Characterization and Influence of a *Multi*-enzymatic Biopreparation for Biogas Yield Enhancement

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A multi-enzymatic biopreparation of *Trichoderma atroviride* G79/11 origin was characterized. The fungus showed relatively high cellulase production in a soybean flour-cellulose-lactose medium. Subsequently, based on its post-culture liquid, the biopreparation of the enzyme mixture was developed and characterized. The liquid form of the enzyme mixture reached 22 U cm^{-3} of cellulolytic activity and its lyophilisate exhibited 1.09 U cm^{-3} at pH 5.1 and 50 °C. The enzyme mixture was characterized by the following activities: xylanase, *β*-glucosidase, carboxymethyl cellulase, polygalactouronase, pectinesterase, amylase, lactase, and protease. A method for an efficient conditioning process of organic waste (fruit processing waste, dairy sewage sludge, corn silage, and grain broth) for biogas yield enhancement using the enzyme mixture was proposed. The enzyme mixture increased the efficacy of biogas production by 30% when the lyophilizate (0.5 mg g^{-1} d.m.) was applied prior to fermentation. A method for conducting the enzymatic conditioning process of organic waste using the enzyme mixture as a pretreatment was proposed. This was part of the optimization of the methane fermentation process to increase the biogas yield. Consequently, after application of the biopreparation, the efficiency of anaerobic digestion of organic waste was improved.

Keywords: Enzyme biopreparation; Cellulolytic activity; Methane fermentation; Trichoderma atroviride

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INTRODUCTION

The constant economic growth, increasing energy demand, and need to ensure energy security are the main driving forces behind research development on the production of clean, environmentally friendly energy. Climate changes associated with the emission of carbon dioxide and other pollutants from the combustion of fossil fuels are the biggest environmental issue, but also there have been economic challenges in recent years (Oleszek *et al.* 2015; Segura *et al.* 2017).

The biological aspect of processing of organic waste multi-compounds is widely taken into consideration by many scientists around the world. Issues that are repeatedly emphasized are focused on scaling up the decomposition of organic biomass, as well as the improvement of pretreatment conditions prior to anaerobic processing aimed at biogas yield enhancement (Alvira *et al.* 2010). A promising strategy for ensuring an efficient renewable energy source is the microbial hydrolysis of waste and anaerobic transformation

of organic and lignocellulose substrates. This is related to the fact that cellulose is the most abundant biopolymer on Earth and, simultaneously, the dominant waste of the agro food industry.

Biogas is a promising source of renewable energy, as the technology of production thereof combines the processes of recycling organic waste to produce methane. Due to the complexity of successive quantitative and qualitative changes in a consortium of microorganisms in a fermentation biomass and the multiplicity of metabolic pathways leading to the production of biogas, the biotechnology of this process has not yet been fully described (Frąc and Ziemiński 2012; Ziemiński and Frąc 2012).

Among the various types of substrates, corn silage is currently most commonly used for the production of biogas, particularly in Western and Central Europe (Mursec *et al.* 2009; Fugol and Prask 2011; Oleszek *et al.* 2015). The mixture of wastes proposed in this paper, in terms of biogas yield, is an alternative to the use of energy crops such as reed canary grass, miscanthus, and Virginia mallow. Moreover, a good yield of energy crops can only be obtained on fertile soils, which *de facto* should be used for food production (Giovannetti and Ticci 2016).

The yields of biogas and methane from the investigated fruit market were reported as 480 m^3 Mg^{-1} d.m. and 250 m^3 Mg^{-1} d.m., respectively (Zieliński *et al.* 2016). The methane content obtained in the process of the anaerobic conversion of dairy sewage sludge was estimated at 61%. However, a rather small disadvantage of organic wastes in the context of the possibility of being used for bioconversion into biogas could be their distribution, as well as the lack of segregation (Igliński *et al.* 2012).

Increasingly, co-fermentation of different types of waste is used as one method for the enhancement of biogas production (Pages Diaz *et al.* 2011). Co-fermentation fits well with the concept of sustainable development and plays an important role in the disposal of waste and increment in methane yields. When selecting substrates for co-fermentation, their chemical composition should be taken into account, especially the content of biodegradable compounds, but also the availability of these substrates on the local market (Montusiewicz and Lebiocka 2011). For example, there are easily available wastes, such as the fruit industry expeller, decoction grain, and dairy sewage sludge. Expellers account for a significant amount of fruit and vegetable industry waste. Wastes from fruit and vegetable processing residues, *i.e.* woody stalks and leaves composed mainly of cell wall polysaccharides, such as pectin, cellulose, and hemicellulose, are suitable for valorization. Simultaneously, the brew is a source of energy, in the form of proteins, fats, and residues of starch and cellulose, which is the preferred starting material in terms of suitability for the gasification of the methane fermentation process. Similarly, dairy sewage sludge that is produced as a by-product of the dairy industry is a valuable substrate for methane production because it is rich in fat and protein (Frąc *et al.* 2014).

Energy production based mainly on waste is beneficial because it reduces greenhouse gas emissions. However, especially the type of biomass containing lignocellulose is not completely biodegradable in the methane fermentation process, due to its structure and chemical composition. This may lead to a lower energy yield of its bioconversion, compared to other substrates. In contrast, lignocellulose biotransformation is difficult, because it is a complex of biopolymers: cellulose, hemicelluloses, and lignin, which are linked together *via* covalent and hydrogen bonds that eventually limit the speed and efficiency of fermentation. An increased use of these components and greater efficacy of biofuel production becomes possible when the compact structure of organic substrates (especially lignocellulose) is relaxed. One of the solutions used for loosening the structure

is to introduce an additional step in biomass bioconversion. The hydrolytic (enzymatic) pretreatment is meant to increase the susceptibility of the substrate to biological degradation, increasing the rate of the methane fermentation process and the efficacy of the biogas obtained. To improve the biodegradability of the wastes, a variety of methods, such as chemical, physicochemical, biological, and combinations thereof, have been employed (Demirbas 2007; Bruni *et al.* 2010; Karki *et al.* 2011; Parawira 2012).

The available methods for conditioning pretreatment of organic biomass are meant to increase the availability of fermentable sugars. As far as enzymatic pretreatment, there is a need to develop multi-enzymatic biopreparations having broad substrate activity directed to complex compounds met in co-substrates (*e.g.* cellulose, pectin, lactose, proteins or starch). According to our best knowledge, commercial biopreparations show relatively narrow specificity (contain one or two types of enzymes).

