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Pre-treatment of lignocellulosic materials by enzymatic mixture to enhance biogas production

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Abstract: The aim of this study was to evaluate the effect of an enzymatic mixture on the increase of biogas production from lignocellulosic materials as rapeseed straw, maize waste, and wheat straw. For efficient application of the enzymatic mixture, conditions of its use were optimized regarding 50 °C, pH 7 and an enzyme dose of 0.25 % w/v. Biogas potential test confirmed positive effect of the enzymatic mixture on anaerobic digestion of already thermally and alkali pre-treated lignocellulosic materials, as significantly higher biogas production was observed after the enzymatic mixture addition for all monitored substrates. Addition of the enzymatic mixture to the most used substrate at biogas plants — maize silage, had also positive effect on biogas production during the biogas potential test. This fact was not proven during long-term operations of the reactors as the values of total cumulative biogas productions for the whole monitored period from reactors for anaerobic digestion of maize silage with and without addition of enzymatic mixture did not differ significantly.

Abbreviations: COD — chemical oxygen demand, FM — fresh material, FPU — filter paper units, OLR — organic loading rate, RS — reducing sugars, TOC — total organic compounds, TS — total solids, U — amount of enzyme catalysing the reaction of 1 μmol of substrate per minute, VFA — volatile fatty acids, VS — volatile solids

Keywords: anaerobic digestion, biomass, long-term operation, maize silage, methane

Introduction

Anaerobic digestion of biodegradable materials yields a rich source of energy — biogas. Such materials include lignocellulosic biomass, which is a substrate with great potential for biogas production in terms of large production in agriculture, forestry, or industry. However, it is difficult to degrade due to the complexity of lignocellulosic biomass structure (Sawatdeenarunat et al., 2015). The goal of lignocellulosic biomass pre-treatment is to eliminate the reasons causing its resistance to the action of anaerobic microorganisms, i.e. lignin and hemicellulose content, cellulose crystallinity, accessible surface area and degree of cellulose polymerization (Zheng et al., 2014). Several pre-treatment methods including chemical (Pelera and Gidarakos, 2018), thermal (Pagliaccia et al., 2019) and physical methods (Kaur and Puthela, 2016; Menardo et al., 2012) have been studied to improve biogas yield from lignocellulosic biomass.

Biological pre-treatment methods including the application of fungi (Wan and Li, 2012), microorganism (Xu et al., 2018) and enzymes have recently been the subject of various studies. They represent potentially more energy-efficient, economical- and environment friendly alternatives to other types of pre-treatments as they do not require the addition of chemicals or high-energy inputs (Koupaie et al., 2019). The topic of our work was to study the effect of enzymatic pre-treatment on biogas production from lignocellulosic biomass, which has received less attention in literature.

Though, several papers have confirmed the positive effect of enzymatic pre-treatment of various lignocellulosic materials. The effect of enzymatic pre-treatment of switchgrass by cellobiase and cellulase was studied by El-Mashad (2015). Anaerobic digestion tests have shown positive effect of such pre-treatment on biogas production resp. methane production when the methane yield from pre-treated switchgrass (274.28 mL/g VS) was by 39 % higher compared to that from untreated material (197.39 mL/g VS).

Schroyen (2015) proved that enzymatic pre-treatment can help to degrade the matrix of lignocellulosic biomass as significant increase of phenolics leaking out of the matrix was observed. Laccase (2 U/g substrate) and versatile peroxidase (1.5 U/g substrate) were used to pre-treat materials such as wheat straw, corn stover, and others, with some additives and incubation at 30 °C for 6 h to increase enzyme activity. When the pre-treated materials were used as substrates in the biomethane potential test, an increase of 24 % and 12 % in methane production from corn stover and wheat straw (compared to the untreated sample) were observed respectively.

In another work, fungal hydrolytic enzyme mixture was used to improve hydrolysis and finally biogas production of selected lignocellulosic materials as these mixtures are increasingly available in the market. First, optimal conditions for enzymatic hydrolysis were determined: temperature of 40 °C, pH 5.4, and enzyme concentration 0.04 g enzyme/g VS of substrate. Application of fungal hydrolytic enzyme mixture is beneficial in large scale processes, as this pre-treatment positively affects the anaerobic digestion of selected materials (Quiñones et al., 2009).

