

## Mesophilic and thermophilic dark fermentation course analysis using sensor matrices and chromatographic techniques

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Received [Dates will be filled in by the Editorial office]

### Abstract

Production of biofuels from biomass is expected to benefit society and the environment. At present bio waste residues processing includes hydrolysis, dark fermentation, photo fermentation, pyrolysis, gasification, and chemical synthesis. As the composition and the chemical structure of organic substances affect the efficiency of mentioned processes, it is believed, that the glucose concentration is a crucial parameter for the evaluation of the efficiency of biological processes. Also, the control of by-products formulated during each stage of biomass processing affects the course of dark fermentation. Therefore model processes regarding mesophilic and thermophilic dark fermentation were carried. Glucose as a sole carbon source was applied as the fermentation broth and Faloye-pretreated activated municipal wastewater sludge was introduced as the source of sporulating microorganisms. Production of hydrogen and methane was controlled by means of sensor matrices. Obtained results are comparable to those obtained using the standard method based on gas chromatography and indicate the suitability of their application for on-line routine analyses of hydrogen and methane during fermentation processes. In addition, the fermentation broth was also examined by means of gas and liquid chromatography in the scope of glucose reduction, and generation of volatile fatty acids and phenols.

**Keywords:** biogas analysis; chromatographic analysis; hydrogen; methane; dark fermentation; sensor matrices

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## Introduction

37

38 The generation of biofuels from lignocellulosic biomass is a complexed process,  
39 including three main stages, i.e. pretreatment, saccharification and fermentation (Kucharska et  
40 al. 2018). The yield and the rate of biogas or hydrogen productivity are affected mainly by  
41 process parameters i.e. pH of the pulp, temperature, composition, biomass pre-treatment  
42 method and digestion time (Gomez et al. 2006; Wang et al. 2009; Chu et al. 2012; Lestinsky  
43 et al. 2017). Several reports regarding the application of activated municipal wastewater  
44 sludge for dark fermentation processes can be found in the literature (Wu and Chang 2007;  
45 Jeppsson et al. 2007; Azbar et al. 2009; Ottaviano et al. 2017). However, the literature lacks  
46 complexed experiments related to the comparison between mesophilic and thermophilic dark  
47 fermentation course when the same sporulating microorganisms obtained during inoculum  
48 pre-treatment from activated sludge were applied (Faloye et al. 2013, 2014).

49 The authors propose to use glucose based fermentation broths for the evaluation of  
50 biofuels efficiency. The analysis of efficiency associated with glucose aims to maximize the  
51 technological, energy and economic benefits in production processes. Energy efficiency is  
52 understood as the ratio of energy obtained from biofuels to the energy consumed in all unit  
53 processes (Wu et al. 2007). In order to compare the dark fermentation process course,  
54 mesophilic and thermophilic conditions were used for the culture (Ivanova et al. 2009; Yasin  
55 et al. 2013). A gas mixture containing hydrogen and carbon dioxide is formed during the dark  
56 fermentation process. However, reports regarding methane formulation are also published  
57 (Levin et al. 2004; Cheng et al. 2010). According to the literature, several differences in the  
58 hydrogen: methane ratio may occur (Lay et al. 1999; Guo et al. 2008; Manish and Banerjee  
59 2008; Wu et al. 2009; Zhu et al. 2010).

60 Hydrogen and methane can be co-generated via anaerobic digestion (AD), a multi-step  
61 process carried out by highly differentiated microorganisms. Anaerobic conditions enable the  
62 transformation of organic matter into carbon dioxide and methane or hydrogen. It is found,  
63 that several types of microbial populations have specific optimal working conditions  
64 regarding pH, temperature, alkalinity, concentration ammonia, sodium and potassium ions,  
65 volatile fatty acids or heavy metals presence (Wilkie et al. 2000; Yang et al. 2006).

66 Biogas is composed of methane (up to 75%), carbon dioxide (up to 40%) and  
67 constituents such as ammonia, hydrogen, hydrogen sulfide, and nitrogen. Usually, consortia  
68 of highly diversified microorganisms enable the generation of biogas and liquid by-products,



69 i.e. volatile fatty acids (VFA) and other metabolic products. It is reported that metabolic  
 70 pathways related to biogas generation are highly complicated (De Gioannis et al. 2013;  
 71 Veluchamy and Kalamdhad 2017). When hydrogen generation is concerned, different  
 72 sporulating bacteria capable of glucose conversion to valuable acids, i.e. propionic acid,  
 73 succinic acid, lactic acid and alcohols, i.e. 2,3-butanediol, ethanol with simultaneous  
 74 liberation of hydrogen is discussed. However, if glucose fermentation is considered, every  
 75 liquid by-product may lead to a decrease of the overall hydrogen or methane yield (Lee et al.  
 76 2006; Wu et al. 2007; Panagiotopoulos et al. 2010).

77 Several types of main-gaseous product and liquid by-product formulation during  
 78 anaerobic digestion are presented in Table 1.

79

80 **Table 1.** Gaseous and liquid products generated during anaerobic digestion on different  
 81 inoculum and broths.

82

<b>Carbon source</b>	<b>Applied microorganisms</b>	<b>Main gaseous products</b>	<b>Main liquid products</b>	<b>References</b>
<b>Glucose/ model process</b>	Mixed anaerobic microflora	Hydrogen	Butyric acid, acetic acid	(Pan et al. 2010)
<b>Lignocellulosic hydrolysate</b>	Anaerobic bacteria	Methane	Lactic acid, citric acid, acetic acid	(Wong et al. 2014)
<b>Barley straw, corn stover and switch grass</b>	<i>Clostridium sp.</i>	Methane-low efficiency	ABE (acetone; butanol; ethanol)	(Qureshi et al. 2010b, a)
Food waste	Sewage sludge	Methane	Acetate, propionate, butyrate, valerate, hexanoic acid	(Cheng et al. 2018)
<b>Waste paper and kitchen waste</b>	<i>Genera 060F05-B-SD-P93</i> and <i>Thermosyntropha</i>	Methane	Ethanol, propionic acid, lactic acid	(Tan et al. 2019)
<b>Food waste</b>	<i>Bifidobacterium, Lactobacillus</i>	Methane	Lactic acid, ethanol, acetic acid, propionic acid, butyric acid, valeric acid	(Feng et al. 2020)
<b>Arthrospira platensis</b>	<i>Clostridium butyricum, Rhodospseudomonas palustris</i>	Methane and hydrogen	Ethanol, acetate, propionate, butyrate, isobutyrate, valerate,	(Ding et al. 2017)



				isovalerate, and caproate	
<b>Biodiesel industry residue</b>	Mixed (from sludge)	cultures activated	Methane	Butyric acid, ethanol, acetic acid, propionic acid, valeric acid	(Kumar et al. 2015)

83

84 When mixed bacterial culture is used in dark fermentation, i.e. bacteria obtained from  
85 mixed activated wastewater sludge, hydrogen is generated in the initial stage of the process  
86 (Pandu and Joseph 2012). However, methane may occur in the final stage of the fermentation  
87 process (Teplyakov et al. 2002). As it can be inferred from the data presented in Table 1, a  
88 large number of anaerobic digestion examples concerning various feeds have been published.

