COMBINATION OF ALKALINE AND ENZYMATIC PRE-TREATMENT TO INCREASE BIO-METHANE PRODUCTION POTENTIAL OF SORGHUM AND WHEAT STRAW

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ABSTRACT: the purpose of this study was to increase methane production by enzymatic and combined alkaline-enzymatic pre-treatments on ensiled sorghum forage and wheat straw. Sodium hydroxide pre-treatment was conducted by soaking samples in a NaOH solution. Enzymatic pre-treatment was comparatively performed employing four commercial preparations. Enzyme preparations were first characterized for their protein, CMCase (endoglucanase), xylanase and avicelase activities, and then added to untreated or alkaline pre-treated substrates. To assess the effect of the applied pre-treatment on the methane production, Biochemical Methane Potential (BMP) tests, before and after the enzymatic and the combined alkaline-enzymatic pre-treatment, were performed. Glucose was the prevailing monosaccharide released by enzymatic treatments, but xylose, mannose and galactose, as well as arabinose and glucuronic acid were also found. Combined alkaline-enzymatic pre-treatment resulted in the highest methane yield increase (+32% and +76% for ensiled sorghum and wheat straw respectively).
Keywords: alkali, biogas, enzyme, lignocellulosic sources, pretreatment.

1 INTRODUCTION

Lignocellulosic biomass is known to represent an interesting source for biogas and ethanol production. In particular, sorghum and wheat straw may be considered suitable substrates for anaerobic digestion in agricultural biogas plants. As for their lignocellulosic nature, anaerobic biodegradability depends on the cellulose, hemicellulose and lignin content. Cellulose and hemicelluloses (holocelluloses), which are the major components of most lignocellulosic materials, are easily degraded by anaerobic microorganisms and can be converted into biomethane. Nevertheless, lignin limits their accessibility to hydrolytic enzymes, preventing their degradation [1, 2].

Thus, the complex structure of lignocellulosic materials could be altered through various methods of pre-treatment. Effective pre-treatment, prior to anaerobic digestion, should break down the linkage between polysaccharides and lignin to make cellulose and hemicelluloses more accessible to hydrolytic enzymes, the final aim being the increase in methane potential of these substrates.

Pre-treatments include mechanical, chemical, thermal, biological processes or a combination of them. Biological pre-treatments are characterised by the use of industrial enzymes, such as cellulase and xylanase or lignolytic enzymes (laccase, lignin and manganese peroxidase), to breakdown all components of lignocelluloses, including lignin [3]. These enzymes can also be produced by micro-organisms such as brown-, white-, and soft-rot fungi that secrete extracellular enzymes [4]. Biological pre-treatment is an energy saving and environmental friendly method of pre-treatment but relative low efficiency, potential loss of carbohydrates and long residence time are the three major disadvantages for enzymatic pre-treatment. Chemical pre-treatments are classified into acidic, alkaline, oxidative, organosolv, and ionic liquids pre-treatments. Among them, alkaline pre-treatments (NaOH, KOH, lime, ammonia, and urea) are efficient in altering the structure of lignin, solubilising hemicelluloses fraction, and increasing efficiently the accessibility of cellulose [5-7].

The purpose of this study was to increase methane production by enzymatic and combined alkaline-enzymatic pre-treatments on ensiled sorghum forage (*sorghum sudanense x sudanense* hybrid) and wheat straw (Aubusson), two types of biomass commonly found in the Northern area of Italy (Lombardy region).

2 MATERIALS AND METHODS

2.1 Substrates

Ensiled sorghum forage (*Sorghum sudanense hybrid*) and wheat straw (*Aubusson*), were collected from a farm near Cremona (Lombardy Region, Italy). After collection, samples were oven dried at 60°C for two days to a moisture content of less than 10%, and ground into particles with a mean diameter of 1 mm by a kitchen blender, and finally stored in air-tight containers prior to use.

2.2 Alkaline pre-treatments

Sodium hydroxide pre-treatment tests were conducted in batch mode by soaking samples in a NaOH solution at 40 °C for 24 h (10 g NaOH/100 gTS), without stirring.

