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Kinetic study of high-solids anaerobic co-digestion of pre-treated organic waste in terms of methane production

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ABSTRACT

Wet anaerobic digestion is the most used stabilisation method in the world. However, it has some limitations resulting from the high hydration of the feed, which requires the construction of large-volume anaerobic digestion chambers, difficulty in managing of digested sludge (digestate), which requires dewatering and drying. For this reason, more worldwide attention is paid to the development of dry technologies, which is supported primarily by the possibility of working with higher organic loading, smaller reactor volume, and the minimum need for digestate dewatering. This study aimed to conduct mesophilic and thermophilic batch high-solids anaerobic digestion (HSAD) of sewage sludge (SS) with grease trap sludge (GTS) and algae biomass (AB). Additionally, to shorten the long digestion time, which is the bottleneck of the process, in this study, various pre-treatment methods were used. The effectiveness of the conditioning methods was assessed based on the results of the biochemical determination of the methane potential and the analysis of the kinetics of the process with the use of five kinetic models. The thermochemical pre-treatment method observed the most pronounced pre-treatment effect on the studied SS/GTS/AB mixture at mesophilic temperature. Its methane yield was 109.48 ± 0.00 N·mL-CH_4/g-VS, which was 110% higher in comparison to the control (52.21 ± 0.00 N·mL-CH_4/g-VS). High-solids co-digestion significantly increased lag phase time (more than 30 d). However, thermochemical pre-treatment improved that parameter by 26.3% compared to the control.

Keywords: Anaerobic digestion; Algae biomass; Kinetic study; Co-digestion; Biogas; Wastewater treatment; Renewable energy; Grease trap sludge; Sewage sludge

1. Introduction

Waste production is intrinsically connected to the life of people as well as development of cities and industrialization. The amount of the produced waste is mainly depended on the development of country in regarding to technological, economical as well as societal aspects 1. Legislative tightening, stimulates changes in waste management and energy conservation practices for many countries and one such change is circular waste management (also known as the circular economy or the "Cradle to Cradle" concept), which imposes a set of requirements for the producers to use various physical, chemical and biological processes to either recycle the waste to obtain the raw materials or neutralize it in the order it could not create a danger for people as well as natural environment. Anaerobic digestion (AD) is one of the many biological methods to stabilize organic waste and it is currently often applied within the scope of the sustainable development agenda and circular economy. It has many advantages, that is, it is safe, which means that the organic waste, often containing various pathogens, is stabilized to produce a safe product that can find its application as a fertilizer after some additional control procedures. Organic waste that is used for AD is also a source of renewable energy, that is, methane [1,2]. There are four

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major sectors, for which AD is applied nowadays, that is, stabilization of the primary as well as secondary sludge that originates from municipal sewage treatment to reduce the volume of wastes and produce biogas; industrial wastewater treatment (i.e., from food processing and fermentation industries, etc.), into which high organically loaded wastewater is produced that is required to be stabilized before disposal; livestock waste treatment for the fertilizer and energy production; treatment of an organic fraction of municipal sewage waste (OFMSW) [3].

Semi-dry-AD and dry-AD, both referred to as high solids anaerobic processes, are possible ways to conduct AD, in which there are used organic wastes that are characterized by higher total solids (TS) content in comparison to wet anaerobic digestion (wet-AD) (for semi-dry-AD the values are 10%–15%, however, in some sources the range is 10%–20% and for dry-AD the values are higher than 20%), so implying that such wastes as an organic fraction of municipal solid waste (OFMSW) and grease trap sludge (GTS) (has TS = 58.43%) can be found applicable [4–8]. GTS is a complex mixture of oils, fats, grease, and water that is characterised by high chemical oxygen demand (COD) (within the range of 138–375 g-COD/L) and has a high energy potential for the production of biodiesel, incineration, and AD [9].

An interesting substrate for high-solids sewage sludge (SS) co-digestion is algae biomass (AB). Algae have the potential to substitute fossil fuels in transportation as well as electricity and heat generation due to their low cellulose and lignin content as well as their high content of easily biodegradable sugars (approximately 60% of carbohydrates which include mannitol, arginine, and laminarin) [10,11]. TS content of macroalgae is within the range of 8.5%–23.2% [12] and 88.70% for *Undaria pinnatifida* as the previous study shows [13], so algae can be good candidates for high-solids co-digestion.

Depending on the substrate and system configuration, the Y_m in high-solids digestion modes ranges from 200 to 600 mL-CH_4/g-VS [6–8]. High-solids AD has many advantages over wet-AD, that is, lower mixing requirements [4]; enables the use of waste with high organic loading rate (OLR), that is, from 5–12 kg-VS/m^3/d, while in the case of wet-AD, OLR is within the range of 2–5 kg-VS/m^3/d [4,8]. Despite the advantages, high-solids anaerobic digestion (HSAD) systems are not used worldwide, but recently have gained the appropriate interest of many researchers, because these methods are cheaper and have many aspects not yet studied [14]. Notwithstanding the above-mentioned fact, for example, the use of dry-AD in Europe increased by 50% between 2010 and 2015, but it accounted for only 35% of all organic waste treated with the method [4]. As it is also in the case of wet-AD, both these operational modes are prone to inhibition which is related to the ammonium and volatile fatty acid (VFA) accumulation, long digestion time due to the small hydrolysis rate (often hydrolysis is a rate-limiting stage) and requirement of a large inoculum volume, whose major cause is low water content, that is way semi-dry-AD is more preferable and economical than dry-AD [1,15]. A problem such as a slow hydrolysis rate can be addressed by application of pre-treatment methods, among which there can be mentioned physical methods (e.g., ultrasound application, mincing and grinding, electrolysis, etc.), chemical methods (e.g., acid/base solution application, ozonation, Fenton reaction, etc.), thermal methods (e.g., high temperature of 100°C–210°C, low temperature and freezing/thawing, etc.) and biological methods (e.g., hydrolytic enzyme addition, double fermentation, and autohydrolytic process, etc.) [16,17]. A relatively new pre-treatment method, Fe^+ autoclaving, was recently found in relation to coking sludge digestion. The improvement in methane content in biogas amounted to 22.1% in comparison to the alkaline pre-treatment method (85.78%CH_4 for Fe^+ autoclaving and 70.24%CH_4 for alkaline pre-treatment were the highest values observed) and 82.9% in comparison to just autoclaving (46.90%CH_4 was the highest value observed for this method). Additionally, volatile solids (VS) removal rate was 81% [18]. That is why, it is a good sign to test this pre-treatment method for other substrates.

Enzymatic pre-treatment is a promising method to treat recalcitrant substrates (e.g., GTS) that may contain complex lipids, proteins, carbohydrates, and fat, and the method usually helps to increase their solubilisation. There are used such enzymes as proteases, lipases, amylases, cellulases, xylanases, and others to break down complex polymers to monomers [19]. There exist few publications, in which the possibility of enzymatic pre-treatment of GTS, in particular, with lipase enzyme application is studied. Few publications also exist, in which the possibilities of semi-dry SS co-digestion with such substrates as GTS and AB are considered, especially in three component co-digestion and at both mesophilic and thermophilic temperatures. Furthermore, the previous study included three component co-digestion of these substrates, however, possibilities of pre-treatment methods' application were not within the scope of that study [20], therefore, there still exists a possibility to improve both Y_B and Y_m as well as methane content in biogas. Thus, the study aimed to conduct semi-dry-AD co-digestion of SS, GTS, and AB at mesophilic and thermophilic temperatures and study the effect of pre-treatment methods on the AD kinetics. In this study, the possibility of bioaugmentation with an application of two types of biostarters was also considered.

2. Materials and methods

2.1. Materials

For the study purposes there was used SS of two types, that is, the mixture of primary and waste activated (excess sludge) sludges as well as microbial inoculum (IN) that was needed for the AD initiation. Both SS types were collected from wastewater treatment plant (WWTP) in the Silesian region, Czestochowa, Poland, which serves a population of 314,835 and treats 90,000 m^3/d (or 16 mln m^3/y) of wastewater. As far as the mesophilic part is concerned, the SS and IN were collected in November 2019 and for thermophilic digestion, the date was January 2020.

As substrates for anaerobic co-digestion (AcD) there were used (Fig. S1):

- GTS, which was collected from grease traps of a meat processing plant, the Silesian region, Poland.

Carbon-to-nitrogen (C/N) ratio of the substrate was 12.8, whereas VS content was 57.80%.

- AB, "Wakame" or scientifically, *Undaria pinnatifida*, referred to as the macroalgae group, is an edible brown seaweed that is native to Japan, China, and Korea but is also abundant in New Zealand, the United States, and other countries. In 2018, it became listed in the top 100 most invasive species in the world [21,22]. As was pointed out in the previous study, the project, entitled "Integrated Technology for Improved Energy Balance and Reduced Greenhouse Gas Emissions at Municipal Wastewater Treatment Plants (BARITECH)" inspired the authors to continue the research of the chosen algae type [20].

SS was dewatered for the study. The dewatering was done using the centrifugation method. Ss dewatering was required to increase the TS parameter up to a value that would properly contribute to the SS/GTS/AB mixture (the final TS value of that mixture after inoculum addition was about 10%–10.5%). SS was centrifugated at 7,000 rpm for 10 min and TS and VS values were 19.16% ± 0.05% and 15.83%, respectively as in Table 1.

2.2. Experimental procedure

The study was divided into biochemical methane potential (BMP) assay and physicochemical analysis. BMP assay was conducted at two operational temperatures, that is, mesophilic (37°C) and thermophilic (51.5°C). The digestion was done in batches. BMP study was done in triplicate. The digestion period for mesophilic temperature was 47 d, whereas for thermophilic it was 30 d. The typical and preferred digestion period for BMP is 30 d, however, high solids mesophilic digestions require a longer time, for the lag phase is often high [23]. The scope of the study also included the application of biostarters, that is, BIOSANiT® (BS) and Bio-Trakt® (BT), that are normally used for the digestion and stabilization of SS originating from septic tanks (Fig. S3).

The prepared SS/GTS/AB mixture (Fig. S1) was pretreated using 5 pre-treatment methods, that is, thermochemical pre-treatment with NaOH and water bath (A1), thermal pre-treatment with a water bath (A2), autoclave pre-treatment (A3), autoclave plus iron powder pre-treatment (A4) and enzymatic pre-treatment with lipase (A5). SS and untreated SS/GTS/AB (control, CN) standalone digestions were also done to provide reference data.

2.2.1. Pre-treatment procedure and BMP assay

The prepared pre-treatment mixtures were analysed through BMP assay to determine the Y_B and Y_m. BMP test is a popular technique for AD. Y_B and Y_m are important parameters that show the amount of biogas or methane that can be produced per a unit mass of VS, or also often COD, from a substrate or waste biomass, that is, these parameters show the degree of biodegradability [23]. The biogas volume was measured indirectly by a liquid displacement system (LDS). the pH of mixtures was adjusted to 7 ± 0.1 by alkaline and acidic solutions to avoid VFA and ammonium accumulation. Fig. S4 shows the LDS method of biogas measurement.