In this paper, selected lignocellulosic materials were pre-treated using a commercial enzymatic mixture. Positive effect of this cheap alternative of enzymatic pre-treatment would lead to considerable savings as using separate enzymes is currently one of the most expensive pre-treatment methods. Optimization of the enzymatic mixture application, i.e. optimal temperature, pH value, and dose of enzymatic mixture, had to be done. Under optimized conditions, this pre-treatment method was further assessed by the biogas potential test using various already thermally or alkali pre-treated lignocellulosic materials such as wheat straw, maize waste, and rapeseed straw. The enzymatic mixture was also applied to the substrate most commonly used at biogas plants — maize silage. The effect of the enzymatic mixture was verified by the biogas potential test as well as by long-term operation of the reactors for anaerobic digestion of maize silage.

Materials and Methods

Substrates and inoculum

Lignocellulosic substrates used in this study to test the biogas potential were maize waste, rapeseed straw, and wheat straw, which are very common agriculture wastes. The commercial enzymatic mixture was also used to increase biogas production of maize silage, which is the most used substrate at biogas plants. The main physicochemical characteristics of the substrates are reported in Table 1.

As inoculum for the biogas potential test, anaerobically stabilized sludge from the wastewater treatment plant in Devínska Nová Ves was used. For long-term reactor operations for anaerobic digestion of maize silage, anaerobic sludge from biogas plant in Hurbanovo was used as inoculum; its characteristics are reported in Table 2.

Enzymatic mixture

The enzymatic mixture Everstar Culturpur, biologically active and ecologically harmless, used to accelerate the decomposition of organic matter in garden compost was used. It contains a mixture of enzymes, bacterial cultures, and nutrients beneficial for the microorganisms' action; cellulase content is < 1 % and it is in form of white to yellowish powder. Since the cellulase content was particularly interesting, the cellulase activity of this mixture was determined.

The use of such an enzymatic mixture for the pre-treatment of studied substrates is a cheaper and more environmentally friendly alternative to the other enzymatic pre-treatment methods, as it is a commercially available mixture.

Determination of enzymatic activity

Specific activity of the enzymatic mixture was determined according to Mandels et al. (1976). As a model cellulose raw material, filter paper (cellulose content 90—95 %) was used. To achieve

Tab. 1. Characteristics of substrates.

	maize waste	wheat straw	rapeseed straw	maize silage
TS (g/g)	0.9485	0.9405	0.9190	0.3565
VS (g/g)	0.9205	0.9225	0.8503	0.3340
COD (g/g)	1.31	1.20	1.24	–
Cellulose (%)	45.15	28.75	22.00	–
TOC (mg/g)	428.09	455.77	496.35	–

Tab. 2. Characteristics of inoculum.

	Inoculum for biogas potential tests	Inoculum for long-term reactor operations
TS (g/L)	25.6	13.1
VS (g/L)	14.1	10.8
VFA (mg/L)	432	3770
COD (mg/L)	3012	14500
N-NH_4 (mg/L)	420	864
P-PO_4 (mg/L)	26	23.4

the required pH value, 20 mL of buffers (0.1 M acetate buffers for pH 4 and pH 5, 0.1 M phosphate buffers for pH 6, pH 7 and pH 8) were added to flasks with weighed dry and ground model material of 1.6 ±0.01 g. Subsequently, the substrates were tempered at the temperatures of 30, 40, 50, 60 and 70 °C for 10 minutes. Hydrolysis mixture was prepared in the ratio of 50 mg of enzyme per 10 mL of water. Enzymatic mixture was dosed to the prepared flasks in the amount of 0.1 g, representing 0.5 % w/v. Samples were taken at various time intervals after the enzymatic mixture dose (2, 4, 6 and 8 h) to determine reducing sugars (RS) according to Somogyi (1952). The determined reducing sugars were used to calculate the enzyme activity and the specific enzyme activity**.** The amount of protein in the commercial enzymatic mixture was 69 mg/g of enzymatic mixture.

Test of biogas potential

Pre-treated materials were used as substrates for biogas potential tests to assess the effect of enzymatic mixture addition for the lignocellulosic materials' pre-treatment on biogas production. Lignocellulosic materials (rapeseed straw, wheat straw, and maize waste) were pre-treated using water or NaOH solutions (0.1 %, 0.25 % or 0.5 %) for 1 hour at 90—100 °C. Enzymatic mixture was added to pre-treated materials under optimized conditions (T = 50 °C, pH = 7, enzyme dose of 0.25 % w/v). The use of the enzymatic mixture to increase biogas production was also assessed for maize silage, where it was added in the amount of 0.2 g or 0.5 g to 5 g of maize silage.

