Highlights

- H_2 supersaturates in the liquid phase at 100 rpm despite hyperthermophilic conditions.
- High H_{2aq} concentrations directly inhibit the specific rates of dark fermentation.
- H_2 supersaturation and H_2 production rate depend on the gas-liquid mass transfer.
- H_2-rich biogas recirculation is an effective method to prevent H_2 supersaturation.
- Gas recirculation increased the hydrogen production rate by 271%.

H_2-rich biogas recirculation prevents hydrogen supersaturation and enhances hydrogen production by *Thermotoga neapolitana cf. capnolactica*

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Abstract

This study focused on the supersaturation of hydrogen in the liquid phase (H_{2aq}) and its inhibitory effect on dark fermentation by *Thermotoga neapolitana cf. capnolactica* by increasing the agitation (from 100 to 500 rpm) and recirculating H_2-rich biogas (GaR). At low cell concentrations, both 500 rpm and GaR reduced the H_{2aq} from 30.1 (± 4.4) mL/L to the lowest values of 7.4 (± 0.7) mL/L and 7.2 (± 1.2) mL/L, respectively. However, at high cell concentrations (0.79 g CDW/L), the addition of GaR at 300 rpm was more efficient and increased the hydrogen production rate by 271%, compared to a 136% increase when raising the agitation to 500 rpm instead. While H_{2aq} primarily affected the dark fermentation rate, GaR concomitantly increased the hydrogen yield up to 3.5 mol H_2/mol glucose. Hence, H_{2aq} supersaturation highly depends on the systems gas-liquid mass transfer and strongly inhibits dark fermentation.

Key words: *Thermotoga neapolitana*; Dark fermentation; Gas recirculation; Sparging; Hydrogen supersaturation; End product inhibition

Declarations of interest: none

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- H_2-rich biogas recirculation is an effective method to prevent H_2 supersaturation.
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Abbreviations

AA	acetic acid
BGR	biomass growth rate
BMY	biomass yield
CSTR	continuously stirred tank reactor
GaR	recirculation of H_2-rich biogas
GCR	glucose consumption rate
H_{2aq}	liquid phase hydrogen
HPR	hydrogen production rate
HY	hydrogen yield
LA	lactic acid

1. Introduction

Hydrogen, a nonpolluting energy carrier can be produced from organic residues in an environmental-friendly biological process called dark fermentation [1,2]. Hydrogen production has been reported in a wide range of temperatures, with thermophilic conditions being generally correlated to higher hydrogen yields (HY) [1,2]. A variety of microbial species are capable of fermentative hydrogen production [3] in both mixed and pure culture applications [4]. The use of mixed cultures is considered to be the simpler and have a more practical approach [4], but often entailing the coexistence of microorganisms which do not produce or even consume hydrogen [5].

Thermotoga neapolitana (hereafter *T. neapolitana*) is a hyperthermophilic microorganism with a high potential for hydrogen production via dark fermentation [6,7]. Some of the key characteristics exhibited by this organism include fast growth kinetics [7], and efficient degradation of a wide range of substrates, e.g. glucose, fructose, xylose, maltose, starch [7,8], molasses, cheese whey [9], algal biomass [10], and carrot pulp [11]. *T. neapolitana* is capable to approach the theoretical HY of 4 mol H_2/mol glucose by optimizing the conditions for the hydrogen forming acetate pathway [12,13]. Additionally, the hyperthermophilic culture conditions of *T. neapolitana* further stimulate the reaction rates [2] and hamper the growth competitive microorganisms [14], also leading to a pasteurization of the used substrate [15].

In dark fermentative hydrogen production, the presence of hydrogen is one of the most crucial factors, as high concentrations of hydrogen are a major inhibitor of the process. To reduce the inhibitory effect of hydrogen on the process, previous experimental studies (Table 1) have employed a variety of techniques, e.g. sparging the headspace, reducing the total pressure, applying vigorous stirring, optimizing the reactor design, or simply increasing

95 the headspace/solution ratio [12]. Regardless of the method used, alleviation of hydrogen
96 inhibition results in increased HYs and/or hydrogen production rates (HPR).
97 The headspace hydrogen concentration has been widely used (Table 1) to predict hydrogen
98 in the liquid phase (H_{2aq}), which *de facto* acts on the microbial cultures. However, poorly
99 soluble gases, such as hydrogen, supersaturate in solution during anaerobic processes
100 [31,32]. Studies, including a direct measurement of H_{2aq} (Table 1), have observed that H_{2aq}
101 can exceed the equilibrium concentration suggested by Henry's law multiple times. As a
102 result, the measurement of headspace hydrogen is considered inappropriate to predict the
103 H_{2aq} value [2,16–18,31,32].

104 Our study focused on the effect of agitation and biogas recirculation on the accumulation of
105 H_{2aq} and the consequent impact on dark fermentation by *T. neapolitana*. The main goal was
106 to demonstrate that the recirculation of the H_2-rich biogas (GaR) into the culture broth is an
107 effective method to improve the gas-liquid mass transfer and prevent H_{2aq} from
108 supersaturating, without diluting the H_2 percentage in the biogas. Furthermore, we wanted
109 to show that keeping the H_{2aq} in equilibrium with the gas phase is sufficient to achieve high
110 hydrogen production yields and rates. In a first assay, the effect of GaR and agitation speed
111 on hydrogen production was evaluated using a highly concentrated *T. neapolitana* culture. In
112 a second assay, a direct measurement of H_{2aq} was included to demonstrate the correlation
113 between hydrogen supersaturation and the performance of dark fermentation.
114

2. Material and methods

2.1. Bacterial culture and medium

A pure culture of *Thermotoga neapolitana cf. capnolactica* [33] was used in all experiments. The cultivation and storage conditions for the culture, the preparation of inoculum for elevated cell concentrations (assay 1) and the modified ATCC 1977 medium used (containing 27.8 mM of glucose) were as described by Dreschke et al. [34]. The pH of the medium was adjusted to 7.5 prior to being autoclaved at 110 °C for 5 min. Prior to the inoculation, the medium was heated at 80 °C for 30 min and sparged with N_2 for 5 min to remove the dissolved oxygen.