Cellulases in such a biopreparation play a significant role, because they catalyze the limiting for biogas production step which is decomposition of cellulose. In general, the efficiency of the hydrolysis of organic matter depends on several factors: the composition of the organic substrate compounds, type of pretreatment, dosage, and the effectiveness of hydrolytic enzymes applied as biopreparations (Alvira *et al.* 2010).

The aim of the study was to characterize the enzymatic properties of the biopreparation of *Trichoderma atroviride* G79/11 origin, including such enzymes as cellulases, xylanases, *β*-glucosidase, carboxymethylcellulase, poligalactouronase, pectinesterase, amylase, lactase and protease. A further goal was to propose a method for the pretreatment of organic waste fermentation co-substrates (fruit processing waste, dairy sewage sludge, corn silage, and grain broth) to enhance biogas yield.

EXPERIMENTAL

Materials

Trichoderma atroviride G79/11

The G79/11 strain was isolated from the dairy sewage sludge and identified as *Trichoderma atroviride*. The nucleotide sequence of segment D2 located within the large ribosomal subunit (Large Subunit, LSU) was deposited in the GenBank database under the accession number KT333455. In addition, the G79/11 strain was deposited in the Collection of Industrial Microorganisms at the Institute of Agricultural and Food Biotechnology in Warsaw, under the number KKP 2056p, as an isolate with high hydrolytic activity. This strain was selected in the Laboratory of Molecular and Environmental Microbiology, Institute of Agrophysics, Polish Academy of Sciences in Lublin.

Enzyme mixture development

The biopreparation was obtained on the basis of a concentrated post-culture liquid after culturing *Trichoderma atroviride* G79/11 on a soy flour-cellulose-lactose medium (MSCL) under the following culture conditions: pH 4.5, temperature 22 ºC, and inoculum *Trichoderma atroviride* G79/11 spores 8×10^9. The MSCL composition was as follows: 10 g dm^{-3} lactose, 5 g dm^{-3} microcrystalline cellulose, 20 g dm^{-3} soy flour, 6.3 g dm^{-3} KH_2PO_4, 9.92 g dm^{-3} $(NH_4)_2NO_3$, 0.82 g dm^{-3} $CaCl_2 \cdot 2H_2O$, 0.82 g dm^{-3} $MgSO_4 \cdot 7H_2O$, 0.15% Tween 80, 5 cm^3 dm^{-3} Antifoam B, 513 mg dm^{-3} $FeSO_4 \cdot 7H_2O$, 166 mg dm^{-3} $MnSO_4 \cdot H_2O$, 8.5 mg dm^{-3} $ZnSO_4 \cdot 7H_2O$, and 5.4 mg dm^{-3} $CoCl_2 \cdot 6H_2O$. The shaking culture

was set up in 120 cm^3 of medium in a shake flask with a capacity of 1 dm^3 on a shaker that was reciprocated at a rotation speed of 160 rpm for 6 days. After this time, the mycelium was separated from the post-culture liquid by repeated filtration through sterile gauze. Then, the suspension was centrifuged at 4000 rpm for 20 min to remove mycelium particles. The concentration of the filtrate (20×) was carried out using an ultrafiltration module Prep/Scale TFF (Merck Millipore, Billerica, United States). The liquid biopreparation obtained was the lyophilized form. This process was performed after freezing (-50 °C) at a pressure of 0.1 mBar (Labconco freeze dryer, FreeZone Letters 12 Console Freeze Dry System, Kansas City, United States). The post-culture filtrate in the form of a concentrated liquid and lyophilizate was termed the enzyme mixture. This biopreparation was used in the subsequent stages of this research to optimize the process of anaerobic digestion. The method for the biopreparation production was described and submitted in the Polish patent application No. P.409138; the trademark *metaferm*, which refers to the enzyme mixture, is protected under the Polish declaration No. z-417652.

Determination of the optimum conditions for cellulolytic activity

The selection of optimal conditions for the cellulolytic activity of *T. atroviride* strain G79/11 included the determination of optimum pH and temperature. The pH optimum was determined in the MSCL medium filtrate. A nonspecific cellulolytic activity assay (FPU) was performed at 50 °C in a McIlvaine buffer at pH 4.3, 4.5, 4.7, 4.9, 5.1, 5.3, 5.5, and 5.7. The selection of optimal temperature conditions was carried out in the following range of temperatures: 4, 10, 20, 30, 40, 50, 60, 70, 80, and 90 °C in a McIlvaine buffer, pH 5.1.

Characteristics of the biopreparation

The thermal stability of the lyophilized enzyme mixture form was determined by an evaluation of the cellulolytic activity, following a Filter Paper Unit (FPU) assay. The suspension of the enzyme mixture (1 mg cm^{-3}) was tested in a McIlvaine buffer at 50 °C and pH of 5.1, after 5 min, 10 min, and 15 min and after 1 h, 3 h, 6 h, 24 h, 48 h, and 72 h of incubation at 4 °C, 20 °C, 30 °C, 40 °C, 50 °C, 60 °C, 70 °C, 80 °C, and 90 °C. The research was conducted to assess the pH stability in the enzyme mixture's lyophilizate. For this purpose, a suspension of 1 mg of the lyophilizate was prepared in 1 cm^3 of Britton-Robinson buffer at the following pH values: 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10. The determination of the cellulolytic activity (FPU) was performed after 0 min, 5 min, 10 min, and 15 min and 1 h, 3 h, 6 h, 24 h, and 48 h of incubation at 4 °C. To determine the pH, a pH-meter CPI-505 with a pH electrode EPP-3 was used. The effect of the storage temperature on the enzyme mixture's cellulolytic activity was evaluated in a lyophilized form of the biopreparation after two weeks of storage in the following temperature conditions: 4 °C, 20 °C, -20 °C, and -70 °C.

Enzymatic activity

The cellulolytic activity of the biopreparation was determined in liquid culture filtrates and based on cellulolytic activity (FPU) after 60 min of incubation at 50 °C and 37 °C (Mandels *et al.* 1976; King *et al.* 2009). The amount of the enzyme that liberated 1 µM of glucose (reducing sugars) during 1 min at 50 °C was assumed as a FPU activity unit (Weldesemayat 2011; Janas and Targoński 2014). The analysis was performed in analytical triplicate for each of the biological duplicate experiments (n = 6). The results were expressed as a percentage of the average cellulolytic activity.

The determination of the activity of other enzymes in the liquid biopreparation and its lyophilized form consisted in determination of the following activities: carboxymethyl cellulase (Hankin and Anagnostakis 1977), lactase (Hall and Hartl 1974), *β*-glucosidase (Deschamps and Huet 1984), xylanase (Miller 1959), amylase (Bernfeld 1955), protease (Anson 1938), pectinesterase (Kertesz 1955), poligalactouronase (Miller 1959), and protein content (Bradford 1976). These assessments were performed in triplicate analysis (n = 3) with colorimetric methods using a spectrophotometric microplate reader Infinite M200PRO (Tecan Group Ltd., Männedorf, Switzerland).