2.3 Enzymatic pre-treatments

Enzymatic pre-treatments were comparatively performed by employing the following commercial preparations: Agazym BGL, Agazym Ultra L (Garzanti Specialties), Pulpzyme HC (Novo Nordisk) and Primafast 200 (Genencor Inc.). Enzymes were added to untreated or alkaline pre-treated substrates at a final concentration of up to 0.40 and 0.12 ml/gTS respectively. Distilled water was used to reach a concentration of 3 ml/gTS, and pH corrected at each appropriate enzyme-specific value.

2.3 Biochemical Methane Production (BMP)

BMP tests were performed in duplicate using a commercial laboratory instrument (AMTPS, Bioprocess control, Sweden). This is a volumetric device consisting of 15 gas-tight glass bottles (0.5 L test volume) placed in a water bath at 35±0.5 °C. Each bottle was continuously mixed with a rotary stirrer. The biogas produced passes through a NaOH solution (3M), for CO_2 absorption.

Methane flows through a liquid-displacement automated measuring unit with a resolution of 11-13 mL. A data acquisition system allows flow-rate data to be recorded continuously. The inoculum used for these tests was obtained by mixing two digested sludge samples: 1) collected from a digester fed on waste activated sludge, with a solid content of 20.2±3.8 gTS/L, 11.9±2.1 gVS/L, and a maximum Specific Methane Activity (SMA) of 22.2 $mLCH_4$/gVS/d, as measured by dosing 1 gCOD/L of acetate; 2) collected from a digester fed on agro-wastes (cattle and poultry manure and corn silage), with a solid content of 55.0±2.6 gTS/L, 37.2±2.3 gVS/L, and a SMA of 13 $mLCH_4$/gVS/d, as measured by dosing 1 gCOD/L of acetate. The mixture was made of 50% each on a VS basis. All sludge characteristics were measured in duplicate. The inoculum was kept under endogenous anaerobic conditions at 35 °C for about 7 days to reduce the non-specific biogas generation. Raw and pretreated samples were mixed with the inoculum obtaining a substrate/inoculum ratio 1 gVS/gVS, as suggested by [8]. Finally, a mineral medium of macronutrients (as suggested by [9]) was added to the bottles. A blank sample was performed by mixing the inoculum and the mineral medium. The BMP test duration was 31 days for all samples. The Biochemical Methane Potential production at 30 days (BMP_{30}) was calculated as follows:

$$BMP_{30}(L_{CH4}/gVS) = (V_{CH4,s} - V_{CH4,blank})(L_{CH4})/VS_s\,(gVS)$$

Where: $V_{CH4,s}$ is the volume of methane produced from the substrate and measured at the end of the test; $V_{CH4,blank}$ is the volume of methane produced from the substrate and measured at the end of the test; ($V_{CH4,s}$-$V_{CH4,blank}$) is the net volume of methane production measured at the end of the test; VS_s is the mass of volatile solids of the added substrate. All gaseous volumes hereafter reported are referred at STP conditions.

2.4 Analytical procedures

Total Solids (TS), Volatile Solids (VS) were determined according to the APHA Standard Methods [10].

Chemical Oxygen Demand (COD) of untreated substrates was determined according to the Italian analytical standard methods [11].

NDF, Neutral Detergent Fibre; ADF, Acid Detergent Fibre; ADL, Acid Detergent Lignin were determined according to the Van Soest method [12] with a ANKOM A220 system (ANKOM Technology) that is based on sequential extraction with neutral and acid detergent, followed by a strong acid extraction. Different fractions are: a) soluble fraction in neutral detergent "SOLU" (1-NDF); b) hemicelluloses "H-CEL" (NDF-ADF); c) cellulose "CEL" (ADF-ADL); d) lignin (ADL).

Fats and proteins of untreated sorghum and wheat straw were determinate with a NIR System (5000 monochromator, Foss).

Endoglucanase (CMCase) enzymatic activity was determined by measuring the amount of glucose released from CMC using the Somogyi-Nelson method with glucose as standard [13].

Xylanase was assayed according to [14] and reducing sugars expressed as xylose determined again through the Somogyi procedure.

Avicelase activity was determined according to [15]. One unit of enzyme (IU) is defined as the amount which releases 1 μmol of reducing sugar (either glucose or xylose) equivalents per minute under the conditions specified above.

Protein content was estimated by the Lowry procedure [16], employing bovine serum albumin as standard.