2.2. Experimental design

All experiments were run in a 3-L fully controlled continuously stirred tank reactor (CSTR) (Applikon Biotechnology, the Netherlands) with a working volume of 2 L. The operating temperature was kept at a constant 80 °C and pH was automatically adjusted to 7 by adding 1M NaOH. GaR was applied by continuously pumping the produced biogas from the headspace to a gas dispersion device at the base of the reactor at a flow rate of 350 mL/min. The produced biogas was released into 500 mL water displacement systems to avoid pressure build-up and quantified every hour. Liquid samples of 2 mL were withdrawn each 30 or 60 min. The fermentation was considered completed when the reactors ceased to produce biogas. Fermentation time was approximated from the inoculation to the end of the fermentation. Each condition was conducted in duplicate to demonstrate reproducibility.

2.2.1. Assay 1 – Effect of agitation speed and gas recirculation on dark fermentation at high cell concentrations

Assay 1 was designed to study the effect of GaR and agitation speed on the dark fermentation process at high cell concentrations of *T. neapolitana*. The reactor was inoculated with 80 mL of concentrated inoculum [34] resulting in a biomass concentration of 0.79 (± 0.03) g cell dry weight (CDW)/L. The following operating conditions were investigated: 300 rpm agitation (300); 300 rpm agitation with gas recirculation (300 + GaR); 500 rpm agitation (500); 500 rpm agitation with gas recirculation (500 + GaR).

2.2.2. Assay 2 – Effect of GaR and agitation speed on H_{2aq}

Assays 2A and 2B were designed to study the effect of agitation speed and GaR on the retention of hydrogen in the liquid phase and its influence on the dark fermentation process. The reactor was inoculated with 20 mL of storage culture (1% v/v) and maintained for 15 h at 100 rpm to acclimatize the culture. Afterwards, a sequence of operating conditions was used with each condition being operated for 2 h as described in Table 2. In assay 2A, 5 min of 500 rpm + GaR (Table 2 – shaded grey cells) was used before each operating condition to remove the accumulated hydrogen from the liquid phase. After 5 or 30 min and at the end of each operating condition, 20 mL of broth was withdrawn to monitor H_{2aq}.

2.3. Analytical Methods

2.3.1. Assay 1

Cell growth was determined by measuring spectrophotometrically (Lambda 365, Perkin Elmer, USA) optical density (OD_{540}) of the liquid samples at 540 nm. Liquid samples were centrifuged at 10,000 rpm for 5 min to collect the supernatant for the determination of glucose, acetic acid (AA), lactic acid (LA) and alanine concentration as described by Dreschke et al. [34]. The glucose concentration was measured by the dinitrosalicylic acid method [35], while AA, LA and alanine were quantified by ^{1}H Nuclear Magnetic Resonance (NMR) with a

600 MHz spectrometer (Bruker Avance 400) as described by Dipasquale et al. [10]. After the completion of the fermentation, 500 mL of culture broth was centrifuged at 3750 rpm for 20 min for the determination of CDW via freeze drying. The concentration of hydrogen in the produced gas was analyzed by gas chromatography as described by Dipasquale et al. [10]. The conversion from volumetric to molar H_2 production was performed using the ideal gas law.

2.3.2. Assay 2

Glucose, AA and LA concentrations were determined using an HPLC (Prominence LC-20A Series, Shimadzu, Japan), equipped with UV/Vis (SPD-20A, Shimadzu Japan) and refractive index (RID-20A, Shimadzu, Japan) detectors, with the method described by Mancini et al. [36] with 0.0065 M of sulfuric acid as the mobile phase. The concentration of hydrogen in the biogas was determined with a Varian 3400 gas chromatograph (GC), equipped with a thermal conductivity detector (TCD) and a Restek packed column using argon as the carrier gas.

For the determination of H_{2aq}, a modified method of Kraemer and Bagley [16,17,37] was applied. Vials with a total volume of 31 mL were closed with silicon septa and depressurized using a plastic syringe. 20 mL of sample was injected into the vials and immediately placed upside down in fridge to equilibrate the liquid and the gas phases. After around 20 h, the sample was heated at room temperature and the negative pressure equilibrated by adding air to the gas phase of the sample. The concentration of hydrogen in the gas phase was measured by the GC described above and referred to the 20 mL of liquid sample.

2.4. Kinetic study of dark fermentation

The rates and lag phases of dark fermentation were evaluated in terms of biomass growth, hydrogen production and glucose consumption and calculated by fitting the experimental data with a modified Gompertz model as described by Dreschke et al. [34].

2.5. Statistical analysis

The statistical significance of the experimental data was determined by the calculation of the p-value applying an unpaired t-test with Microsoft Excel 2016 (Microsoft Corporation, USA).

3. Results

3.1. Effect of agitation speed and GaR on dark fermentation at high cell concentrations (assay 1)

Fig. 1. shows the glucose consumption (A), cumulative hydrogen (B) and biomass growth (C) during batch fermentation with an initial cell concentration of 0.79 (± 0.03) g CDW/L of *T. neapolitana* using an agitation of 300 or 500 rpm with and without GaR.

Increasing the agitation significantly enhanced the specific rates of biomass growth [mg CDW/h/g CDW], hydrogen production [mL H_2/h/g CDW], and glucose consumption [mmol glucose/h/g CDW] from 73 (± 11), 294 (± 44), and 4.4 (± 1.3) at 300 rpm to 266 (± 9) (p-value: 0.003), 695 (± 46) (p-value: 0.012), and 10.1 (± 0.6) (p-value: 0.028) at 500 rpm (Fig. 2, Table 3), respectively. Due to the accelerated process, the fermentation time and lag phase of hydrogen production were reduced from 11 h and 2.9 (± 0.2) h at 300 rpm to 6 h and from 1.3 (± 0.1) h at 500 rpm (Table 3), respectively. The HY increased from 3.0 (± 0.0) at 300 rpm to 3.2 (± 0.1) mol H_2/mol glucose at 500 rpm, while the biomass yield (BMY) increased from approximately 16.7 to 21.9 g CDW/mol glucose (Table 4). The composition of the end products, expressed in terms of an LA/AA ratio, decreased from 0.31 at 300 rpm to 0.21 at 500 rpm (Table 5).