89 The fermentation process is usually monitored by pH, biogas production rate, redox  
90 potential, concentration of volatile fatty acids (VFA), total phenolic content (TPC) and gas  
91 composition, in order to ensure the correctness of the process. Among these indicators, VFA  
92 concentration in fermentation broth, as well as biogas compositions, are widely considered as  
93 the two most crucial and direct indicators of the biogas production process due to the fact that  
94 the dark fermentation process leads mainly to the formation of VFA followed by gasses  
95 production (hydrogen and carbon dioxide) which, in the last step, are transformed into  
96 methane. However, the increase in VFA concentration is linked to the methanogenesis  
97 inhibition or organic overloading and implies a risk of reducing the efficiency of biogas  
98 production (Rosecrance et al. 2013). In addition, several studies have also observed that the  
99 formation of phenols may also adversely affect the fermentation process (Fenske et al. 1998;  
100 Luo et al. 2002; Per Persson et al. 2002).

101 For the determination of VFA in fermentation broths, the techniques of fluorescence  
102 spectroscopy, near-infrared spectroscopy, titration, high performance liquid chromatography  
103 (LC) and gas chromatography (GC) are mainly used. The concentration of phenolic  
104 compounds could be analysed using UV-Vis spectroscopy (Madsen et al. 2011), GC and  
105 HPLC analysis (Nilvebrant et al. 2001; Quéméneur et al. 2012). The analysis of gas  
106 formulated during anaerobic digestion is usually carried using gas chromatography, for the  
107 determination of the gas content and composition (Rosales-Colunga et al. 2010).

108 However, GC measurement has several disadvantages i.e.: manual injections and long-  
109 time analysis (Isobe et al. 2011). To analyze the processes occurring during dark  
110 fermentation, a sensor matrices consisted of sensors selective for hydrogen, methane, carbon  
111 dioxide, hydrogen sulphide and ammonia may be applied (Hoff et al. 2006; Gebicki 2016;

112 Gebicki and Dymerski 2016). Nowadays, sensor arrays in environmental applications are  
113 mainly used for air analysis. This technique belongs to dynamically developing instrumental  
114 techniques and it is increasingly applied for monitoring and evaluation of the effectiveness of  
115 deodorization of unpleasant odours generated by different fields of human activity  
116 (Szulczyński et al. 2017).

117 However, they can also be used to on-line analysis of the biogas composition. In such  
118 cases, the biogas characteristics can be detected using metals oxide based MOS sensors.  
119 These sensors should be selective for hydrogen, methane and inorganic compounds, i.e.  
120 hydrogen sulphide, ammonia, oxygen, carbon dioxide, as well as organic compounds, toluene,  
121 benzene or VFA (e.g. acetic acid, butyric acid). In addition, the sensors should be  
122 characterized by good selectivity for a given gas and a lack of sensitivity to the interaction of  
123 other gases contained in the mixture (Ponzoni et al. 2017). In addition, the sensor matrices  
124 require careful design and testing for which model conditions are used and then perform tests  
125 on real samples. Continuous biogas measurements using sensor matrices are possible using  
126 the flow configuration of the measurement system.

127 The paper presents a comprehensive evaluation of the mesophilic and thermophilic  
128 dark fermentation processes in model conditions. As a source of carbon, glucose was selected  
129 because it may be sole carbon source for most the microorganisms. The fermentation broth  
130 was examined by means of gas and liquid chromatography in the scope of glucose reduction  
131 as well as generation of dark fermentation by-products (i.e. VFA and TPC). The possibility of  
132 using sensory matrices to investigate the composition of biogas was also examined. The  
133 results obtained with sensor matrices were compared with gas chromatography. Then the  
134 correlation matrices were created to better understand the course of fermentation processes.

135

136

## Experimental

137

### 138 *Materials and Methods*

139

#### 140 *Chemicals*

141

142 For the purposes of analytical methods, the standard substances: D (+) Glucose  
143 ( $\geq 99.5\%$  Sigma Aldrich), Sodium Hydroxide (99%, Sigma Aldrich), Dichloromethane  
144 ( $\geq 99.9\%$ , Sigma Aldrich), Buffered Peptone Water (Biomaxima, Poland), Syringol (99%  
145 Sigma Aldrich), Formic Acid (80% POCH), Acetic Acid ( $>99\%$  Sigma Aldrich), Propionic



146 Acid ( $\geq 99\%$  Sigma Aldrich), Butanoic Acid ( $\geq 99\%$  Sigma Aldrich), Isobutanoic Acid ( $\geq 99\%$   
147 Sigma Aldrich), were used in the study.

148 Anaerobic conditions during dark fermentation were created by purging the bioreactor  
149 with nitrogen – purity N5 (Linde Gas, Poland).

150 Hydrogen – purity N 5.5 from a Packard 9400 hydrogen generator (Packard, USA)  
151 was used in the gas chromatography. During the analysis of the sensor matrices, N5 purity  
152 compressed air was used (Linde Gas, Poland). An eluent consisting of aqueous 0.2% HCOOH  
153 (POCH, Poland) was used for the high-performance liquid chromatography analysis.

154

#### 155 *Dark fermentation*

156

157 Dark fermentation was carried out in sterile 1200 mL glass bioreactors with working  
158 volume of 1000 mL). The initial fermentation broth was composed of 900 mL of 20 g/L  
159 solution of Buffered Peptone Water (Biomaxima, Poland) and 5.5 g/L of glucose (POCH,  
160 Poland) as a sole carbon source. Dark fermentation was carried out with the use of activated  
161 sludge after Faloye procedure. The Faloye procedure was used for inoculum preparation. The  
162 pH of the activated sludge was adjusted to 8.93 with 1 M NaOH solution and further  
163 autoclaved (15 minutes, 121 °C). After autoclaving the pre-treated sludge was thermostated  
164 for 20 h at 37 °C with constant stirring to stabilize the culture of microorganisms.

165 The fermentation broth was adjusted to pH = 7.00 (1 M NaOH) and a constant pH was  
166 maintained throughout the process, using Arduino Data Logger. The anaerobic conditions  
167 were created by purging the reactor with sterile nitrogen for 20 to 60 min. After establishing  
168 anaerobic conditions, inoculations were carried out using 100 mL of activated sludge after the  
169 Faloy'e procedure. The fermentation in bioreactors were carried at 35 °C (mesophilic process)  
170 and 65 °C (thermophilic process) with magnetic stirring of 150 RPM. Fermentation was  
171 carried for 115 hours. Due to exploitation of the carbon source, after 80 hours of the process,  
172 3.0 g of glucose was added to stimulate the further biogas production.