Methods

Physicochemical properties of fermentation substrates, biomass, and biogas

The substrate for anaerobic digestion was made up of a mixture of organic wastes (MOO) (average size of particles was 2 mm to 4 mm), which was composed of fruit waste (25%), dairy sewage sludge (25%), corn silage (12%), and grain decoction (38%). The proportion of ingredients was chosen based on preliminary analysis of single substrates and different mixture of co-substrates. The highest biogas efficiency was obtained from the above mentioned mixture; therefore it was selected for the biopreparation testing. The chemical composition of the applied mixture and anaerobic process conditions are shown in Table 1. The anaerobic precipitate was acquired from an agricultural biogas plant in Konopnica (near Łodź, Poland). For two months, the sludge was adapted to the conditions of the process *via* storage at 37 °C, and then anaerobic sludge (20%) was mixed with the mixture of organic wastes. The retention time in the adaptation period was 40 days.

Table 1. The Chemical Composition of the Waste Mixture and Anaerobic Sludge

Parameter	Organic Waste Mixture	Anaerobic Sludge
D.M. (%)	14.1 ± 0.81	5.40 ± 0.25
Organic dry matter (% d.m.)	90.7 ± 0.52	76.5 ± 0.05
Ash (% d.m.)	13.0 ± 1.13	12.6 ± 0.54
COD (g O_2 kg^{-1} d.m.)	1.45 ± 0,08	-
Kjeldahl nitrogen (% d.m.)	4.90 ± 0.06	-
Phosphorus (g kg^{-1} d.m.)	6.87 ± 0.08	-
pH	4.28 ± 0.01	7.67 ± 0.02
C/N	36.16	-
Explanations: ± standard deviation, d.m. – dry matter, COD - Chemical oxygen demand		

The analysis of the cell wall polysaccharides (hemicelluloses and cellulose) was performed in a mixture of organic wastes (MOO) and post-fermentation mass, where the enzyme mixture (0.05 cm^3 g^{-1} d.m) was applied prior to fermentation (24 h hydrolysis at 37 °C) (MPB) and in post-fermentation mass without enzyme mixture application (MPK), using an Agilent gas chromatograph (Agilent 7890S Series GC Custom, Santa Clara, USA), according to Cyran and Dynkowska (2014). Prior to the analysis, the samples were de-starched and de-proteinated with α-amylase from *Bacillus licheniformis* (EC 3.2.1.1), amyloglucosidase from *Aspergillus niger* (EC 3.2.1.3), and protease from *B. licheniformis* (EC 3.4.21.14) purchased from Megazyme Inc. (Wicklow, Ireland). The polysaccharide material was isolated by precipitation with 80% ethanol. The hemicelluloses and cellulose were assayed after one-step (1 M, 100 °C, 2 h) and two-step (12 M, 30 °C, 1 h and 1 M, 100 °C, 2 h) hydrolysis with sulfuric acid, respectively (Theander *et al.*1955)

The determination of dry weight, organic dry matter, and ash (minerals) in the substrate for fermentation (before and after hydrolysis involving the biopreparation) was carried out in accordance with the Polish norm PN-C-04616-01: 1975 (1975). The determination of the chemical oxygen demand (COD) in the substrate for anaerobic digestion (before and after hydrolysis involving the biopreparation) was performed using dichromate according to the Polish standard PN-74/C04578.03:1974 (1974), taking into account the modification for solid samples (Raposo *et al.* 2008). The determination of the total organic carbon content (TOC) in the substrate for anaerobic digestion (before and after hydrolysis involving the biopreparation) was performed using a Coulomat 702 Li/C (Strohlein Instruments, Staufenberg, Germany). The total nitrogen was determined with the Kjeldahl method, according to the Polish standard PN-EN 13342: 2002. The content of ammonia nitrogen was determined using the Nessler method (Morrison 1971) with a spectrophotometer HACH DR/2000 (Hach Inc., Loveland, United States) with software version 3.0. Absorbance was measured at a wavelength λ = 425 nm. The contents of methane and carbon dioxide were determined with gas chromatography (Agilent 7890 GC, Agilent Technologies, Inc., Santa Clara, USA, with a TCD detection system). The split was made using the 2D technique with pneumatically switched columns.

Anaerobic digestion process

A fermentation batch was prepared in a glass digester with a volume of 2 dm^3. The content of the chambers was stirred at 4 rpm. The process was performed in mesophilic conditions at a temperature of 37 °C. The daily volume of biogas was evaluated by the measuring system that consisted of an electronic flow meter Aalborg® type GFM1 (Aalborg®, North Jutland, Denmark), with continuous measurement of the temperature during the process. The heating of the fermentation was provided by a thermostat connected to the outer shell of the fermenter. The analyses were performed in a continuous system of periodically sealed reactors in mesophilic (37 °C) or thermophilic (50 °C) conditions. To measure the biogas evolved in the anaerobic digestion, the system was connected to the gas meter. It consisted of two connected bottles with hydraulic closings. The inoculum was anaerobic sludge from the biogas plant (in Konopnica) added to the reactor (20 g d.m. dm^{-3}).

Organic waste pretreatment using the biopreparation and anaerobic digestion

The effect of organic waste pretreatment with the addition of the biopreparation (in liquid form) was measured. The waste mixture was treated with the liquid enzyme mixture form at doses of 0.01 cm^3 g^{-1} d.m., 0.02 cm^3 g^{-1} d.m., and 0.05 cm^3 g^{-1} d.m. In the second experiment, the organic waste mixture was subjected to preliminary enzymatic hydrolysis prior to entering the batch bioreactor (NORMAG Labor- und Prozesstechnik GmbH, Ilmenau, Germany). The research was conducted at two temperatures: 37 °C and 50 °C, with enzyme mixture doses of 0 cm^3 g^{-1} d.m., 0.05 cm^3 g^{-1} d.m., and 0.1 cm^3 g^{-1} d.m. After mixing the enzyme mixture with organic waste, anaerobic digestion was performed under mesophilic conditions. The fermentation time was 35 days. The effect of the organic waste mixture pre-treatment using enzyme mixture in the lyophilized form was assessed. The anaerobic digestion process was run in mesophilic conditions (37 °C). The enzyme mixture was added directly to the organic waste (mixed thoroughly) in an amount of 0.1 mg g^{-1} d.m. and 0.5 mg g^{-1} d.m., and then incubated at 37 °C for 24 h. After this time, the biomass was mixed with anaerobic sludge (20%) and introduced into the batch bioreactor. The fermentation was carried out for 35 days. In the second step, 0.1 cm^3 g^{-1} d.m. and 0.5 mg

g^{1} d.m. of the enzyme mixture's lyophilizate was added to the organic waste mixture. The biomass prepared in this way was introduced together with the anaerobic sludge directly into the batch bioreactor (according to the procedure described above). The batch bioreactor load for each dose was 4.96 g dm^{-3} d.m.