A combination of GaR and agitation speed at 300 rpm further accelerated the dark fermentation. The specific biomass growth rate reached 423 (± 9) mg CDW/h/g CDW (p-value: 0.004), while the specific rates of hydrogen production and glucose consumption increased up to 1090 (± 91) mL/h/g CDW (p-value: 0.032) and 13.3 (± 0.8) mmol glucose/h/g CDW (p-value: 0.040), respectively, over those obtained at 500 rpm without GaR (Fig. 2, Table 3). The fermentation was completed within 4 h with a lag phase of 0.4 (± 0.0) h (Table 3), a HY of 3.5 (± 0.2) mol H_2/mol glucose, an approximate BMY of 24.8 g CDW/mol glucose (Table 4), and a LA/AA ratio of 0.11 (Table 5).

Increasing the agitation to 500 rpm while applying GaR had no significant effect on the process, as shown by the similar performance obtained at 300 rpm + GaR and 500 rpm + GaR (Fig. 1). At 500 rpm + GaR, the specific rates of biomass growth, hydrogen production and glucose consumption were 431 (± 9) mg CDW/h/g CDW, 1016 (± 22) mL H_2/h/g CDW and 12.7 (± 0.2) mmol glucose/h/g CDW (Fig. 2, Table 3), respectively. The HY reached 3.3 (± 0.1) mol H_2/mol glucose (Table 4) coupled with a LA/AA ratio of 0.13 (Table 5). The fermentation time and lag phase of hydrogen production remained at 4 h and 0.5 (± 0.1) h (Table 3), respectively, with an approximate BMY of 27.7 g CDW/mol glucose.

The highest volumetric HPRs of 850 (± 71) and 813 (± 18) mL/h/L were obtained when GaR was applied at 300 and 500 rpm, respectively (Table 3). The hydrogen concentration in the biogas produced was not affected by the use of GaR or the agitation speed and remained constant at 65 (± 2)% (data not shown) under all studied conditions. In all bioassays, glucose was consumed up to 82 - 89 % (Table 4) with AA and LA as the main fermentation end products in the digestate and alanine constituting a minor proportion of 2% (Table 5). A mass balance based on the stoichiometric equations 1 and 2 was performed to validate the experimental results. The dark fermentation model suggests the formation of two moles of

fermentation end products (AA and LA) per mole of glucose with the concomitant production of 2 moles of hydrogen per mole of AA. Under all operating conditions, the sum of fermentation products in the digestate reached 92% or more of the theoretical value calculated from the glucose consumption and 2.08 (± 0.01) moles H_2 per mole of AA were averagely produced.

$$C_6H_{12}O_6 + 4\,ADP + 4\,H_2PO_4^- \leftrightarrow 2\,CH_3CO_2H + 2\,CO_2 + 4\,ATP + 4\,H_2 + 2\,H_2O \quad (1)$$

$$C_6H_{12}O_6 + 2\,ADP + 2\,H_2PO_4^- \leftrightarrow 2\,CH_3CH(OH)CO_2H + 2\,ATP + 2\,H_2O \quad (2)$$

3.2. Effect of GaR and agitation speed on H_{2aq} and HPR (assay 2)

3.2.1. Assay 2A – Use of different agitation speeds

Assay 2A assessed the effect of the agitation speed on H_{2aq} and HPR. After the initial 15 h at 100 rpm, H_{2aq} accumulated up to 30.1 (± 4.4) mL/L (Fig. 3B). Then, using 500 rpm + GaR for 5 min before each operating condition reduced H_{2aq} to an average of 7.5 (± 1.0) mL/L (Fig. 4A). The subsequent agitation of the culture at 300 and 500 rpm without GaR for 2 h led to a H_{2aq} of 17.0 (± 2.3) and 7.4 (± 0.7) mL/L, respectively (Fig. 3B).

To better demonstrate the effect of hydrogen retention in the liquid phase on the HPR, the 2 h duration of each operating condition was subdivided in 3 phases and the HPR was calculated for each individual phase: 1 – removal phase, i.e. period from 0 to 5 min after changing the operating condition when additional GaR was applied (“HPR GaR”); 2 – retention phase, i.e. period from 5 to 30 min after changing the operating condition (“HPR post GaR”); 3 – equilibrium phase, i.e. period from 30 to 120 min after changing the operating condition (“HPR”) (Fig. 3A). At 300 rpm, a significantly higher “HPR GaR” of 316 (± 54) mL/L/h (Fig. 3A) was observed than “HPR” of 54 (± 13) mL/L/h (p-value: 0.0002) and

252 “HPR post GaR” of 19 (± 17) mL/L/h (p-value: 0.0017), respectively. In contrast, at 500 rpm,
253 the HPR remained similar before, during, and after GaR. The “HPR” reached 112 (± 22)
254 mL/L/h, which was not significantly different from “HPR post GaR” (100 (± 16) mL/L/h, p-
255 value: 0.397) and “HPR GaR” (120 (± 11) mL/L/h, p-value: 0.614) (Fig. 3A). Changing the
256 agitation from 300 to 500 rpm roughly doubled the “HPR”, and concomitantly increased the
257 glucose consumption rate (GCR) from 3.6 (± 0.4) to 4.7 ± (0.3) mmol/L/h (p-value: 0.016)
258 (data not shown).

259 ### 3.2.2. Assay 2B – Gas sparging

260 Assay 2B was run to assess the effect of GaR on H_{2aq} and HPR at a constant agitation at 200
261 rpm. Similar to assay 2A, the highest H_{2aq} of 26.8 (± 3.6) mL/L (Fig. 4B and C) was obtained
262 after 15 h of operation at 100 rpm. Applying 200 rpm + GaR rapidly decreased the H_{2aq} to 9.0
263 (± 1.8) mL/L in 5 min (Fig. 4B and C) reaching a final value of 7.2 (± 1.2) mL/L after 2 h (Fig.
264 4B). The 200-rpm agitation without GaR caused an accumulation of H_{2aq} up to a
265 concentration of 16.4 (± 0.9) mL/L in 30 min, which was 91% of 18.0 (± 0.9) mL/L reached
266 after 2 h (Fig. 4B and C). The “HPR” at 200 rpm + GaR of 91 (± 20) mL/L/h was significantly
267 higher than 47 (± 8) mL/L/h (p-value: 0.017) obtained at 200 rpm without GaR (Fig. 3A).
268 Concomitantly, the GCR reached values of 4.2 (± 0.1) and 3.1 (± 0.8) mmol/L/h (p-value:
269 0.077), at 200 rpm + GaR and 200 rpm without GaR, respectively (data of GCR not shown).
270 When agitation was increased from 100 to 200 rpm after 15 h in the absence of GaR, H_{2aq}
271 decreased from 29.4 to 24.8 mL/L in 2 h (Fig. 4C), which was higher than 18.7 (± 4.6) mL/L
272 obtained at 200 rpm agitation at 21 h after applying GaR between 17 and 19 h.