The test of biogas potential was performed according to the method of Angelidaki et al. (2009). Substrates and inoculum (Table 2) were loaded to gas-tight flasks in triplicate. Flasks containing only inoculum and controls with pre-treated materials without enzymatic mixture addition were also tested. Before hermetically sealing the flasks with rubber septum, the sludge mixture was bubbled through with nitrogen for 1 minute. Biogas production was measured daily at given time intervals by volumetric method.

During the test, biogas was also collected in gas sampling bags and a gas analyser GA 2000 Plus (Geotechnical Instruments, UK) was used to determinate the biogas composition.

Long-term reactor operation

Long-term monitoring of anaerobic reactors for anaerobic digestion of maize silage without and with addition of the enzymatic mixture were performed under mesophilic conditions (37 °C) in semi-continuous reactors with a useful volume of 6.76 L. Anaerobic sludge from biogas plant Hurbanovo, which processes maize silage, was used as inoculum for the reactors' start-up (Table 2). Initial OLR (organic loading rate) of the reactors was 0.5 kg VS/($m^3 \cdot d$), which corresponds to a dose of maize silage of 10 g FM (fresh material). After 8 days of operation, OLR increased to 1 kg VS/($m^3 \cdot d$). When the sludge water parameters of both reactors were approximately the same, on the 36th day of operation, maize silage with the enzymatic mixture were added to one of the reactors. The enzyme dose was 0.04 g of enzyme/g FM of maize silage.

During the long-term operation of the reactors, parameters such as pH, total solids (TS), volatile solids (VS), chemical oxygen demand (COD), ammonia nitrogen (N-NH_4), phosphate phosphorus (P-PO_4), and volatile fatty acids (VFA) of sludge water were monitored. Biogas production was monitored daily and the biogas composition was determined by a gas analyser GA 2000 Plus (Geotechnical Instruments, UK) on the 29th and 52nd day of operation.

Analytic methods

Determination of COD, TS, VS, N-NH_4 and P-PO_4 concentrations was performed according to APHA (2017) and pH values during the experiments were determined using an ION-type ACTIVITY METER MS 20 pH meter. To determine VFA, three-point titration method according to Kapp (1984) was used. The Biuret method was used to determine the total protein concentration (Zheng et al., 2017).

Results and Discussion

Optimization of conditions for the use of a commercial enzymatic mixture

The most suitable enzymatic hydrolysis conditions for efficient use of the commercial enzymatic mixture were determined. Influence of temperature, pH, and concentration of enzymes resp. the ratio of the enzymes mass to the solution volume were studied to obtain the highest possible yield of fermentable sugars. The amounts of reducing sugars in mg released during enzymatic hydrolysis from 1.6 g of filter paper at different pH and temperatures are shown in Figure 1. Calculated specific enzymatic activities at the specified hydrolysis conditions are recorded in Table 3.

The largest amount of reducing sugars from 1.6 g of filter paper was released at 50 °C and pH 7; pH 7 seems to be the most suitable for enzyme activity at all examined temperatures. At 60 °C, the same amount of reducing sugars was released at pH 7 and pH 8 but the amount of sugars is by 50 % lower than that released at 50 °C and pH 7. At each