As a heating system, there were used two water baths, whose temperatures were kept at the desired values. The fermentation bottles were connected to the respective LDS columns and the biogas was free to feel the column overnight (Fig. S2). The water bath application for this study was required because of the low working volume and the possibility to have high internal pressure. Table 2 presents information regarding the organic composition of the pre-treatment mixtures.

There were used 250 mL fermentation bottles, to which 50 ± 0.2 g and 25 ± 0.2 g of the prepared mixtures were added respectively for mesophilic and thermophilic digestions. There was added inoculum of 95 ± 0.2 g for the mesophilic digestion trial and 25 ± 0.2 g for the thermophilic one. Organic loading (OL) of mixtures for mesophilic digestion was 31.6–39.63 g-VS/L, whereas for the thermophilic digestion trial 14.85–19.25 g-VS/L. It was decided to decrease OL and inoculum volume for thermophilic digestion because the bottles' internal pressure increased dramatically because of their small volume and some leakages occurred during the first part of the study.

In terms of digestion with biostarters, they were added along with inoculum to some of the samples (CN, A1, A3, and A4 were chosen for that substudy). That digestion was conducted at thermophilic temperature in incubators parallelly during the thermophilic digestion trial of the main samples. OL for mixtures with BT was 18.01–19.31 g-VS/L whereas for BS it was 18.16–18.21 g-VS/L and I/S for mixtures with BT was 0.573–0.614 g-VS/g-VS, for BS the ratio was 0.608–0.610 g-VS/g-VS. The organic composition of the prepared biostarter mixtures was assumed to be the same as was for the main thermophilic digestion mixtures. There were used recommended doses (Table S1) for biostarter solution's preparation as a reference to determine the necessary doses, that is, in terms of Bio-Trakt®, there was added 0.25 mL of the biostarter solution, whereas of BIOSANiT® there was added 0.00675 g (referring to the dose for septic tanks of 2 m^3, to which 12 measures are supposed to be added.

Prior to the thermophilic digestion, inoculum was introduced to a reactor whose temperature was gradually

Table 1
Information regarding to the organic composition of the feedstock and selected substrates

Substrate	Total solids (%)	Volatile solids (%)	C/N (–)	Volatile solids/total solids (–)
Raw SS (mesophilic)	4.54 ± 0.04	3.73	–	0.82
Raw SS (thermophilic)	9.45 ± 0.91	7.52	–	0.79
Grease trap sludge	58.40	57.80	12.8	0.99
Algae biomass (*Undaria pinnatifida*)	88.70	60.08	9.36	0.67

increased (about 1°C–1.5°C) for about 15–20 d (starting from 37°C) in order to acclimatize microorganisms for thermophilic temperature (51.5°C was applied for the study). The stirring intensity was 120 rpm. On each fifth day, a fresh portion of inoculum was introduced and the old one removed to obtain high quality inoculum. Thermophilic microorganisms are very susceptive to temperature changes, that is why, a gradual increase in temperature was required for the process stability. Fig. 1 presents the scheme of the experiment.

Table 2
Organic characteristics of the prepared pre-treatment mixtures

Stage	Mixture	Total solids (%)	Volatile solid (%)	Volatile solids/total solids (–)	C/N (–)	NPOC (g/L)	Total carbon (mg/g)
Mesophilic	CN	24.11 ± 0.03	18.36	0.78	11.67	0.9211	452.70 ± 7.92
	A1	24.61 ± 0.18	19.18	0.78	10.62	1.79	449.00 ± 0.57
	A2	25.06 ± 0.48	19.77	0.79	11.19	1.18	432.25 ± 1.06
	A3	24.62 ± 0.26	19.61	0.80	11.19	1.38	446.45 ± 6.58
	A4	25.51 ± 0.37	19.83	0.78	11	1.18	420.35 ± 3.46
	A5	24.11 ± 0.03	18.86	0.78	11.67	0.9211	452.70 ± 7.92
	SS	19.24 ± 0.29	15.83	0.82	–	0.2471	–
	IN	2.23 ± 0.01	1.36	0.61	–	0.3709	–
Thermophilic	CN	22.54 ± 0.12	18.13	0.8	–	1.14	–
	A1	22.57 ± 0.76	18.01	0.79	–	1.73	–
	A2	23.33 ± 0.92	18.78	0.80	–	1.37	–
	A3	23.83 ± 0.31	19.23	0.81	–	1.89	–
	A4	22.99 ± 1.02	18.31	0.80	–	1.8	–
	A5	22.54 ± 0.12	18.13	0.80	–	1.14	–
	SS	18.38 ± 1.01	14.81	0.81	–	0.7001	–
	IN	4.69 ± 0.16	2.90	0.61	–	0.7057	–

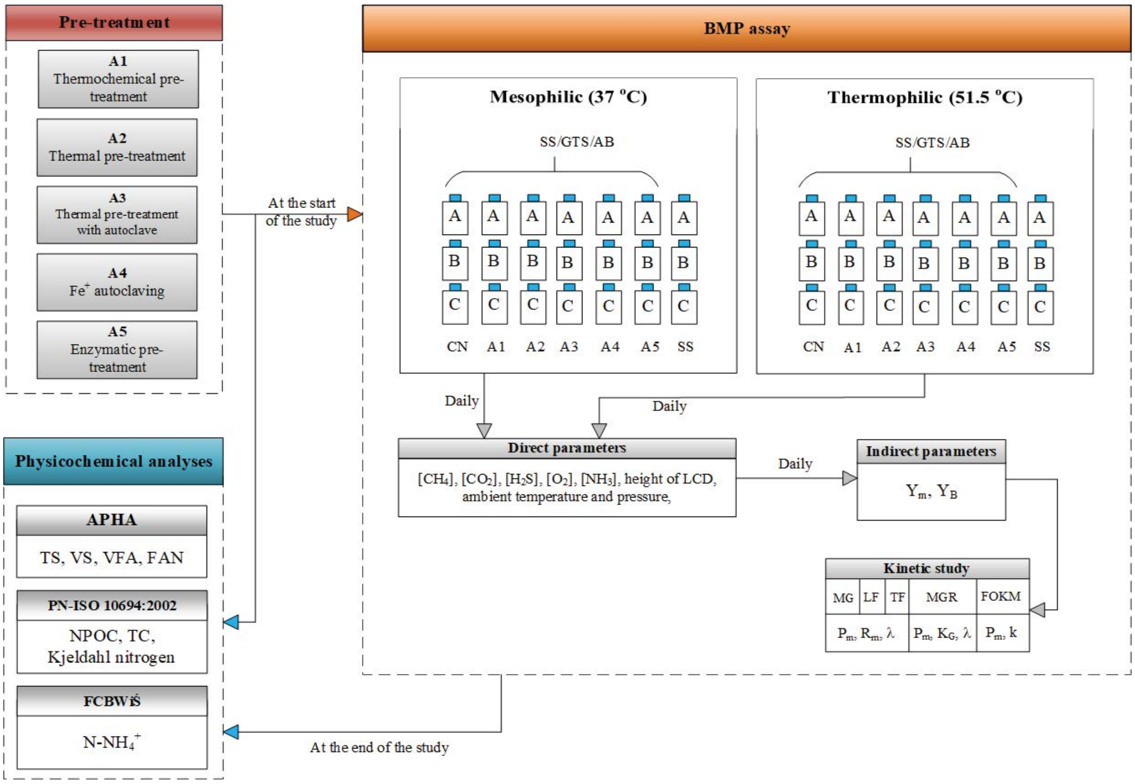


Fig. 1. Scheme of the experiment.

In terms of pre-treatment procedure, the prepared SS/GTS/AB mixture of 250 g was pre-treated with the chosen methods.

Alkaline thermochemical pre-treatment (A1) was performed with NaOH solution. The heating was provided with a water bath, the temperature of which was adjusted to 70°C during the process. The mixtures were also slightly stirred to provide a uniform heat displacement along the pre-treatment material and the stirring intensity was 100 rpm. The added amount of NaOH was proportionally calculated as 0.9 g based on the study of Grosser and Neczaj [24], that is, (3.6 g/0.25 mL)/1,000 mL = 0.9 g. The solution was prepared as 0.9/10 mL, then added to the 250 g SS/GTS/AB mixture and mixed after the addition.

Waterbath thermal pre-treatment (A2) was done with the same water bath as in the case of the thermochemical pre-treatment. The duration of the pre-treatment was 1 h.

Autoclave thermal pre-treatment (A3) was done at the following conditions: the operational temperature was 121°C, pressure 1 atm, and the duration of the treatment was 30 min.

Autoclave thermal pre-treatment with iron powder (A4) was done at the same operational conditions as it was with mono autoclave pre-treatment. There was added 0.71 g of iron powder before the procedure. The dose was calculated proportionally based on the dose and operational conditions by the study of Yang et al. [18], that is, 2.4 g of iron powder and autoclave operational conditions were 121°C, pressure 1 atm. For the calculation it was assumed that the coking sludge density is 1,400 kg/m^3 and, taking into account the sample volume in the reference study (0.6 L), the dose was calculated as: (2.4 g/0.25 L)/(0.6 L/(0.001 m^3/1 L)/1,400 kg/m^3) = 0.71 g.

In terms of the enzymatic pre-treatment (A5) there was used lipase enzyme that was bought from Sigma-Aldrich.com [25]. Fig. 2 provides a summarizing information regarding to the applied pre-treatment methods and conditions.

2.2.2. Physicochemical analysis of mixtures

Physicochemical analyses were done with the following parameters, that is, TS, VS, ionized ammonium nitrogen (N–NH$_4^+$), VFA, non-purgeable organic carbon (NPOC), total carbon (TC), Kjeldahl nitrogen (KN). The inoculum was also studied to the above-mentioned parameters. N–NH$_4^+$, and VFA served as indicators of the process stability. TS and VS showed the organic composition of the prepared mixtures, and VS was used for Y_B and Y_m calculations.

TC represents the total carbon in both inorganic and organic forms that is present in an analysed sample. NPOC is the part of total organic carbon that remains after the moment when acidified analysed sample is purged with gas [26].

The physicochemical analyses were done twice per study stage (mesophilic and thermophilic), that is, at the beginning and at the end of the experiment. VFA, TS, VS, KN were determined in accordance with APHA [27], whereas NPOC, TC were determined in accordance to the norm PN-ISO 10694:2002. N–NH$_4^+$ was determined under the method mentioned by the study of Hermanowicz et al. [28].

A5 samples did not require to be analysed, because the results would be identical to the CN samples. The enzymatic activity was not supposed to be observed at this stage but only during digestion. After shaking, the samples were filtered with filter paper. The samples at the end of each digestion were first centrifugated at 11,200 rcf for 15 min to obtain supernatant. It was assumed that chosen mixtures for the biostarter digestion had the same results at the beginning as those obtained at the start of the main digestion at mesophilic temperature and therefore, they did not require the VFA, KN, N–NH$_4^+$, NPOC, TC analyses. Table S2 shows the summary of the applied methodology for analyses to the mentioned parameters.

