *Bacillus subtilis* 152, β-glucosidase of *B. pumilus* PS213 and cellobiosidase of *X. axonopodis* pv *glycines* NCPPB3658. The resulting PCR products were checked for quantity and purity by agarose 1% gel electrophoresis, cloned into the pTOPO PCR cloning vector (Thermo Fisher Scientific) and confirmed by sequencing. These fragments were then excised from pTOPO by corresponding restriction enzymes, and cloned into the corresponding restriction sites of the 6xHis-tag expression vector pQE30 yielding pQEBGL, pQECBS and pQEEGC constructs. Expression and purification of 6xHis-tagged BGL, CBS and EGC were carried out in *E. coli* M15 (pREP4) (pQEBGL), (pQECBS) and (pQEEGC) according to the instruction of the supplier (Qiagen).

Enzyme activity

Cellulase activity was determined by using the Nelson Somoygi method and several types of cellulose as substrates: medium fibers cellulose (Sigma), short fibers

cellulose from tissue paper industry waste (Papelera del Plata, Zarate, Argentina), corn stalk powder and corn cob powder. A commercial cellulase from *Aspergillus* sp. was used as the positive control. The cellulase activities were expressed in international units; one unit of enzyme activity was defined as the amount of enzyme that catalyzes the formation of 1 µmol of product per min under the specified assay conditions.

Optimal conditions for enzyme activity and stability

The optimal pH and temperature for the purified cellulase enzyme were determined in the range from pH 3 to 9 (50 mM Na acetate, pH 3 to 5.5; Na phosphate, pH 6.0 to 7.0; and Tris-HCl buffer, pH 7.5 to 9.0) and from 25 to 50°C, respectively. For the pH stability determination, samples were incubated in buffers from pH 3.0 to 9.0 at room temperature for 10 days. The thermal stability was determined at the optimal pH and temperature, so samples were incubated at pH 6.0 and 40°C and 50°C for 10 days. In both cases, the residual activity was measured and pH and thermal stability calculated.

Large scale production of purified endoglucanase

Ten ml culture of *E. coli* M15 pREP4+pQEEGC-endoglucanase was grown in LB supplemented with 0.4% glucose, 50 µg/ml kanamycin and 100 µg/ml ampicillin in 100 ml Erlenmeyer flask up to OD_{600} 0.1. This culture was used to inoculate the 5 liter-glass bioreactor Biostat B Plus (Sartorius) containing 2 liters of LB medium supplemented with 0.4% glucose, 50 µg/ml kanamycin and 100 µg/ml ampicillin. Fermentation parameters were the following: stirring from 300 to 800 rpm; $pO_2 \geq 30\%$; aeration, 0.5-2.5 vvm; pH 6.9; temperature 37°C. The pH and foam were controlled using 2M NH_4OH and 10% simethicone antifoam, respectively. When the glucose concentration was close to zero, 0.5 ml of 1M IPTG was added to induce the expression of the cellulase gene and 0.2 ml of 1 M IPTG was added after 30 minutes. The level of dissolved oxygen was controlled and kept above 30% varying automatically the level of agitation and airflow rate. After two hours from the first induction, fermentation was stopped and the culture broth centrifuged at 10000 rpm at 4°C for 10 minutes in a Sigma centrifuge model 6K15. The supernatant was discarded and the pellet obtained resuspended in the following lysis buffer: 50 mM $NaHPO_4$, 300 mM CaCl, and 20 mM imidazole, pH 8.0. The pellet was kept at -70°C until processing for lysis. The sample volume was kept as small as possible to reach an $OD_{600} \leq 30$. For lysis, the pellet was thawed and sonicated keeping it in ice until complete cell lysis. Before the purification process, the lysate was centrifuged and the supernatant obtained was filtered sequentially through the 0.8 and 0.45 µm filters. Purification of recombinant endoglucanase was achieved using an XK 16/20 Fractogel EMD chelate resin column (Merck) and an Äkta Purifier chromatograph (General Electric). Four hundred ml of sample, previously filtered through 0.45 µm, was loaded onto the column, followed by two-column volume of washing buffer (50 mM NaH_2PO_4, 300 mM NaCl 20 mM imidazole, pH 8.0). The sample was eluted by applying a linear gradient of elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole, pH 8.0), from 10 to 100%. Fractions of 10 ml were collected. Enzymatic activity was determined as described above. Positive fractions were also analysed by SDS PAGE.