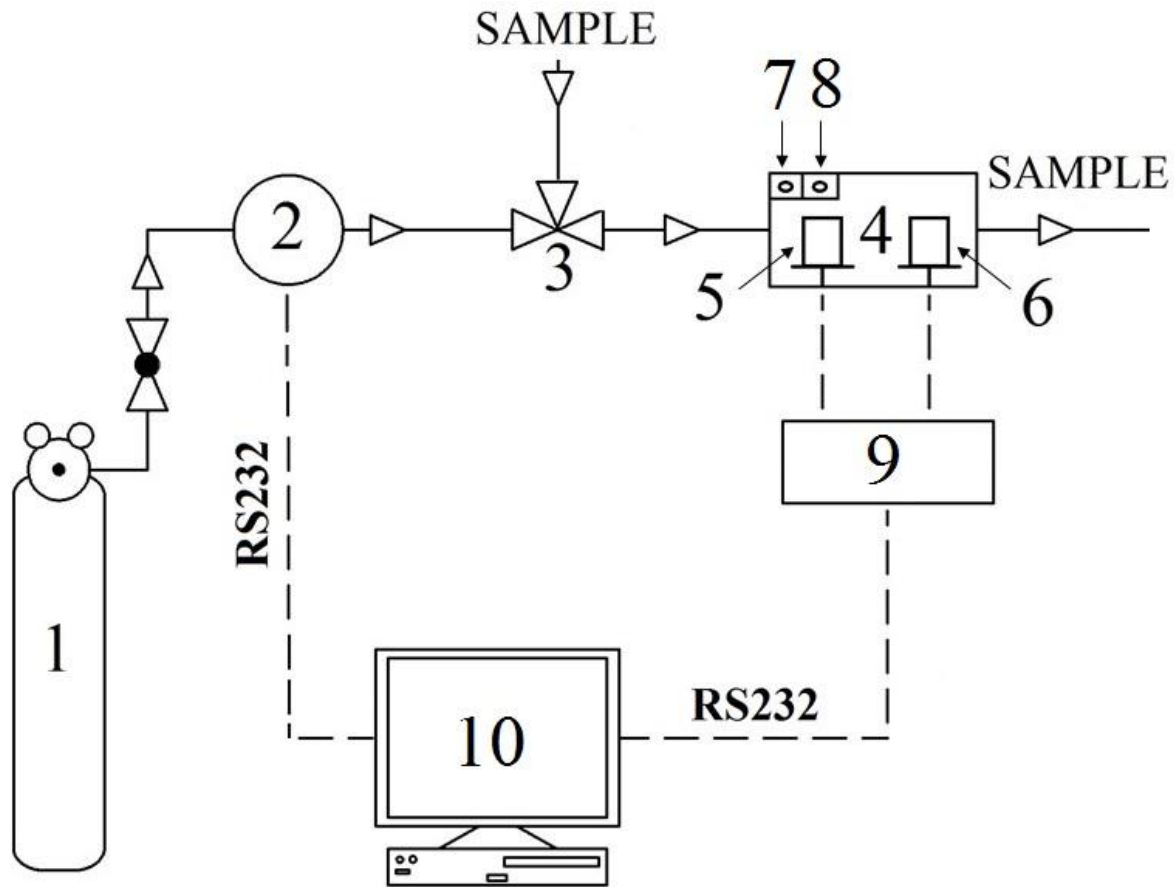
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#### 174 *Sensors analysis – gas phase analysis*

175

176 The biogas samples were analyzed using a self - constructed sensor matrix (SM). The  
177 device was equipped with commercial sensors selective for methane and hydrogen  
178 manufactured by Figaro Engineering (TGS2611, TGS2600). In the figure 1. it the scheme of  
179 the measurement system is shown. A stream of clean air flows through the measuring

180 chamber at a constant flow rate of 100 mL/min. The flow stream is controlled by an ADM  
 181 1000 flow meter (Agilent Technologies, USA). By changing the position of the valve (see  
 182 Fig. 1 - point 3), the biogas sample was directed to the measurement chamber. The volume of  
 183 the analyzed sample was 5.0 mL while the time of dosing the sample was equal to 30 s. After  
 184 this time the clean air was returned to the measurement chamber for the regeneration of the  
 185 sensors by changing the position of the valve. Signals from the sensors were recorded using  
 186 an AD (analog – to – digital) converter (Simex SIAi-8). Data analysis were performed using  
 187 SigmaPlot 11.0 software .  
 188



189  
 190 **Fig. 1** Measurement system: 1 – air, 2 – flow meter, 3 – valve 4 – sensor chamber, 5 –  
 191 methane sensor, 6 - hydrogen sensor, 7- temperature sensor, 8- humidity sensor, 9- analog-to-  
 192 digital converter (ACD Converter), 10 – computer.

193

194 *Gas chromatography analysis - gas phase analysis*

195

196 The biogas was also analyzed by means of gas chromatography (Perkin-Elmer  
 197 AutoSystem XL) with a Porapak Q column (100-120 mesh length 6.5 m, diameter 1/8 inch)  
 198 and an oven temperature of 60 °C. The following conditions were used during analysis: flame



199 ionization detector (FID, temperature 220 °C) and thermal conductivity detector (TCD,  
200 temperature 100 °C). Nitrogen with a flow of 30 mL/min was used as the carrier gas. The  
201 volume of the analyzed sample was 0.5 mL. The total analysis time was 12 minutes. During  
202 the analysis, Turbochrom software was applied.

### 203 *Gas chromatographic analysis - fermentation broth analysis*

204

205 Samples from dark fermentation process were taken to determine the changes in the  
206 concentration of individual and total content of volatile fatty acids and phenols. During the  
207 fermentation process, 2 mL samples were collected and stored frozen in the temperature of -  
208 18 °C. For analysis the samples melted and centrifuged (Hitachi EBA 8S) for 5 min at 3000  
209 RPM, an initial removal of the solid phase was realised. The aqueous phase (1.0 mL) was  
210 filtered through a 0.45 µm hydrophilic-cellulose filter (Hahnemühle FineArt HmbH,  
211 Germany) and transferred to a 1.5 mL vial. 10 µL of hydrochloric acid was added to the  
212 sample to adjust the pH to 2.0 and then 300 µL of dichloromethane (DCM) was added. The  
213 sample was shaken vigorously in the vial for 1 minute and then centrifuged (3000 RPM) for 5  
214 minutes for liquid-liquid extraction. The obtained organic phase was transferred in a volume  
215 of 150 µL by means of an automatic pipette into 2.0 mL vials. The extracted sample (1 µL)  
216 was introduced into GC-FID. Individual VFA were analyzed by gas chromatography (Varian  
217 CP 3800) with a DB-WAX column (30 m x 0.53 mm x 1.0 µm). The following  
218 chromatographic conditions were used: oven temperature 100 °C (5 min) – ramped at 10  
219 °C/min to 250 °C (10 min); injection port temperature 280 °C; injection volume 1 µL;  
220 injection mode: split 1:20; FID detector temperature 200 °C; carrier gas N5 nitrogen (flow 1  
221 mL/min). During the analysis System Control software - Varian Star was used.

222

### 223 *High-performance liquid chromatography analysis - fermentation broth analysis*

224 For the determination of the glucose and TPC content in fermentation broth, liquid  
225 chromatography was applied. The filtered sample (50 µL) of fermentation broth was directly  
226 introduced into the HPLC system. The analysis was provided by means of liquid  
227 chromatograph (Merck - Hitachi, Germany) equipped with a pump L-7100 with the so-called  
228 low-pressure gradient system was applied. The Shodex SH1011, (7 µm, 8 x 300 mm) column  
229 was used. It was thermostated by means of the ACS thermostat. The system had two detectors  
230 connected in series: Spectrophotometric (L-7450 - Merck - Hitachi, Germany) in the UV-VIS  
231 range using photodiode (DAD) and differential refractometric sensors (RID - RI Detector  
232 2100 - Knauer, Germany). In addition, the apparatus had a valve to change the direction of the





233 mobile phase flow in the back-flush column (Merck, Germany), controlled manually. HSM  
234 software was used to record and process the results.