2.3. Kinetic study and statistical analysis

In this study there were applied 5 mathematical kinetic models, that is, modified Gompertz model (MG), logistic function (LF), transference function (TF), first-order kinetic

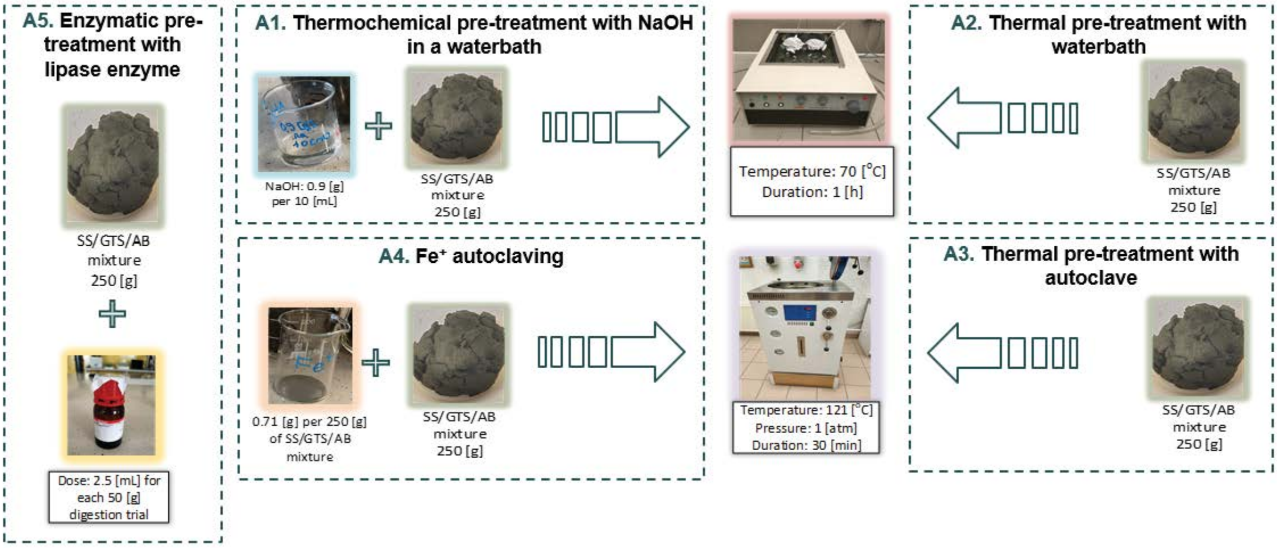


Fig. 2. Summarizing information regarding the pre-treatment methods.

model (FOKM), and reparametrized modified Gompertz model (MGR), to describe AcD and determine kinetic parameters such as potential methane yield (P_m), maximum rate of methane (R_m) and lag phase time (λ) and others. For that purpose, there was used aOriginLab 2018 as well as StatSoft STATISTICA 10, Additionally, factorial ANOVA was done. The equation below presents MG model [5]:

$$Y_m = P_m \cdot \exp\left\{-\exp\left[\frac{\left(R_m \cdot e\right)}{P_m}\right] \cdot \left(\lambda - t\right) + 1\right\} \tag{1}$$

where Y_m is cumulative specific methane yield in N·mL-CH$_4$/g-VS; P_m is potential specific methane yield in N·mL-CH$_4$/g-VS; R_m is maximum rate of specific methane yield and expressed in N·mL-CH$_4$/g-VS/d; t represents time of a measurement and is expressed in days; e is base of the natural logarithm; λ represents lag phase time that is expressed in days.

The equation that represents LF is given as:

$$Y_m = \frac{P_m}{\left\{1 + \exp\left[\frac{\left(4R_m\left(\lambda - t\right)\right)}{P_m}\right] + 2\right\}} \tag{2}$$

The equation that represents TF is given below:

$$Y_m = P_m \cdot \left\{1 - \exp\left[-\frac{\left(R_m\left(\lambda - t\right)\right)}{P_m}\right]\right\} \tag{3}$$

The equation that represents FOKM is given as:

$$Y_m = P_m \cdot \left(1 - \exp\left(-k \cdot t\right)\right) \tag{4}$$

where k is biogas rate kinetic constant in 1/d.

The equation that represents MGR is given as follows:

$$Y_m = P_m \cdot \exp\left(-\exp\left(-K_G \cdot \left(t - 1\right)\right)\right) \tag{5}$$

where K_G is growth-rate coefficient in 1/d.

3. Results and discussion

3.1. Physicochemical analysis

There was conducted mesophilic and thermophilic SS/GTS/AB batch co-digestion with five pre-treatment methods applied. The mesophilic digestion lasted for 47 d, whereas thermophilic 30 d. Along with the main thermophilic digestion, there was also conducted additional digestion of some chosen mixtures (CN, A1, A3, and A4) to which two types of biostarters were added. Two main control parameters were analysed at the start of the digestions and the end, that is, VFA and N–NH$_4^+$. These parameters served as an indicator of the overall process stability. Accumulation of both VFA and N–NH$_4^+$ leads to inhibition as suggested by some researchers [29,30]. All the pre-treatment, as well as control mixtures, had appropriate VFA and N–NH$_4^+$ concentrations at the start of both mesophilic and thermophilic digestion with maximum values for mesophilic digestion of CN and A5, that is, 5.14 ± 0.00 mg/g, respectively for VFA (Table 3). The range of values 30–60 mg/g for VFA (for acetate and propionate) is considered inhibitory [31].

Mixture A3 had the highest N–NH$_4^+$ concentration at the start, that is, 2.8 ± 0.00 mg/g, which was close to the inhibitory range (3–8 mg/g) [4]. In other literature, the inhibitory range of N–NH$_4^+$ is reported as 29–38 mg/g [31].

Corresponding maximum values for VFA and N–NH$_4^+$ at the start of thermophilic digestion were 4.57 ± 0.00 mg/g for VFA and 0.46 ± 0.00 mg/g for N–NH$_4^+$. Inoculum, however, had the highest concentrations to those parameters at the start at both temperatures and the N–NH$_4^+$ values surpassed the inhibitory range. However, at the end of each digestion, the concentrations of VFA and N–NH$_4^+$ dramatically increased which was associated with the microbial activity, that is, hydrolysis of the available organic matter to the simpler compounds such as organic acids and amino acids [32].

The maximum value for the VFA parameter at the end of the mesophilic digestion was achieved by the mixture that contained the enzyme (A5), that is, 177.08 ± 2.18 mg/g and it can be attributed to the increased hydrolysis intensity due to the enzyme addition. The maximum value for thermophilic digestion to that parameter was 245.57 ± 0.61 mg/g for CN and 245.14 ± 3.64 mg/g again for A5. In terms of biostarter digestion, the highest VFA value was obtained by mixture A3-BT (191.14 ± 4.85 mg/g). In this study VFA concentrations were much higher than those obtained in other studies that were mentioned in the literature review, that is, 10–120 and 20–80 mg/g and it can be attributed to the study scale, that is, batch, which does not allow to either introduce new substrate into the system or remove the used substrate that contains inhibitory substances from the system to stabilize the process [4,33]. In regard the N–NH$_4^+$, concentrations were within the range of inhibitory ones that were mentioned in the literature review, that is, 15–30 mg/g, especially A1 at mesophilic temperature (26.54 ± 0.40 mg/g), however, it did not much inhibit the methane production, for this mixture had the highest methane content in biogas (Fig. S7) and the possible explanation to that phenomenon can be the microorganism adaptation.

Overall, N–NH$_4^+$ values for all mixtures at the end of thermophilic digestion were much higher than at mesophilic one as is reported in the literature and that is the main reason why thermophilic temperature can be unstable [34,35]. N–NH$_4^+$ values in this study are much higher than those in the previous study (N–NH$_4^+$ values were within the range of 10.38–12.62 mg/g in the previous study) [20].

Table S3 presents results to the organic composition of the studied mixtures after digestion. At both mesophilic and thermophilic temperatures, NPOC parameter increased about 10 times as an average from all mixtures. VS parameter decreased as an average for all mixtures at 76.16% at mesophilic temperature and 69.08% after thermophilic one. Reduction of the TS parameter was observed the most at mesophilic temperature, that is, 33.5%, whereas at thermophilic only 19.3%.

3.2. BMP assay

In terms of Y_B parameter at mesophilic temperature (Fig. 4), the highest value was obtained by mixture

Table 3
Results of the physicochemical analysis

		Main digestion					
Stage	Mixture	Mesophilic			Thermophilic		
		VFA	$N–NH_4^+$	KN	VFA	$N–NH_4^+$	KN
Start-up	CN	5.14 ± 0.00	0.26 ± 0.00	38.78 ± 0.20	4.00 ± 0.00	0.37 ± 0.00	38.08 ± 0.00
	A1	2.29 ± 0.00	0.75 ± 0.26	42.28 ± 1.98	4.57 ± 0.00	0.28 ± 0.00	37.66 ± 0.59
	A2	3.43 ± 0.00	1.03 ± 0.13	39.62 ± 0.20	3.42 ± 0.00	0.46 ± 0.00	42 ± 0.79
	A3	4.57 ± 0.00	2.8 ± 0.00	39.90 ± 0.20	3.42 ± 0.00	0.28 ± 0.00	40.6 ± 0.79
	A4	4.00 ± 0.00	0.32 ± 0.00	38.22 ± 0.20	4.00 ± 0.00	0.18 ± 0.00	40.46 ± 0.99
	A5	5.14 ± 0.00	0.26 ± 0.00	38.78 ± 0.20	4.00 ± 0.00	0.37 ± 0.00	38.08 ± 0.00
	SS	4.29 ± 0.00	0.21 ± 0.00	43.66 ± 0.18	2.29 ± 0.81	0.09 ± 0.00	40.4 ± 0.2
	IN	7.43 ± 0.00	10.08 ± 0.26	44.17 ± 2.32	6.00 ± 1.21	11.25 ± 0.20	41.3 ± 2.57
After digestion	CN	87.25 ± 2.67	26.15 ± 0.08	–	245.57 ± 0.61	37.03 ± 0.98	–
	A1	111.94 ± 1.7	26.54 ± 0.40	–	228.43 ± 13.94	35.14 ± 0.19	–
	A2	126.51 ± 0.97	26.09 ± 0.23	–	207.43 ± 6.06	35.56 ± 0.39	–
	A3	126.34 ± 2.18	23.18 ± 0.23	–	228 ± 2.42	31.43 ± 0.98	–
	A4	115.54 ± 2.42	22.79 ± 0.16	–	180.86 ± 1.21	31.99 ± 0.98	–
	A5	177.08 ± 2.18	21.84 ± 0.31	–	245.14 ± 3.64	34.58 ± 0.00	–
	SS	125.14 ± 1.21	18.34 ± 0.03	–	77.57 ± 0.61	16.03 ± 0.09	–
	IN	–	–	–	–	–	–
		Subdigestion					
Stage	Mixture	Bio-Trakt®			BIOSANiT®		
After digestion	A1	129 ± 0.60	28.87 ± 0.04		–	–	–
	A3	191.14 ± 4.84	31.57 ± 0.89		–	–	–
	A4	133.71 ± 0.00	29.92 ± 0.05		–	–	–
	CN	173.57 ± 3.03	34.37 ± 0.098	–	–	–	–
	CN	–	–	–	125.57 ± 1.81	29.68 ± 0.19	–

All values are expressed in (mg/g).