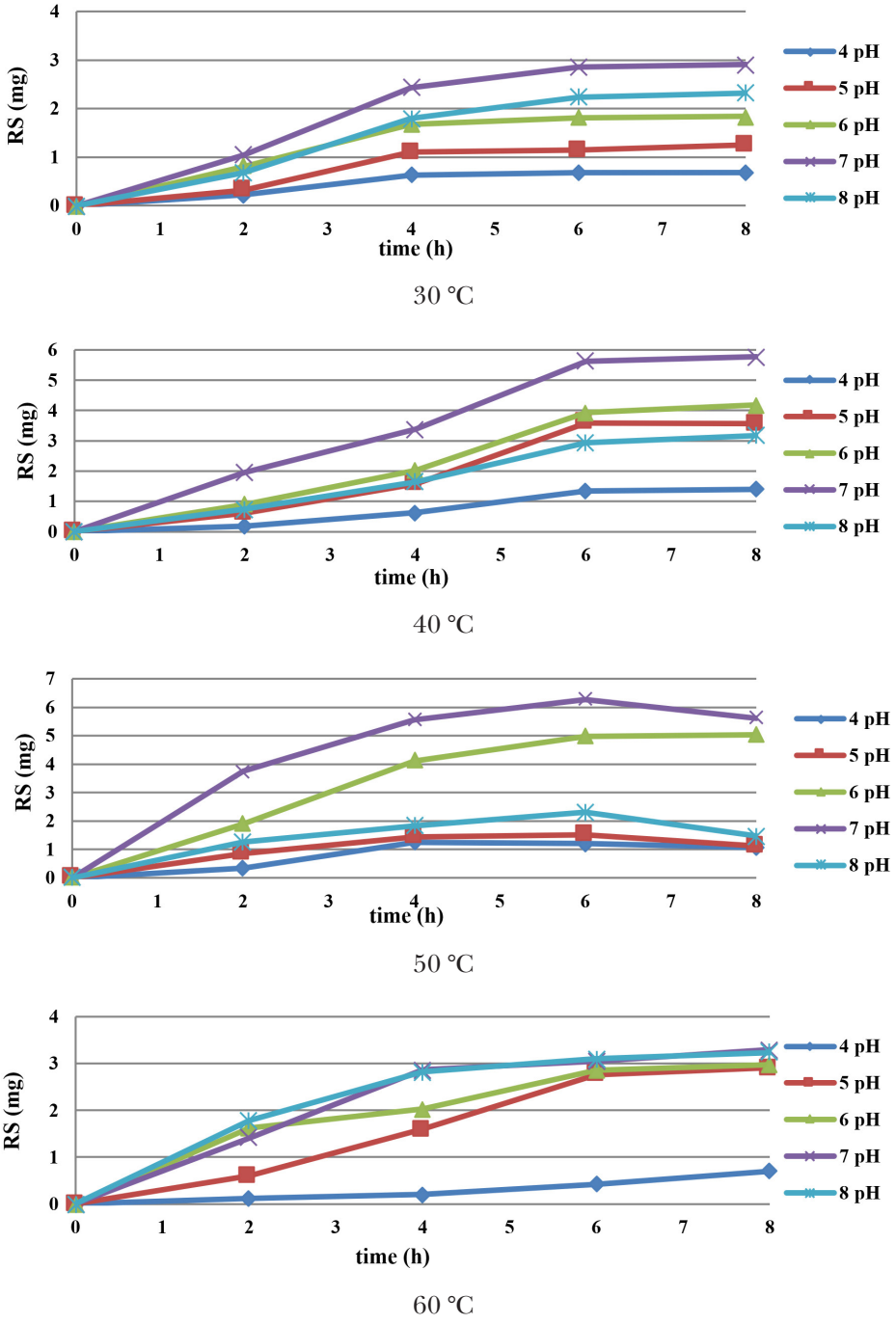


Fig. 1. Amount of reducing sugars released during enzymatic hydrolysis of filter paper.

Tab. 3. Specific enzyme activity at different conditions (temperature and pH).

specific activities (pkat/mg)	pH 4	pH 5	pH 6	pH 7	pH 8
30 °C	25.71	35.81	91.80	118.40	76.63
40 °C	19.75	69.87	100.97	223.00	85.01
50 °C	38.99	96.74	216.11	426.74	143.05
60 °C	13.79	68.03	184.69	159.69	203.13

studied temperature, the lowest amount of released reducing sugar was at pH 4.

After determining the optimal temperature and pH (50 °C, pH 7) of enzymatic hydrolysis, optimal dose of enzymes (ratio of enzymes to solution based on released reducing sugars during enzymatic hydrolysis) was determined. Results of the specific activities reported in Table 4 show that the highest specific activity was achieved at 0.25 % w/v and the lowest at 2.5 % w/v, which indicates lower required amount of the enzymatic mixture to be applied. This result is also consistent with the research of Ziemiński et al. (2012), where only slight increase of reducing sugars concentration in sugar beet pulp hydrolysates was observed with increasing dose of enzymes from 0.15 FPU/g TS to 0.75 FPU/g TS. Similarly, in Thulluri et al. (2014), the release of sugars increased with the increase in the enzyme dose from 5 to 25 U/g of substrate, but reached maximum concentration of released sugar at the enzyme loading of 20 U/g and no significant difference in sugar release was found between the enzyme loadings of 20 U/g and 25 U/g. A decrease in the total reducing sugar yield caused by increased enzyme concentration may be due to the lower amount of substrate available for enzyme attack (Thani et al., 2019).