Statistical evaluation

The statistical analysis of the results was performed with an ANOVA analysis using Statistica 10.0 (StatSoft, Inc., Tulsa, United States), with a significance level $\alpha = 0.05$. To determine significant differences between the mean values of the studied parameters, the Tukey test ($p < 0.05$) was used.

RESULTS AND DISCUSSION

The Characteristics of the Biopreparation

The efficiency of cellulases involved in the hydrolysis of organic wastes depends *inter alia* on the specificity of their performance and efficacy of the reaction catalyzed in certain conditions. The rate of the enzymatic reaction depends on a number of factors, particularly pH and temperature. Achievement of high activity of the enzyme is possible in a limited pH range, which ensures proper conditions for the reaction related to the presence and distribution of the electric charge in the active center and the rest of the protein (Guerriero *et al.* 2016). In contrast, the inactivation of the biocatalyst by changing the acidity of the environment or increasing the temperature causes loss of catalytic activity. The studies conducted on the cellulolytic biopreparation included an evaluation of the effect of the pH range of 4.3 to 5.7 (Fig. 1a) and the temperature in the range from 4 °C to 90 °C (Fig. 1b). The results revealed that enzyme mixture exhibited optimum activity at a pH of 5.1 and 50 °C. Relatively high cellulase activity was also found at the lower pH (4.7, 4.9, and 4.5) levels, which remained at the level of 82%, 68%, and 63% of the maximum activity, respectively. At higher pH values (> 5.1), the cellulolytic activity of enzyme mixture was greatly decreased, which demonstrated inactivation of the enzyme caused by the change in the acidity of the environment.

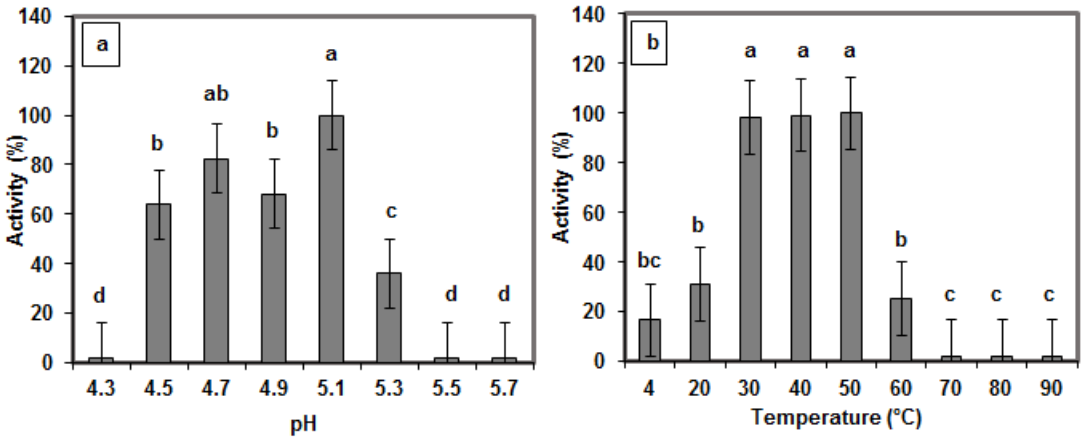

Fig. 1. Cellulolytic activity of the enzyme mixture depending on the pH (a) and temperature (b); Error bars indicate standard error, n = 3; different letters above the bars indicate the significant differences ($\alpha = 0.05$) between the mean values (Tukey's test ($p < 0.05$)); Cellulolytic activity (U cm^{-3}) was calculated as a % of maximum activity obtained for the experiment

The results indicated that *Trichoderma atroviride* G79/11 is a mesophilic strain, producing cellulolytic enzymes that are stable in the temperature range of 30 °C to 50 °C (Fig. 1b). At the temperature of 20 °C, there was a 31% reduction in the level of the enzymatic activity, and only 25% of the enzyme activity was found at 60 °C. The increase in temperature to 70 °C, 80 °C, and 90 °C resulted in complete inactivation of cellulases due to denaturation of the enzyme protein. For example, Woon *et al.* (2016) also described microbial strains that are able to produce cellulases at the temperature of 50 °C; however thermophilic microbial strains described in the literature grow at higher temperatures (de Oliveira *et al*. 2016).

To perform the hydrolysis of an organic waste mixture containing lignocellulose, efficient enzymes that have high cellulolytic activity are needed. The molecular weight of most cellulases ranges from 25 kDa to 75 kDa; therefore, a filter module with a molecular weight cut-off membrane for proteins < 5 kDa was used. The post-culture liquid of the G79/11 strain from the culture on MSCL media was concentrated 20×. As a result, the cellulolytic activity of the enzyme mixture reached 22 U cm^{-3}. The liquid enzyme mixture was subjected to lyophilization, and 1 mg cm^{-3} of the biopreparation had an activity of 1.089 U cm^{-3}, as shown in Table 2. The biopreparation did not differ in terms of nonspecific cellulolytic activity from commercially available enzyme preparations, which could potentially be used for organic biomass pretreatment prior to anaerobic bioconversion. The enzymes are as follows: Celustar XL (Dyadic International Inc., Florida USA) xylanase and endoglucanase, FPU 28.82 U cm^{-3} from *Trichoderma longibrachiatum*; CellusoftConc.L (Novozymes, Denmark), endoglucanase, FPU 17.96 U cm^{-3} from *Trichoderma longibrachiatum*; Optimash^{TM}VR (Genecor, San Francisco, USA) carboxymethylocellulase, endoglucanase, FPU 16.94 U cm^{-3} from *Penicillium funiculosum*; Agropect pomace (Danisco Poland Sp. z o. o.), pectinase and endoglucanase, FPU 7.05 U cm^{-3} as summarized by Kowalska-Wentel (2015) or Pectinex Ultra SP-L (Novozyme, Bagsvaerd, Denmark *β*-galactosidase activity immobilized onto Eupergit C) from *Aspergillus aculeatus* (Aslan and Tanrıseven, 2007). The enzyme mixture was characterized by the presence of other enzymes with relatively wider range and high activity, which is its advantage over others biopreparations mentioned above.