273 ## 4. Discussion

274 ### 4.1. Effect of agitation speed and gas recirculation on H_{2aq} concentration

In this study, GaR and agitation speed were examined as strategies to lower H_{2aq} and, thus, stimulate H_2 production by *T. neapolitana*. Agitating at 100 rpm led to the highest H_{2aq}, i.e.30.1 (± 4.4) mL/L, which was approximately 3 times higher than the theoretical dissolved hydrogen concentration (i.e. 9.7 mL/L) in equilibrium with a gas phase containing 65% H_2 at 80 °C according to Henry's Law (Eq. 3):

$$H(T) = H^{\Theta} * \exp\left(\frac{d\ln(H)}{d(1/T)} * \left(\frac{1}{T} - \frac{1}{T^{\Theta}}\right)\right) \quad (3)$$

where H(T) and H^{Θ} are Henry's constants expressed in mol/m^3/Pa at temperature T and T^{Θ} (= 298.15 K), respectively; H^{Θ} equals 7.7 x 10^{-6} [38]; *d*ln (*H*)/*d*(1/T) equals 500 K [38] and is the temperature dependence factor of Henry's constant; and T is the temperature in K.

Increasing the agitation speed to 500 rpm effectively improved the gas-liquid mass transfer, decreasing the H_{2aq} concentration (Fig. 4 and 3B) until reaching values below the theoretical equilibrium concentration. Similarly, GaR maintained the hydrogen partial pressure of gas and liquid phase in equilibrium (Fig. 3B).

The extent of H_{2aq} accumulation is determined by the HPR and the mass transfer from liquid to gas phase (Eq. 4) [17,32].

$$\frac{dH_{aq}}{dt} = HPR - k_L a\,(H_{aq} - H_{dis}) \quad (4)$$

with H_{2aq} being the hydrogen concentration in the liquid phase [mL/L]; H_{dis} the concentration of dissolved hydrogen at the thermodynamic equilibrium; HPR the volumetric production of hydrogen in the liquid phase [mL/L/h]; k_La the global mass transfer coefficient [1/h] made up of two terms, i.e. k_L (the "film" coefficient) and *a* (the specific interfacial area per unit of liquid volume in the reactor).

When the HPR exceeds the gas-liquid mass transfer of the system, H_{2aq} supersaturates. However, elevated H_{2aq} concentrations inhibit the HPR, which again reduce the degree of

298 H_{2aq} supersaturation (Eq. 4) until HPR and H_{2aq} reach an equilibrium. Because of this
299 feedback inhibition, the HPR of a production system is in many cases primarily determined
300 by an insufficient gas-liquid mass transfer instead of the kinetic potential of the culture.

301 The evolution of H_{2aq} accumulation and its dependence on the HPR and the mass transfer
302 was clearly shown in the 3 phases of assay 2A at 300 rpm (Fig. 4A). In the retention phase, a
303 low "HPR post GaR" of 19 (± 17) mL/L/h (Fig. 3A) was obtained as HPR was strongly affected
304 by the fast retention of hydrogen in the liquid phase (Fig. 4A). The "HPR" during the
305 equilibrium phase was instead higher, reaching 54 (± 13) mL/L/h (Fig. 3A). Contrary to the
306 "HPR post GaR", "HPR" was no longer affected by hydrogen retention but rather by the
307 prevalent H_{2aq} of 17.0 (± 2.3) mL/L, which was determined by the mass transfer of the
308 system (Fig. 3B). The subsequent application of GaR after 2 h removed the accumulated
309 hydrogen from the liquid phase, leading to a high "HPR GaR" of 316 (± 54) mL/L/h and a
310 decrease of H_{2aq} to 7.5 (± 1.0) mL/L (Fig. 3A and B). The length of the retention phase was
311 based on the results of assay 2B, where a cultivation at 200 rpm after GaR increased the H_{2aq}
312 to 16.4 (± 0.9) mL/L in 30 min, i.e. 91% of 18.0 (± 0.9) mL/L measured after 2 h (Fig. 4B and
313 C). The accumulation of H_{2aq} was, therefore, considered completed at the end of the
314 retention phase and the HPR in equilibrium with H_{2aq} as explained in section 4.1 (Eq. 4). At
315 500 rpm, hydrogen did not accumulate in the liquid phase as demonstrated by the low H_{2aq}
316 of 7.4 (± 0.7) mL/L after 2 h (Fig. 3B) and the statistically similar values of "HPR post GaR",
317 HPR" and "HPR GaR", i.e. 110 (± 18) mL/L/h (Fig. 3A).
318 In conclusion, at low cell concentrations the sole agitation at 500 rpm as well as GaR
319 provided an adequate mass transfer to remove the produced hydrogen from the liquid to
320 the gas phase and prevent hydrogen supersaturation.

321 **4.2. Enhancement of dark fermentation kinetics**

322 Independent of the technique applied, the reduction of H_{2aq} in assay 2 resulted in a
323 significant increase of the HPR. Adding GaR increased the "HPR" by 94% from 47 (± 8) to 91
324 (± 20) mL/L/h at 200 rpm, whereas increasing the agitation from 300 to 500 rpm enhanced
325 the "HPR" by 107% from 54 (± 13) to 112 (± 22) mL/L/h (Fig. 3A), both accompanied by a
326 simultaneous increase of the GCR.
327 In assay 1, we accelerated the dark fermentation process by using a higher cell concentration
328 (0.79 g CDW/L). At the lowest agitation of 300 rpm, we observed a specific HPR of 294 (± 44)
329 mL H_2/h/g CDW. Increasing the agitation to 500 rpm accelerated the dark fermentation
330 process, enhancing the specific HPR by 136% to 695 (± 46) mL H_2/h/g CDW. As observed in
331 assay 2, the higher HPR obtained at 500 rpm was likely due to a decreased H_{2aq}
332 concentration. However, the addition of GaR was capable to further increase the HPR to
333 1090 (± 91) and 1016 (± 22) mL H_2/h/g CDW at 300 + GaR and 500 + GaR, respectively, i.e.
334 271 and 246% higher than the HPR achieved at 300 rpm in the absence of GaR. This indicates
335 that, in contrast to what was observed at low cell concentrations, 500 rpm agitating was
336 likely insufficient to completely prevent hydrogen supersaturation at high cell
337 concentrations. In contrast, GaR effectively enhanced the gas-liquid mass transfer (by
338 increasing the specific interfacial area *a* in Eq. 4) and maintained the H_{2aq} low, leading to the
339 highest values of specific HPR. The similar reactor performance at 300 + GaR and 500 + GaR
340 furthermore shows that hydrogen supersaturation was the crucial factor limiting the process
341 as, once it was prevented by GaR, the agitation speed had no influence on the process.
342 The present results are in line with those obtained by Beckers et al. [29], who observed an
343 enhancement of the HPR from 0.14 to 0.26 L/h when agitation was increased from 0 to 400
344 rpm in 2-L reactors using *Clostridium butyricum* at 30°C and glucose as a substrate at a feed

