

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

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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

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



				isovalerate, and caproate	
Biodiesel industry residue	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

Materials and Methods

139

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.

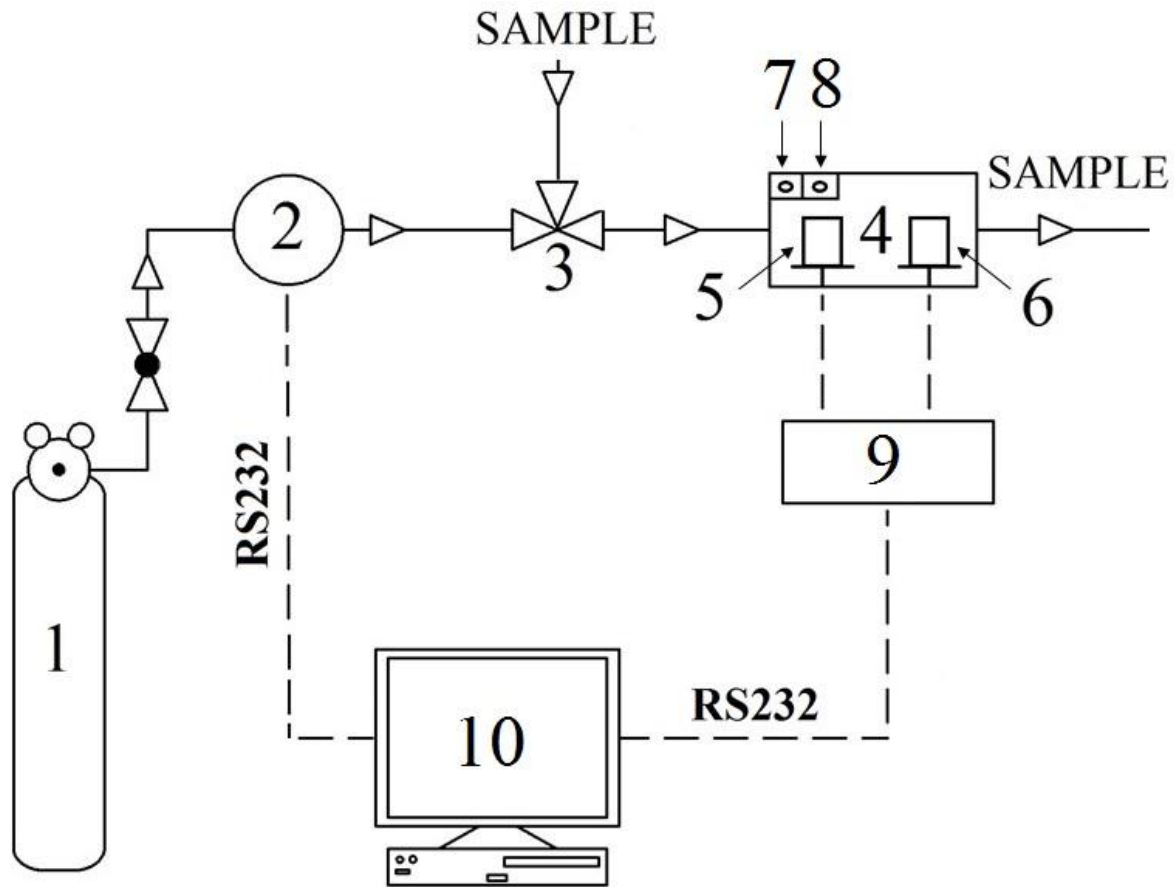
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174 *Sensors analysis – gas phase analysis*

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: H₂O + 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.