Table 2. Enzymatic Activities of the Enzyme Mixture

Enzymatic Activity	Liquid Form	Lyophilized Form
Celullases (U cm^{-3})	22.27 ± 0.30	1.09 ± 0.10
Pectinesterase (μmol min^{-1} cm^{-3})	719.0 ± 32	248 ± 8.0
Poligalactouronase (μmol min^{-1} cm^{-3})	1374 ± 80	182 ±10
Amylase (μmol min^{-1} cm^{-3})	623.0 ± 25	53 ± 4.0
β-Glucosidase (nmol min^{-1} cm^{-3})	1056 ± 63	141 ± 6.0
Carboximethylcellulase (μmol min^{-1} cm^{-3})	1246 ± 31	28 ± 3.0
Xylanase (μmol min^{-1} cm^{-3})	5663 ± 695	1478 ± 7.0
Lactase (g min^{-1} cm^{-3})	0.010 ± 0.00	0.03 ± 0.0
Protease (μmol min^{-1} cm^{-3})	0.410 ± 0.01	0.23 ± 0.0
Explanations: ± standard deviation		

The lyophilization process did not result in the loss of cellulolytic activity. However, both the liquid biopreparation and its lyophilized form can be used to hydrolyze organic wastes, and, therefore, increase the biogas yield. The observed reduction in the activity of the lyophilizate over the liquid biopreparation is normally a result of the freeze-drying process of lyophilization. An advantage of the lyophilized formulation is the

possibility of long-term storage in a much smaller area. The higher activity of the liquid biopreparation is an advantage; however, the biopreparation has a shorter expiration date, which indicates the maximum maintenance of its catalytic activity (Wesolowska-Trojanowska and Targonski 2014). Nonetheless, motivations for using onsite-produced enzymes in biorefineries are to avoid expenditures and the loss of its activity during lyophilization (Kiran *et al.* 2014).

Biopreparations usually have a narrow spectrum of activity; therefore, their use in the process of hydrolysis of organic waste may not be effective. An advantage of the enzyme mixture (both liquid and lyophilized) is the wide array of activities. The activity of xylanase, *β*-glucosidase, and carboxymethyl cellulase reached the levels of 5663 μmol min^{-1} cm^{-3}, 1056 nmol min^{-1} cm^{-3}, and 1246 μomol min^{-1} cm^{-3}, respectively. Polygalactouronase, pectinesterase, amylase, lactase, and protease activities were 719 μmol min^{-1} cm^{-3}, 1374 μmol min^{-1} cm^{-3}, 623 μmol min^{-1} cm^{-3}, 0.010 μmol min^{-1} cm^{-3}, and 0.410 μmol min^{-1} cm^{-3}, respectively. The level of the enzymatic activity of the lyophilizate suspension (0.1%) was as follows: 0.98 μmol min^{-1} cm^{-3} nonspecific cellulolytic activity, 1478 μmol min^{-1} cm^{-3} xylanase, 141 nmol min^{-1} cm^{-3} *β*-glucosidase, and 28 U cm^{-1} carboxymethyl cellulase (Table 2). Other enzymatic activities, even if they were not relatively high, can contribute a great added value, especially for complex wastes. The variety of enzymes remarkably reduces the need for mixing different biopreparations to achieve the desired hydrolytic effect on the hydrolysis of lignocellulose-containing organic wastes (Zieliński *et al.* 2016). The enzyme mixture obtained in the present work was lyophilized without the use of additional stabilizers and was found to retain relatively high cellulolytic activity after two weeks of storage at 4 °C. The introduction of stabilizing agents to biopreparations could further increase the production costs of the final products.

Liquid enzyme biopreparations are usually stabilized with sorbitol, glycerol, or propylene glycol (van den Tweel *et al.* 1993). These substances are added in an amount from 10% to 50% for maximum stabilization of the enzyme biopreparation activity for several months. It is also important to protect the biopreparation against the growth of microorganisms. To this aim, preservatives, such as sodium chloride, sodium benzoate, and potassium sorbate, can be added. Buffers and surfactants can also be added for this purpose (Bayer *et al.* 2004). Although no preservatives were applied to the enzyme mixture, no microbial contamination was observed during the experiments.

An important parameter characterizing enzymes is their thermal stability (Moraïs *et al.* 2016). Figure 2 shows the stability of the lyophilized biopreparation of cellulolytic fungus G79/11 at temperatures ranging from 4 °C to 90 °C. It was shown that, if the temperature was increased to 60 °C and higher, the enzyme activity decreased within the first few minutes of incubation. An 80% decrease in the activity of the biopreparations was noted after 1 h of incubation at 70 °C, 80 °C, and 60% at 60 °C, and a 40% decrease was observed after 24 h. For the temperatures of 4 °C, 20 °C, 30 °C, 40 °C, and 50 °C, there was a 40% to 75% increase in the activity after the first 6 h of incubation. This may have been related to conformational changes in the protein enzyme.

The stability of the enzyme mixture was comparable to the stability of other cellulolytic strains described in previous literature. For example, Kumar and colleagues (Kumar *et al.* 2008) showed the highest stability at 50 °C for cellulases derived from a culture of species, such as *Penicillium purpurogenum*, *Pleurotus xorida*, and *Pleurotus cornucopiae*. The authors revealed that the activity decreased (even up to 98%) after 48 h incubation at 40 °C. However, there are reports of obtaining a highly thermostable enzyme

with activity at low pH values. Zarafeta *et al.* (2016) characterized thermostable (70 °C) and highly halotolerant cellulases derived from a hot spring isolate.