Determination of the biochemical methane potential (BMP) of enzymatically-treated feedstock

A second-generation Automatic Methane Potential Test System (AMPTS-II, Bioprocess Control AB) was used to determine the BMP of corn silage and digestate, before and after the enzymatic pre-treatment. The UNI/TS 11703/2018 method for the assessment of potential production of methane from anaerobic digestion in wet conditions was followed. Each test was performed in triplicate and values mediated. The volume of each anaerobic reactor was 500 ml. Each reactor was connected to a 100 ml bottle containing 3N NaOH solution to eliminate CO_2 from the generated biogas and allow the measurement of the methane volume only, according to the instruction of the instrument's supplier and the UNI standard.

Enzymatic pre-treatment was performed in the 500 ml glass anaerobic digestors by suspending approximately 15 g of feedstock, either corn silage or digestate, in 75 ml of 0.2 M potassium phosphate buffer pH 6.0. The reaction was carried out at 40°C for two days with agitation, after the addition of 2 mg of the purified enzyme with an enzymatic activity of 251 IU/mg of carboxymethyl cellulose (CMC). The negative control was the same reaction mixture without enzyme. The anaerobic digestion process started with the addition of 400 g of inoculum, a digestate from various biogas plants, sieved with a 5 mm mesh, and pre-incubated. The starting pH of the test was 7.8 and the initial concentration of volatile solids of the inoculum was 2.2%. The process was conducted at 40°C for 30 days.

Results and Discussion

Three cellulolytic enzymes-encoding genes were heterologously expressed in *E. coli:* the endocellulase (endoglucanase) from *B. subtilis*, the cellobiohydrolase (cellobiosidase) from *X. axonopodis* pv *glycines* and the β-glucosidase from *B. pumilus* (Figure 1). However, only the endoglucanase was soluble in the cell-free crude extract obtained in the experimental conditions. Among the three bacterial donors, two are soil bacteria and one is a bacterial plant pathogen, all three are well known for their ability to attack plant cell wall components, although Bacilli preferentially operate in consortia [23].

The heterologous expression of recombinant proteins in *E. coli* is a well established technology for production of valuable enzymes and therapeutic products also at industrial level, although in some cases the production might be low or absent and requires development of new

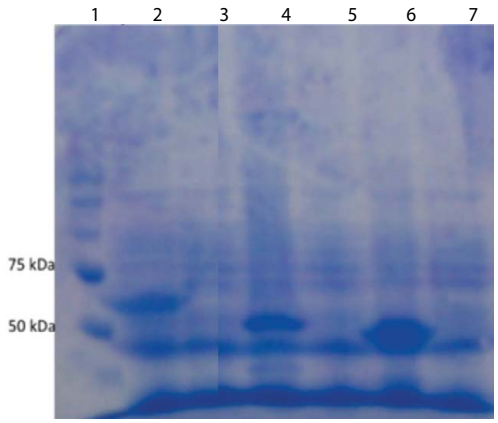


Figure 1. SDS PAGE of cell-free crude extract from *E. coli* cultures. 1. Molecular markers; 2, 4 and 6, IPTG induced cultures expressing cellobiohydrolase, β-glucosidase and endoglucanase, respectively; 3, 5 and 7, non-induced cultures of E. coli harboring pQECBS, pQEBGL and pQEEGC, respectively.

approaches to improve the recombinant protein expression [24]. Protein toxicity can arise if the heterologous protein has a detrimental function in the host. In addition it might be possible to see the formation of inclusion bodies: this happens because the microenvironment of *E. coli* is different from the one of the parental host. Incorrect folding mechanisms and disulfide bond formation might occur, and high levels of expression correspond to high concentrations of hydrophobic stretches in the polypeptide, which lead to protein aggregation and insolubility [25,26]. In addition, the expressed protein can be inactive because the architecture of the active site is not properly formed to be suitable for activity [27].