Test of biogas potential of pre-treated lignocellulosic materials with addition of enzymatic mixture

To determine whether the dosing of enzymes into the mixture of anaerobic microorganisms influences substrate degradation or anaerobic microorganisms' activity and thus the result biogas production, biogas potential tests was performed. The enzymatic mixture was applied to lignocellulosic materials already pre-treated by environment friendly methods, water and NaOH at low concentration.

Tab. 4. Specific enzyme activity at different enzyme doses.

dose of enzyme (% w/v)	0.25	0.5	2.5
specific activities (pkat/mg)	691.47	426.74	86.53

After 1 hour of thermal and thermochemical pre-treatment with alkalis and subsequent enzymatic hydrolysis, the investigated materials were used as substrates for biogas potential test. The effect of single and combined pre-treatment (with enzymatic hydrolysis) on biogas production was compared. Table 5 shows the specific biogas production (biogas production based on VS of substrates) of substrates pre-treated by the selected methods / specific biogas productions of substrates pre-treated by the selected methods with the addition of the enzymatic mixture. The table shows a summary of specific biogas productions from solid and liquid fractions of pre-treated materials.

A comparison of individual specific biogas productions shows that the addition of an enzymatic mixture increased the production of biogas from pre-treated substrates except for one case, maize waste pre-treated by 0.25 % NaOH. Addition of the enzymatic mixture to thermally pre-treated materials increased the biogas yield for rapeseed straw by 23 %, that of wheat straw by 50 % and that of maize waste by 42 % compared to the biogas yields from thermally pre-treated substrates without the addition of the enzymatic mixture. The specific biogas production of materials pre-treated with 0.1 % NaOH and enzymatic mixture increased by 48 % for rapeseed straw, 85 % for wheat straw and by 58 % for maize waste compared to specific biogas production of pre-treated materials without the addition of the enzymatic mixture.

Pre-treatment with 0.25 % NaOH together with the enzymatic mixture resulted in up to 38 % and 40 % increase in specific biogas production of rapeseed straw and wheat straw, but a slight decrease (5 %) was observed for maize waste. Application of the enzymatic mixture to materials pre-treated with 0.5 % NaOH also showed positive effect on biogas production; the specific biogas production of the studied materials (rapeseed straw, wheat straw and maize waste) increased by 4 %, 25 % and 62 %, respectively.

Compared to untreated substrates, the largest increase (by 302 %) in biogas production was measured in for wheat straw pre-treated with 0.5 % NaOH with the addition of an enzymatic mixture. The method using 0.5 % NaOH and the addition of an enzymatic mixture was proved to be a very

Tab. 5. Results of biogas potential tests.

	Specific biogas production (mL/g VS)				
	untreated	**water/+E**	**0.1 % NaOH/+E**	**0.25 % NaOH/+E**	**0.5 % NaOH/+E**
rapeseed straw	64.1	75.1/92.3	122.1/180.1	140.5/193.5	222.5/231.3
wheat straw	71.0	90.3/135.4	127.4/235.0	182.4/255.3	227.6/285.4
maize waste	109.3	108.7/154.3	211.2/334.1	245.1/233.9	257.0/415.1

effective pre-treatment method as a significant increase in biogas production also for other substrates (261 % for rapeseed straw and 280 % for maize waste) was observed.
The biogas potential test confirmed that this enzymatic mixture has positive effect on the action of anaerobic microorganisms and the decomposition of lignocellulosic biomass, which structure was already disturbed by previous pre-treatment.
The great potential of the combination of alkaline (5 % NaOH) and enzymatic pre-treatment of lignocellulosic biomass degradation and anaerobic digestion was also proved by Michalska et al. (2015). There was significant increase in biogas production when a combination of methods was used compared to the biomass pre-treated only with NaOH. As the cumulative biogas production for Miscanthus and Sida pre-treated both chemically and enzymatically was 421.5 Ndm^3/kg TS and 316.3 Ndm^3/kg TS and for biomass pre-treated only chemically it was 8.7 Ndm^3/kg TS and 57.4 Ndm^3/kg TS. The low biogas production from biomass pre-treated by 5 % NaOH is justified by the high concentration of phenolic compounds released as the results of lignin degradation.