concentration of 27.8 mM. However, in that study the H_{2aq} was observed to remain high at approximately 4 times the equilibrium concentration despite 400 rpm agitation.

In our experiments, the specific rates of glucose consumption and biomass growth were closely linked to the HPR (Table 3), showing a similar enhancement when the different measures to prevent H_{2aq} supersaturation were applied. The specific GCR and biomass growth rate (BGR) at high cell concentrations increased by 202% and 479% from 4.4 (± 1.3) to 13.3 (± 0.8) mmol glucose/h/g CDW and from 73 (± 11) to 423 (± 9) mg CDW/h/g CDW at 300 rpm and 300 rpm + GaR, respectively. This acceleration of the process led to a reduction of the total fermentation time from 11 and 6 h with the sole agitation at 300 and 500 rpm, respectively, to 4 h when GaR was applied independent from the agitation speed.

Ljunggren et al. [17] successfully increased the HPR by decreasing H_{2aq} through N_2 headspace sparging using *Caldicellulosiruptor saccharolyticus*. The authors stated that the culture self-regulates the growth rate as a response to high H_{2aq} to slow down the process, decrease the HPR and consequently prevent the H_{2aq} from reaching inhibitory levels (Eq. 4). However, in our study the effect on the specific HPR, BGR and GCR was simultaneous (Fig. 2), indicating that H_{2aq} directly acts on all rates of dark fermentation.

4.3. Hydrogen and biomass yields under different operating conditions and H_{2aq} concentrations

Despite the inhibition by H_{2aq} discussed in section 4.2, the HY remained high at 3.0 (± 0.0) mol H_2/mol glucose (Table 4) when applying 300 rpm agitation without GaR in assay 1. The HY increased even further to 3.5 mol H_2/mol glucose coupled with a decrease of the LA/AA ratio (Table 4) when GaR maintained the H_{2aq} in equilibrium with the gas phase.

T. neapolitana predominately ferments glucose via the AA and the LA pathways, with the former leading to hydrogen production and one additional mole of ATP [7].

The AA pathway is thereby energetically more challenging [1], because the formation of hydrogen requires a high free Gibbs energy change as protons are poor electron acceptors [39]. The reaction to gaseous hydrogen is favored by low hydrogen concentrations and high temperatures as explained in detail by Verhaart et al. [39]. This indicates that the temperature was most likely responsible for the high HYs at sole agitation of 300 rpm. The additional decrease of H_{2aq} by GaR caused an additional energetical advantage and allowed the further shift towards the AA pathway leading to the increase of the HY.

A similar effect on the HY was obtained by Beckers et al. [29], measuring H_{2aq} in 200 mL serum bottles inoculated with *Clostridium butyricum* at 30°C. Unstirred cultures experienced a supersaturation up to 7 times the equilibrium concentration resulting in a reduced HY of 1.16 mol H_2/mol glucose, in comparison to 1.52 mol H_2/mol glucose obtained when agitating at 120 rpm. The inhibition by hydrogen has also been demonstrated previously in *T. neapolitana* cultivation using closed 120 mL serum bottles. d'Ippolito et al. [12] were able to increase the HY by reducing the culture/headspace ratio leading to a lower hydrogen partial pressure in the gas phase.

When GaR was applied at high cell concentrations, a lower LA/AA ratio indicates that more energy was gained by the culture per unit of substrate. Concomitantly, a higher BMY was observed, which suggests that biomass production did not, as often assumed, compete with the final product formation, but rather increased simultaneously when the environmental conditions were optimized. The decrease of the biomass concentration at 500 rpm depicted in Fig. 1C was caused by a change of *T. neapolitana* cell morphology in response to nutrient limitations. This phenomenon interferes with the optical density measurement as described in more detail by Dreschke et al. [34], whereas the actual biomass concentration is not affected. In our study, the variation of HY and BMY was much lower than that of HPR under

different operating conditions, indicating that hydrogen inhibition primarily acts on the process rates. We assume that a reduction of the dark fermentation rate by the culture at high H_{2aq} concentrations aims to prevent the inhibition of the AA pathway which allows the higher energy yield.

4.4. Biogas recirculation as a strategy to prevent hydrogen supersaturation

A large number of studies used headspace sparging with gases such as N_2 or CO_2 (Table 1) to counteract hydrogen inhibition by lowering H_{dis} (Eq. 4). For instance, Sonnleitner et al. [26] observed an increase of HPR from 25 to 119 mL/L/h when sparging the headspace of a *Caldicellulosiruptor saccharolyticus* culture with N_2 at 1 L/L/h. However, while sparging with external gases is generally successful to improve the process performance (Table 1), it inevitably leads to an undesired dilution of the produced hydrogen and consequently an increase of costs for gas purification. In contrast, the concentration of hydrogen in the produced gas remains high when GaR is applied.