The *E. coli* clone expressing the soluble endocellulase (endoglucanase) from *B. subtilis* was grown and the protein expressed, purified and characterized from the biochemical point of view in order to find the optimal conditions for its activity/use. The endoglucanase activity has already been reported in *Bacillus spp*. [28], and the endoglucanase of *B. subtilis* was also expressed in *E. coli* [29]. Here we report the expression of 6xHis tagged endoglucanase in *E. coli* M15 (pREP5) and its purification to homogeneity in a single step-affinity chromatography under native conditions (Figure 2). The expression of correct protein was confirmed by MS analysis of trypsin-digest that identified peptides which covered 45% of the expected amino acid sequence (Figure 3). The specific activity was also measured on different substrates and results are shown in Table 1.

The optimal temperature and pH for activity of purified endoglucanase enzyme were determined (Figure 4A and 4B) which were found to be 40C and pH 6, respectively. In addition, the enzyme was stable at the optimal pH and temperature conditions for almost three days, with approximately 90% of residual activity (Figure 4C). This information was used as a basis for setting the experimental conditions for feedstock and digestate pre-treatment in 500 ml anaerobic reactors.

Fed batch fermentation for recombinant expression of endoglucanase in *E. coli* was performed using 2 liters working volume in a 5-liters fermenter. This resulted in 30.9 g of fresh cell-pellet, corresponding to OD_{600} of 14.9. The single step affinity chromatography allowed the purification of approximately 95 mg of enzyme, with a specific activity of 250 U/mg. The purified 6xHis-tagged protein was used to perform the BMP analysis of corn silage and digestate, with and without enzymatic treatment. The BMP curves of untreated corn silage and digestate, compared to the enzyme-treated samples are shown in Figure 5. The metrological uncertainty and the dispersion of the single values of the BMP tests were analyzed according to the Italian norm UNI/TS 11703. Since the methane production of the blank reactors was negligible, there is no error amplification factor. The instrumental error margin is then the one stated by the instrument's manufacturer, i.e. 1%. The average BMP values of both substrates are hence accurate. Nevertheless, the heterogeneity of the substrates under test induces some dispersion between the methane production of the single reactors. Table 2 shows the standard deviation of each test.

The dispersion for both treated and untreated corn silage is high, so such tests are accurate but not precise. Such dispersion of the corn silage's BMP is in line with the literature [30], and is caused just by the heterogeneity of the samples. The correct way of expressing the BMP values, according to the ISO/IEC Guide 98-1:2009 "Uncertainty of measurement" is shown in Table 3.

The samples of untreated silage and digestate were also analysed at the Laboratory of Fodder Analysis of the University of Parma (Italy). The volatile solids of the silage had the following composition: 4.20% ashes, 8.37% proteins, 3.18% lipids, 40.16% NDF (neutral detergent fibre), 22.35% ADF (acid detergent fibre), 3.03% ADL (acid detergent lignin). Since the ADL is undigestible for anaerobic bacteria, and NDF and ADF are both carbohydrates, the theoretical

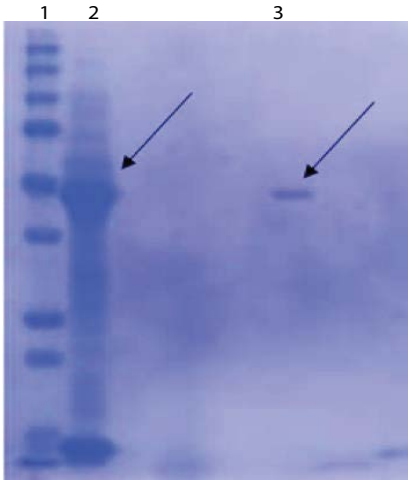
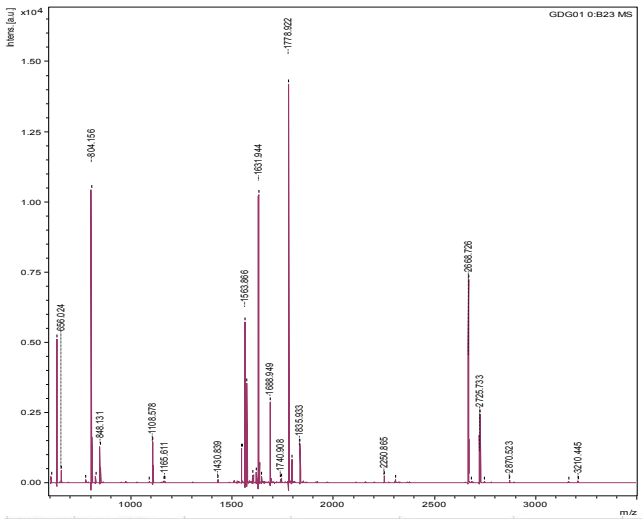