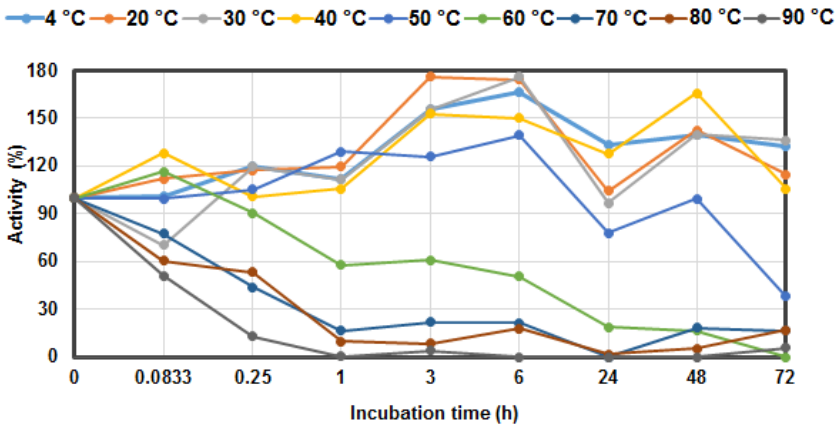

Fig. 2. The thermostability of a biopreparation based on cellulolytic activity; please see Fig. 1

The results obtained in this research showed that the enzyme mixture was less efficient and considerably less stable in alkaline conditions. Figure 3 shows the difference in pH stability of the biopreparation at a pH of 2.0 to 10.0. Similar to the determination of thermal stability, marked fluctuations of cellulolytic activity in the early hours of incubation of the lyophilizate were revealed. The highest activities were obtained for pH 3.0, 5.0, 6.0, 4.0, and 2.0 at 24 h of incubation. The cellulolytic activity obtained for the variants mentioned above differed by approximately 10%. The highest activity was observed when the enzyme mixture was suspended in a buffer with a pH of 3.0. The results of cellulolytic activity at pH 8.0 to 10.0 showed a noticeable decrease (at pH 8.0) or total loss of activity (at pH 9.0 and pH 10.0).

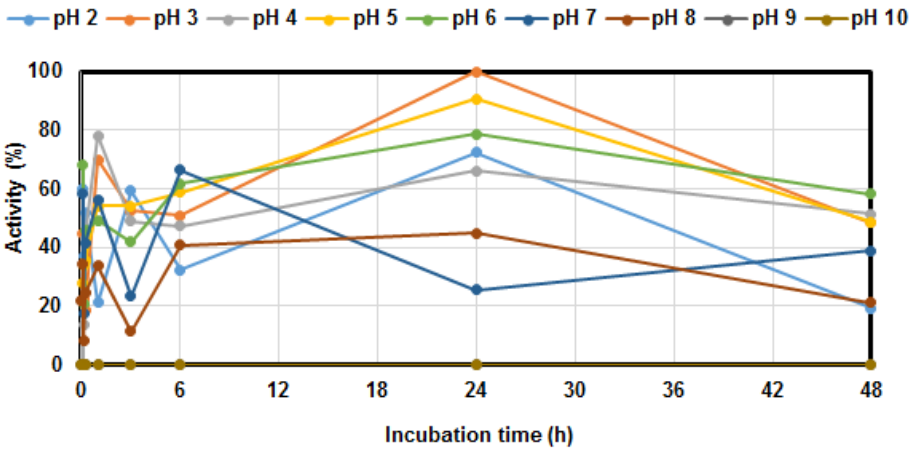

Fig. 3. The pH-stability of the enzyme mixture based on cellulolytic activity; please see Fig. 1

The influence of storage conditions on cellulolytic activity of the lyophilizate is shown in Fig. 4. It was revealed that the two-week storage of the lyophilized biopreparation at 20 °C, compared to 4°C, freezing (-20 °C), or low-temperature freezing (-70 °C), yielded a higher level of cellulolytic activity, *i.e.* by 28%, 16%, and 53%, respectively.

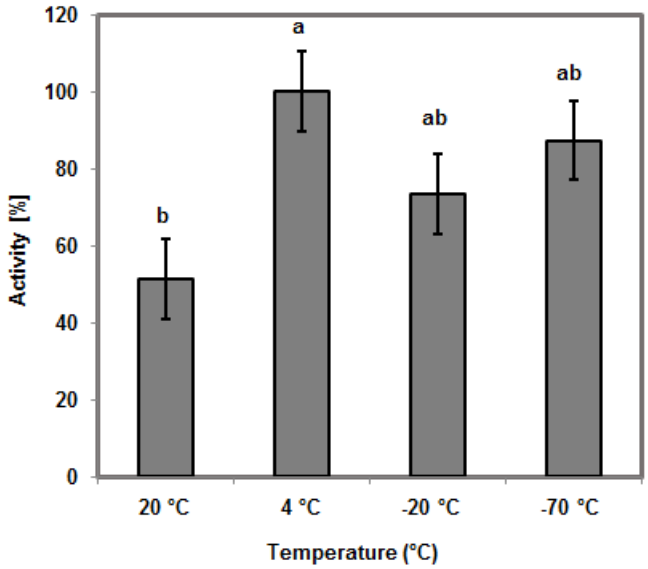

Fig. 4. The temperature influence on the enzyme mixture's lyophilizate cellulolytic activity during storage; please see Fig. 1

Efficiency of Biogas Production after Organic Waste Conditioning

The effect of liquid enzyme mixture on biogas production introduced directly into the digester with a waste mixture or pretreatment of wastes was evaluated. The enzyme mixture most effectively fulfilled its function at 50 °C (Table 3).

Table 3. Efficiency and Composition of Biogas in Methane Fermentation Process after Addition of Enzyme Mixture

Treatment	Dose (cm^3 g^{-1} d.m. /mg g^{-1} d.m.)	Yield (m^3 Mg^{-1})				Biogas Composition		
		Biogas		Methane				
		COD	d.m.	COD	d.m.	CH_4 (%)	CO_2 (%)	H_2S (ppm)
Control	0.00	340	493	242	350	71	29	101
Liquid enzyme mixture addition, 24 h hydrolysis at 37 °C	0.05	370	540	266	389	72	28	110
	0.10	369	542	266	390	72	28	115
	0.15	372	551	268	397	72	28	115
Liquid enzyme mixture addition, 24 h hydrolysis at 50 °C	0.05	373	545	273	398	73	27	115
	0.10	379	557	277	407	73	27	115
	0.15	391	578	285	422	73	27	115
Lyophilized enzyme mixture addition prior to fermentation, 24 h hydrolysis at 37 °C	0.1	389	554	277	410	71	29	108
	0.5	437	642	319	456	71	29	108
Lyophilized enzyme mixture addition directly into the fermenter	0.1	371	535	263	380	71	29	110
	0.5	410	594	291	422	71	29	110

For the same dose (*e.g.,* 0.15 cm^3 g d.m.$^{-1}$), there was a higher content of reducing sugars formed by the hydrolysis of cellulose at 50 °C than at 37 °C, which reached 40.04 mg cm^{-3} and 35.91 mg cm^{-3}, respectively.