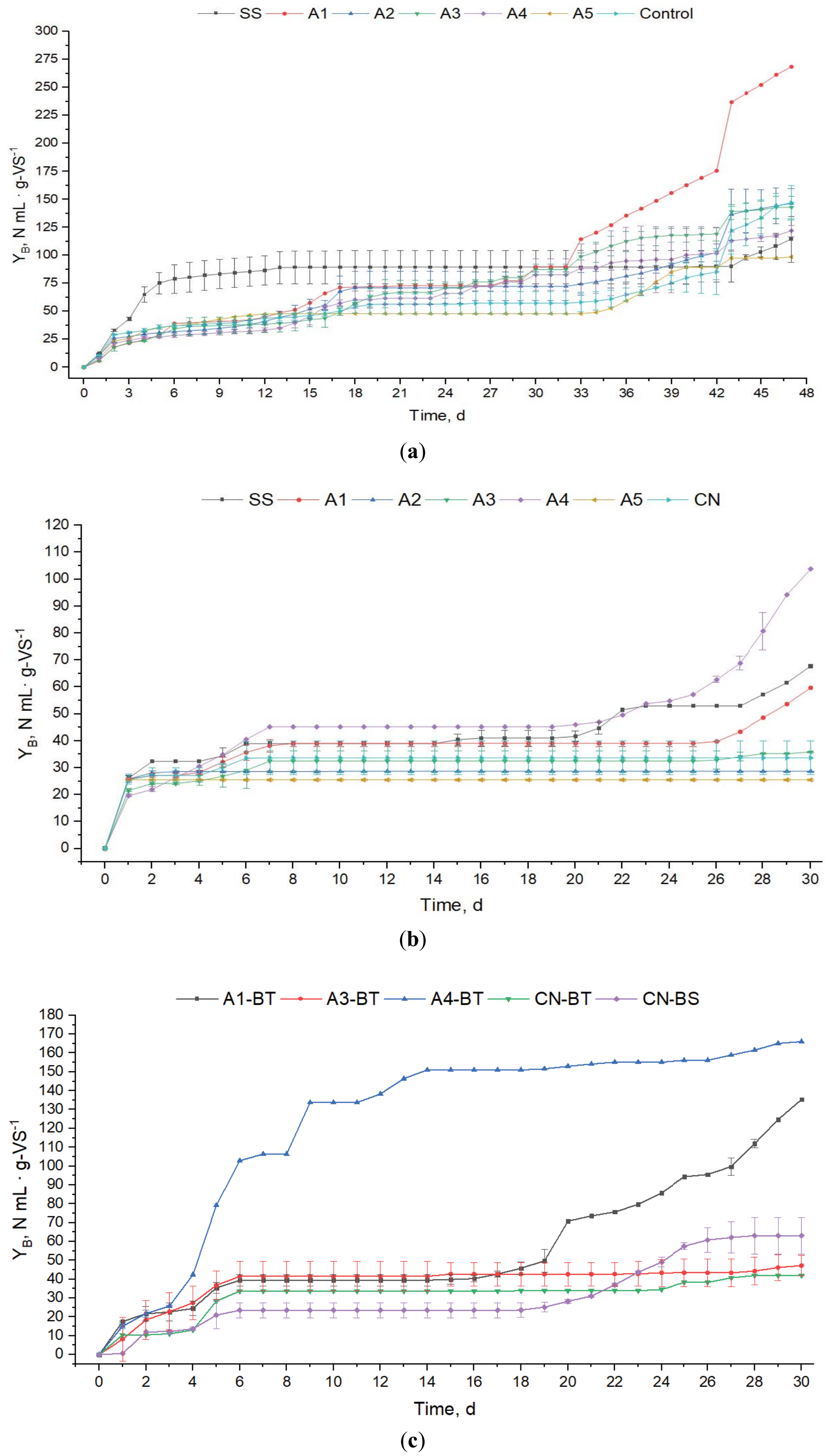
A1, that is, 268.59 ± 0.00 N·mL/g-VS which was higher for 83% and 134% in comparison to CN and SS, respectively. Their corresponding values were 147.13 ± 15.48 and 114.72 ± 20.65 N·mL/g-VS. In this study, the Y_B value obtained for this mixture (even being pre-treated) was much lower than that was obtained in the previous study related to the SS/GTS/AB and SS/OFMSW/AB wet co-digestion (i.e., 646.11 ± 34.08 N·mL/g-VS for mesophilic digestion and 678.19 ± 23.57 N·mL/g-VS for thermophilic one) [20]. The value obtained in this study was, however, close to that which was obtained for SS/GTS wet mesophilic co-digestion (i.e., 310–450 N·mL/g-VS) in the literature [36]. Mixtures A2 and A3 had very similar values as was obtained for CN (i.e., 146.45 N·mL/g-VS for A2 and 142.95 N·mL/g-VS for A3) and overall all other mixtures except A1 had lower values to Y_B parameter, which can imply little effect that was done by the pre-treatment methods applied.

Application of pre-treatment methods for co-digestion mixture SS/GTS/AA did not improve results compared to SS, however, as was shown in the study [37], co-digestion of these substrates led to 9%–27% increase in terms of methane yield (Fig. 3).

The highest value of Y_B during thermophilic digestion was obtained by mixture A4, that is, 103.84 ± 0.00 N·mL/g-VS which was 14.98% lower than the value obtained by the same mixture but at mesophilic temperature. That value was 2 times higher than CN (33.66 ± 6.47 N·mL/g-VS) and 53% higher than SS (67.74 ± 0.00 N·mL/g-VS) at thermophilic temperature. All the Y_B values that were obtained at this temperature were overall lower in comparison to mesophilic temperature.

In terms of thermophilic biostarter digestion, the highest Y_B value was also obtained by mixture A4 containing BT (A4-BT), that is, 166.06 ± 0.00 N·mL/g-VS which was about 4 times higher than CN-BT (41.94 ± 0.00 N·mL/g-VS) and 2.6 times higher than CN-BS (63.12 ± 9.72 N·mL/g-VS). It was also 59.93% higher than A4 during thermophilic digestion and 35.97% higher than A4 at mesophilic temperature, that is, 122.14 ± 5.14 N·mL/g-VS. These highest values that were obtained during main thermophilic digestion and subdigestion were much lower than the value obtained in the previous study [20].

In regarding to Y_m, as Fig. 4 presents, the highest Y_m at mesophilic temperature was obtained by A1 mixture, that

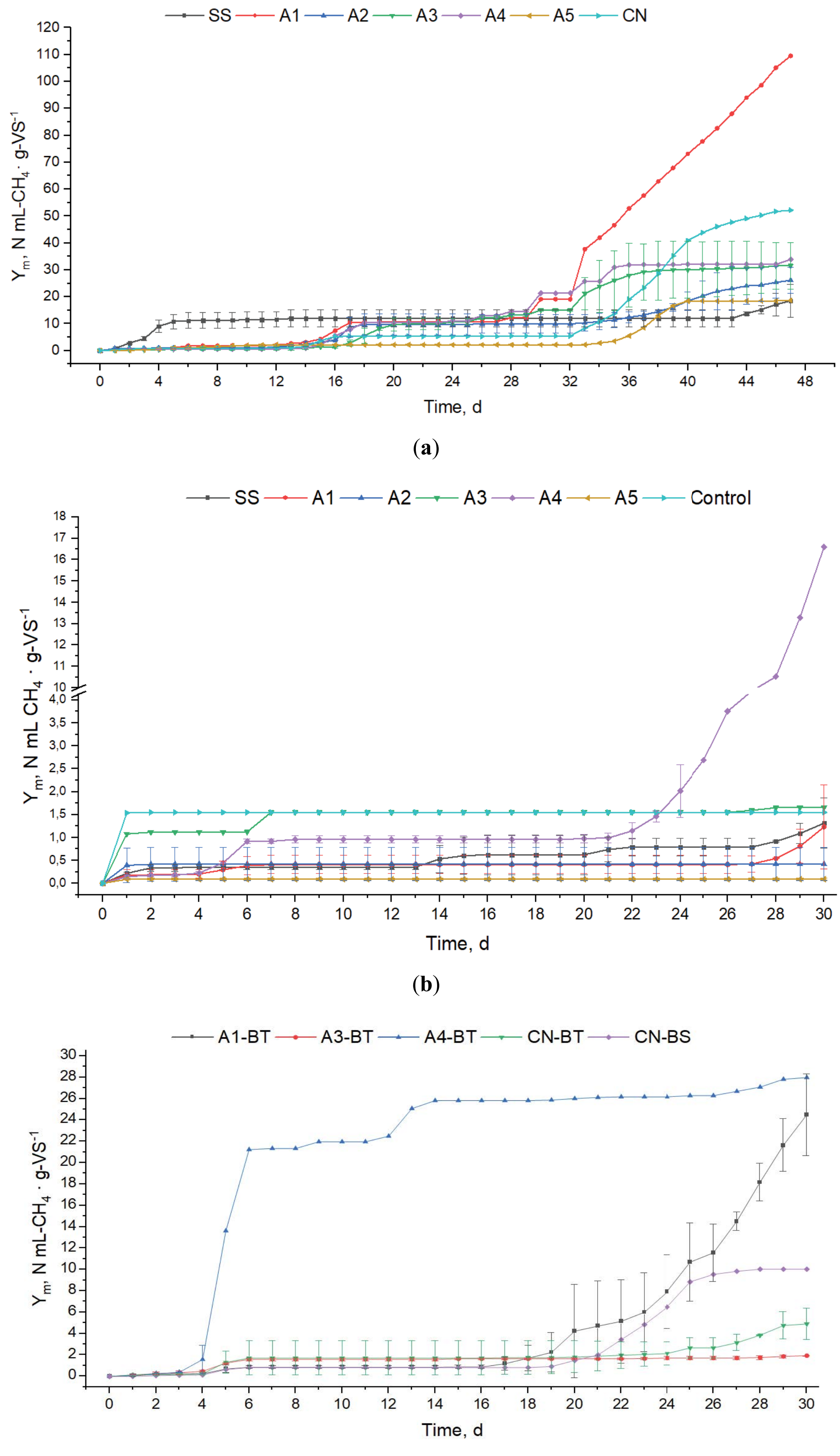


(**a**)

(**b**)

(**c**)

Fig. 3. Y_B: (a) mesophilic temperature, (b) thermophilic temperature and (c) biostarters.



