The quest for improved biohydrogen formation: are heat shocks the solution?

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Highlights

- The entire reactor content of 3.5 litre was heat shocked.
- Heat shocks economically boosted dark fermentation hydrogen yield.
- Heat pumps with COP of 4.3 would allow energetic net gain due to heat shocks.
- Oven and microwave-based heat shocks are not significantly different.
- Microwaves heat sludge 6.4 times faster than ovens.
- Heat shocks inhibited methane production completely.
- Heat shocks increased particularly *Clostridium sensu stricto* 1 and 11.
- Hydrogen could be enriched from sucrose, but not from lipids or proteins.

Abstract

Heat shocks are a favoured method used to trigger hydrogen production in dark fermentation. However, these treatments are often restricted to a very small volume, which is then inoculated into synthetic medium. Moreover, heat shocks are usually applied with an oven. This study explored the feasibility of applying heat shocks to the entire seed sludge using a microwave, a method not previously investigated. The experiments were conducted on a technical scale with 5 litre tank reactors. The highest productivity was achieved with an oven (0.95 mol H_2/mol hexose), but the microwave was not significantly different and had the advantage of heating the sludge 6.4 times faster. The study also emphasized the repeatability of heat shocks, with microwave-assisted experiments showing low variation coefficients averaging 29%. The approach is already economically sustainable with high-temperature heat pumps with a COP of 4.3, enhancing its practical relevance.

1 Introduction

Anaerobic digestion is a technology, which allows the production of methane from multiple organic resources and residues, as for example straw and corn stover (Croce et al., 2016), chicken dung (Bayrakdar et al., 2017), food waste (Xu et al., 2018) and other. The possibility to produce methane from biodegradable remnants is intriguing, although it is hard to compete to existing technologies, which are based on fossil fuels. In Germany, the monetary shortcoming is counterbalanced based on the German Renewable Energy Sources Act. Therefore, to make anaerobic digestion a self-sustained technology, technological and conceptual advancements are needed. A possibility to achieve this is to intertwine anaerobic digestion with existing bio-based industries creating synergetic effects. To give some examples: Chen et al. (2018) suggested to integrate anaerobic digestion and microalgae cultivation. A review article from Sawatdeenarunat et al. in 2016 discusses additionally the possibility to produce valuable side products from extracted solids from the digestate or from the biogas itself, as for example syngas, methanol, butanol and other. A particular promising approach to produce valuable side products is the application of two stage digesters with a separated acidification stage. Acidic anaerobic digestion allows the accumulation of short chain fatty acids (C2 – C5), which are important platform chemicals for the chemical industry (Uludag-Demirer et al., 2019). Under specific conditions even a chain elongation can be achieved during the digestion process, yielding medium chain carboxylic acids (Groof et al., 2019). Another promising metabolite formed during anaerobic digestion is hydrogen (Khan et al., 2018). Particularly, a two-stage digestion process is well suited for microbial hydrogen production (Cooney et al., 2007). Using acidic pretreatment stages of anaerobic digesters for hydrogen production is a process, which is very similar to dark fermentation. Dark

73 fermentation approaches are extensively described in the literature and already in late 80s,
74 mixed culture fermentation was applied for microbial hydrogen production
75 (Roychowdhury et al., 1988). However, particularly when applying hydrogen production
76 with mixed cultures and in combination with a methane yielding digester, several
77 problems can occur. Anaerobic digestion involves around 300 operational taxonomic
78 units, which covered 80 percent of all reads from 16S rRNA gen amplicon high
79 throughput sequencing of samples belonging to 32 full-scale digester plants (Kirkegaard
80 et al., 2017). Such a high diversity makes it difficult to achieve a stable microbiome that
81 yields a predictable outcome of hydrogen. A study by Alibardi et al. (2012) is noteworthy
82 in the context of high microbial diversity. This research emphasized that heat-shock
83 applications could enhance hydrogen yield, thereby increasing its predictability. Another
84 problem that occurs when coupling a dark fermentation stage with a methanogenic reactor
85 is the immigration of methanogens. Although high organic loading rates (OLR) and,
86 therefore, low pH levels tend to supress methanogenesis (Bertucci et al., 2019; Senturk
87 et al., 2014), the formation of methane during dark fermentation has been described
88 before (Słupek et al., 2019). Interestingly, heat-shocks can help to optimize this problem
89 too. Although methanogenesis becomes not fully supressed, it has been indicated recently
90 that heat-shocks can reduce the amount of methane, which is produced in separated
91 acidification stages (Abendroth et al., 2018). The fact that heat-shocks can simultaneously
92 supress methanogenesis and contribute to an enrichment of hydrogen forming bacteria
93 makes them particularly interesting. Although multiple research articles already address
94 the application of heat-shocks to improve hydrogen formation, the authors of the present
95 work have barely detected works, where the complete seed sludge was heat shocked. An
96 enrichment culture is unlikely to prevail in a complex anaerobic sludge. In respect to an

industrial application, therefore, the treatment of the complete seed sludge would be more practical. The presented work tries to close this gap by investigating the industrial suitability of heat-shocks for separated acidification stages heating up the entire seed sludge. For the first time, the application of microwaves was assessed in parallel to oven-based heat-shocks. Further, the impact of heat-shocks on the underlying microbiome was explored.

2 Material and Methods

2.1 Inoculum and Substrate

Dark fermentation experiments were performed, using customary sucrose as substrate and sewage sludge as seed sludge. The sewage sludge was retrieved from a digester of a municipal wastewater treatment plant in Saxony, Germany. At mesophilic temperatures (30 – 35°C), the average solids retention time (SRT) was kept at 16.5 days with an average pH of 7.6. The amount of total solids (TS) was at 2.78 g/l with a median ammonium content (NH_4-N) of 1109 mg/l. The sludge contained 57.52 g/l total organic solids. During the experiments, the solubilized chemical oxygen demand of the seed sludge was 1220 mg/l and the median of the VFAs was 34.71 mg/l. The dissolved organic carbon (DOC) was 329.85 mg/l. The seed sludge contained further 118.45 mg/l calcium, 33.71 mg/l magnesium, 178.35 mg/l potassium and 16.46 mg/l sodium. Gelatine, mixed rye bread, coconut oil and sucrose were tested as substrates and based on that sucrose was chosen as the carbon source for the ongoing experiments.

2.2 Experimental set-up

For dark fermentation, customized tank reactors (CTRs) from the company Lehmann-UMT GmbH (Germany) were applied (Figure 1A). These CTRs had a volume of 5 litre

121 with a working volume of 3,3 litre maximum. Each fermentation lasted for 7 days. Before
122 transferring the seed sludge into the respective reactors, 50, 75 or 100 g/l of sucrose were
123 added. The process temperature during fermentation was 38 ± 1 °C.
124 During the experiments, the impact of heat shocks on hydrogen formation was
125 investigated. Heat shocks were applied before the feeding substrate was added. Different
126 methods of heating were tested. For the heat shock, the slurry was heated by means of
127 both oven and microwave. By oven, the sludge was incubated at 130°C in 1 litre Schott
128 flasks. By microwave, the sludge was heated in 5 litre measuring cups at 1000 watts. Both
129 methods were used to heat the sludge to 80-90°C, to eliminate hydrogen consuming
130 microorganisms. Incubation times varied from 25 minutes to 3 hours (Figure 1B)
131 depending on the used method. After the heat shock, the sludge was cooled down to 40°C.
132 Zhu and Béland (2006) reported that heat shocks are often performed in the range of 80
133 to 104°C and with exposure times of up to 120 min. Here, the exposure times are much
134 shorter and the temperature of the heat shock is only up to 80°C ± 1°C. For the microwave
135 these were 5-10 minutes and in the oven 10-20 minutes. These lower conditions were
136 chosen following the experiments of Wong et al. (2014) which showed that effective heat
137 shock pretreatment of anaerobic sludge can be achieved at lower temperatures (65-85 °C)
138 and durations of 45-60 min. Taking into account that the entire seed sludge was heat
139 treated in the present work, lower temperatures and shorter incubation times were
140 preferred to be more energy efficient.
141 For each condition tested, fermentation experiments were repeated minimum 6 times
142 which resulted in a total amount of 69 reactions, that were evaluated. Control experiments
143 accounted for 29, oven heat pretreatment for 20, and microwave heat shock pretreatment
144 for 20 tests.