To our knowledge, only two studies have so far focused on the recirculation of the H_2-rich biogas in dark fermentation. Kim et al. [22] used GaR at flow rates ranging from 100 to 400 mL/min in a CSTR with a mixed culture at 35°C. They obtained similar HPRs and HYs, i.e. 0.77–0.86 and 0.77 mol H_2/ mol hexose, respectively, with and without GaR. This suggests that at the prevalent reactor conditions, hydrogen did not supersaturate in the liquid phase which rendered GaR ineffective. Bakonyi et al. [23] recirculated the internal biogas at two different H_2 concentrations into a CSTR using a mixed culture at 35°C. The recirculation of the less concentrated gas (50% H_2) resulted in a HPR of 8.9 – 9.2, whereas the use of a more concentrated gas (66% H_2) reduced the HPR to 2.7 – 3.0 L H_2/L/d, compared to 7.4 L H_2/L/d obtained without recirculation. Unfortunately, a direct measurement of H_{2aq} was not included in any of the two studies, making the correlation of the reactor performance to H_{2aq}

417 impossible. In our study, we directly demonstrate the impact of stirring speed and GaR on
418 H_{2aq} and correlate it to the process performance. GaR has proven highly effective to provide
419 an adequate gas-liquid mass transfer, that can be readily adjusted by adapting the
420 recirculation flow to meet the requirements of the system. Furthermore, the results confirm
421 that maintaining H_{2aq} in equilibrium with the gas phase is sufficient to reach high HY up to
422 3.5 mol H_2/mol glucose (Table 4) and a high specific HPR of 1090 ± 91 mL/h/g CDW (Table 3).

423 **Conclusions**

424 This study confirms that hydrogen supersaturates in the liquid phase and strongly inhibits
425 dark fermentation by *T. neapolitana cf. capnolactica*. GaR and the sole agitation at 500 rpm
426 efficiently reduced the H_{2aq} (i.e. 30.1 ± 4.4 mL/L) observed at low cell concentration and
427 agitation of 100 rpm to below the equilibrium value suggested by Henry´s law (i.e. 9.7 mL/L).
428 At high cell concentrations (i.e. 0.79 g CDW/L), 500 rpm agitation did not provide sufficient
429 gas-liquid mass transfer to prevent H_{2aq} supersaturation, which was instead counteracted by
430 GaR. High H_{2aq} concentrations led to thermodynamic constraints and the reciprocal influence
431 of H_{2aq} and HPR, which directly hampered the dark fermentation rates. In this line, we
432 observed the specific HPR increasing by 271% when adding GaR at 300 rpm, as well as a HY
433 increase from 3.0 to 3.5 mol H_2/mol glucose. Hence, we conclude that recirculation of H_2-
434 rich biogas is an efficient method to prevent hydrogen supersaturation and allow high
435 production rates and yields without negatively effecting the hydrogen content of the
436 produced biogas.

437

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Fig. 1: Glucose consumption (A), cumulative hydrogen production (B) and biomass growth (C) throughout the dark fermentation of 27.8 mM of glucose by *T. neapolitana cf. capnolactica* using 300 and 500 rpm agitation speed, excluding or including recirculation of the produced gas (GaR).

Fig. 2: Specific rates of biomass growth (BGR), glucose consumption (GCR) and hydrogen production (HPR) obtained during the dark fermentation of 27.8 mM of glucose by 0.79 (± 0.03) g CDW /L of *T. neapolitana cf. capnolactica* using 300 and 500 rpm agitation speed, excluding or including recirculation of the produced gas (GaR). Specific rates were calculated based on the initial biomass concentration in the reactor expressed as cell dry weight (CDW).

Fig. 3: Hydrogen production rate (HPR) (A) and concentration of hydrogen in the liquid phase (H_{2aq}) (B) at different operating conditions using 100, 200, 300 and 500 rpm as well as 200 rpm and 500 rpm with gas recirculation (GaR) for 2 h during the dark fermentation of 27.8 mM of glucose by *T. neapolitana cf. capnolactica*. ("HPR post GaR" – retention phase, i.e. 25 min after applying GaR; "HPR" – equilibrium phase, i.e. 90 min before the subsequent GaR; "HPR GaR" – removal phase, i.e. when applying GaR for 5 min). The dashed line (----) represents the H_{2aq} in equilibrium with the gas phase at 65% H_2 at 80 °C, i.e. 9.7 mL/L.

Fig. 4: Cumulative hydrogen production (•) and H_{2aq} evolution (□) during the dark fermentation of 27.8 mM of glucose by *T. neapolitana cf. capnolactica* under different operating conditions: (A) 100, 300 and 500 rpm agitation speed with 5 min of gas recirculation (GaR) between each condition; (B) and (C) 100, 200 and 200 rpm + GaR. The dashed line (----) represents the H_{2aq} in equilibrium with the gas phase at 65% H_2 at 80 °C, i.e. 9.7 mL/L.

Table 1: Different techniques applied to decrease the H_{2aq} and reduce its inhibition on dark fermentation. A "x" was used to indicate if H_{2aq} was directly measured in the referenced study or an effect on H_2 yield and production rate was observed.

Table 2: Operating conditions during assay 2. 100, 200, 300 and 500 represent the agitation speed in rpm.

Table 3: Hydrogen production, biomass growth and glucose consumption rates during the dark fermentation of 27.8 mM of glucose by *T. neapolitana cf. capnolactica* at 300 and 500 rpm, excluding or including recirculation of the produced gas (GaR). The rates and lag phase were determined through data fitting with a modified Gompertz model. The adequateness of the fit is illustrated by the coefficient of determination (R^2). Specific rates are calculated based on the initial biomass concentration in the reactor.

Table 4: Hydrogen yield (HY), biomass yield (BMY) and glucose consumption obtained in a 2 L batch reactor inoculated with 0.79 (± 0.03) g CDW/L of *T. neapolitana cf. capnolactica*, performing the dark fermentation of 27.8 mM of glucose at 300 and 500 rpm agitation speed, in the presence or absence of recirculation of the produced gas (GaR).

Table 5: Composition of the digestate at the end of the fermentation of 27.8 mM of glucose by *T. neapolitana cf. capnolactica* using 300 and 500 rpm agitation speed with or without the recirculation of the produced gas (GaR).

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H_2-rich biogas recirculation prevents hydrogen supersaturation and enhances hydrogen production by *Thermotoga neapolitana cf. capnolactica*

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Abstract

This study focused on the supersaturation of hydrogen in the liquid phase (H_{2aq}) and its inhibitory effect on dark fermentation by *Thermotoga neapolitana cf. capnolactica* by increasing the agitation (from 100 to 500 rpm) and recirculating H_2-rich biogas (GaR). At low cell concentrations, both 500 rpm and GaR reduced the H_{2aq} from 30.1 (± 4.4) mL/L to the lowest values of 7.4 (± 0.7) mL/L and 7.2 (± 1.2) mL/L, respectively. However, at high cell concentrations (0.79 g CDW/L), the addition of GaR at 300 rpm was more efficient and increased the hydrogen production rate by 271%, compared to a 136% increase when raising the agitation to 500 rpm instead. While H_{2aq} primarily affected the dark fermentation rate, GaR concomitantly increased the hydrogen yield up to 3.5 mol H_2/mol glucose. Hence, H_{2aq} supersaturation highly depends on the systems gas-liquid mass transfer and strongly inhibits dark fermentation.