Sugars were also determined by HPLC using a Merck Polyspher OA-KC column with a refractive index (RI) detector, eluted at 0.4 ml/min with 5 mM H_2SO_4 at 30°C.

3 RESULTS AND DISCUSSION

3.1 Chemical composition of raw substrates

Chemical composition of sorghum and wheat straw, after drying and milling, are given in Table I. Both samples have an average COD/VS value of almost 1.2, which is close to the typical value for carbohydrates. A similar content of cellulose and hemicelluloses was observed both for ensiled sorghum forage and wheat straw. Sorghum has a higher protein and fat content and a lower lignin content than wheat straw. Despite the high variability, the results of the chemical composition are in agreement with literature values both for sorghum forage and wheat straw [17, 18].

Table I. Composition of ensiled sorghum forage and wheat straw (all analytical determinations were performed in duplicate).

Parameter	**Ensiled sorghum forage**	**Wheat Straw**
TS (% wet weight)	93.0±3.9	93.9±3.9
VS (%TS)	86.6±0.4	92.7±0.4
COD/VS	1.21	1.15
Protein (%VS)	9.3±3.3	4.0±1.0
Fat (%VS)	1.8±0.3	0.9±0.8
Cellulose (%VS)	48.9±1.4	49.1±1.5
Hemicelluloses (%VS)	35.1±1.8	34.1±1.8
Lignin (%VS)	4.1±0.0	6.5±0.0

3.2 Characterization of enzymatic preparations

Enzymatic treatment was carried out employing four types of commercial enzymatic preparations: Agazym BGL and Ultra L (Garzanti Specialties), Pulpzyme HC (Novo Nordisk) and Primafast 200 (Genencor Inc.).

Agazym BGL is an enzymatic preparation especially formulated to favour the breakdown of plant cell walls to extract tissue components during industrial processing of cereals.

Agazym Ultra L instead is recommended to perform alcoholic fermentation of red wines, when must is fermented in contact with grape husk, to facilitate pigments and flavors extraction.

Pulpzyme is used during the process of bleaching and deinking for the production of recycled paper, while Primafast is recommended for clothes processing such as depilling, softening and to obtain the so-called "stone-washed look".

Enzyme preparations were first characterized for their protein, endoglucanase (CMCase), xylanase and

avicelase contents (Table II).

3.3 Enzymatic and alkaline-enzymatic pre-treatments

Because of their highest xylanase and endoglucanase (CMCase) content, BGL and Primafast were chosen for the prosecution of the research. These preparations were then added to samples of untreated or alkaline-treated sorghum or wheat straw, to evidence any possible hydrolytic activity towards lignocellulosic components. In this phase of the research attention was focused on finding out the best reaction conditions, i.e concentration of enzyme/s applied, time and temperature of incubation. Concentrations were chosen according to the technical instructions provided by the supplier, in particular BGL was tested at 0.04 - 0.1 - 0.2 mL/gTS, Primafast at 0.12 - 0.2 mL/gTS. Samples were for incubated for 24 h at each appropriate pH (7.0 for Primafast, 4.5 for BGL) and temperature (50°C). At appropriate intervals, samples were taken and the release of total and reducing sugars, as well as monomers characterization, was determined via HPLC. Results are reported in Table III. Glucose was the most important monosaccharide released, and its concentration increased when using the enzymatic pre-treatment. Nevertheless also xylose, mannose and galactose, as well as arabinose and glucuronic acid were found. The combined use of BGL and Primafast (0.12 and 0.2 ml/gTS, respectively) yielded the highest release of total soluble sugars, that reached values of 31-40 g/L in the applied condition, with respect to values of 3-4 g/L evidenced for raw substrates.

3.4 Biochemical Methane Production (BMP)

Specific methane production of untreated and pretreated ensiled sorghum forage and wheat straw are represented in Figure 1. The methane production of untreated wheat straw (0.188±0.010 L_{CH4}/gVS) was lower than that of ensiled sorghum forage (0.282±0.010 L_{CH4}/gVS), due to their different chemical composition.