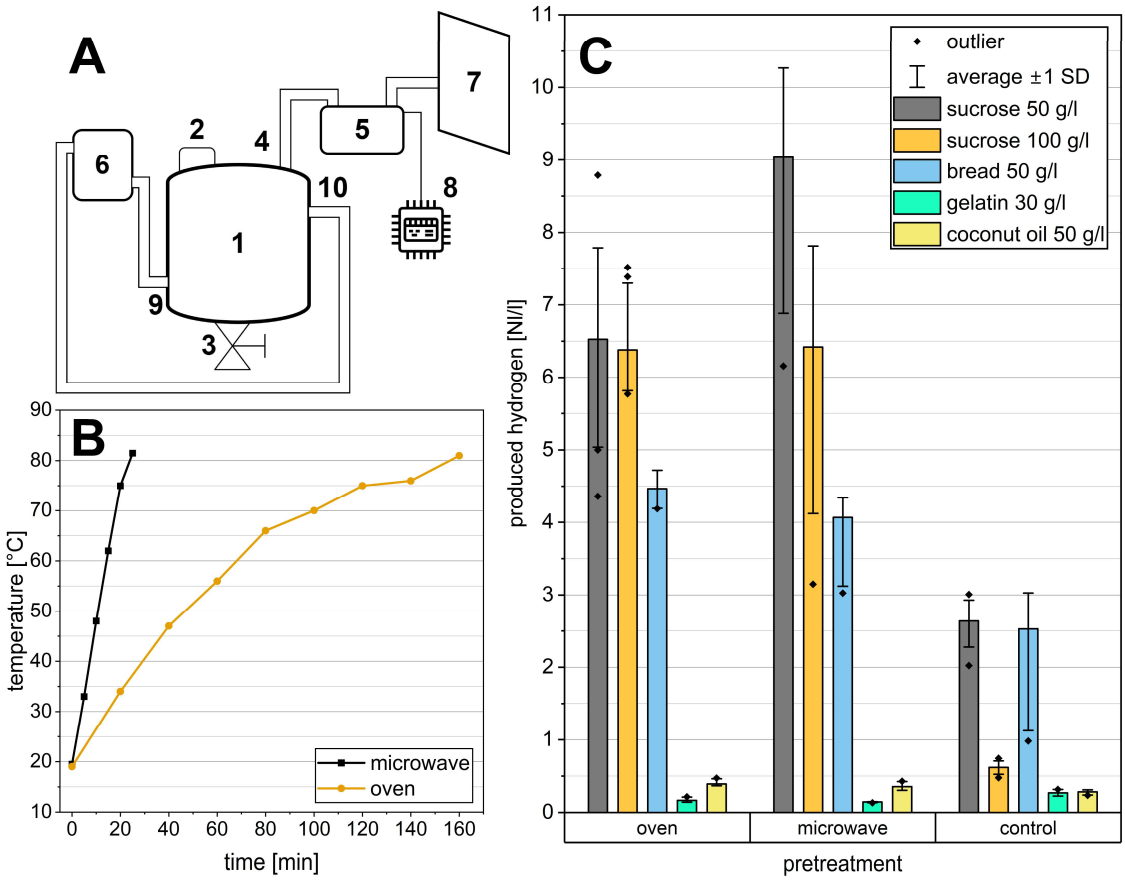

Figure 1: ***(A)*** *Experimental set up: double-walled reactors (1) with a volume of 5L each. Each reactor was filled through an input port (2) and acidified hydrolysate was removed through a port at the bottom (3). For heating, a water pipe was connected to the input (9) and output (10) ports of the double-wall clearance. Water was heated using a thermostat (6). Produced gas was measured by a MilliGascounter (5), connected with a tube (4), and stored in a gas bag (7). The time resolved gas production was recorded with an Arduino (8) connected to the gas counter.* ***(B)*** *Average incubation time for heat shocking up to a minimum of 80°C for pre-treatment with oven (set to 130°C) and microwave (set to 1000 Watt).* ***(C)*** *Preliminary tests: Comparison of hydrogen production with sucrose, mixed rye bread, gelatine and coconut oil as feeding substrates and sewage sludge as inoculum. Both control and heat shock (oven and microwave) scenarios were tested.*

157 *2.3 Sampling and chemical analysis*

158 During the fermentation, produced biogas was collected in gas bags (Tesseraux,
159 Germany) and quantified using a RITTER MilliGascounter (Bochum, Germany). Based
160 on the guideline VDI 4630 from the Association of German Engineers (2016), the gas
161 volume was normalized to standard temperature (273 K) and standard pressure (1013
162 hPa).

163 The solubilized COD was measured in the liquid phase after centrifugation at 1.200 g and
164 subsequent vacuum filtration through a 0.2 µm cellulose-acetate filter (Sartorius AG,
165 Göttingen, Germany). The COD was determined using the Spectroquant COD kit (VWR,
166 Germany) according to the manufacturer's guidelines. The spectrum of VFAs (lactic acid,
167 formic acid, acetic acid, propionic acid, iso-butyric acid, butyric acid and valeric acid)
168 was determined by ion chromatography. For this, a cation exchange column (Metrosep
169 Organic Acids 250/7.8 column; Model: 882 Compact IC plus, Metrohm AG, Herisau,
170 Switzerland) was applied. The mobile phase had a concentration of 0.6 mmol/l of
171 perchloric acid and 10 mmol/l of lithium chloride. The detection limit was 0.25 mg/l. The
172 inoculation sludge had no lactic and valeric acid and only very small amounts of propionic
173 and butyric acid (1.54 and 2.75 mg/l). Formic acid and acetic acid were present in the
174 initial substrate at 7.17 mg/l and 13.42 mg/l, respectively. Total volatile fatty acids
175 (TVFAs) were analysed as the sum of all detected VFAs. Initially, heavy metals were
176 measured by microwave digestion and ICP (PerkinElmer Optical-Emission Spectrometer
177 Optima 8000/ S10 Autosampler). Nitrogen content (TKN and NH_4-N) was determined
178 according to ISO 5663.