Figure 2. *B. subtilis* endoglucanase expressed in *E. coli*. 1. Molecular markers; 2. Cell-free crude extract from IPTG induced culture before purification; 3. Endoglucanase purified by affinity chromatography.



Database: **User Protein**
Considered modifications: | **Acetyl (Protein N-term) | Gln->pyro-Glu (N-term Q) | Met-loss (Protein N-term M) | Met-loss+Acetyl (Protein N-term M) | Oxidation (M) |**
Digest Used: **Trypsin**
Max. # Missed Cleavages: **1**
User AA Formula 1: **C2 H3 N1 O1**
Constant Modification: **Carbamidomethyl (C)**
Minimum Digest Fragment Mass: **800**
Maximum Digest Fragment Mass: **4000**
Minimum Digest Fragment Length: **5**

COVERAGE 45%

Index Number: **1**
pI of Protein: **7.3**
Protein MW: **52363**
Amino Acid Composition: **A34 C2 D36 E18 F14 G45 H10 I25 K41 L31 M3 N40 P17 Q22 R13 S32 T31 V26 W14 Y19**

1	GSAAGTKTPV	AKNGQLSIKG	TQLVNRDGKA	VQLKGISSHG	LQWYGDFVNK	DSLKWLRDDW	GITVFRAAMY	TADGGYIDNP
81	SVKNKVKEAV	EAAKELGIYV	IIDWHILNDG	NPNQNKEKAK	EFFKEMSSLY	GNTPNVIYEI	ANEPNGDVNW	KRDIKPYAEE
161	VISVIRKNDP	DNIIIVGTGT	WSQDVNDAAD	DQLKDANVMY	ALHFYAGTHG	QSLRDKANYA	LSKGAPIFVT	EWGTSDASGN
241	GGVFLDQSRE	WLNYLDSKNI	SWVNWNLSDK	QESSSALKPG	ASKTGGWPLT	DLTASGTFVR	ENIRGNKDST	KDGPETPAQD
321	NPTQEKGVSV	QYKAGDGRVN	SNQIRPQLHI	KNNGNATVDL	KDVTARYWYN	AKNKGQNFDC	DYAQIGCGNL	THKFVTLHKP
401	KQGADTYLEL	GFKTGTLSPG	ASTGNIQLRL	HNDDWSNYAQ	SGDYSFFQSN	TFKTTKKITL	YHQGKLIWGT	EPH

Figure 3. Mass spectrometry analysis of the trypsin-digested heterologous expressed endoglucanase and peptides identified.

Table 1. Specific activity on some cellulose-containing substrates.

Substrate	Activity (IU/mg)
Cellulose (Sigma)	251.1
Short-fiber cellulose waste	142.1
Corn cob powder	74.8
Corn stalk powder	69.5