It is well-known that cellulase activity is inhibited by the products of cellulose hydrolysis such as glucose and cellobiose. Ximenes *et al.* (2011) found that, in the case of a crude cellulase biopreparation produced by a *Trichoderma reesei* strain, inhibition of the hydrolysis of cellulose occurred when the glucose and cellobiose concentrations in the reaction mixture were 69 mg cm^{-3} and 3.3 mg cm^{-3}, respectively. Accordingly, even the highest dose proposed in this study did not inhibit the hydrolysis process, which was demonstrated by the reducing sugar content at the higher enzyme doses. To check the efficiency of the enzyme mixture, the smallest dose was used, so as not to further increase the costs of the entire fermentation process. The cellulolytic enzyme can be inhibited by various compounds, such as surfactants, phenolic compounds, and solvents, which may be present in the biomass subjected to hydrolysis (Ximenes *et al.* 2011). Therefore, the efficiency of enzyme biopreparations vary depending on the type of biomass subjected to hydrolysis. In contrast, the above-mentioned compounds must protect enzymes already associated with cellulose. However, the cellulolytic enzymes have limited availability due to the porous structure of cellulose. This phenomenon is called substrate resistance (Sun and Chesunng 2002).

The analysis of the composition of the organic waste mixture used in the study (Table 1) allowed a conclusion that the mixture was characterized by a high content of organic matter (> 92% d.m.), which indicated high potential for biogas production. However, the low pH value (4.28) and at the same time the high concentration of nitrogen (the ratio of carbon to nitrogen equaled to 36) could have had an inhibiting effect on the methane fermentation process, but could have accelerated the hydrolysis involving the enzyme mixture, which was most intensive in the pH range of 4.0 to 5.0.

The methane content in the biogas accounted for 71% (Table 3). The enzyme mixture added into the digester in an amount of 0.01 cm^3 g^{-1} d.m. (0.22 g FPU g^{-1}d.m. (FPU = μmol min^{-1} cm^{-3} = U cm^{-3})) resulted in an approximately 4% increase in the biogas yield, compared to the fermentation waste mixture without the enzyme mixture. The subsequent doses of the enzyme mixture introduced into the fermenter, *i.e.* 0.02 cm^3 g^{-1} d.m. and 0.05 cm^3 g^{-1} d.m., resulted in an improvement of the biogas efficiency compared to the control by approximately 7% and 9%, respectively (Table 3).

It was found that the methane fermentation of waste subjected to pretreatment with the enzyme mixture depended not only on the dose, but also on the hydrolysis variant. Similar results were obtained by Zhang *et al.* (2006). In this study, regardless of whether the enzyme mixture (at a dose of 0.05 cm^3 g^{-1} d.m.) was applied directly to the digester or used to treat the organic waste prior to introduction into the fermenter, the yield of the biogas from 1 mg d.m. of the waste was the same, and amounted to 540 m^3 (Table 3). Substantially better results were obtained by subjecting the substrate to hydrolysis at 50 °C rather than at 37 °C. This is the optimum temperature for the enzyme mixture, which is shown in Fig. 2.

A comparison of the effects of fermentation of the pre-hydrolyzed organic waste mixture at a temperature of 37 °C and 50 °C demonstrated that there were no differences in the biogas yield for the lowest biopreparation dose used. The highest tested dose of 0.15 cm^3 g^{-1} (Table 3) had the most remarkable influence on the amount of biogas obtained. Compared with the hydrolysis without the pretreatment of samples subjected to hydrolysis

at 37 °C, there was an 11.6% increase in the biogas yield and the value was 17.2% higher for the hydrolysis at a temperature of 50 °C.

The methane fermentation process was also optimized in the experiment based on the organic waste mixture with lyophilized enzyme mixture. The main components of the organic waste mixture were arabinoxylan (60.30 mg g^{-1}) and non-cellulosic polysaccharides (rhamnose, fucose, mannose, and galactose), which represented 20.62 mg g^{-1} (Table 4).

The presence of cellulose (2.58 mg g^{-1}) was found in the organic waste mixture, which was expressively lower (0.81 mg g^{-1}) than in the mass after the application of the biopreparation (Fig. 5). The lower cellulose content in the post-fermentation biomass, when the conditioning process (hydrolysis) was performed using the enzyme mixture, showed the degradation of the polymer by the cellulolytic enzymes contained in the enzyme mixture.

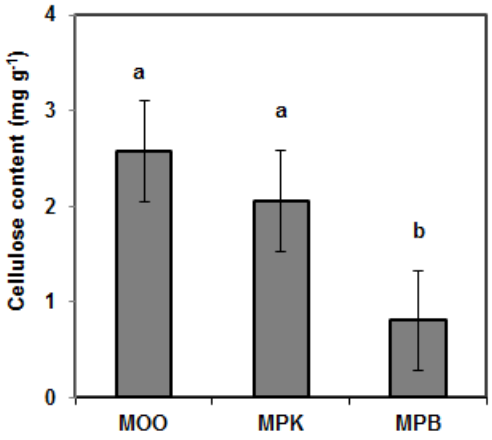

Fig. 5. The effect of lyophilized biopreparation on the cellulose content of the organic waste mixture and post-fermentation biomass. Error bars indicate standard error; different letters above the bars indicate errors on the significance of differences (α = 0.05) between the mean values (Tukey's test ($p < 0.05$)); MOO - a mixture of organic waste (substrate for methane fermentation); MPK - the post-fermented biomass- control the process without conditioning using biopreparation; MPB - the post-fermentation biomass, a process with the conditioning step using biopreparation

Table 4. Hemicellulose Sugar Composition in Organic Waste Mixture and Post-fermentation Mass

Sugar (mg g^{-1})	Organic Waste Mixture (MOO)	Post-fermentation Mass	
		Enzyme mixture (MPB)	Control (MPK)
Rhamnose	2.10 ± 0.08	2.09 ± 0.03	2.26 ± 0.08
Fucose	1.54 ± 0.04	1.43 ± 0.10	1.72 ± 0.10
Mannose	5.16 ± 0.08	5.22 ± 0.57	5.26 ± 0.06
Galactose	6.94 ± 0.31	5.80 ± 0.46	6.19 ± 0.32
Glucose	3.27 ± 0.12	1.20 ± 0.08	2.01 ± 0.20
Arabinose	18.44 ± 1.59	16.30 ± 0.39	17.61 ± 1.67
Xylose	42.32 ± 3.51	44.01 ± 1.24	41.53 ± 3.35
Ara/Xyl	0.44	0.37	0.42
Explanations: Please see Table 1			