As for methane production of untreated substrates, these experimental data are well in agreement with literature values. Previous studies have indicated a specific methane yield of untreated straw in the range of 0.162 to 0.241 L_{CH4}/gVS [19-21]. Jerger et al. [22] and Chynoweth et al. [23] have indicated a specific methane yield of untreated sorghum (0.8 mm size) in the range of 0.260 to 0.390 L_{CH4}/gVS. Enzymatic pre-treatment had an effect on sorghum hydrolysis, as confirmed by the increase in sugars released (Table III). However the decrease in methane production compared to untreated sample was probably due to a loss of organic matter and thereafter a production of CO_2 during the pre-treatment, as also showed by [24]. Nevertheless, further tests are needed to confirm these data. [24] also studied the effects of the addition of enzyme products containing cellulase, hemicellulase, and ß-glucosidase to anaerobic digestion systems using Jose Tall Wheat Grass, which has an higher lignin content (around 20%) than ensiled sorghum forage. They showed that the pre-treatment had a significant effect on hydrolysis; however, it did not produce a significant effect in biogas and methane production. Enzymatic pre-treatment had also an effect on wheat straw hydrolysis (Table III), but not on its methane production.

Sodium hydroxide pre-treatment, prior to enzymatic pre-treatment, could alter the structure of lignin to make cellulose and hemicelluloses more accessible to hydrolytic enzymes. Therefore, a combination of NaOH and enzymatic pre-treatment showed an increase (up to +32% and +76% for ensiled sorghum and wheat straw respectively).

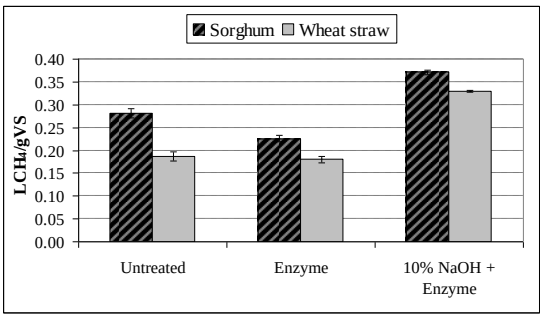

Figure 1. Specific methane production of untreated and pretreated sorghum and wheat straw at 30 days.

4 CONCLUSION

The effect of enzymatic and combined alkaline-enzymatic pre-treatments was investigated in order to increase the methane production of ensiled sorghum forage and wheat straw. Biochemical methane potential tests revealed that enzymatic pre-treatments did not show any improvement in the methane yields. An improvement in methane production (up to +32% and +76% for ensiled sorghum and wheat straw respectively) compared to untreated sample, was reached after the alkaline and combined alkaline and enzymatic pre-treatment, probably due to the lignin reduction. The combined use of BGL and Primafast enzymatic preparations yielded the highest release of sugars. The promising results obtained combining the use of chemical and enzymatic pre-treatments will pave the way to the application of these procedure in bio-methane production through anaerobic digestion.

Table II. Characterization of enzyme preparations.

Enzymatic activity	Enzyme preparation			
	BGL	**Ultra L**	**Pulpzyme HC**	**Primafast**
Protein (mg protein/ml)	120.3 ± 5.9	69.3 ± 3.3	21.5 ±0.6	167.0 ± 9.5
CMCase (IU/ml)	235.7 ± 24.3	613.2 ± 42.9	10.9 ± 2.7	2063.4 ± 0.8
Xylanase (IU/ml)	126.5 ± 10.6	108.1 ± 12.2	106.8 ± 1.9	282.8 ± 5.7
Avicelase (IU/ml)	0.3-1.7	1.7 - 6.1	traces	1.85 - 3.5

Table III. Composition of soluble sugars (g/L) present in samples of sorghum and wheat straw untreated or pretreated with alkali and added with enzyme preparations (data are mean of three replicates, CV in the range 8-17%).

Sample	Glucose	Xylose-Mannose-Galactose	Glucuronic acid	Arabinose	Ramnose	Fucose
Sorghum	0.28	0.40	<0.05	0.10	0.13	< 0.05
+ Enzyme	2.44	2.79	0.11	0.67	0.60	0.17
+ 10%NaOH + Enzyme	7.59	3.84	0.15	0.66	<0.05	<0.05
Wheat straw	0.21	0.32	<0.05	<0.05	<0.05	<0.05
+ Enzyme	5.96	2.73	<0.05	0.52	<0.05	<0.05
+ 10%NaOH + Enzyme	7.41	4.77	0.13	0.64	<0.05	<0.05

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