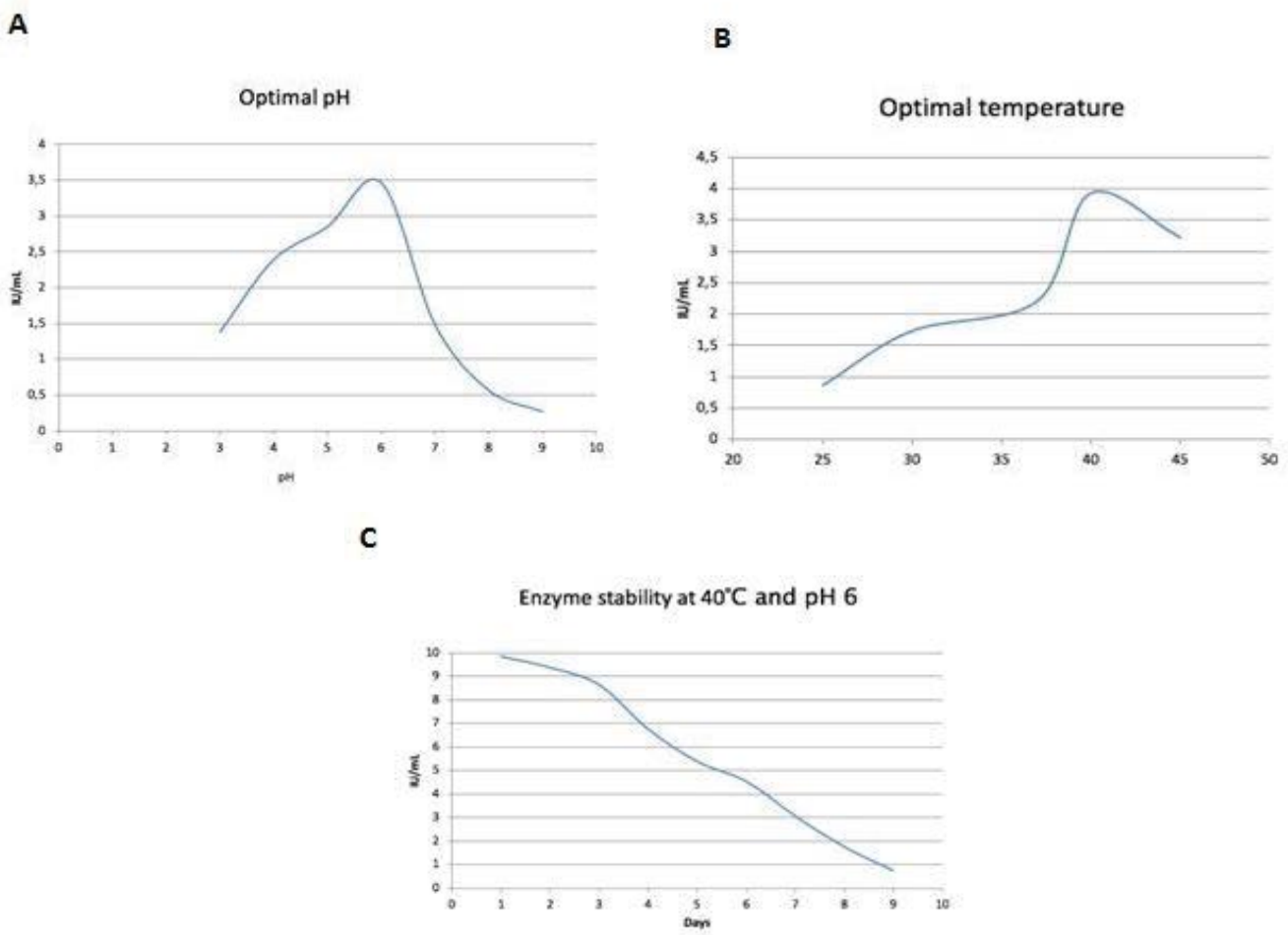


Figure 4. A) Optimal pH B) Optimal temperature C) Enzyme stability at 40° and pH 6.