235 In the HPLC studies, the eluent used was: H<sub>2</sub>O + 0.2% HCOOH at a flow rate of 1.2  
236 mL/min. The temperature of the thermostated column was 60 °C. The total analysis time was  
237 30 minutes. HPLC analysis was carried out of 50 µL sample. After 7.6 minutes, a back-flush  
238 was used to elute the TPC that were determined relative to the syringol standard. The total  
239 TPC content was determined with reference to the syringol calibration curve (TPC standard)  
240 in the range from 0.9 to 6.5 mg/mL according to previous work (Słupek et al. 2018).

241

242

### Results and discussion

243 The objective of this paper is to present the application of sensor matrices as an  
244 alternative method for gas analysis. To analyze the products obtained during mesophilic and  
245 thermophilic dark fermentation, a sensor matrices consisted of sensors selective for hydrogen  
246 and methane were constructed.

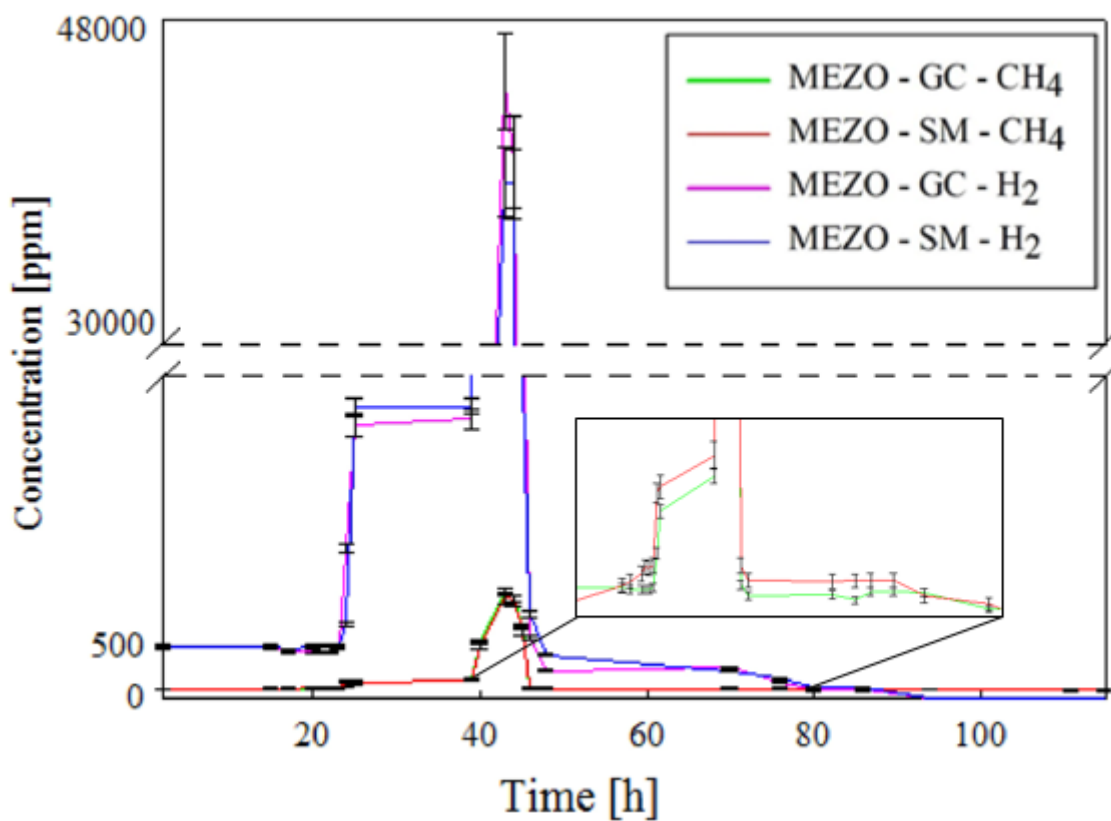
247 The changes in the composition of gases generated during dark fermentation are  
248 presented in Fig. 2 and Fig. 3. Significantly higher concentrations of hydrogen and methane  
249 were obtained for fermentation under mesophilic conditions compared to thermophilic  
250 conditions. The highest concentrations of hydrogen and methane were obtained in the range  
251 of 40 to 45 hours of the mesophilic process and in the range of 20 to 24 hours of the  
252 thermophilic process. In both processes, the concentration of methane produced remains  
253 constant, while the hydrogen concentration changes significantly during the process. In order  
254 to effectively use the activated sludge after the Faloye process, 3.0 g of glucose was added for  
255 stimulation of the microspheres of bacteria responsible for methanogenesis. After 85 h of both  
256 processes, the termination of hydrogen production is visible, while methane production  
257 remains at a constant level.

258 It was noticed, that the application of sensor matrices allows to obtain an on-line gas  
259 analysis and with its application it is possible to obtain two different streams during anaerobic  
260 digestion. In the first stage of the process the main gaseous products are hydrogen and carbon  
261 dioxide, while after 80 hours of the process, the second stage starts and methane is formed. It  
262 can be assumed that methanogens consume the acids generated by hydrogenogenic bacteria in  
263 the first stage of the process. Separation of the streams may allow decreasing the costs related  
264 to the separation of biohydrogen from biomethane. In order to accurately determine the end of  
265 the stage production biohydrogen and the start of the stage production biomethane, on-line  
266 gas analysis is necessary.