Key words: *Thermotoga neapolitana*; Dark fermentation; Gas recirculation; Sparging; Hydrogen supersaturation; End product inhibition

Declarations of interest: none

Highlights

- H_2 supersaturates in the liquid phase at 100 rpm despite hyperthermophilic conditions.
- High H_{2aq} concentrations directly inhibit the specific rates of dark fermentation.
- H_2 supersaturation and H_2 production rate depend on the gas-liquid mass transfer.
- H_2-rich biogas recirculation is an effective method to prevent H_2 supersaturation.
- Gas recirculation increased the hydrogen production rate by 271%.

Abbreviations

AA	acetic acid
BGR	biomass growth rate
BMY	biomass yield
CSTR	continuously stirred tank reactor
GaR	recirculation of H_2-rich biogas
GCR	glucose consumption rate
H_{2aq}	liquid phase hydrogen
HPR	hydrogen production rate
HY	hydrogen yield
LA	lactic acid

1. Introduction

Hydrogen, a nonpolluting energy carrier can be produced from organic residues in an environmental-friendly biological process called dark fermentation [1,2]. Hydrogen production has been reported in a wide range of temperatures, with thermophilic conditions being generally correlated to higher hydrogen yields (HY) [1,2]. A variety of microbial species are capable of fermentative hydrogen production [3] in both mixed and pure culture applications [4]. The use of mixed cultures is considered to be the simpler and have a more practical approach [4], but often entailing the coexistence of microorganisms which do not produce or even consume hydrogen [5].

Thermotoga neapolitana (hereafter *T. neapolitana*) is a hyperthermophilic microorganism with a high potential for hydrogen production via dark fermentation [6,7]. Some of the key characteristics exhibited by this organism include fast growth kinetics [7], and efficient degradation of a wide range of substrates, e.g. glucose, fructose, xylose, maltose, starch [7,8], molasses, cheese whey [9], algal biomass [10], and carrot pulp [11]. *T. neapolitana* is capable to approach the theoretical HY of 4 mol H_2/mol glucose by optimizing the conditions for the hydrogen forming acetate pathway [12,13]. Additionally, the hyperthermophilic culture conditions of *T. neapolitana* further stimulate the reaction rates [2] and hamper the growth competitive microorganisms [14], also leading to a pasteurization of the used substrate [15].

In dark fermentative hydrogen production, the presence of hydrogen is one of the most crucial factors, as high concentrations of hydrogen are a major inhibitor of the process. To reduce the inhibitory effect of hydrogen on the process, previous experimental studies (Table 1) have employed a variety of techniques, e.g. sparging the headspace, reducing the total pressure, applying vigorous stirring, optimizing the reactor design, or simply increasing

95 the headspace/solution ratio [12]. Regardless of the method used, alleviation of hydrogen
96 inhibition results in increased HYs and/or hydrogen production rates (HPR).
97 The headspace hydrogen concentration has been widely used (Table 1) to predict hydrogen
98 in the liquid phase (H_{2aq}), which *de facto* acts on the microbial cultures. However, poorly
99 soluble gases, such as hydrogen, supersaturate in solution during anaerobic processes
100 [31,32]. Studies, including a direct measurement of H_{2aq} (Table 1), have observed that H_{2aq}
101 can exceed the equilibrium concentration suggested by Henry's law multiple times. As a
102 result, the measurement of headspace hydrogen is considered inappropriate to predict the
103 H_{2aq} value [2,16–18,31,32].

104 Our study focused on the effect of agitation and biogas recirculation on the accumulation of
105 H_{2aq} and the consequent impact on dark fermentation by *T. neapolitana*. The main goal was
106 to demonstrate that the recirculation of the H_2-rich biogas (GaR) into the culture broth is an
107 effective method to improve the gas-liquid mass transfer and prevent H_{2aq} from
108 supersaturating, without diluting the H_2 percentage in the biogas. Furthermore, we wanted
109 to show that keeping the H_{2aq} in equilibrium with the gas phase is sufficient to achieve high
110 hydrogen production yields and rates. In a first assay, the effect of GaR and agitation speed
111 on hydrogen production was evaluated using a highly concentrated *T. neapolitana* culture. In
112 a second assay, a direct measurement of H_{2aq} was included to demonstrate the correlation
113 between hydrogen supersaturation and the performance of dark fermentation.
114

2. Material and methods

2.1. Bacterial culture and medium

A pure culture of *Thermotoga neapolitana cf. capnolactica* [33] was used in all experiments. The cultivation and storage conditions for the culture, the preparation of inoculum for elevated cell concentrations (assay 1) and the modified ATCC 1977 medium used (containing 27.8 mM of glucose) were as described by Dreschke et al. [34]. The pH of the medium was adjusted to 7.5 prior to being autoclaved at 110 °C for 5 min. Prior to the inoculation, the medium was heated at 80 °C for 30 min and sparged with N_2 for 5 min to remove the dissolved oxygen.

2.2. Experimental design

All experiments were run in a 3-L fully controlled continuously stirred tank reactor (CSTR) (Applikon Biotechnology, the Netherlands) with a working volume of 2 L. The operating temperature was kept at a constant 80 °C and pH was automatically adjusted to 7 by adding 1M NaOH. GaR was applied by continuously pumping the produced biogas from the headspace to a gas dispersion device at the base of the reactor at a flow rate of 350 mL/min. The produced biogas was released into 500 mL water displacement systems to avoid pressure build-up and quantified every hour. Liquid samples of 2 mL were withdrawn each 30 or 60 min. The fermentation was considered completed when the reactors ceased to produce biogas. Fermentation time was approximated from the inoculation to the end of the fermentation. Each condition was conducted in duplicate to demonstrate reproducibility.