179 For microbiome analysis with 16S-rRNA gene amplicon high-throughput sequencing, an
180 aliquot of 3 ml of each sample stored in ethanol was centrifuged and washed with sterile

181 phosphate-buffered saline (PBS) until the supernatant was clear. DNA was extracted from
182 the resulting pellets using a Qiagen DNeasy PowerSoil Kit. DNA was quantified using
183 the Qubit 1x dsDNA Kit from ThermoFisher. The extracted metagenomic DNA was used
184 to amplify the hypervariable region V3-V4 of the 16S ribosomal RNA gene. The
185 conserved regions V3 and V4 (470 bp) of the 16S rRNA gene were amplified using PCR
186 cycling: initial denaturation at 95 °C for 3 min; 25 amplification cycles (20 s at 98 °C, 15
187 s at 60 °C, 30 s at 72 °C); and 5 min extension at 72 °C. The following primers were used:

188 - 341F (5' ACACTCTTCCCTACACGACGCTCTTCCGATCT NNNNN
189 CCTAYGGGRBGCASCAG 3') and
190 - 806R (5' GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
191 GGACTACNNGGGTATCTAAT 3').

192 Amplification was performed using the Thermo Scientific Phusion High-Fidelity DNA
193 polymerase kit. The purification of the amplicons was carried out after each PCR with
194 the 1x v/v AmPure XP beads from Beckmann Coulter. The purified 16S amplicons were
195 indexed with a second PCR containing TruSeq Illumina Indexing Primers (cycling
196 conditions 98C 30sec denaturation, 98C 15sec, 65C 75sec, 10 cycles, final elongation
197 5min 65C, 4C overnight). Final libraries were again purified with 1x v/v AmPure XP
198 beads and quality-assessed on an Agilent FragmentAnalyzer (1-6000bp NGS Kit).
199 Libraries are then equimolarly pooled and sequenced. Sequencing was performed using
200 2X250pb or 2 × 300pb paired-end cycle runs on an Illumina MiSeq instrument. The
201 Illumina raw sequences were loaded into Qiime2 (v. 2021.2.0) (Bolyen et al., 2019) . The
202 quality of the sequences was checked with the Demux plugin and the DA-DA2 pipeline
203 integrated in Qiime2 was used to trim and join the sequences, remove chimaeras and
204 detect amplicon sequence variants (ASVs) (> 99.9 % similarity). The taxonomy of each

sequence variant was determined using SILVA (Quast et al., 2013) as the reference database for taxonomic assignment.

2.4 Analysis of hydrogen content

Produced gas was collected in gas bags (Tesseraux, Germany). The gas volume was determined and normalised after measuring temperature and air humidity. Carbon dioxide (CO_2), oxygen (O_2), methane (CH_4) and hydrogen sulphide (H_2S) were detected with a X-AM 8000 gas detector by Dräger (Lübeck). Nitrogen and hydrogen cannot be detected directly by the analyser. At the beginning the reactor was filled with 3.3 litre of sewage sludge and 1.7 litre air, consisting of nitrogen by 79 %. The nitrogen content of the initial gas phase was deducted of the gas production. The amount of hydrogen was estimated as the remaining part after subtracting the percentages of nitrogen, oxygen, carbon dioxide and methane. The amount of hydrogen sulphide was very low (usually a few ppm), and therefore, it was neglected. The gas contained in the headspace was also considered. The formula to estimate the yield of hydrogen considers the density and the molar mass of hydrogen, the input mass and molar mass of the substrate (sucrose) and the number of hexose molecule per molecule sucrose. The formula assumes that the substrate is consumed completely.

229 $$y = \frac{V(H_2) \times \rho(H_2) \times M(Suc)}{(M(H_2) \times m(Suc) \times N(Suc)}$$

230 *y… yield [mol H_2/mol Hexose]*

231 *V(H_2)… generated volume of hydrogen [Nl]*

232 *ρ(H_2)… density of hydrogen [g H_2/Nl]; (0,0899 g H_2/Nl)*

233 *M(Suc) … molar mass of sucrose [g Suc/mol Suc]; (342,3 g Suc/mol Suc)*

234 *M(H_2)… molar mass of hydrogen [gH_2/molH_2]; (2,01588 g H_2/mol H_2)*

235 *m(Suc) … mass of sucrose [g Suc/l Input]*

236 *N(Suc)… number of hexose molecules per sucrose molecule; (1.9)*

237

238 *2.5 Statistical Analysis*

239 For the basic statistical evaluation of the test series, each with at least 6 measured values,
240 the median was selected in order to better represent the tendency of the individual test
241 results. To test the experimental groups for statistically significant group differences, the
242 Levene test was first used as a test procedure for homogeneity of variance. Unlike the
243 Bartlett test, this test is significantly more robust when populations are not normally
244 distributed (Eid et al., 2017). For experimental groups that did not have homogeneity of
245 variance, a WELCH ANOVA was performed (Sadooghi-Alvandi et al., 2012; Eid et al.,
246 2017). Groups with homogeneity of variance were compared using classic ANOVA.
247 Pairwise experimental group differences were determined using post-hoc tests. For this
248 purpose, the Tukey-Kramer test (Ruxton and Beauchamp, 2008; Shingala and Rajyaguru,
249 2015) and, for groups without homogeneity of variance, the Games Howell test was used
250 (Lee and Lee, 2018). For all tests, the significance level was set at $\alpha = 0.05$.

251 **3 Results and Discussion**

252 *3.1 Dark mixed culture fermentation with different substrates*

253 The main substrate in the present work is sucrose. Usually, dark fermentation experiments
254 are published with carbohydrates as substrates. Originally, it was planned to work also
255 with substrates rich in proteins and fat to assess the suitability of substrates other than
256 carbohydrates. Therefore, in preliminary tests, various feeding substrates (namely
257 sucrose, mixed rye bread, gelatine and coconut oil) were evaluated regarding to their
258 hydrogen formation efficiency. These substrates were mixed with sewage sludge. The
259 room loadings were between 30 g/l for gelatine and 50 g/l for bread, coconut oil and
260 sucrose, and an additional 100 g/l for sugar. Each mixture was filled into 1 litre SIMAX
261 glass bottles, which were prefilled with digested sewage sludge. To trigger H_2 formation,
262 heat shocks were performed using an oven and a microwave as described in material and
263 methods. In every experiment, a reaction with untreated sludge was used as control. The
264 highest amount of hydrogen was obtained in the experiments, where sludge was heat
265 shocked using an oven and with a loading rate of 50 g/l of sucrose (Figure 1C). This
266 resulted in a yield of 9 litre hydrogen per litre sludge. High amounts of hydrogen were
267 also obtained with 100 g/l sucrose in both oven and microwave heat shocks. Mixed 50 g/l
268 rye bread performed well compared to 30 g/l gelatine and 50 g/l coconut oil, but it could
269 not match the hydrogen production achieved with sucrose (Figure 1C). These results align
270 with findings from other studies on dark fermentation, where substrates high in
271 carbohydrates yielded significantly better gas rates (Lay et al., 2003; Kannaiah Goud et
272 al., 2014; Luo et al., 2010). As depicted in Figure 1C, as twice as much biogas was
273 produced in the heat-treated trials compared to the untreated control.

274

275 *3.2 Dark mixed culture fermentation of sucrose*

276 As shown in Figure 1C, fat and proteins are hardly suited for dark mixed culture
277 fermentation. Therefore, subsequent experiments were focused on sucrose as substrate.
278 Acidification of sucrose was performed in 5 litre CTRs, using digested sewage sludge
279 from a local wastewater treatment plant in Saxony as inoculum. Different loading rates
280 (50, 75 and 100 g/l) were compared in terms of hydrogen production, to determine the
281 optimal substrate concentration (Figure 2). Assuming an idealized uniform consumption
282 of the substrate, these would correspond to loading rates of 7, 11 and 14 g COD/l*d. The
283 highest average hydrogen yield in mol H_2 per mol hexose was achieved in experiments
284 with 50 g/l sucrose (Figure 2C). Control and pretreated sludges with the microwave or
285 the oven showed hydrogen formation efficiencies of 0.14, 0.51 and 0.95 mol H_2/mol
286 hexose respectively. These values correspond to the median. With untreated substrate,
287 the lowest median hydrogen formation was observed at 100 g/l sucrose, where only 0.69
288 litre H_2 per litre of working volume was formed. This corresponds to a formation
289 efficiency of just 0.06 mol H_2/mol hexose.