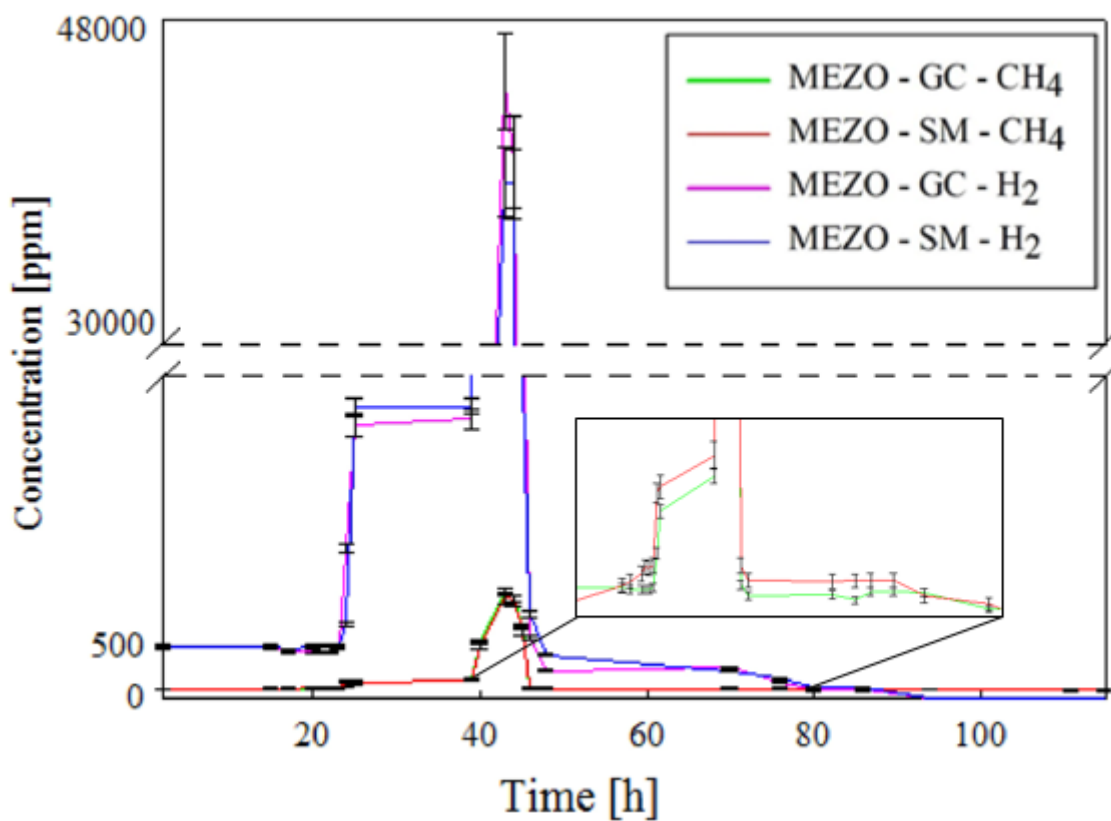
247 The changes in the composition of gases generated during dark fermentation are
248 presented in Fig. 2 and Fig. 3. Significantly higher concentrations of hydrogen and methane
249 were obtained for fermentation under mesophilic conditions compared to thermophilic
250 conditions. The highest concentrations 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.



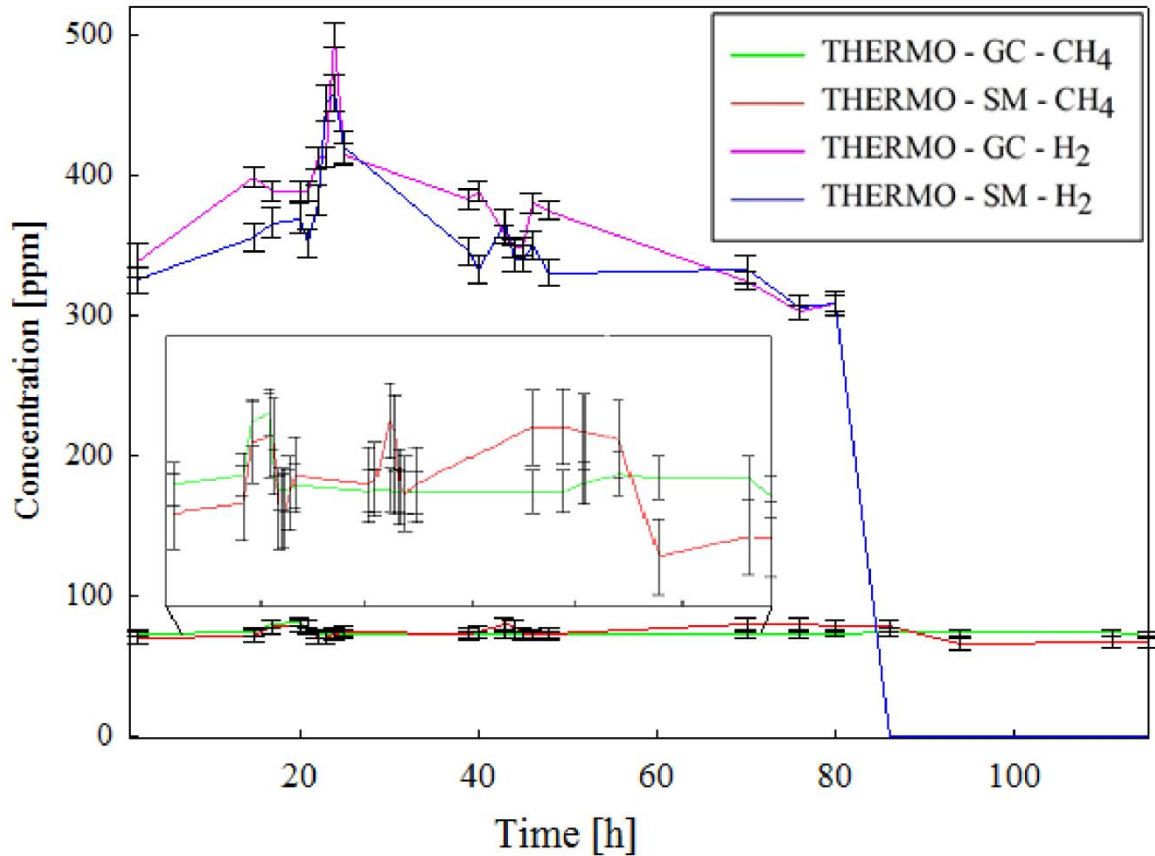
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269