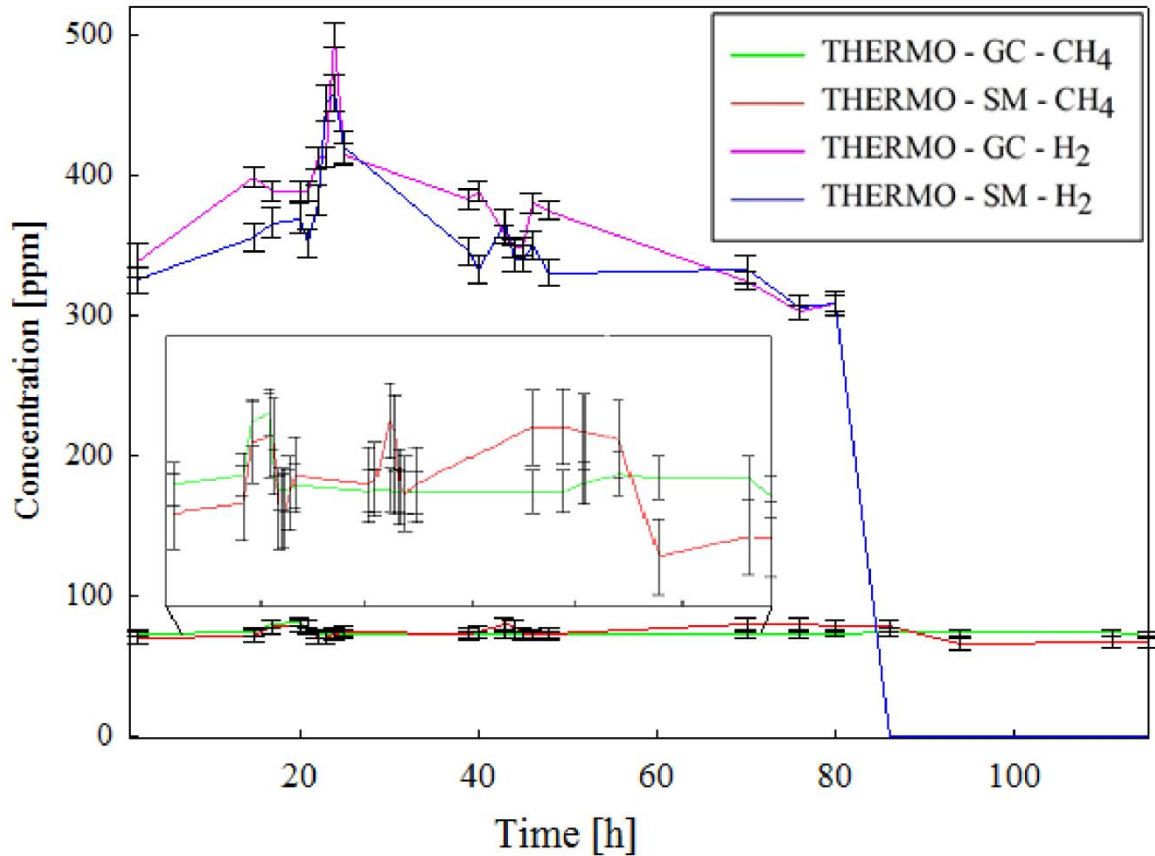
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269

270 **Fig. 2** Changes in the gas composition (hydrogen and methane) occurring during dark  
 271 fermentation with respect to the mesophilic process determined by means of gas  
 272 chromatography (GC – green and violet line) and sensor matrices (SM – red and blue line)  
 273 (n=3).



274

275 **Fig. 3** Changes in gas composition (hydrogen and methane) occurring during dark  
 276 fermentation with respect to the thermophilic process done by means of gas chromatography  
 277 (GC – green and violet line) and sensor matrices (SM – red and blue line) (n=3).

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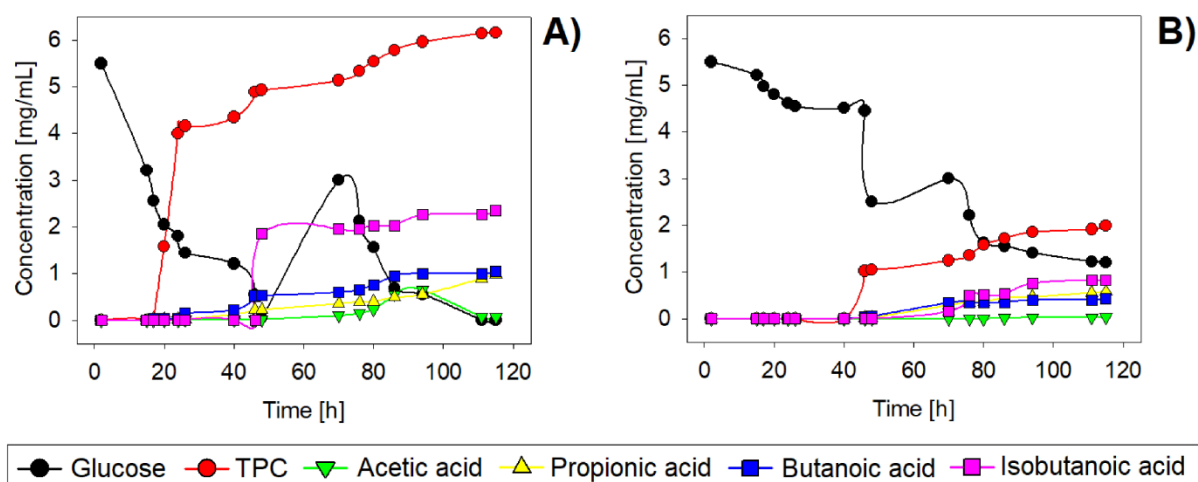
279 The results obtained by commercially available selective sensors for methane and  
 280 hydrogen were used and the results were compared with the results obtained during GC  
 281 analysis. It can be concluded that the results obtained using sensor matrices (see blue and red  
 282 line, Fig. 2 and Fig. 3) correspond with the gas chromatography results (see violet and green  
 283 line, Fig. 2 and Fig. 3).

284 The repeatability of the analytical procedure for sensor matrices and gas  
 285 chromatography was determined by means of the standard deviation value (RSD) obtained as  
 286 a result of three analysis operations of the reference gas sample at 1000 ppm methane and  
 287 hydrogen. As a result of comparison of repeatability of both analytical procedures, RSD =  
 288 2.82% (methane) and RSD = 3.54% (hydrogen) were obtained for sensor matrices, RSD =  
 289 1.59% (methane) and RSD = 1.81% (hydrogen) for gas chromatography. In the real process,  
 290 the minimum and maximal concentration differences of the resulting biogas were found  
 291 between the results obtained from sensor matrices and GC. The lowest difference for the

292 methane concentration in the mesophilic process was 0.35 ppm, while the highest 52.66 ppm.  
 293 In the thermophilic process, the lowest difference was 0.20 ppm and the highest difference  
 294 was 6.52 ppm. The calculations were also made for hydrogen concentration where the lowest  
 295 differences of 9.47 ppm were obtained in the mesophilic process, while the highest - 1053.46  
 296 ppm. In the thermophilic process, the lowest concentration was 0.85 ppm, while the highest  
 297 difference was 56.15 ppm. The average standard deviation between the obtained results from  
 298 the sensor matrix and GC, which was at the RSD level = 3.89% (methane) and RSD = 8.95%  
 299 (hydrogen) was calculated. In addition, the results were analyzed by analysis of variance  
 300 (ANOVA) (Table S1-S4). In ANNOVA, the values of statistical parameter p were used as  
 301 criteria at a 95% confidence level. All the obtained results using both methods (GC and SM)  
 302 were found to be statistically insignificant due to the p-value higher than 0.05. For the  
 303 thermophilic process, the p-value was 0.68 and 0.74 for methane and hydrogen respectively  
 304 and for the mesophilic process, the p-value was 0.99 and 0.97 for methane and hydrogen  
 305 respectively. The obtained differences in the values are acceptable and indicate the usefulness  
 306 of sensor matrices in the on-line control of the dark fermentation process. However, the  
 307 correctness of the results obtained by sensors matrices should be periodically checked using  
 308 gas chromatography.