290 In the heat-treated experiments, the lowest median hydrogen yield was produced with 100
291 g/l sucrose, respectively 0.39 and 0.33 mol H_2/mol hexose for microwave and oven
292 pretreatment. The reduced yield per gram of substrate indicates overfeeding. As depicted
293 in Figures 3A and B, an increase in feeding rates led to a rise in both the total volume and
294 the absolute quantity of hydrogen, but only in the experiments that underwent microwave
295 pretreatment. Conversely, in the oven-treated experiments, the volumes decreased,
296 leading to an inverse effect. Consequently, the efficiency of hydrogen formation in the
297 oven experiments dropped significantly by approximately two-thirds from 50 g/l to 100
298 g/l. However, the decrease in hydrogen yield with microwave pretreatment was only 28%.

299 In the control experiments that were untreated, there was only a slight change in gas
300 volumes.
301
302 *3.3 Application of heat shocks for optimization of hydrogen production*
303 After the initial characterization of sucrose, it was now tried to optimize the hydrogen
304 production due to thermal pretreatment. Processes involving heterogeneous microflora
305 are deemed more suitable than homogeneous pure cultures due to their simpler process
306 control and efficient substrate conversion, provided that the medium undergoes
307 pretreatment (Rafieenia et al., 2018). As per Saady (2013), inhibiting H_2-consuming
308 microorganisms such as hydrogenotrophic methanogens, homoacetogens, lactic acid
309 bacteria, propionate-producing bacteria, and sulphate reducers is a crucial step in H_2
310 production by dark fermentation in mixed microbial communities. In the here presented
311 experiments, heat shocks were employed as a pretreatment to the seed sludge prior to the
312 addition of the respective substrate to enhance hydrogen yield. Many studies use heat
313 shocks to generate small volume of enriched hydrogen, which is than inoculated in a
314 medium. To increase industrial relevance, digested sewage sludge was used as seed
315 sludge and the entire seed sludge was pretreated. This strategy allows for the selection of
316 spore-forming hydrogen-producing microorganisms and the inhibition of non-spore-
317 forming hydrogen consumers. Different incubation times were necessitated by the
318 varying heat shock methods. The microwave pretreatment took approximately 35 minutes
319 for the slurry temperature to reach at least 80°C, while in the oven, it took nearly 3 hours
320 for the slurry to attain the desired temperature (Figure 1B). Once the temperature was
321 achieved, the sludge was immediately removed and allowed to cool down to 40°C. Figure
322 2A illustrates that compared to the untreated control experiments, the heat shocks

323 significantly augmented both the total gas volume and absolute hydrogen production
324 (Figure 2B), as improved hydrogen formation efficiency (Figure 2C). In the heat shock
325 trials (both oven and microwave), methane production was successfully eliminated
326 (Figure 2D). For instance, Hasyim et al. (2011) conducted a shock at 105°C for 20 min
327 in an autoclave, while Wang et al. (2019) performed a 70°C heat shock for 60 min with
328 free ammonia assistance to fully inhibit methane production using glucose as a substrate.
329 The recent experiments have demonstrated that even shorter and less energy-intensive
330 heat shocks can completely eliminate methane production and facilitate more efficient
331 biological hydrogen production from sewage sludge. However, it needs to be considered
332 that the experiments were batch-wise. In a continuous reactor or with sludge recirculation
333 in a two-stage process, it would still be conceivable that methanogens could gradually
334 adapt to the temperature shocks.
335 As a result of the heat treatments, the relative standard deviation of the hydrogenation
336 efficiency increased by 49% on average in the microwave pretreatment and by 70% in
337 the oven pretreatment. The standard deviation of the oven tests was on average 32%
338 higher than in the microwave tests. Hydrogen yield could potentially be increased further
339 by pH controlling, since pH values of 4.0 to 4.5 have a negative effect on hydrogen
340 production (O-Thong et al., 2009). The pretreatment with the oven was overall related to
341 higher gas production compared to the microwave. However, the boxplots of the
342 pretreated series of experiments show that the microwave shocks reduced the scatter of
343 the results, an effect that was not observed in the experiments with oven treatment.
344 Distinct observations were made at a loading rate of 50 g/l sucrose. Here, the oven
345 demonstrated a significant increase in absolute hydrogen production and consequently in
346 hydrogen formation efficiency (Figure 2B and 2C). A median of 5.92 l H_2/l sludge was

347 produced, corresponding to a hydrogen yield of 0.95 mol H_2/mol hexose. The maximum
348 hydrogen yields of 1.09 and 1.05 mol H_2/mol hexose are comparable to the experimental
349 results of O-Thong et al. in 2009 (1.16 mol H_2/mol hexose) and Baghchehsaraee et al. in
350 2008 (1.32 to 2.12 mol H_2/mol hexose). The high HBut/HAc molar ratios for the heat
351 shocked experiments (Figure 4B) indicating that hydrogen was produced via the butyrate
352 pathway (Pineda-Muñoz et al., 2020). Without heat pretreatment, the HBut/HAc ratio
353 shows that hydrogen was accordingly formed via the acetate pathway. For both heat
354 shock methods, the median hydrogen formation efficiency shows a decreasing trend as
355 the loading rate increases. This pattern is reflected in the total volume and hydrogen
356 volume for the oven-based heat shock experiments. In contrast, specific hydrogen
357 production increases with increasing feeding rate in the microwave pretreatment
358 experiments (Figure 2C). Concomitantly, the microwave assisted hydrogen production
359 efficiency remains more constant, decreasing by only 22% from 50 g/l to 100 g/l (65%
360 decrease for oven pretreatment). The distribution of gas volume indicates that in all
361 experiments involving heat shock pretreatment, the total hydrogen content in the gas
362 increased to approximately 40% for microwave and 41% for oven pretreated. This is a
363 significant increase when compared to the median of the control at 18% H_2 (Figure 2D).
364 Fang and Liu (2002) investigated the effect of pH on hydrogen formation as well as the
365 composition of the digestate and showed that within a short time 90% of the glucose was
366 metabolized. They managed to increase the hydrogen content in the biogas to 64% with
367 a pH value, which was optimally adjusted to 5.5. While in microwave-treated approaches
368 the relative hydrogen fraction increases slightly from 38% to 42%, it remained constant
369 at 40% to 42% in the oven-treated experiments. In both heat shock pretreated methods,
370 the highest relative hydrogen content was obtained in the experiments with 100 g/l