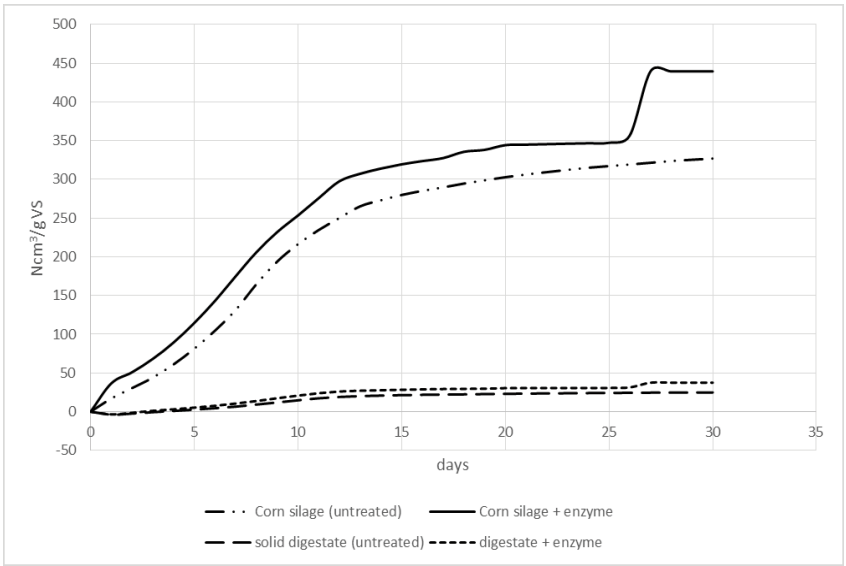


Figure 5. BMP of untreated and enzyme-treated corn silage and digestate. Average of three replicate tests of each substrate.

Table 2. Standard Deviation of each substrate test.

Sample	R1 (Ncm^3)	R2 (Ncm^3)	R3 (Ncm^3)	σ (Ncm^3)	σ (%)
Corn silage (untreated)	1114.5	1486.6	1799.1	342.7	23%
Digestate (untreated)	135.5	133.1	143.1	5.3	4%
Corn silage+enzyme	1846.2	2667.0	1364.0	658.8	34%
Digestate+enzyme	206.7	199.8	163.3	23.3	12%

Table 3. Average BMP and absolute uncertainty margins (E), in Nm^3/ton SV.

Sample	BMP		E
Corn silage (untreated)	327	±	76
Digestate (untreated)	25	±	1
Corn silage+enzyme	440	±	148
Digestate+enzyme	37	±	5

BMP can be estimated with the following formula (from the norm UNI/TS 11703, Annex B2, Table 1):

BMP_{th}=((0.4016+0.2235) × 0.415+8.37 × 0.496+0.0318 × 1.114)/0.95=0.601 Nm^3/kg SV=601 Nm^3/kgSV

The maximum BMP measured during this test (R2 with enzyme-treated silage) was 588 Nm^3/ton SV, which is smaller than the benchmark for theoretical BMP. Although the measured value is higher than the most commonly found in the literature (Rosato, 2017), it is anyway physically possible/achievable.

The methane production corresponded to the usual sigmoid curve for all three reactors with untreated silage. The reactors with enzyme-treated silage produced more methane and with an unusual pace: the cumulated gas volume curve follows a sigmoid but with a higher production rate than the untreated samples, and presents a “step” (diauxic growth) on the 26th day. The reactors with the treated digestate showed the same step before the final plateau of the curve, but it was not so high. Since none of the blank reactors, nor the untreated sample reactors, showed any anomalous behaviour, the only possible explanation for cause of the diauxia is the enzymatic treatment/activity. The dynamics of anaerobic bacteria population and substrate degradation that lead to such an unconventional BMP curve should be further investigated.

Conclusions

The average BMP values of the enzyme-treated samples are systematically higher than those of the untreated samples. Nevertheless, this does not mean that such differences are significative. The BMP of the enzyme-treated corn silage is 34% higher than the untreated one, but the dispersion margin of the triplicate test is of the same order of magnitude. Hence, the difference is not statistically significative. In other words, it is not possible to conclude that the higher (average) BMP of the treated samples is the result of the enzyme’s effect: it could be just the result of random errors adding (property related to a particular sample). On the other hand, the comparison of the average measured BMP with the maximum value calculated with the theoretical formula is well below the theoretical limit, which indicates that there were no errors in the tests and hence, the treatment with the enzyme indeed increases the anaerobic degradability of the silage. The calculated 34% increase is then an acceptable result. In the case of the digestate, the difference between treated and untreated samples is 48%, while the standard deviation is 12%. Hence, in the worst case, it is possible to assume that the enzymatic treatment increased the BMP of the digestate, by at least 36%.

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