309 Biogas production is a sensitive process because there are strong correlations of many factors  
 310 (such as substrate concentration, composition of fermentation broth, temperature and pH  
 311 value) that affect the efficiency of the production of biohydrogen and biomethane. These  
 312 additional parameters were also monitored and controlled throughout the dark fermentation.  
 313 Total glucose concentration used as the sole carbon source in the initial fermentation broth  
 314 was set at 5.5 g/L for each of the processes (mesophilic and thermophilic). Hence, the glucose  
 315 content for each analyzed process corresponds proportionally with the data presented in Fig. 4  
 316 as well as Tables S5 and S5. One way dark fermentation can occur is the conversion of  
 317 glucose to hydrogen and acetic acid (Eq. 1). This reaction occurs spontaneously with a  
 318 maximum theoretical production of 4 moles of hydrogen per mole of glucose as soon as acetic  
 319 acid is one of VFA. In addition, other VFAs may be formed in fermentation processes such  
 320 propionic acid (Eq. 2), which reduce the efficiency of the process (Manish and Banerjee 2008;  
 321 Luo et al. 2010).





324

325 Fig. 4 Changes in the glucose, total phenolic compound (TPC) and selected volatile fatty  
 326 acids (VFA) concentration in fermentation broth during dark fermentation A) mesophilic  
 327 process, B) thermophilic process.

328

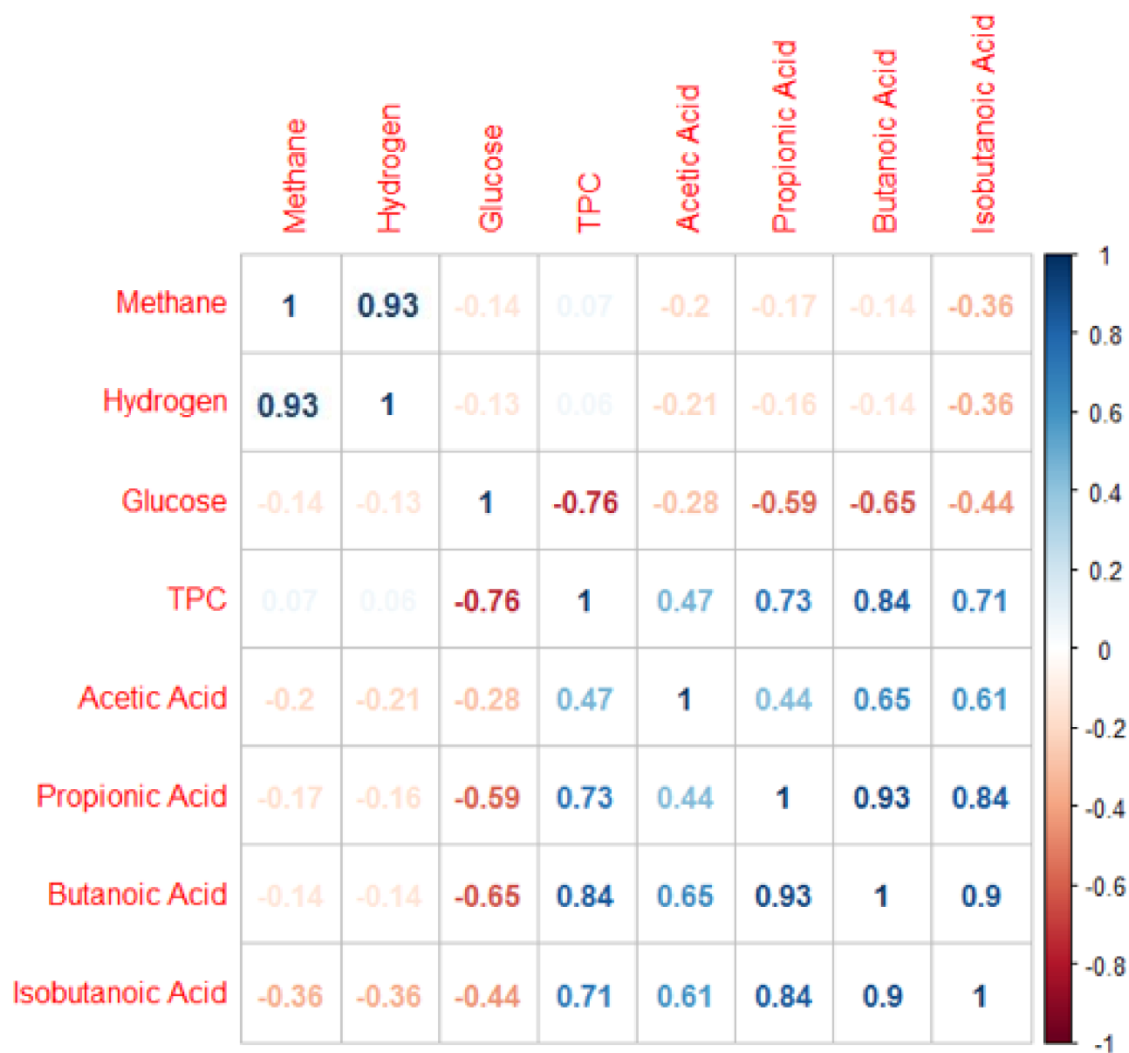
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The production of biogas under anaerobic conditions in the digester requires the joint  
 330 action of many populations of microorganisms that have been extracted from the activated  
 331 sludge. In the fermentation processes, it can be observed that microorganisms not only  
 332 produce biogas but also VFA (acidogenesis stage). In the next fermentation stage  
 333 (acetogenesis) propionic, butanoic, and isobutanoic acids are converted to octanoic acid and  
 334 phenolic compounds (Fig. 4). In the mesophilic process, VFA accumulated much faster in the  
 335 fermentation chamber, than in thermophilic conditions. It may be a consequence of a much  
 336 faster loss of glucose in the mesophilic process, which led to faster biogas production but also  
 337 resulted in the formation of more inhibitors of dark fermentation. Previous studies showed  
 338 that the optimal pH in terms of biohydrogen production is within a range of 5.0–7.0 which  
 339 favors the activity of the hydrogenases and is also suitable for microbial development in dark  
 340 fermentation (Li and Fang 2007; Szulczyński et al. 2019). During the fermentation process,  
 341 growth of the bacteria that contribute to the formation of volatile organic acids, resulting in  
 342 decrease in pH. However, after a few days of the process of reaching an increase in pH due to  
 343 the conversion of organic acids to methane after multiplication of methanogens. Rapid pH  
 344 changes can adversely affect stability and efficiency process. Therefore, the process was  
 345 carried out with pH control (Cieślik et al. 2016).

346

Based on the data presented in Tables S5 and S6, a correlation matrix for the  
 347 formation of hydrogen and methane, as well as for glucose, one of VFAs and TPC was

348 prepared. The correlation matrix was created by means of R Studio software (Fig. 5 and Fig.  
349 6) (RStudio 2016; RCore 2018).



350

351 **Fig. 5** Correlation matrix for the formation of hydrogen and methane, but also for glucose,  
352 individual inhibitors and total phenolic compound (TPC) - mesophilic process.

353



354

355 **Fig. 6** Correlation matrix for the formation of hydrogen and methane, but also for glucose,  
 356 individual inhibitors and total phenolic compound (TPC) - thermophilic process.