371 sucrose. Methane was not produced in any of the heat-treated trials. The residual portion
372 of the gas mixture consisted entirely of carbon dioxide.
373 In general, thermal pretreatment increased the hydrogen production capacity of anaerobic
374 sludge in dark fermentation experiments by at least a factor of 3.6 for microwave and a
375 factor of 5.3 for oven compared to untreated experiments. The maximum increase of 6.8
376 times was obtained in oven-treated trials at 75 g/l loading rate and in microwave assisted
377 trials by 6.5 times at 100 g/l loading rate (microwave: 50 g/l - 3.6 times, 75 g/l - 5.4 times;
378 oven: 50 g/l - 6.6 times, 100 g/l - 5.3 times). Hence, these findings align with those
379 reported by Baghchehsaraee et al. (2008) and O-Thong et al. (2009), who reported
380 enhancement rates of 5.1 (80°C for 30 min) and 7.9-fold (100°C for 1 h) as a result of
381 thermal pretreatments. The greatest increase of 1.9-fold in the oven-treated trials over
382 those with microwave treatment was achieved at a loading rate of 50 g/l. However, this
383 did not result in statistically significant differences between microwave and oven
384 pretreatment in terms of overall hydrogen production (Figure 4A). Only at 100 g/l, no
385 significant group differences for biogas volume and hydrogen content could be
386 determined between control and oven. In both the 50 g/l and 75 g/l loading rate
387 experiments, there were statistically significant differences between oven/microwave and
388 control for the hydrogen volume, hydrogen fraction, efficiency, and biogas volume. In a
389 few cases, hydrogen yields similar to those achieved by Tao et al. in 2007 (2.64 and 3.20
390 mol H_2/mol sucrose) were obtained. They carried out experiments with cattle dung and
391 longer and hotter heat shocks (112° for 4.5 h). Pineda-Muñoz et al. (2020) also succeeded
392 in producing high hydrogen yields by combining heat shock and ultrasonic treatment but
393 failed to completely suppress methane production despite nearly identical heat shock
394 conditions.

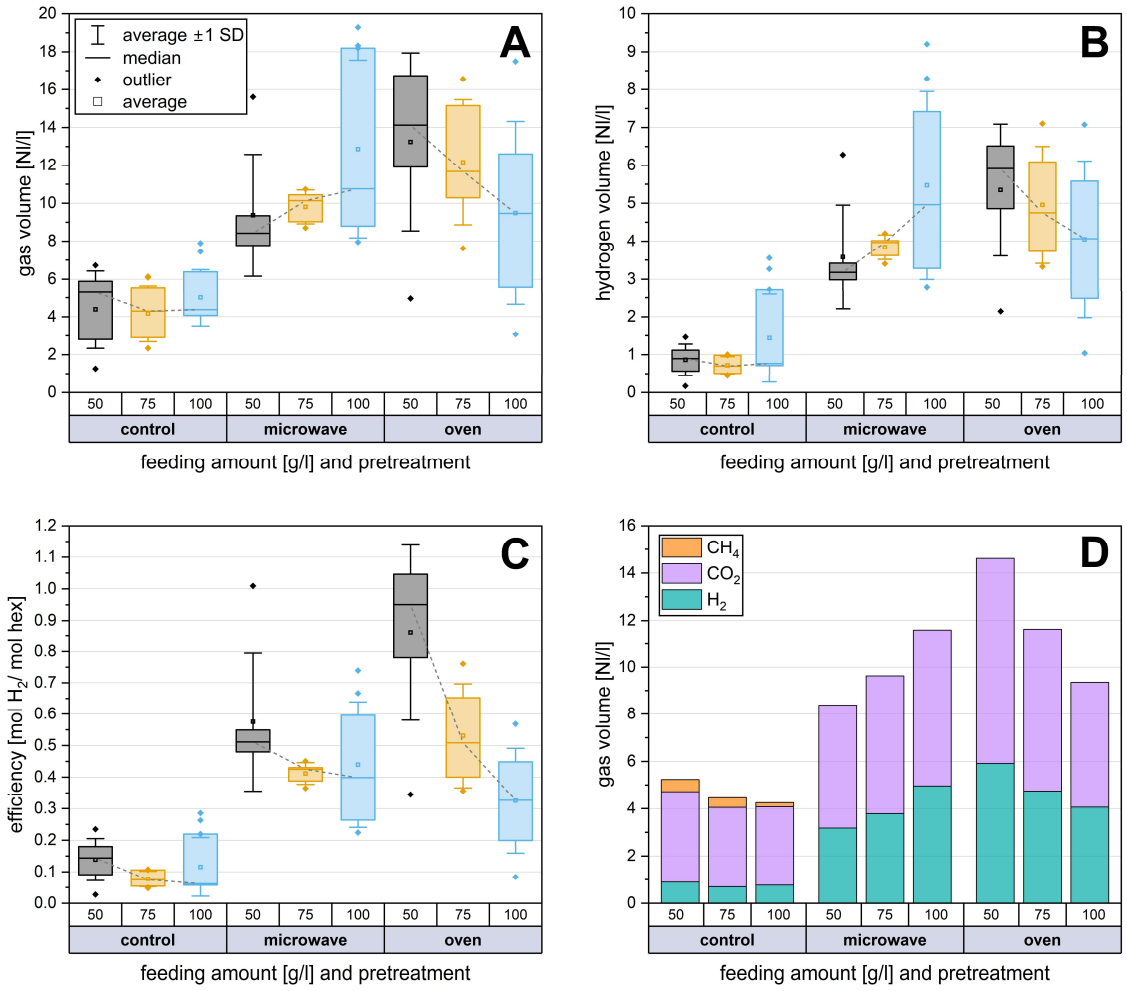

Figure 2: Application of heat shocks to optimize hydrogen productivity: Different concentrations of substrate (sucrose) and different pretreatments (oven and microwave) were compared. ***(A)*** *Maximal biogas productivity and* ***(B)*** *the highest hydrogen production were reached with substrate concentration of 50 g* L^{-1} *and pretreated with oven heat shock. All fermentations were performed at least 5 times.* ***(C)*** *The highest hydrogen efficiency was achieved with 50 g/l sucrose both in microwave and oven experiments.* ***(D)*** *shows the gas composition depending on the feeding amount and pretreatment. To better indicate the impact of different loading rates, median values have been connected with a trend line.*

406 *3.4 Taxonomic shifts due to heat shocks*

407 Using 16S rRNA gene amplicon high-throughput sequencing, the effect of microwave
408 heat shock on the taxonomic profile and diversity of the microbiome at 25 g/l sucrose was
409 determined to reflect the stressor of overfeeding less strongly. Microwave treatment
410 effectively eliminated non-spore formers and homogenized the microbiome towards
411 *Clostridia*. In more severely overfed experiments, a similar effect was observed, but with
412 lower abundances of *Clostridia*. With a loading rate 25 g/l sucrose *Clostridia*
413 predominantly colonized the microwave-treated substrates (average abundance of 79%).
414 As depicted in Figure 3A, the microbial growth community in the microwave-treated
415 reactions was composed of 60% to 69% *Clostridium sensu stricto 1*. In contrast,
416 *Streptococcus* dominated the control experiments, making up 46% to 56% of the total
417 microbiome. *Paeniclostridium* was only present in the heat-treated experiments,
418 suggesting that it either benefited from the heat shock or was activated, as it must have
419 already been present in the inoculum. The heat shock was successful in completely
420 suppressing *Lactobacillus*, a lactic acid producer. Heat shock pretreatment resulted in a
421 reduction of species diversity by an average of 7% compared to the control, as shown in
422 Figure 3B. While the Shannon index showed no difference in diversity, the Simpson
423 index indicated a reduction in species diversity in the microwave-treated trials. Both
424 scenarios exhibited low evenness, pointing to a highly uneven distribution of species. The
425 strong shift towards *Clostridium sensu stricto 1* and the decrease in absolute species
426 number in the heat-treated experiments led to a more uneven distribution of species. This
427 generally low equal distribution is also mirrored in the taxonomic profile, which shows
428 that 20% to 28% of the microbiome consists of smaller groups of microorganisms with
429 less than 5% relative abundance.