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(**c**)

Fig. 4. Y_m: (a) mesophilic temperature, (b) thermophilic temperature and (c) biostarters.

is, 109.48 ± 0.00 N·mL-CH_4/g-VS which was 2 times higher in comparison to CN (52.21 ± 0.00 N·mL-CH_4/g-VS) and about 6 times higher than SS (18.51 ± 6.08 N·mL-CH_4/g-VS) at the same temperature. The pre-treatment effect of $NaOH^+$ water bath in this study was much more considerable than that reported in the study related to SS/GTS co-digestion, that is, 36% increase (from 134.75 mL-CH_4/g-VS for SS control to 184.28 mL-CH_4/g-VS for SS pre-treated) and the value for A1 was close to that range [36]. The effect of the thermal-alkaline pre-treatment was also much higher compared to the study [38], where microalgae were co-digested with wheat straw (e.g., 1/1 microalgae + wheat straw mixture had Y_m of 299 ± 15 N·mL-CH_4/g-VS when was pre-treated and 289 ± 3 N·mL-CH_4/g-VS for untreated).

Contrary to the study [18], addition of Fe^+ to mixtures for autoclaving did not improve the results of Y_m at both temperatures, however, in terms of Y_B, the improvement was noticeable, especially at thermophilic temperature.

The lowest Y_m among pre-treatment mixtures was obtained by A5 (18.65 ± 0.00 N·mL-CH_4/g-VS) which was very close to SS and 64% lower than CN. In terms of thermophilic digestion, the highest value of Y_m was obtained by A4 (16.59 ± 0.00 N·mL-CH_4/g-VS) which is more than 10 times higher in comparison to CN (1.55 ± 0.00 N·mL-CH_4/g-VS) and SS (1.31 ± 0.00 N·mL-CH_4/g-VS). Thermophilic digestion of A1 gave extremely small Y_m results in comparison to mesophilic temperature (1.23 ± 0.91 N·mL-CH_4/g-VS). Addition of biostarters improved the situation in a certain degree, especially for mixture A1-BT (24.49 ± 3.84 N·mL-CH_4/g-VS) and A4-BT (27.97 ± 0.00 N·mL-CH_4/g-VS), however, it is still much lower than the values obtained at mesophilic temperature, in particular, in terms of A1, whose difference is 77.62%. The next table (Table 4) presents summary of the result for Y_B and Y_m parameters with the result of the Tukey HSD test based on one-way ANOVA (letters (a, b, c, d, e, and f) represent homogeneous groups).

Fig. S6 shows methane content in biogas for main and biostarter digestions. Notwithstanding the fact, that the values for Y_B and Y_m were much lower than those obtained in the previous study related to the SS/GTS/AB and SS/OFMSW/AB mesophilic and thermophilic co-digestions, the methane content in biogas, in particular at mesophilic temperature, was much higher in this study than in the previous one [13]. Mixture A1 started to have a stable 69.16% CH_4·v/v as an average from day 33 up to 47, which corresponds to the end of the lag phase. The highest methane content in biogas during that period was 76.4% CH_4·v/v for that mixture. In terms of other pre-treatment mixtures, the highest methane content in that period was achieved by mixture A3, that is, 63.74% CH_4·v/v, although its average value during that period was 36.09% CH_4·v/v. In other cases, CN had better results in comparison to remaining mixtures (44.004% CH_4·v/v as an average in the period from 33 to 47 d and 57.95% CH_4·v/v the maximum value obtained during that period). In terms of thermophilic digestion, the highest methane content in biogas had mixture A4 which was close to 35% CH_4·v/v and its methane content started to grow profoundly from day 25. The pre-treatment effect of this method in this study regarding the methane content in biogas was much lower (for 59%) than that reported in the literature (85.78% CH_4·v/v was reported), however, that study concerned wet digestion of coking sludge [18]. The highest methane content in biogas during thermophilic biostarter digestion had mixture A1, that is, 34% CH_4·v/v, however, mixture A4 had also a similar value (31.8% CH_4·v/v). As can be seen from that figure, there are three distinctive time ranges, during which the results were observed, that is, from day 2 to 6, 11 to 13, and 16 up to 30.

3.3. Kinetic study

The full results of the kinetic study are presented in Tables S4 and S5. Fig. 5 shows the difference in the actual and measured specific methane yields based on the most precise fit as well values of other parameters such as maximum rate of methane yield and lag phase time. Most of the mixtures had high lag phase time as can be seen from the figures discussed in section 3.2 (Figs. 3 and 4). Even

Table 4
Summary of the results to the Y_B and Y_m parameters including Tukey HSD test results (values marked with the same letter in the graph are not significantly different to Tukey test, $p > 0.05$)

Mixture	Y_B (N·mL/g-VS)		Y_m (N·mL-CH_4/g-VS)	
	M	T	M	T
SS	114.72 ± 20.65 a	39.97 ± 8.38 ab	18.51 ± 6.08 a	0.58 ± 0.33 a
A1	268.60 ± 0.00 b	33.68 ± 9.31 ab	109.48 ± 0.00 c	0.29 ± 0.25 a
A2	146.45 ± 13.69 a	21.61 ± 12.2 a	26.16 ± 4.87 a	0.42 ± 0.36 a
A3	142.96 ± 10.38 a	32.63 ± 2.79 ab	31.56 ± 8.64 ab	0.82 ± 0.63 a
A4	122.14 ± 5.14 a	39.08 ± 6.93 ab	33.89 ± 0.00 ab	0.99 ± 0.1 a
A5	98.62 ± 0.00 a	23.53 ± 3.38 a	18.65 ± 0.00 ab	0.08 ± 0.02 a
CN	147.13 ± 15.48 a	30.77 ± 6.77 ab	52.21 ± 0.00 b	0.84 ± 0.65 ac
A1-BT	–	56.65 ± 13.13 bc	–	6.36 ± 1.38
A3-BT	–	33.70 ± 16.15 ab	–	1.65 ± 0.07 ab
A4-BT	–	72.59 ± 6.88 c	–	26.00 ± 0.00 d
CN-BT	–	16.66 ± 1.88 a	–	2.88 ± 0.02 b
CN-BS	–	24.98 ± 5.70 a	–	1.49 ± 0.75 ab

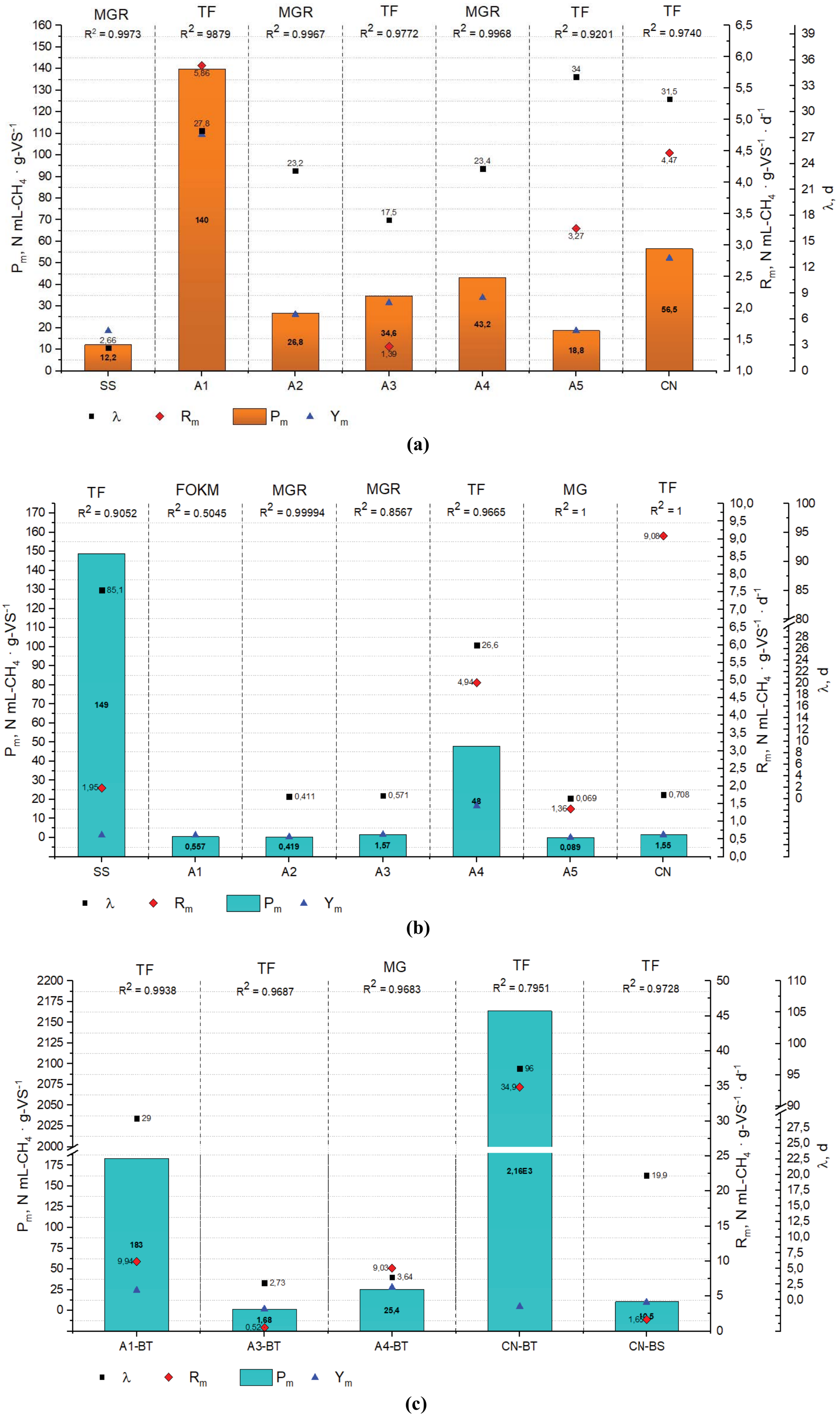

Fig. 5. Most precise fit: (a) mesophilic temperature, (b) thermophilic temperature and (c) biostarters.

when pre-treated, mixture A1 at mesophilic temperature had a lag phase of 27 d, after which a considerable increase in the production started to appear. Similar to the studies [39,40], thermal pre-treatment (autoclaving) improved start-up period by 44.4% (mixture A3, CN had 31.5 d and A3 had 17.5 d) compared to the results for the treated duckweed + waste AS mixture (59%, 14.8 d for the treated and 6 d for untreated). The addition of Fe$^+$ during autoclaving improved the results even further at mesophilic temperature, that is, 25.6% (CN has 31.5 d and A4 has 23.4 d).

In terms of mesophilic temperature, such models as MGR and TF fitted the experimental results the best (judging by R^2 coefficient) in comparison to other studied models, and the highest R^2 was obtained for MGR model fitted to SS results ($R^2 = 0.9973$). However, for SS the difference in P_m from the experimental data was 34%, which was the highest among other mixtures. In terms of mesophilic temperature, λ for standalone SS was the smallest one (λ = 2.66 d) in comparison to the pre-treatment mixtures as well as CN, which indicated that the addition of GTS and AB makes it difficult for microorganisms to properly hydrolyse the substrates in high-solids conditions. However, in comparison to the results that were obtained in the previous study related to the wet co-digestion of SS/GTS/AB and SS/OFMSW/AB, in that study λ was much lower for all mixtures including standalone SS at both studied temperatures and SS/GTS/AB mixture λ was 0.595 d (with $R^2 = 0.99908$) at mesophilic temperature and 1.486 d (with $R^2 = 0.99944$) [20]. In this study, λ the parameter among the pre-treatment mixtures at mesophilic temperature was within the range of 17.5–34 d.