357

358 Correlation analysis consists in examining whether two variables (expressed in  
 359 numbers) are significantly related to each other. The calculated determination ratio varies  
 360 from -1 to 1. A positive correlation appears when the increase in the value of one variable  
 361 corresponds with the increase in the value of the second variable, while negative correlation  
 362 occurs when the increase in the value of one variable corresponds with the decrease in the  
 363 value of the second variable. A value of (0) means a total lack of correlation between the two  
 364 factors (Zhu et al. 2017). Unexpectedly, it was found that the production of hydrogen and  
 365 methane is negatively correlated with the concentration of glucose in the growth medium (see  
 366 Fig.5) - the mesophilic process, with respect to the increase of the glucose concentration.  
 367 However, there is a positive correlation between hydrogen and methane generation and the

368 concentration of glucose during the process carried out in thermophilic conditions.  
369 Thermophilic process course corresponds with the tendencies presented in the literature, for  
370 diversified biofuels generation (Wilkie et al. 2000; Eskicioglu et al. 2011; Cieřlik et al. 2016;  
371 Łukajtis et al. 2018). The authors suppose, that these untypical results are related with the  
372 sudden changes in the broth composition, i.e a significant decrease in glucose concentration  
373 after 46 hours of the process, due to glucose supplementation. In the mesophilic process the  
374 decrease in glucose concentration is observed after 15 h of the process. In addition, a strong  
375 positive correlation is observed for methane and hydrogen generation, which indicates the  
376 simultaneous formation of both gases under mesophilic and thermophilic conditions. During  
377 the dark fermentation process organic compounds break down into small molecules which are  
378 substrates for the hydrogen generation by hydrogenogenes, which are also able to generate  
379 acetic acid. In the first stage of biogas production, hydrogen, methane, and TPC are produced.  
380 The formulated acetic acid is used by methanogenes to produce methane (Bateni et al. 2017).  
381 Therefore, for this stage of the process, it may be crucial to consider acetic acid as a second  
382 carbon source, besides glucose in order to provide conclusions regarding chemometrical  
383 analysis of the processes. Higher concentrations of acetic acid are obtained during mesophilic  
384 process and therefore, its effect on the mesophilic process (Fig.4a) course is noticeable.  
385 Preparing a procedure for carbon balance in the system may be a required step to be  
386 considered in further research. Methane and hydrogen productivity correlation during the  
387 thermophilic process is lower in comparison with the mesophilic process. The correlation  
388 matrices for the mesophilic and thermophilic process demonstrate a strong negative  
389 correlation of glucose concentration with TPC and VFA concentration during  
390 hydrogenogenesis. Decreasing glucose concentration and an increase in TPC concentration  
391 result in a decrease in biogas productivity.

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393

394

## 394 **Conclusions**

395

396 The paper presents the use of chromatographic techniques and sensor matrices for the  
397 monitoring of hydrogen and methane production during the dark fermentation process carried  
398 out under mesophilic and thermophilic conditions (model conditions). In order to understand  
399 the changes occurring during the whole dark fermentation process, gas phase (methane,  
400 hydrogen) studies and fermentation broth (glucose, VFA, TPC) studies were carried out. In  
401 the first stage of dark fermentation, the production of hydrogen was mainly observed. The





402 second stage was initiated, which consisted of redirecting the process to methanisation. The  
403 results indicate significantly higher concentrations of hydrogen and methane during the dark  
404 fermentation process under mesophilic conditions than in the process under thermophilic  
405 conditions. The concentration of biogas (methane and hydrogen) is closely related to the  
406 content of glucose in the nutrient solution. In the mesophilic process, a significant decrease in  
407 glucose concentration was observed. Microorganisms in the first stage of the fermentation  
408 process, convert glucose to biogas, and after 17 hours to VFA, while after 20 h other  
409 fermentation inhibitors (TPC) was also created. Similarly, in the thermophilic process,  
410 initially, glucose is converted by bacteria into gases, in turn, both phenolic compounds and  
411 VFA are formed after 46 hours of the process. In both mesophilic and thermophilic processes,  
412 the decrease in the production efficiency of hydrogen and methane is associated with an  
413 increase in the concentration of fermentation inhibitors (VFA and TPC). Microorganisms  
414 cease to produce both hydrogen and methane after consumption of glucose.

415 Correlation of factors enabled also the selection of significant variables that should be  
416 controlled on-line during processes carried out in actual real conditions. The most important  
417 parameters – concentration of methane and hydrogen was monitored on-line during  
418 fermentation processes by sensor matrices. The results obtained from sensor matrices are  
419 comparable to those obtained with gas chromatography coupled with a TCD and FID. The  
420 results indicate suitability of sensors matrices for on-line routine analyses of hydrogen and  
421 methane during fermentation processes. Moreover, sensor matrices based analysis enables  
422 finding the point at which the hydrogen generating bacteria culture is terminated and the  
423 fermentation process tends to redirect to the anaerobic digestion and the production of  
424 methane. Hydrogen and methane production using one process allows a better use of the  
425 potential of bacteria contained in the activated sludge, and also significantly reduces the cost  
426 of biogas production compared to individual processes. In addition, the use of sensor matrices  
427 allows immediate correction of the fermentation broth composition, which allows to improve  
428 the efficiency of biogas production. The use of GC techniques in "off-line" or "in-line" mode  
429 results in a long delay in the results obtained, which prevents immediate action to correct the  
430 process or eliminate potential system failures.

431 In the case of biogas production, i.e. from landfills, the obtained biogas stream  
432 contains much more pollutants (i.e. hydrogen sulphide, ammonia, carbon dioxide, volatile  
433 organic compounds) which can affect the process of dark fermentation and the operation and  
434 correctness of results obtained from sensor matrices. Therefore, in the future, it is planned to



435 create sensor matrices in which additional temperature, humidity, and selective pollution  
436 sensors will be considered.

437

438 **Author Contributions:** Edyta Słupek, Karolina Kucharska conceived and designed the  
439 experiments. Edyta Słupek, Patrycja Makoś carried the experiments. Edyta Słupek, Patrycja  
440 Makoś, Karolina Kucharska and Jacek Gębicki wrote the paper.

441

442 **Conflicts of Interest:** The authors declare no conflict of interest. The founding sponsors had  
443 no role in the design of the study; in the collection, analyses, or interpretation of data; in the  
444 writing of the manuscript, and in the decision to publish the results.

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## *Electronic Supplementary Material*

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### **Mesophilic and thermophilic dark fermentation course analysis using sensor matrices and chromatographic techniques**

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**Table S1** Analysis of variance (ANOVA) for the methane in thermophilic process.