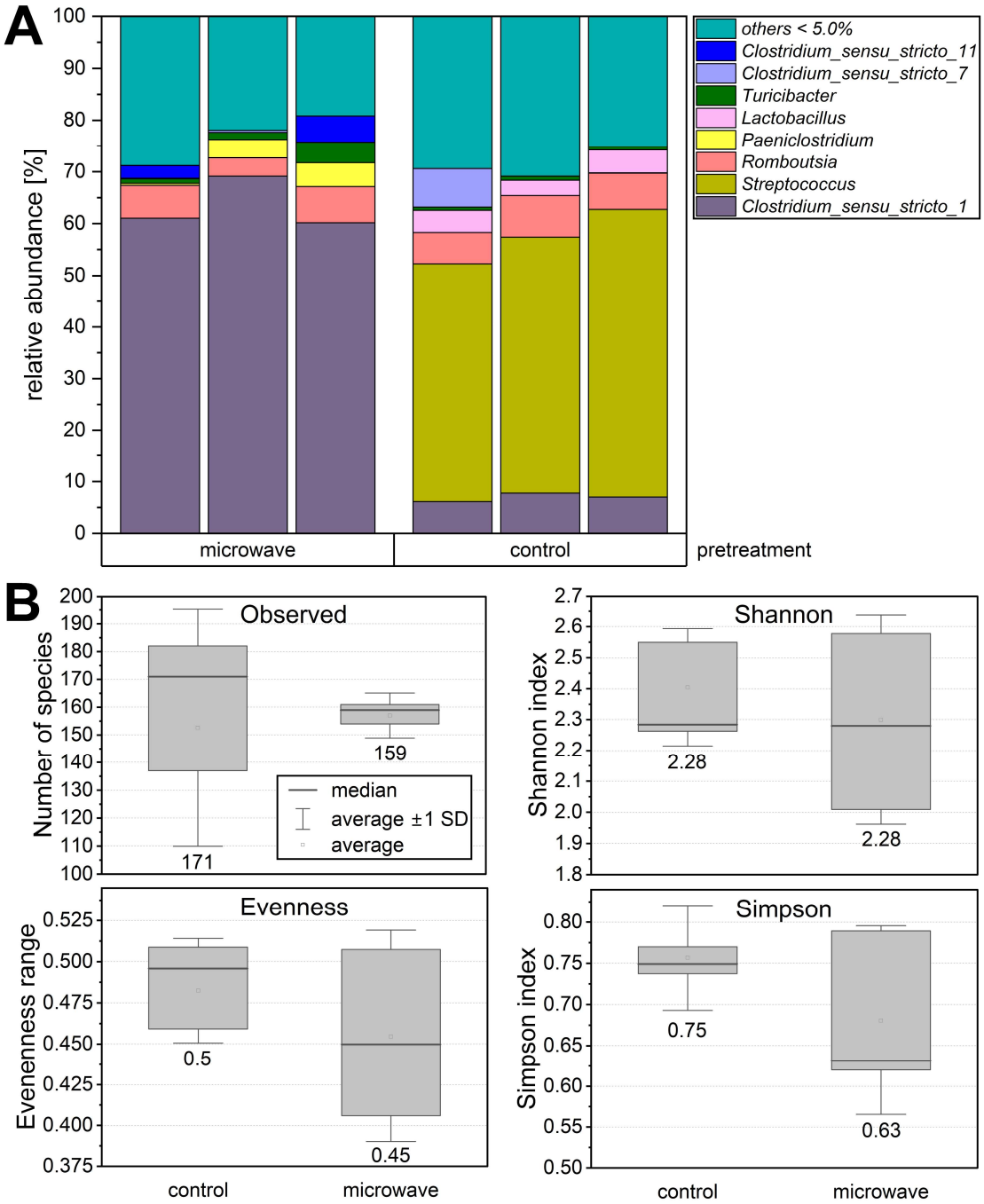

Figure 3: ***(A)*** *Microbial community as relative abundances differentiated according to pretreatment. Only the most abundant microorganisms with abundance >5% are presented. Others with abundances below 5% are summarized as "others < 5.0%".* ***(B)*** *Alpha Diversity of the microbiome for genus level data of microwave and control samples, loading rate 25 g/l of sucrose.*

436 *3.5 Production of volatile fatty acids*

437 In general, the TVFA production was approximately 30 to 40 g/l for heat shocked
438 experiments and between 35 and 55 g/l for non-pretreated ones. The thermal pretreated
439 experiments demonstrated a significant shift of the acid balance towards butyric acid. In
440 heat shocked experiments, the molar ratio of HBut/HAc was in a range from 1.13 to 2.05
441 (Figure 4B). Without thermal pretreatment, the HBut/HAc ratio was between 0.34 and
442 0.59. With the loading rate of 50 g/L and due to microwave assisted pretreatment, butyric
443 acid concentration increased 2.77-fold up to 7.55 g/l. In contrast, the concentration of
444 formic acid decreased to 24.02 g/l (0.75-fold). With the oven assisted pretreatment,
445 butyric and formic acid reached similar concentrations with values of 10.80 g/l and 28.08
446 g/l respectively. Of the seven VFA analysed, formic, acetic and butyric acid were the
447 most abundant ones (in sum >95%). Lactic and propionic acid showed rather low
448 concentration, which is similar to results of Fang and Liu (2002). 2.73 g/l butyric acid
449 and 31.97 g/l formic acid were formed in the untreated control reactions, with the same
450 loading rate of 50 g/l. In none of the experiments were larger amounts of propionic or
451 lactic acid present, although their formation is typical for processes with high loading
452 rates (Oh et al., 2004). It can therefore be assumed that only a few propionic acid formers
453 were already present in the seed sludge. Notable concentrations of iso-butyric acid (0.019
454 g/l) and valeric acid (0.007 g/l) could not be determined in any of the tests either. The
455 formation of acetic acid was hardly influenced by the pretreatment, neither by the oven
456 or the microwave. After an experimental period of seven days, pH values of 3.98 on
457 average were measured in all experiments. At the start of the experiments, the pH value
458 was generally 7.56. Based on Pineda-Muñoz et al. (2020), it can be assumed that acetic
459 acid and as well the formic acid were increasingly formed with decreasing pH. Therefore,

460 it can be assumed that the ratio of butyric acid would be even higher if the pH would be

461 regulated.