Table 5
Results of factorial ANOVA in relation to the main digestion (results for the final digestion day were chosen for the analysis)

Parameter	Effect	Factor		
		Temp.	Mix.	Temp. × Mix.
Y_B	F	46.0306	2.3877	2.1093
	p	0.000000	0.054613	0.083892
Y_m	F	19.44289	1.08000	1.13306
	p	0.000139	0.398058	0.369215

Temp. – temperature; Mix. – mixture (pre-treatment method).

Table 6
Results of factorial ANOVA for subdigestion in relation to CN-BT

Parameter	Effect	Factor						
		Time	Bio.	Mix.	Time × Bio.	Time × Mix.	Bio. × Mix.	Time × Bio. × Mix.
Y_B	F	2.1008	2.1895	8.3574	0.2499	0.5959	2.7307	0.7126
	p	0.108858	0.143862	0.000091	0.861083	0.795726	0.051059	0.695314
Y_m	F	1.61847	10.79638	4.14510	0.41371	0.50072	2.07082	0.74027
	p	0.193803	0.001653	0.009522	0.743718	0.868610	0.112842	0.670703

Temp. – temperature; Mix. – mixture (pre-treatment method); Bio – biostarter.

Table 7
Results of Tukey HSD test for Y_B and Y_m parameters

Parameter	Factor	Mixture													
Y_B	Mixt.	T	T	T	T	T	T	M	T	M	M	M	M	M	M
	Temp.	A2	A5	CN	A3	A1	SS	A5	A4	A2	CN	A4	A3	A1	SS
	Mean	21.59	23.53	30.77	32.42	33.53	37.40	37.52	38.45	52.52	61.90	67.07	72.22	79.55	85.68
	a	****	****	****	****	****	****	****	****						
	b			****	****	****	****	****	****	****					
	c						****		****	****	****				
	d									****	****	****	****		
	e										****	****	****	****	
	f											****	****	****	****
Y_m	Mixt.	T	T	T	T	T	T	T	M	M	M	M	M	M	M
	Temp.	A5	A1	A2	SS	A3	CN	A4	A5	A2	CN	A4	A3	SS	A1
	Mean	0.080	0.29	0.42	0.47	0.82	0.84	0.97	2.99	6.51	7.44	10.31	10.50	10.85	15.16
	a	****	****	****	****	****	****	****	****	****	****	****			
	b		****	****	****	****	****	****	****	****	****	****	****		
	c				****	****	****	****	****	****	****	****	****	****	
	d									****	****	****	****	****	****

**** – results of Tukey HSD test from STATISTICA software; Temp. – temperature; Mixt. – mixture.

Table 8
Observed pre-treatment effect for the studied mixture

Pre-treatment method	Observed effect		
	Mesophilic temperature	Thermophilic temperature	Thermophilic temperature + biostarters
A1: Thermochemical pre-treatment with NaOH in waterbath	- Y_B: 134% increase in comparison to SS and 83% increase in comparison to CN - Y_m: 6-fold increase in comparison to SS and 110% increase in comparison to CN - λ: 11.7% decrease in comparison to CN	- Y_B: 12% decrease in comparison to SS and 77% increase in comparison to CN - Y_m: no substantial observed effect in comparison to SS and 20% decrease in comparison to CN - λ: Nd	- Y_B: 2-fold increase in comparison to CN-BT and 114.39% increase in comparison to CN-BS - Y_m: 4-fold increase in comparison to CN-BT and 144% increase in comparison to CN-BS - λ: 69.78% decrease in comparison to CN-BT and 46% increase in comparison to CN-BS
A2: Thermal pre-treatment in waterbath	- Y_B: no observed effect in comparison to CN and 27.67% increase in comparison to SS - Y_m: 41% increase in comparison to SS and 50% decrease in comparison to CN - λ: 26.3% decrease in comparison to CN	- Y_B: 58% decrease in comparison to SS and 15% decrease in comparison to CN - Y_m: 68% decrease in comparison to SS and 73% decrease in comparison to CN - λ: 41.9% decrease in comparison to CN (not reliable)	–
A3: Autoclaving	- Y_B: no substantial observed effect in comparison to CN and 24.62% increase in comparison to SS - Y_m: 70% increase in comparison to SS and 40% decrease in comparison to CN - λ: 44.5% decrease in comparison to CN	- Y_B: 47% decrease in comparison to SS and no substantial observed effect in comparison to CN - Y_m: 26% increase in comparison to SS and no substantial observed effect in comparison to CN - λ: 19.4% decrease in comparison to CN (not reliable)	- Y_B: 12.59% increase in comparison to CN-BT and 25.18% decrease in comparison to CN-BS - Y_m: 60.45% decrease in comparison to CN-BT and 80.66% decrease in comparison to CN-BS - λ: 97% decrease in comparison to CN-BT and 86.2% decrease in comparison to CN-BS
A4: Fe^+ autoclaving	- Y_B: 17% decrease in comparison to CN and no substantial observed effect in comparison to SS - Y_m: 83% increase in comparison to SS and 35% decrease in comparison to CN - λ: 25.6% decrease in comparison to CN	- Y_B: 53% increase in comparison to SS and 2-fold increase in comparison to CN - Y_m: 11-fold increase in comparison to SS and 10-fold increase in comparison to CN - λ: 36-fold increase in comparison to CN (not reliable)	- Y_B: 3-fold increase in comparison to CN-BT and 163% increase in comparison to CN-BS - Y_m: 5-fold increase in comparison to CN-BT and 178.8% increase in comparison to CN-BS - λ: 96.2% decrease in comparison to CN-BT and 81.66% decrease in comparison to CN-BS
A5: Enzymatic pre-treatment	- Y_B: 33% decrease in comparison to CN and 14% decrease in comparison to SS - Y_m: no substantial observed effect in comparison to SS and 64% decrease in comparison to CN - λ: 8.2% increase in comparison to CN	- Y_B: 62% decrease in comparison to SS and 24% decrease in comparison to CN - Y_m: 93%–94% decrease in comparison to both SS and CN - λ: 90.3% decrease in comparison to CN (not reliable)	–

The highest λ was for mixture A5, that is, 34 d, followed by A1 (λ = 27.8 d). The highest R_m was predicted for mixture A1, that is, 5.86 N·mL-CH_4/g-VS/d whereas the lowest one was for mixture A3 (1.39 N·mL-CH_4/g-VS/d).

In terms of mesophilic temperature, the least precise models judging by the coefficient of determination were FOKM and LF and the worst fitting was obtained for CN with FOKM (R^2 = 0.5575).

In terms of thermophilic temperature, overall, the results were too low to be properly described by the kinetic models. For mixtures, A2, CN, and A5 (the latter mentioned especially) the fermentation did not start and there was, therefore, a straight line on the Y_m and Y_B graphs, resulting in $R^2 = 1$ fitting, which is incorrect from the point of view of the process but not mathematically. Therefore, it was impossible to determine the close to real λ values for those mixtures. The most trustworthy fit was obtained for mixture A4 with $R^2 = 0.9665$ which was determined λ as 26.6 d and R_m as 4.94 N·mL-CH_4/g-VS/d. The most precise fitting was obtained by MGR, TF, FOKM, and MG, however, their fitting is questionable, especially for A5 and CN.

The most precise fit in relation to the biostarter digestion was obtained for mixture A4-BT ($R^2 = 0.9683$) and CN-BS ($R^2 = 0.9728$) judging by the closeness of P_m values in comparison to Y_m, that is, P_m = 25.42 N·mL-CH_4/g-VS and Y_m = 27.97 N·mL-CH_4/g-VS for A4-BT (difference of about 9.1%), and P_m = 10.03 N·mL-CH_4/g-VS and Y_m = 10.50 N·mL-CH_4/g-VS for CN-BS (difference of about 4.66%). The worst fit among the most precise values was obtained for mixture CN-BT ($R^2 = 0.7951$) that determine P_m as 2163.89 N·mL-CH_4/g-VS and λ as 96 d. The most precise models, in this case, were TF and MG.

3.4. Statistical analysis

Tables 5 and 6 present the results of factorial ANOVA analysis for main digestion and subdigestion. The confidence limit was 0.95 whereas the significance level was $p = 0.05$. As can be seen from Table 5, in terms of all parameters the factor "temperature" influenced the results the most, especially in terms of Y_B parameter (p within the range 0–0.000139 and $F = 46.0306$). However, the mutual connection of two factors, that is, "mixture" and "temperature" were not within the range of the significance level.

In terms of the biostarter digestion in relation to CN-BT (Table 6), "biostarter" as a factor had significant correlation to Y_m parameter ($p = 0.001653$ and $F = 10.79638$), whereas factor "mixture" was significant for all studied parameters, especially for Y_B ($p = 0.000091$ and $F = 8.3574$). So, addition of the biostarters as well as application of pre-treatment methods influenced the methane production. Neither of the factor connections had the significant correlation, however, in terms of Y_B "biostarter" and "mixture" factor connection was close to the significance level ($p = 0.051059$ with $F > 1$, $F = 2.7307$).

Table 7 presents results for Tukey HSD test that was performed for only those results that were obtained at the fourth digestion week. The letters a, b, c, d, e and f mean homogeneous groups.

Table 8 presents the summarising information regarding to the pre-treatment effect that was observed for the studied SS/GTS/AB mixture in relation to Y_B, Y_m and λ parameters.

4. Conclusion

The following conclusion can be drawn:

- The most pronounced pre-treatment effect to the studied SS/GTS/AB mixture was observed at mesophilic temperature for the thermochemical pre-treatment method using NaOH and water bath (A1). Its Y_m was 109.48 ± 0.00 N·mL-CH_4/g-VS which was 2 and 5 times higher compared to control and standalone SS, respectively (52.21 ± 0.00 N·mL-CH_4/g-VS and 18.51 ± 6.08 N·mL-CH_4/g-VS).
- The second most conspicuous pre-treatment effect was attributed to the Fe^+ autoclaving at thermophilic temperature. Its Y_m was 16.59 ± 0.00 N·mL-CH_4/g-VS, which was respectively 11 and 10-fold higher in comparison to SS and CN (1.31 ± 0.54 and 1.55 ± 0.00 N·mL-CH_4/g-VS).
- The least pronounced pre-treatment effect at both studied temperatures was for thermal pre-treatment with water bath (A2), autoclaving (A3) and enzymatic pre-treatment (A5).
- In the case of main digestion, temperature influenced the results the most (p within the range 0–0.000139), in case of biostarter digestion both pre-treatment method as well as biostarter addition separately influenced the results (p within the range 0.000091–0.009965), however, their mutual influence was not confirmed.
- SS/GTS/AB dry co-digestion significantly increased λ the parameter (more than 30 d), however, thermochemical pre-treatment improved that parameter by 26.3% in comparison to CN.