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).

278

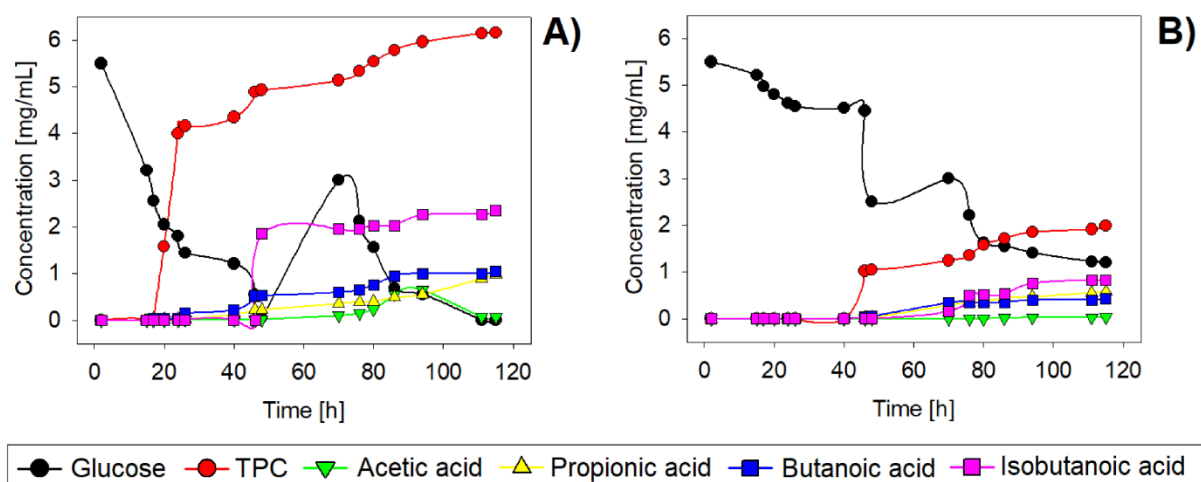
279 The results obtained by commercially available selective sensors for methane and
 280 hydrogen were used and the results were compared with the results obtained during GC
 281 analysis. It can be concluded that the results obtained using sensor matrices (see blue and red
 282 line, Fig. 2 and Fig.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