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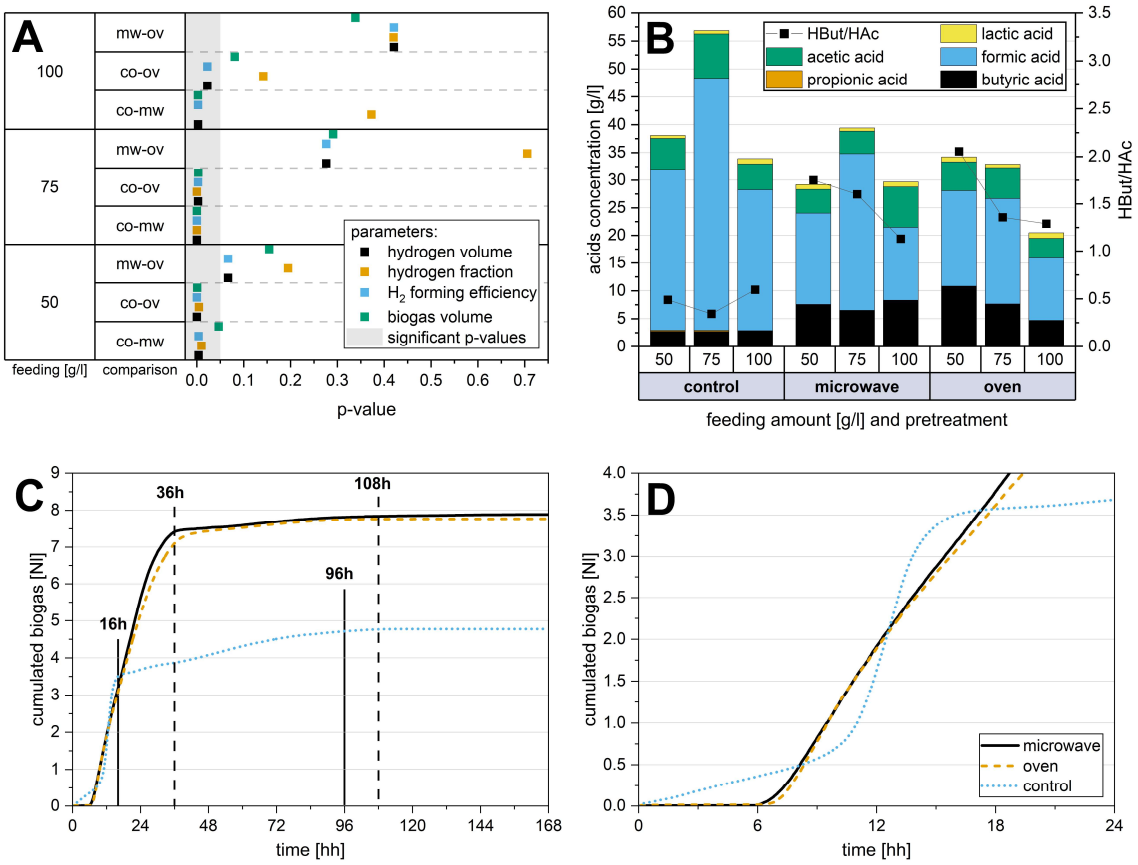

463

464 *Figure 4:* ***(A)*** *Graphical representation of statistical significance (significant for p<0.05)*

465 *of group comparisons for hydrogen and biogas volumes, hydrogen formation efficiency*

466 *and hydrogen fraction. Marker in grey area means a significant difference between the*

467 *respective comparison groups.* ***(B)*** *Production of volatile fatty acids (median)*

468 *differentiated by treatment method and feeding amount. Volatile fatty acids were*

469 *analysed in triplicates in all cases.* ***(C)*** *Exemplary course of gas production over 7 days*

470 *and* ***(D)*** *first 24 hours for untreated (control) and heat shock treated (oven and*

471 *microwave). Performed with sewage sludge and 75 g/l sucrose as feeding substrate.*

472

473 *3.6 The kinetics of hydrogen production after heat-shock application*

474 In the untreated experiments, gas production commences immediately upon the
475 introduction of the feeding substrate, albeit at a slow pace for the initial 9 hours (Figure
476 4C and 4D, with 50 g/l sucrose as substrate). This was succeeded by a swift escalation
477 over the subsequent 7 hours. After approximately 16 hours of experimentation, there is a
478 noticeable plateau in gas production, which nearly halts entirely after about 96 hours. In
479 contrast, in the heat shock experiments, biogas production does not initiate until 6 hours
480 post-feeding but then ascends steeply and continuously until the 36th hour. The
481 subsequent rise is considerably less steep compared to the control experiment and nearly
482 ceases entirely after 108 hours. This pattern was observed with both pretreatment, oven
483 and microwave. The rate of gas formation in the heat-treated trials was less steep
484 compared to the untreated control (Figure 4C), yet the robust biogas formation remained
485 active for almost twice as long (30 hours) compared to the control trials (16 hours). The
486 experiments demonstrated that heat shock pretreatment results in a more uniform and
487 prolonged biogas production, significantly augmenting the total volume of biogas, albeit
488 with a delay in gas production for the initial six hours.

489 To better highlight, whether differences between the different treatments are significant,
490 statistical significance was assessed based on an ANOVA (Figure 4A). For example, at a
491 loading rate of 100 g/L of sucrose, there is no significant difference between microwave
492 and oven assisted pretreatment regarding hydrogen volume, biogas volume, efficiency
493 and hydrogen percentage.

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3.7 Industrial relevance

The research conducted in this study indicates that one litre of heat-treated sludge can yield an average of 5.92 m^3 of hydrogen per m^3 sludge at a loading rate of 50 g/L. Subtracting the average values for the control from this, the heat treatment results in an additional gain of 5.02 m^3 hydrogen per m^3 of sludge. The thermal energy required to heat the seed sludge from 38°C to 80°C is 49.04 kWh/m^3, considering that it takes 4.2 kJ to increase the temperature of 1l sludge by 1°C. The additional energy produced in form of hydrogen due to the heat shocks corresponds to 54.18 kJ/l sludge, which corresponds to 15.06 kWh/m^3 sludge. Based on the 49.04 kWh/m^3 for the heat shock, the energy loss for the heat shock process is 33.98 kWh/m^3. This amount of energy must at least be recovered using a heat pump, to make this concept energetically attractive.

Approved high-temperature heat pumps, which are currently available on the market, can achieve a coefficient of performance (COP) of 4.3 when heating to 80°C from a starting temperature of 20°C (as per data from ENGIE Refrigeration GmbH in 2018). This implies that 1 kWh of electrical energy can produce up to 4.3 kWh of thermal energy. To generate 49.04 kWh of thermal energy, a heat shock requiring 11.4 kWh electrically would be needed, assuming a coefficient of performance (COP) of 4.3. By this COP an additional energy output of 3.66 kWh/m3 sludge compared to the calorific energy of the extra hydrogen is produced by the heat shock. The process would also be energetically profitable due to further energy losses during the heat exchange process with the sludge, which are not considered here. Future technological improvements that raise the COP could make enhanced hydrogen production through heat shocks even more sustainable.

Considering the varying costs of hydrogen and electric energy, the economic appeal of the concept could remain intact even if the hydrogen equivalent of one kilowatt-hour is

priced higher than electricity itself. Based on Machhammer et al. (2015) and Bukold (2020), a good benchmark for costs of hydrogen production is the average price for steam reforming of 1.70€/kg H_2. The energy cost for a heat shock using a high temperature heat pump with a COP of 4.3 would be 0.4€/m^3 of sludge, (based on an average industrial electricity price of 35€/MWh. Selling the produced hydrogen at the same price of 1.70€/kg, would generate an average revenue of 0.91€ from the amount of hydrogen from one cubic meter of heat shocked sludge. This translates to a profit of 0.51€/m^3 of sludge. Despite a net energy loss, the production of hydrogen through heat shocks proves to be more cost-effective compared to other alternative methods of hydrogen production. The current hydrogen costs of hydrogen production without steam reforming range from 5.20€/kg, as reported by Gerloff in 2023, to 7.60€/kg, according to Machhammer et al. in 2015. Lowering the heat shock temperature could significantly decrease the energy requirement and thus the essential costs, while potentially yielding similar or even higher hydrogen yields (Baghchehsaraee et al., 2008). These calculations are based on ideal assumptions and the successful scaling of trials in an industrial setting, with a particular focus on electricity costs.