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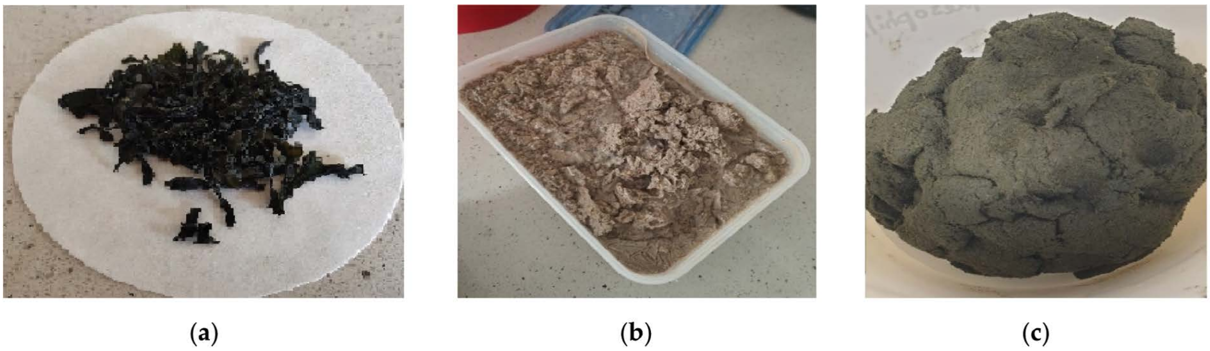
Supplementary information:



(**a**) (**b**) (**c**)

Fig. S1. Substrates that were used in the study: (a) algae substrate “Wakame”, *Undaria pinnatifida*, (b) grease trap sludge from the meat processing plant, Silesian region, Poland and (c) SS/GTS/AB mixture (without inoculum added).

(a) (b)

Fig. S2. Biostarters that were applied within the scope of the study: (a) Bio-Trakt® and (b) BIOSANiT®.

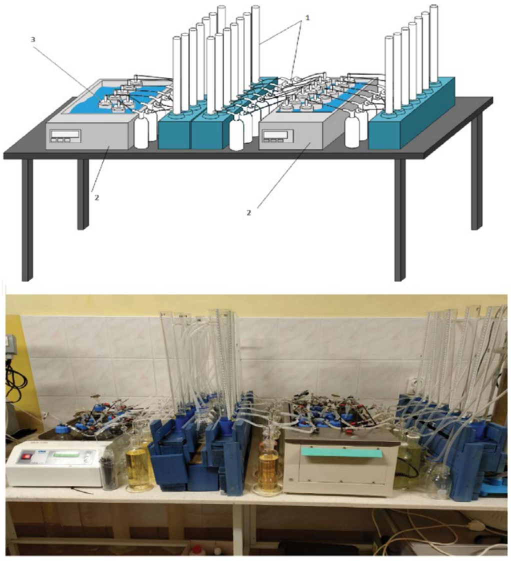


Fig. S3. Research stand: 1 – LDS elements; 2 – two water baths for heating; 3 – BMP bottle with a mixture.

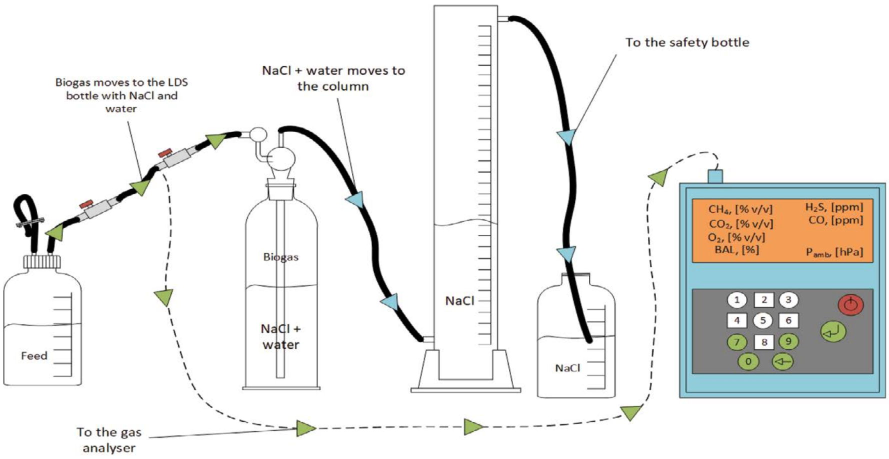


Fig. S4. LDS method of biogas measurement.

Table S1
Information regarding to the composition and recommended dose of the applied biostarters

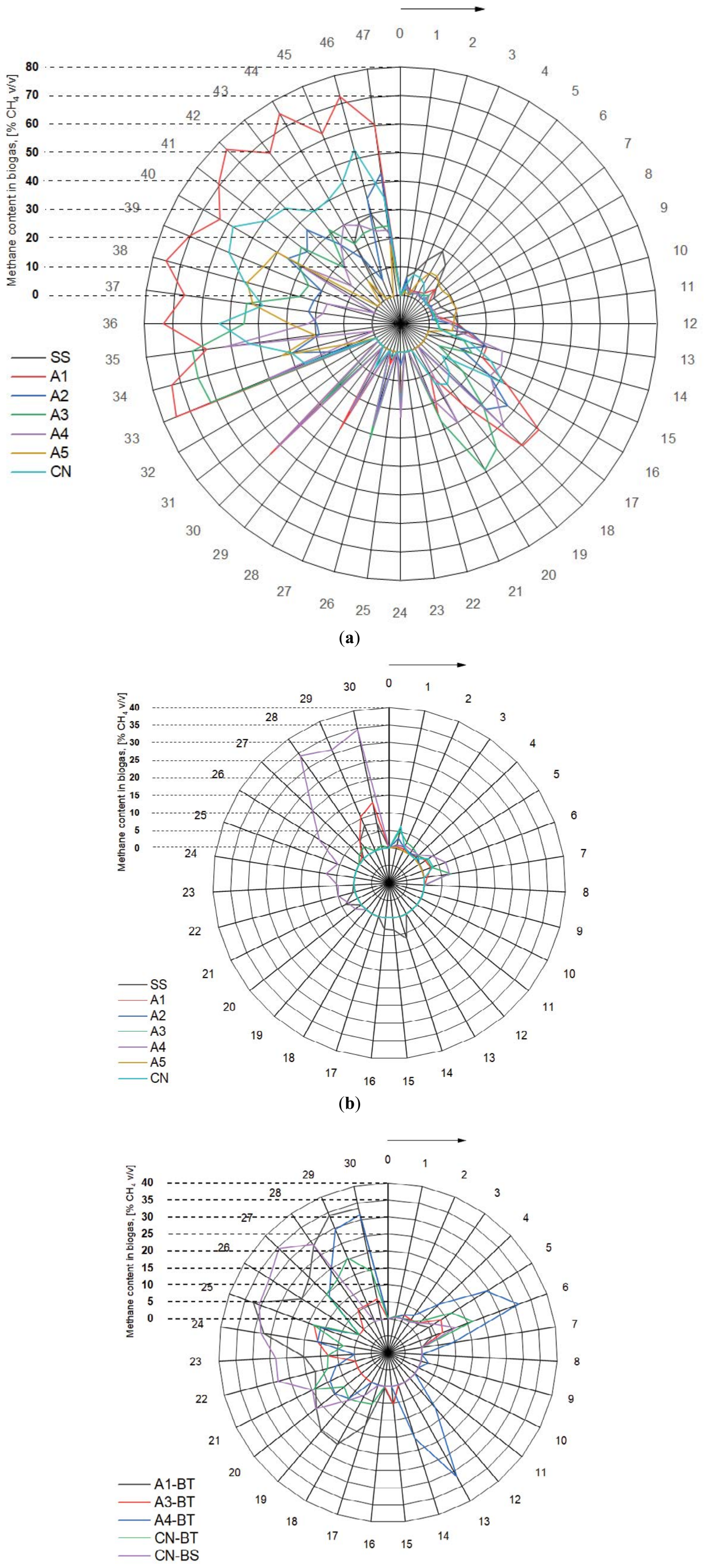
Bio-Trakt®		BIOSANiT®	
Composition	Recommended dose	Composition	Recommended dose
Lactic fermentation bacteria: min. 1.1 × 10^7 CFU/mL *Bacillus subtilis*: min. 4.4 × 10^6 CFU/mL Cyanobacteria and phototropic bacteria Yeast: 2.6 × 10^5 Ethanol Acetic acid Organic sugar cane molasses Pure non-chlorinated water	1 L of biostarter per 1 m^3 of SS originating from a septic tank or a sewage chamber	Nd	[Volume]/[dose in a measure[a]]: Septic tanks with drainage: 1) [2–4]/[12] 2) [5–7]/[18] 3) [9–11]/[22] Sealed septic tanks: 1) [2–4]/[12] 2) [5–7]/[20] 3) [9–11]/[24]

Nd – no information
[a]according to the information that is presented on the package, one measure is equal to 7.5 mL or about 4.5 g.

Table S2
Information regarding to the methods that were used for the physicochemical analyses

APHA			PN-ISO 10694:2002		FCBWiŚ (Physicochemical examination of water and wastewater)	
Parameter	Section	Method	Parameter	Method	Parameter	Method
Volatile fatty acids	2310, B and 2320, B	Titration method	Total carbon	Total carbon content and high-temperature combustion method	N–NH_4^+	Determination of ammoniacal nitrogen after preliminary distillation
Total solids	2540, B	Total solids dried at 103°C–105°C				
Volatile solids and FS	2540, E	Fixed and volatile solids ignited at 550°C	NPOC			
Kjeldahl nitrogen	4500-Norg, B	Macro-Kjeldahl method				

Fig. S5. Lipase enzyme that was bought from Sigma-Aldrich.com.



(**a**)

(**b**)

(**c**)

Fig. S6. Methane content in biogas: (a) mesophilic temperature, (b) thermophilic temperature and (c) biostarters.