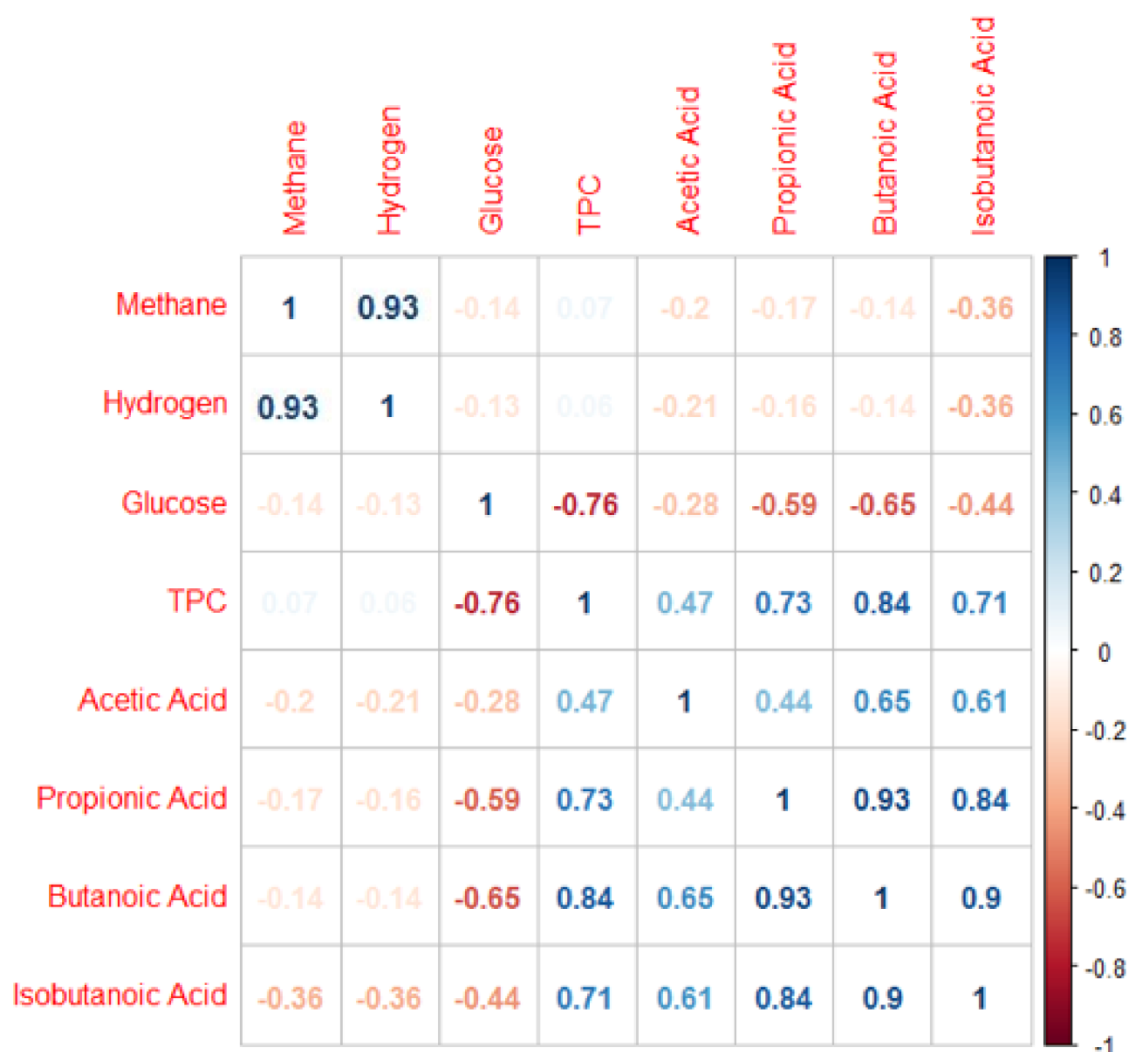
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329 The production of biogas under anaerobic conditions in the digester requires the joint
 330 action of many populations of microorganisms that have been extracted from the activated
 331 sludge. In the fermentation processes, it can be observed 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

347 Based on the data presented in Tables S5 and S6, a correlation matrix for the
 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.

392

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

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439 experiments. Edyta Słupek, Patrycja Makoś carried the experiments. Edyta Słupek, Patrycja
440 Makoś, Karolina Kucharska and Jacek Gębicki wrote the paper.

441

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Electronic Supplementary Material

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 ₂	MS- H ₂	GC- CH ₄	MS- CH ₄	GC- H ₂	MS- H ₂	GC- CH ₄	MS- CH ₄
[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 CH₄ for MS; RSD = 3.54% and LOD = 0.001 mg/L values calculated for a concentration of 1000 mg/mL H₂ for MS, RSD = 1.59% and LOD = 0.002 mg/L values calculated for a concentration of 1000 mg/mL CH₄ for GC; RSD = 1.81% and LOD = 0.001 mg/L values calculated for a concentration of 1000 mg/mL H₂ for GC

*mg – (H₂ or CH₄) / L (total gas phase)