The hemicelluloses were mainly represented by xylose and arabinose (Table 4). The other polysaccharides present in this fraction were galactose, mannose, glucose, rhamnose, and fucose. The slightly higher content of polymeric xylose that was found in MPB (44.01 mg g^{-1}), compared to the content in MOO (42.32 mg g^{-1}) and in MPK (41.53 mg g^{-1}), did not correspond with the enzyme mixture xylanase activity level evidenced in Table 2. The other major components of organic matter than arabinoxylan were more efficiently utilized upon the enzyme mixture treatment, as shown by the organic matter analysis shown in Table 1. Hence, the arabinoxylan level in MPB was not indicative of the degradation of the xylan backbone by a synergistic action of both endo-*β*-D-xylanase and *β*-D-xylosidase. In contrast, the arabinose-to-xylose (Ara/Xyl) ratio that illustrates the degree of substitution of the xylan backbone with arabinose side units, clearly points to partial degradation of this polysaccharide. The Ara/Xyl ratio in MOO reached 0.44 and its lower value was found in MPB (0.37). This implied a decrease in the number of arabinose side substituents on the xylan chain as a result of the hydrolytic action of *α*-L-arabinofuranosidase present in the enzyme mixture. It has been shown that even relatively small changes in the arabinoxylan Ara/Xyl ratio (0.01 to 0.02) cause substantial changes in its macromolecular characteristics (Cyran and Dynkowska 2014). The considerable reduction in the arabinoxylan Ara/Xyl ratio observed in MPB (0.07) implied a much lower number of arabinose substituents that form a steric barrier, which suggested its increased susceptibility to the action of xylan degrading enzymes. Although released xylose is a good substrate for the methane fermentation process (Cheng *et al.* 2012), this compound might have not been fully used for the production of methane (Table 4).

The enzyme mixture lyophilizate addition directly into the fermenter and 24 h hydrolysis of the organic waste mixture in both doses improved the efficiency of the biogas fermentation process (Table 3). Better results were recorded when the enzyme mixture was added 24 h before fermentation and hydrolysis continued at 37 °C, compared to the addition of the enzyme mixture lyophilizate directly into the fermenter. At a lower dose of the lyophilizate (0.1 mg g^{-1} d.m), there was an increase in the yield obtained, *i.e.* 12% and 8%, after 24 h incubation and upon the direct addition, respectively. The higher dose of the lyophilizate (0.5 mg g^{-1} d.m.) was reported to contribute to an improvement of biogas yield, reaching approximately 30% when 24 h hydrolysis was applied and 20% without the hydrolysis step. For example, there are easily available wastes on the market, such as the fruit industry expeller, decoction grain, and dairy sewage sludge. Expellers account for a significant amount of the waste from the fruit and vegetable industry. Wastes from fruit and vegetable processing residues, *i.e.* woody stalks and leaves composed mainly of cell wall polysaccharides, such as pectin, cellulose, and hemicellulose, are suitable for valorization. Simultaneously, the brew is a source proteins, fats, and residues of starch and cellulose, which is the preferred starting material in terms of suitability for the gasification of the methane fermentation process. Similarly, dairy sewage sludge that is produced as a by-product of the dairy industry is a valuable substrate for methane production because it is rich in protein. Even though there are several methods to increase biogas production involving physical, chemical, and biological processes, the enzymatic pretreatment is regarded as the most environmentally friendly (Ziemiński *et al.* 2012). Especially acid or base hydrolysis requires troublesome processing before application in fermentation processes. Degradation of organic waste by microbial enzymes outperforms chemical hydrolysis because enzymes display high substrate and reaction specificity, do not generate by-products, and operate under mild conditions (Micard *et al.* 1997). The enzyme mixture represented great variety of enzymatic specificity, as shown in Table 2. In the light of

composed substrates to be hydrolyzed listed above, its function mechanism is a joint action of cellulases (among others *β*-glucosidase and/or carboximethylcellulase) degrading cellulose, pectinesterase and poligalactouronase for pectin, amylase for starch, and xylanase, lactase, protease hydrolyzing respectively hemicellulases (xylan and mannan), lactose, and protein, leading eventually to biogas yield enhancement (Table 3).

The content of biogas did not depend on the dose of the enzyme mixture and accounted for 71% of methane. This methane content in the biogas was relatively high and indicated high potential biogas profitability of the methane fermentation substrate proposed in this article (a mixture of organic wastes). Normally, the methane content in biogas, in efficient methane fermentation, can reach up to 75% (Oleszek *et al.* 2015). Similar research on the conditioning of substrates for anaerobic digestion in the methane fermentation process with enzyme biopreparations were conducted, *e.g.* by Ziemiński *et al*. (2012); however, the substrate in their study included beet pulp. The authors applied preparations, such as Celustar XL, Agropect pomace, and Optimash VR and Agropect pomace, in different volumetric ratios yielding doses of 0.03 FPU g^{-1} d.m. to 0.75 FPU g^{-1} d.m. This corresponds with the FPU quantities used in this study. Ziemiński *et al.* (2012) found a synergy of the activities of mixed biopreparations related to the fact that enzymes with different catalytic specificity were used in the mixture. As a result of prolonged enzymatic hydrolysis of substrates (up to 8 days), the authors received increased yields of biogas. However, the costs associated with extending the process may outweigh its effect. Therefore, in this study, the hydrolysis duration was limited, because the stability of enzymes in the enzyme mixture notably decreased after 24 h of incubation (Figs. 2 and 3).

Cerda *et al.* (2016) used the Pectinex Ultra SP-L biopreparation (pectinolytic activity) and Celluclast 1.5 L (cellulolytic activity) at 2 mg g^{-1} d.m. for conditioning berry expellers. The process was performed in two modes, *i.e.* by hydrolysis before fermentation and by the addition of enzyme preparations directly to the fermenter. The authors obtained a 28% increase in biogas production upon the addition of pectinolytic enzymes directly into the digester. No significant increase in biogas production was reported for Celluclast. The application of the relatively high dose of enzymes resulted in a yield of biogas similar to that obtained in this study at an application of 0.5 mg g^{-1} of the enzyme mixture (Table 3).

Efficient substrate conditioning of complex biomass using enzyme biopreparations particularly requires the use of a broad spectrum of enzymatic activities. Commercially available biopreparations showed a narrow spectrum of activity. According to manufacturers, commercial biopreparations usually exhibit one or two types of activity. The complexity of the hydrolytic properties of the enzyme mixture accounted for its relatively high potential in the application.

CONCLUSIONS

1. A method for preparing a multi-enzyme biopreparation and the method to optimize the process of methane fermentation of the waste mixture (fruit waste, dairy sewage sludge, corn silage, and grain broth) using the enzyme mixture was proposed. A wide range of enzyme activities exhibited by the enzyme mixture contributed to its competitiveness in relation to commercial biopreparations. The enzyme mixture exhibited high potential for organic waste mixture degradation.

2. The use of 0.5 mg g^{-1} d.m. of lyophilizate from the enzyme mixture resulted in an elevation of biogas yield up to 30%.

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