Application of enzymatic mixture to maize silage

The enzymatic mixture was also applied to maize silage, which is the most used substrate at biogas plants. The effect of enzymatic mixture dosing to maize silage was assessed based on the biogas potential test and long-term operation of semi-continuous reactors.
Results of the biogas potential test after 16 days (Table 6) show that addition of the enzymatic mixture to the substrate increased the final biogas production. With the addition of 0.2 g of enzyme, the production of biogas increased by 32 % compared to that from maize silage without enzyme addition. At 0.5 g of used enzyme, the biogas production increased by only 13 %. Also, a lower dose of the enzymatic mixture proved to be more suitable in this experiment.
Addition of the enzymatic mixture to a substrate (maize silage) in long-term operation of laboratory models of semi-continuous reactors was further investigated to compare whether it affects biogas production on a long-term basis. Based on the results of the previous biogas potential test, the enzyme dose in long-term operation was in a lower ratio (0.04 g enzyme/g silage (FM)).
Results of long-term reactors' operation are shown in the course of daily biogas production from both reactors (Figure 2), where the start of enzymatic mixture dosing is also marked (from the 36th day) as it significantly affected the amount of daily biogas production. The increase in biogas production, by 25 % higher on average, lasted only for 14 days. After 50 days of operation, the biogas production values in both reactors returned to comparable values. Ultimately, the values of the total cumulative biogas productions for the whole monitored period did not differ significantly (Figure 3).
This can be explained by the microbial diversity probably occurring after the addition of the enzymatic mixture, which could support the growth of specific bacteria. Similar results are reported in Romano et al. (2009), where addition of the enzymatic mixture Novozyme 342 (with a high proportion of cellulases) with wheat grass directly into the anaerobic digestion process was studied. Their results show that the rate of biogas production was significantly affected but it decreased significantly after 10 days. Even the enzyme products showed positive effects on the solubilization of wheat grass when used alone, there were no significant improvements in the final biogas yield of the anaerobic digestion systems.
Similarly, methane content of biogas (Table 7) in the period before and after the start of the addition of the enzymatic mixture did not show significant differences between the reactors. Even in the reactor without the addition of enzymatic mixture showed higher methane content after 52 days.
Average values of the monitored parameters (Table 8) in sludge water from the reactors during operation did not differ significantly even when an enzymatic mixture was added to one of the reactors. Also, the values of VS in both reactors were about 70 %, which reveals that the enzymes did not cause higher decomposition of maize silage.
Despite the previous result of the biogas potential test, which showed increased biogas production, this fact was not proven during long-term reactors' operation.
For comparison, in Dubrovskis and Plume (2017), where the enzyme mixture of cellulase, b-gluconase

Tab. 6. Results of biogas potential test.

dose of maize silage (g FM)	–	**5**	**5**	**5**
dose of enzymatic mixture (g)	–	–	**0.2**	0.5
total biogas production (mL)	108	800	**1180**	920
specific biogas production (mL/g VS)	–	479	**707**	551

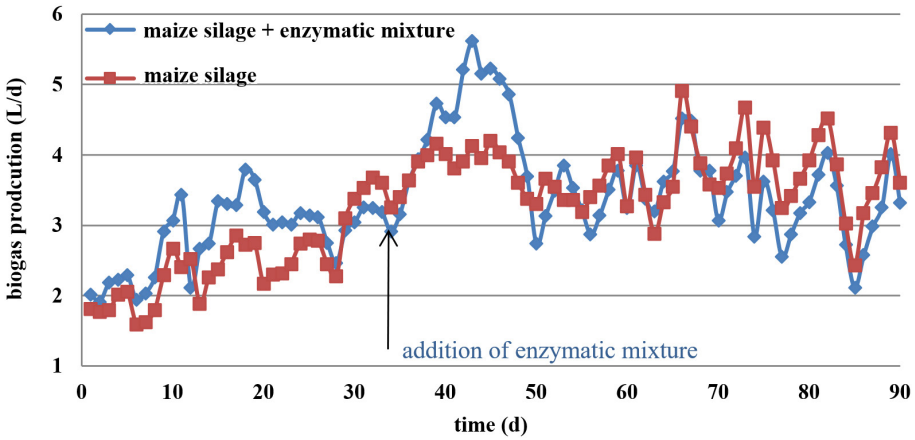


Fig. 2. Daily biogas production.