Source of variation	SS	df	MS	F	p-value	F-critical
Between groups	2.109149	1	2.109149	0.16484	0.686707	4.061706
Within groups	562.9854	44	12.79512			
Total	565.0945	45				

**Table S2** Analysis of variance (ANOVA) for the hydrogen in thermophilic process.

Source of variation	SS	df	MS	F	p-value	F-critical
Between groups	2390.174	1	2390.174	0.108959	0.742899	4.061706
Within groups	965205.1	44	21936.48			
Total	967595.3	45				

**Table S3** Analysis of variance (ANOVA) for the methane in mesophilic process.

Source of variation	SS	df	MS	F	p-value	F-critical
Between groups	4.28611	1	4.28611	5.97E-05	0.99387	4.061706
Within groups	3158609	44	71786.58			
Total	3158614	45				

**Table S4** Analysis of variance (ANOVA) for the hydrogen in mesophilic process.

Source of variation	SS	df	MS	F	p-value	F-critical
Between groups	154985.6	1	154985.6	0.001415	0.970167	4.061706
Within groups	4.82E+09	44	1.1E+08			
Total	4.82E+09	45				

**Table S5** Changes in the glucose, total phenolic compounds (TPC) and selected volatile fatty acids (VFA) concentration in fermentation broth during dark fermentation - mesophilic process

Time	Glucose	TPC	Acetic Acid	Propionic Acid	Butanoic Acid	Isobutanoic Acid
[h]	[mg/mL]					
2	5.50	<LOD	<LOD	<LOD	<LOD	<LOD
15	3.21	<LOD	<LOD	<LOD	<LOD	<LOD
17	2.56	<LOD	<LOD	0.020	0.034	<LOD
20	2.05	1.582	<LOD	0.020	0.031	<LOD
24	1.80	3.991	<LOD	0.022	0.031	<LOD
26	1.44	4.160	0.011	0.022	0.147	<LOD
40	1.21	4.350	0.012	0.123	0.222	<LOD
46	0.55	4.888	0.015	0.228	0.525	<LOD
48	0.05	4.932	0.015	0.228	0.529	1.845
70	3.00	5.136	0.095	0.358	0.598	1.948
76	2.12	5.340	0.150	0.390	0.658	1.955
80	1.56	5.545	0.240	0.399	0.758	2.020
86	0.68	5.786	0.550	0.490	0.950	2.029
94	0.55	5.958	0.650	0.555	0.999	2.255
111	<LOD	6.145	0.071	0.898	1.001	2.268
115	<LOD	6.15	0.074	0.969	1.041	2.345

LOD – limit of detection;

LOD = 0.01 mg/mL. RSD = 2.15% - values calculated for a concentration of 2.5 mg/mL (glucose). LOD = 0.073 mg/mL. RSD = 1.23% - values calculated for a concentration of 3.6 mg/mL (TPC). LOD = 0.001 – 0.003 mg/mL. RSD = 2.13% - values calculated for a concentration of 5 µg/mL (organic acids)

**Table S6** Changes in the glucose, total phenolic compounds (TPC) and selected volatile fatty acids (VFA) concentration in fermentation broth during dark fermentation- thermophilic process

Time	Glucose	TPC	Acetic Acid	Propionic Acid	Butanoic Acid	Isobutanoic Acid
[h]				[mg/mL]		
2	5.50	<LOD	<LOD	<LOD	<LOD	<LOD
15	5.21	<LOD	<LOD	<LOD	<LOD	<LOD
17	4.97	<LOD	<LOD	<LOD	<LOD	<LOD
20	4.80	<LOD	<LOD	<LOD	<LOD	<LOD
24	4.61	<LOD	<LOD	<LOD	<LOD	<LOD
26	4.55	<LOD	<LOD	<LOD	<LOD	<LOD
40	4.51	<LOD	<LOD	<LOD	<LOD	<LOD
46	4.45	1.021	<LOD	0.020	0.030	<LOD
48	2.50	1.044	<LOD	0.020	0.049	<LOD
70	3.00	1.245	<LOD	0.289	0.339	0.154
76	2.21	1.355	<LOD	0.350	0.340	0.495
80	1.62	1.579	<LOD	0.359	0.339	0.513
86	1.55	1.714	0.010	0.450	0.349	0.526
94	1.41	1.849	0.020	0.468	0.398	0.759
111	1.22	1.912	0.021	0.555	0.400	0.815
115	1.20	1.985	0.030	0.581	0.434	0.828

LOD – limit of detection;

LOD = 0.01 mg/mL. RSD = 2.15% - values calculated for a concentration of 2.5 mg/mL (glucose). LOD = 0.073 mg/mL. RSD = 1.23% - values calculated for a concentration of 3.6 mg/mL (TPC). LOD = 0.001 – 0.003 mg/mL. RSD = 2.13% - values calculated for a concentration of 5 µg/mL (organic acids)

**Table S7** Changes in the glucose, total phenolic compounds (TPC) and selected volatile fatty acids (VFA) concentration in fermentation broth during dark fermentation A) mesophilic process. B) thermophilic process.

Time	Mesophilic Process				Thermophilic Process			
	GC- H <sub>2</sub>	MS- H <sub>2</sub>	GC- CH <sub>4</sub>	MS- CH <sub>4</sub>	GC- H <sub>2</sub>	MS- H <sub>2</sub>	GC- CH <sub>4</sub>	MS- CH <sub>4</sub>
[h]	[mg /L]*							
2	0.043	0.042	0.061	0.054	0.030	0.029	0.052	0.050
15	0.043	0.042	0.061	0.062	0.036	0.032	0.053	0.051
17	0.038	0.040	0.060	0.064	0.035	0.033	0.057	0.056
20	0.039	0.043	0.060	0.068	0.035	0.033	0.058	0.056
21	0.038	0.043	0.060	0.070	0.035	0.031	0.053	0.056
22	0.038	0.044	0.060	0.070	0.037	0.034	0.052	0.050
23	0.043	0.044	0.061	0.071	0.037	0.040	0.052	0.050
24	0.124	0.061	0.077	0.105	0.045	0.041	0.052	0.052
25	0.225	0.241	0.096	0.108	0.037	0.038	0.052	0.053
39	0.231	0.241	0.112	0.122	0.034	0.031	0.052	0.052
40	1.649	1.743	0.372	0.341	0.035	0.030	0.052	0.053
43	3.327	3.074	0.697	0.661	0.032	0.033	0.052	0.057
44	3.126	3.072	0.658	0.646	0.031	0.030	0.052	0.056
45	0.350	0.308	0.478	0.440	0.031	0.030	0.052	0.052
46	0.050	0.069	0.062	0.071	0.034	0.031	0.052	0.052
48	0.023	0.035	0.057	0.064	0.033	0.029	0.052	0.052
70	0.025	0.023	0.058	0.064	0.029	0.030	0.052	0.057
76	0.013	0.015	0.055	0.064	0.027	0.027	0.052	0.057
80	0.006	0.008	0.059	0.064	0.028	0.028	0.052	0.056
86	0.006	0.008	0.059	0.064	0.000	0.000	0.053	0.056
94	0.000	0.000	0.059	0.057	0.000	0.000	0.053	0.047
111	0.000	0.000	0.051	0.054	0.000	0.000	0.053	0.048
115	0.000	0.000	0.051	0.051	0.000	0.000	0.051	0.048

LOD – limit of detection; RSD = 2.82% and LOD = values calculated for a concentration of 1000 mg/mL CH<sub>4</sub> for MS; RSD = 3.54% and LOD = 0.001 mg/L values calculated for a concentration of 1000 mg/mL H<sub>2</sub> for MS, RSD = 1.59% and LOD = 0.002 mg/L values calculated for a concentration of 1000 mg/mL CH<sub>4</sub> for GC; RSD = 1.81% and LOD = 0.001 mg/L values calculated for a concentration of 1000 mg/mL H<sub>2</sub> for GC

\*mg – (H<sub>2</sub> or CH<sub>4</sub>) / L (total gas phase)