4 Conclusions

The presented experiments indicate that thermal shock pretreatment of the entire seed sludge can trigger biohydrogen production from digested sewage sludge. Microwave heat shocks, which were statistically equivalent to oven heat shocks in terms of hydrogen production via dark fermentation, led to a less diverse microbiome and a strong increased Clostridia growth. By using already available high-temperature heat pumps and successful upscaling, heat shocks are energetically sustainable and economically competitive with electrolysis for hydrogen production. A significant byproduct of

545 microwave heat shocks was the production of substantial amounts of butyric and formic
546 acid as potential future green platform chemicals.

547 **Acknowledgements**

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549 and Climate Action (grant numbers 49MF190057, 16KN070128, 16KN070126 and
550 16KN070163).

551 **Data availability**

552 The genome data will be published in the gene bank database of the National Center for
553 Biotechnology Information after acceptance of the paper.

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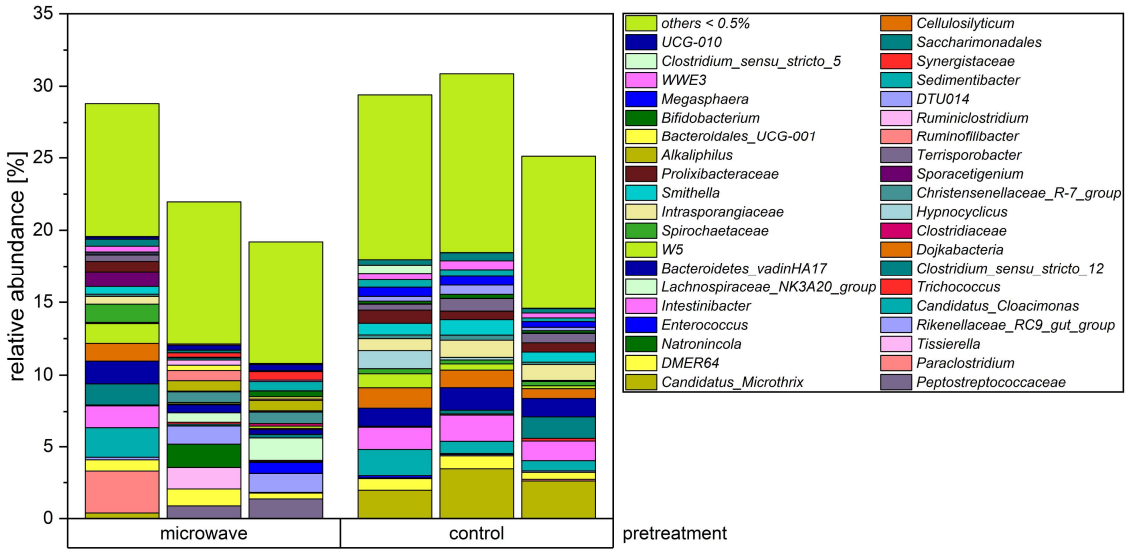
626 **Appendices**



627

628 *Figure S1: Microbial community as relative abundances differentiated according to*

629 *pretreatment (loading rate 25 g/l). Only the microorganisms with abundance < 5% are*

630 *presented. Others with abundances below 0.5% are summarized as "others < 0.5%".*

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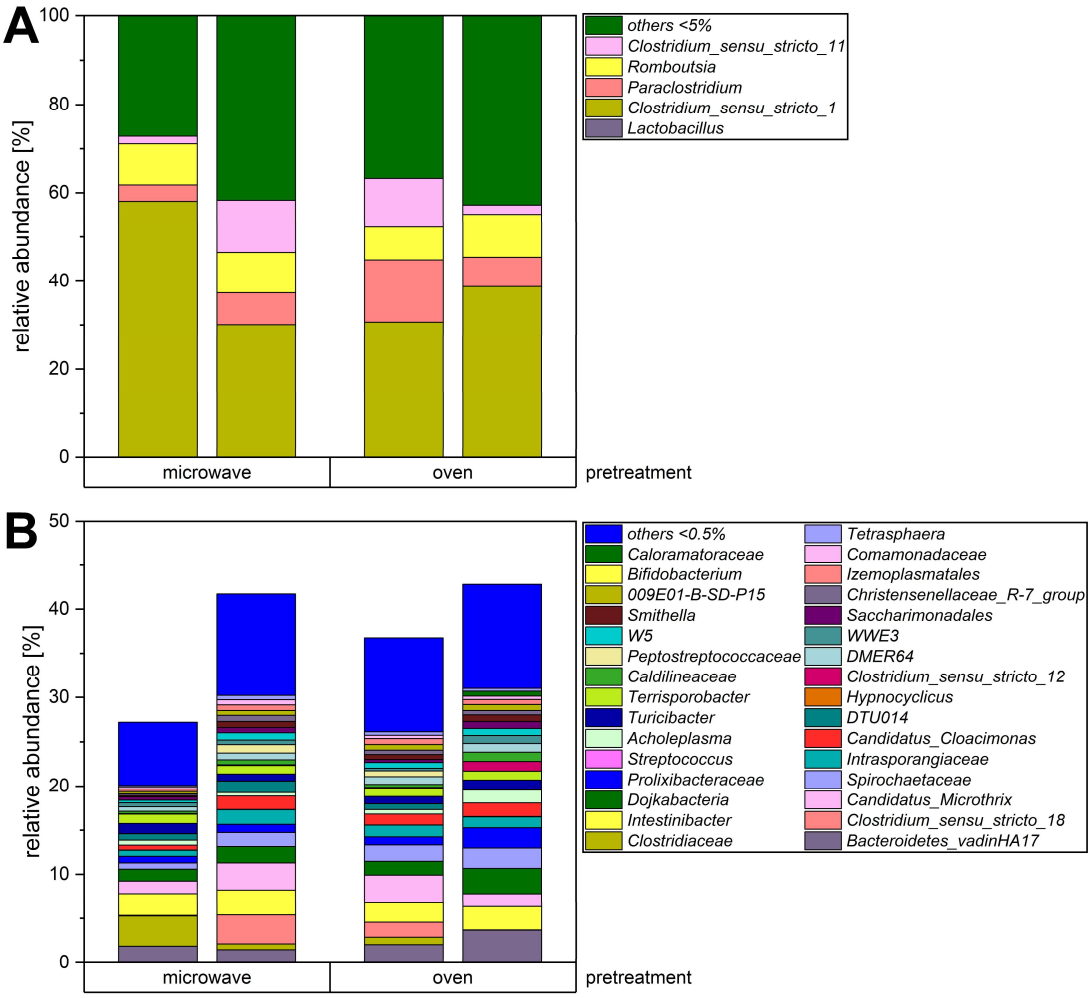

632

633 *Figure S2: Microbial community as relative abundances differentiated according to*

634 *pretreatment. Loading rate was at 100 g/l sucrose.* ***(A)*** *Only the most abundant*

635 *microorganisms with abundance > 5% are presented. Others with abundances below 5%*

636 *are summarized as "others < 5.0%.* ***(B)*** *Breakdown of minor microorganisms below 5%*

637 *abundance. Organisms with abundances below 0.5% are summarized as "others<0.5%".*

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