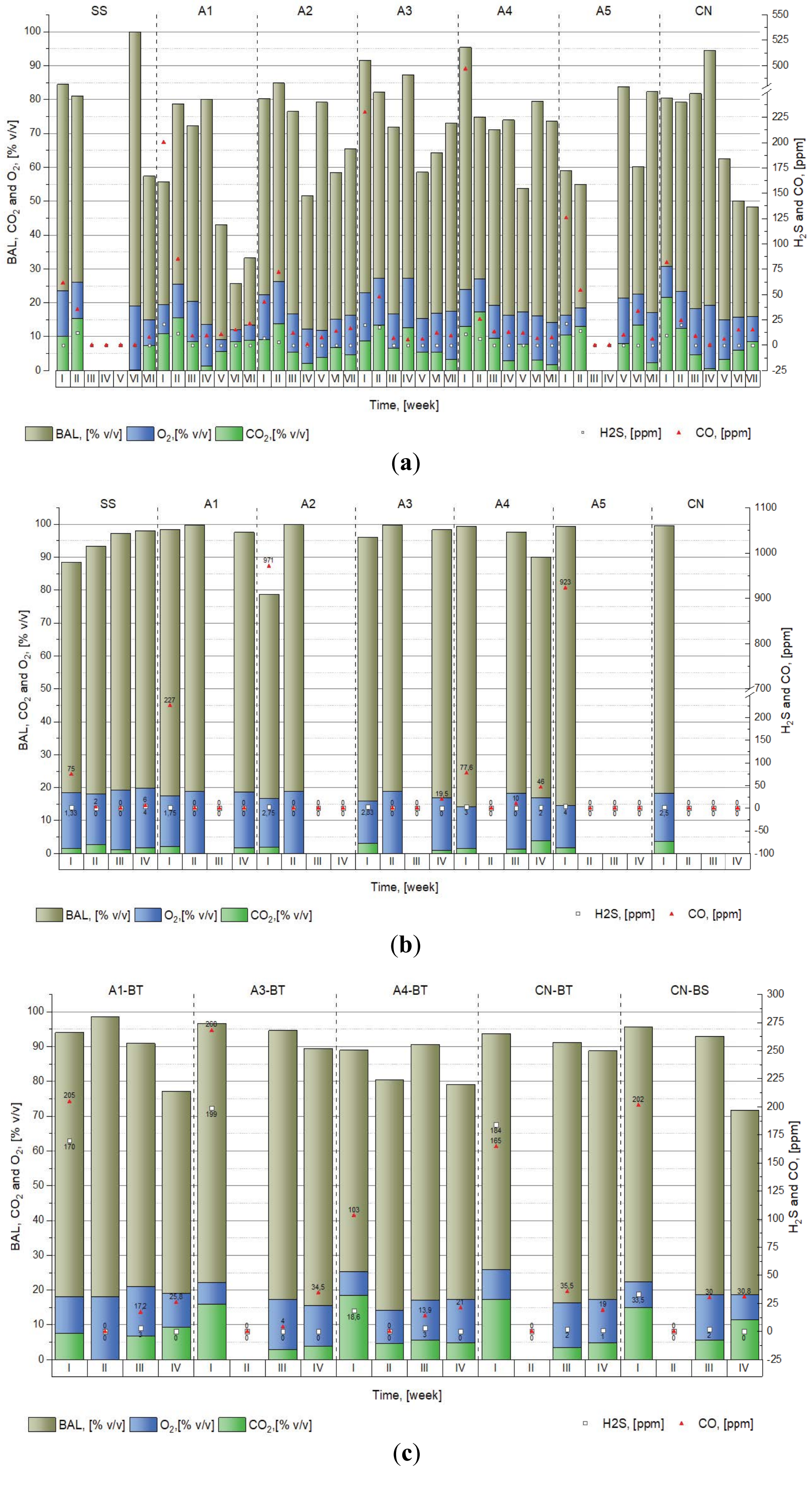


Fig. S7. Content of other components in biogas besides methane: (a) mesophilic temperature, (b) thermophilic temperature and (c) biostarters.

Table S3
Organic composition of the studied pre-treatment mixtures after each digestion part

Main digestion					
Stage	Mixture	Total solids (%)	Volatile solids (%)	Volatile solids/total solids (–)	NPOC (g/L)
Mesophilic	CN	6.72 ± 0.08	4.46	0.66	7.53
	A1	6.26 ± 0.26	4.04	0.64	8.67
	A2	6.90 ± 0.02	4.62	0.66	9.64
	A3	6.66 ± 0.02	4.38	0.65	8.53
	A4	6.68 ± 0.29	4.37	0.66	7.50
	A5	7.63 ± 0.01	5.43	0.71	13.42
	SS	5.71 ± 0.07	4.04	0.71	8.64
Thermophilic	CN	7.71 ± 0.06	5.29	0.68	15.54
	A1	7.20 ± 0.07	4.83	0.67	15.68
	A2	7.63 ± 0.04	5.28	0.69	16.42
	A3	8.65 ± 0.24	6.07	0.70	14.59
	A4	10.10 ± 0.16	6.72	0.66	13.94
	A5	10.28 ± 0.05	7.62	0.74	22.82
	SS	4.54 ± 0.08	3.22	0.70	7.25
Biostarter digestion					
Bio-Trakt®	A1	8.95 ± 0.02	6.09	0.68	13.91
	A3	9.41 ± 0.08	6.65	0.70	–
	A4	9.26 ± 0.02	5.97	0.64	11.38
	CN	9.81 ± 0.05	6.74	0.68	13.55
BIOSANiT®	CN	9.13 ± 0.02	6.31	0.69	12.15

Table S4
Results of the kinetic study in relation to the main digestion

Mixture	Model	P_m (mL-CH$_4$/g-VS)		R_m (mL-CH$_4$/g-VS/d)		λ (d)		R^2 (–)		k (1/d)		K_G (1/d)	
		M	T	M	T	M	T	M	T	M	T	M	T
SS	MG	12.17	388.11	3.12	1.15	1.20	125	0.8323	0.9041	–	–	–	–
	LF	12.34	–1.75E^{+12}	3.47	0.028	0.39	–4.917	0.8101	0.8762	–	–	–	–
	TF	12.13	148.84	3.37	1.952	1.46	85.07	0.8321	0.9052	–	–	–	–
	FOKM	12.37	2.764	–	–	–	–	0.8031	0.8166	0.25	0.015	–	–
	MGR	12.15	36,886	–	–	2.66	524.67	0.9973	0.9047	–	–	0.7781	0.0047
CN	MG	60.76	1.548	4.54	4.75	31.41	0.299	0.9659	1	–	–	–	–
	LF	–7.08E^{12}	1.548	1.015	7.51	10.64	7.922	0.6871	1	–	–	–	–
	TF	56.54	1.548	4.474	9.08	31.47	0.708	0.974	1	–	–	–	–
	FOKM	421,176	1.548	–	–	–	–	0.5575	1	1.60E$^{–6}$	4.85	–	–
	MGR	60.81	1.548	–	–	36.33	0.431	0.9659	1	–	–	0.0485	8.525
A1	MG	365.34	700.22	6.56	1.38	29.92	203	0.9834	0.5011	–	–	–	–
	LF	4.68E^{14}	–2.109	2.08	0.015	10.11	–11.38	0.7729	0.4814	–	–	–	–
	TF	139.73	342.89	5.86	–3.053	27.79	–1.329	0.9879	–9.04E-05	–	–	–	–
	FOKM	6.93E^{6}	0.5574	–	–	–	–	0.8120	0.50453	2.03E$^{–7}$	0.1170	–	–
	MGR	346.65	398,624	–	–	49.44	911.51	0.9834	0.5036	–	–	0.0503	0.0029
A2	MG	935	0.4191	4.385	0.7321	68.92	0.2	0.9290	0.99983	–	–	–	–
	LF	–3.52E^{13}	0.4192	0.5	1.1785	5.05	–1.007	0.8910	0.99988	–	–	–	–
	TF	142.24	75.82	2.19	–1.7647	38.65	–3,466	0.9270	–2.9784	–	–	–	–
	FOKM	268,618	0.4192	–	–	–	–	0.8598	0.99988	1.59E$^{–6}$	2.811	–	–
	MGR	26.81	0.419	–	–	23.19	0.411	0.9967	0.99994	–	–	0.0483	4.747

Table S4 (Continued)

Table S4

Mixture	Model	P_m (mL-CH$_4$/g-VS)		R_m (mL-CH$_4$/g-VS/d)		λ (d)		R^2 (–)		k (1/d)		K_G (1/d)	
		M	T	M	T	M	T	M	T	M	T	M	T
A3	MG	41.44	1.566	1.22	0.2352	15.51	–1.877	0.9746	0.814	–	–	–	–
	LF	$1.85E^{14}$	1.5631	0.82	0.5753	7.86	–0.5117	0.9207	0.8472	–	–	–	–
	TF	34.64	1.5677	1.39	0.1805	17.48	–2.835	0.9772	0.7957	–	–	–	–
	FOKM	525,914	1.5454	–	–	–	–	0.8460	0.8252	$1.17E^{-6}$	0.5052	–	–
	MGR	37.46	1.5662	–	–	24.83	0.571	0.9755	0.8567	–	–	0.0723	0.4078
A4	MG	40.58	34,305	1.31	498.86	14.15	56.016	0.9705	0.964	–	–	–	–
	LF	$6.93E^{13}$	7.5962	0.87	0.2792	7.04	6.477	0.9276	0.4393	–	–	–	–
	TF	35.32	47.996	1.49	4.937	16.19	26.59	0.9748	0.9665	–	–	–	–
	FOKM	473,392	93,784	–	–	–	–	0.9293	0.3745	$1.44E^{-6}$	2.047	–	–
	MGR	43.23	116,380	–	–	23.40	93.17	0.9968	0.96421	–	–	0.0476	0.0344
A5	MG	18.84	0.089	3.85	1.356	34.35	0.069	0.9125	1	–	–	–	–
	LF	$-3.38E^{13}$	0.08936	0.36	3.219	8.63	–9.287	0.6246	1	–	–	–	–
	TF	18.75	17.1584	3.27	–0.8496	34.04	–25.98	0.9201	1	–	–	–	–
	FOKM	136,584	0.089	–	–	–	–	0.5669	1	$1.95E^{-6}$	36.104	–	–
	MGR	18.85	0.0893	–	–	36.15	0.095	0.9125	1	–	–	0.5521	41.033

Table S5
Results of the kinetic study in relation to the biostarter digestion

Biostarter	Mixture	Model	P_m (mL-CH$_4$/g-VS)	R_m (mL-CH$_4$ · g-VS^{-1} · d^{-1})	λ (d)	R^2 (–)	k (1/d)	K_G (1/d)
BT	A1	MG	31,453	291.40	68.15	0.9929	–	–
		LF	–5.302	0.5243	6.90	0.6431	–	–
		TF	183.14	9.9363	29.01	0.9938	–	–
		FOKM	148,308	–	–	0.7584	2.304	–
		MGR	88,491	–	123.77	0.9931	–	0.02235
	A3	MG	1.6806	0.5421	2.719	0.9607	–	–
		LF	1.7508	0.3822	0.6125	0.9043	–	–
		TF	1.676	0.5201	2.7293	0.9687	–	–
		FOKM	1.7678	–	–	0.8915	0.1884	–
		MGR	1.6807	–	3.8594	0.9607	–	0.8767
	A4	MG	25.429	9.032	3.643	0.9683	–	–
		LF	27.755	4.545	1.121	0.9083	–	–
		TF	25.233	10.74	3.870	0.9636	–	–
		FOKM	28.507	–	–	0.8831	0.1295	–
		MGR	20.677	–	30.644	8.8817	–	–59.944
	CN	MG	12,174	32.67	178.07	0.7882	–	–
		LF	–2.9170	0.1101	–1.951	0.7452	–	–
		TF	2,163.89	34.86	96.01	0.7951	–	–
		FOKM	8,021.4	–	–	0.7352	$1.505E^{-5}$	–
		MGR	3.2308	–	745.06	0.7916	–	0.00387
BS	CN	MG	10.933	1.6761	19.757	0.9704	–	–
		LF	3.5768	0.3387	6.6207	0.6939	–	–
		TF	10.501	1.6632	19.87	0.9728	–	–
		FOKM	85,861.26	–	–	0.5992	2.6766	–
		MGR	10.9388	–	22.16	0.9704	–	0.4163