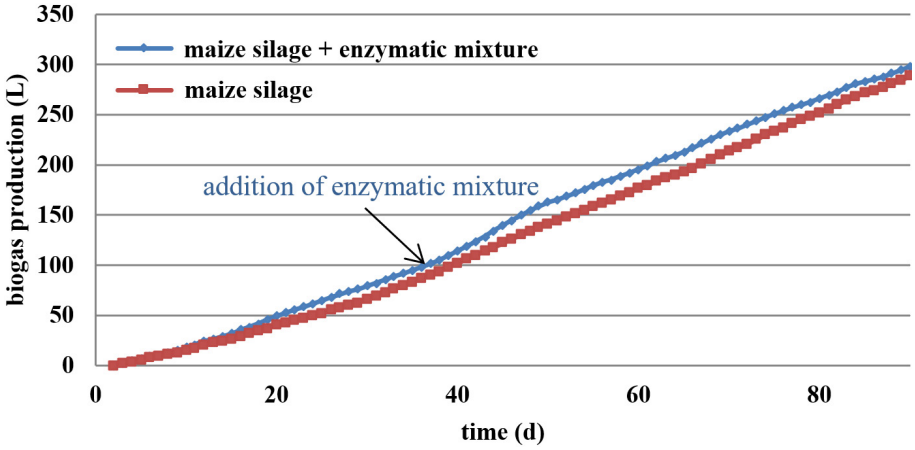


Fig. 3. Cumulative biogas production.

Tab. 7. Biogas composition on the 29th and 52nd days of long-term reactors' operation (R1 — maize silage with the addition of enzymes from 36th day, R2 — maize silage without the addition of enzymes).

	29th day		52nd day	
	R_1	R_2	R_1	R_2
CH_4 (%)	49.2	49.4	52.6	57.2
CO_2 (%)	49.4	48.3	46.6	42.2
O_2 (%)	0.4	0.5	0.2	0.3
H_2 (ppm)	29	31	109	112
H_2S (ppm)	76	231	276	104

Tab. 8. Average concentrations of monitored parameters in sludge water from reactors (R1 — maize silage with the addition of enzymes from 36th day, R2 — maize silage without the addition of enzymes).

days	pH		COD (mg/L)		VFA (mg/L)		N-NH_4 (mg/L)		P-PO_4 (mg/L)	
	R_1	R_2	R_1	R_2	R_1	R_2	R_1	R_2	R_1	R_2
1—36	7.2	7.3	12 379	10 675	6733	5386	1009	1041	57	56
37—50	7.3	7.4	15 802	15 127	5650	5358	921	945	16	14
51—90	7.4	7.3	17 410	12 808	2516	2517	905	903	52	48

and xilanase enzymes was used to improve the process of anaerobic digestion of maize silage, a slight increase in biogas production was observed. When maize silage and an enzymatic mixture, 0.03 g/g silage (FM), were added to a bioreactor with inoculum, the specific biogas production was 0.681 L/g VS while the specific biogas production from bioreactor without added enzymes was 0.632 L/g VS. Specific methane yield from the bioreactor with maize silage with enzyme mixtures was by 7.76 % higher compared to that obtained from the bioreactor with maize silage without the addition of enzymes. Also, the difference in biogas methane content was not significant, i.e. 53.6 % after the addition of the enzyme and 53.2 % without the enzyme addition.

Conclusion

Application of an easily available commercial enzymatic mixture under optimized conditions (50 °C, pH 7 and an enzyme dose of 0.25 % w/v) to already pre-treated lignocellulosic substrates has resulted in a significant increase in biogas production from lignocellulosic materials such as wheat straw, maize waste, and rapeseed straw. The highest increase in specific biogas production after enzymatic mixture addition (by 85 %) was observed for wheat straw pre-treated with 0.1 % NaOH. Also, for other materials and pre-treatment methods, the biogas potential test confirmed that this enzymatic mixture has positive effect on the decomposition of lignocellulosic biomass and thus biogas production. The addition of the enzymatic mixture to maize silage also showed positive effect on the biogas production during the biogas potential test. However, since this positive effect did not show during long-term reactor operation, it cannot be confirmed that the enzymatic mixture support biogas production on a long-term basis.

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