2.2.1. Assay 1 – Effect of agitation speed and gas recirculation on dark fermentation at high cell concentrations

Assay 1 was designed to study the effect of GaR and agitation speed on the dark fermentation process at high cell concentrations of *T. neapolitana*. The reactor was inoculated with 80 mL of concentrated inoculum [34] resulting in a biomass concentration of 0.79 (± 0.03) g cell dry weight (CDW)/L. The following operating conditions were investigated: 300 rpm agitation (300); 300 rpm agitation with gas recirculation (300 + GaR); 500 rpm agitation (500); 500 rpm agitation with gas recirculation (500 + GaR).

2.2.2. Assay 2 – Effect of GaR and agitation speed on H_{2aq}

Assays 2A and 2B were designed to study the effect of agitation speed and GaR on the retention of hydrogen in the liquid phase and its influence on the dark fermentation process. The reactor was inoculated with 20 mL of storage culture (1% v/v) and maintained for 15 h at 100 rpm to acclimatize the culture. Afterwards, a sequence of operating conditions was used with each condition being operated for 2 h as described in Table 2. In assay 2A, 5 min of 500 rpm + GaR (Table 2 – shaded grey cells) was used before each operating condition to remove the accumulated hydrogen from the liquid phase. After 5 or 30 min and at the end of each operating condition, 20 mL of broth was withdrawn to monitor H_{2aq}.

2.3. Analytical Methods

2.3.1. Assay 1

Cell growth was determined by measuring spectrophotometrically (Lambda 365, Perkin Elmer, USA) optical density (OD_{540}) of the liquid samples at 540 nm. Liquid samples were centrifuged at 10,000 rpm for 5 min to collect the supernatant for the determination of glucose, acetic acid (AA), lactic acid (LA) and alanine concentration as described by Dreschke et al. [34]. The glucose concentration was measured by the dinitrosalicylic acid method [35], while AA, LA and alanine were quantified by ^{1}H Nuclear Magnetic Resonance (NMR) with a

600 MHz spectrometer (Bruker Avance 400) as described by Dipasquale et al. [10]. After the completion of the fermentation, 500 mL of culture broth was centrifuged at 3750 rpm for 20 min for the determination of CDW via freeze drying. The concentration of hydrogen in the produced gas was analyzed by gas chromatography as described by Dipasquale et al. [10]. The conversion from volumetric to molar H_2 production was performed using the ideal gas law.

2.3.2. Assay 2

Glucose, AA and LA concentrations were determined using an HPLC (Prominence LC-20A Series, Shimadzu, Japan), equipped with UV/Vis (SPD-20A, Shimadzu Japan) and refractive index (RID-20A, Shimadzu, Japan) detectors, with the method described by Mancini et al. [36] with 0.0065 M of sulfuric acid as the mobile phase. The concentration of hydrogen in the biogas was determined with a Varian 3400 gas chromatograph (GC), equipped with a thermal conductivity detector (TCD) and a Restek packed column using argon as the carrier gas.

For the determination of H_{2aq}, a modified method of Kraemer and Bagley [16,17,37] was applied. Vials with a total volume of 31 mL were closed with silicon septa and depressurized using a plastic syringe. 20 mL of sample was injected into the vials and immediately placed upside down in fridge to equilibrate the liquid and the gas phases. After around 20 h, the sample was heated at room temperature and the negative pressure equilibrated by adding air to the gas phase of the sample. The concentration of hydrogen in the gas phase was measured by the GC described above and referred to the 20 mL of liquid sample.

2.4. Kinetic study of dark fermentation

The rates and lag phases of dark fermentation were evaluated in terms of biomass growth, hydrogen production and glucose consumption and calculated by fitting the experimental data with a modified Gompertz model as described by Dreschke et al. [34].

2.5. Statistical analysis

The statistical significance of the experimental data was determined by the calculation of the p-value applying an unpaired t-test with Microsoft Excel 2016 (Microsoft Corporation, USA).

3. Results

3.1. Effect of agitation speed and GaR on dark fermentation at high cell concentrations (assay 1)

Fig. 1. shows the glucose consumption (A), cumulative hydrogen (B) and biomass growth (C) during batch fermentation with an initial cell concentration of 0.79 (± 0.03) g CDW/L of *T. neapolitana* using an agitation of 300 or 500 rpm with and without GaR.

Increasing the agitation significantly enhanced the specific rates of biomass growth [mg CDW/h/g CDW], hydrogen production [mL H_2/h/g CDW], and glucose consumption [mmol glucose/h/g CDW] from 73 (± 11), 294 (± 44), and 4.4 (± 1.3) at 300 rpm to 266 (± 9) (p-value: 0.003), 695 (± 46) (p-value: 0.012), and 10.1 (± 0.6) (p-value: 0.028) at 500 rpm (Fig. 2, Table 3), respectively. Due to the accelerated process, the fermentation time and lag phase of hydrogen production were reduced from 11 h and 2.9 (± 0.2) h at 300 rpm to 6 h and from 1.3 (± 0.1) h at 500 rpm (Table 3), respectively. The HY increased from 3.0 (± 0.0) at 300 rpm to 3.2 (± 0.1) mol H_2/mol glucose at 500 rpm, while the biomass yield (BMY) increased from approximately 16.7 to 21.9 g CDW/mol glucose (Table 4). The composition of the end products, expressed in terms of an LA/AA ratio, decreased from 0.31 at 300 rpm to 0.21 at 500 rpm (Table 5).

A combination of GaR and agitation speed at 300 rpm further accelerated the dark fermentation. The specific biomass growth rate reached 423 (± 9) mg CDW/h/g CDW (p-value: 0.004), while the specific rates of hydrogen production and glucose consumption increased up to 1090 (± 91) mL/h/g CDW (p-value: 0.032) and 13.3 (± 0.8) mmol glucose/h/g CDW (p-value: 0.040), respectively, over those obtained at 500 rpm without GaR (Fig. 2, Table 3). The fermentation was completed within 4 h with a lag phase of 0.4 (± 0.0) h (Table 3), a HY of 3.5 (± 0.2) mol H_2/mol glucose, an approximate BMY of 24.8 g CDW/mol glucose (Table 4), and a LA/AA ratio of 0.11 (Table 5).

Increasing the agitation to 500 rpm while applying GaR had no significant effect on the process, as shown by the similar performance obtained at 300 rpm + GaR and 500 rpm + GaR (Fig. 1). At 500 rpm + GaR, the specific rates of biomass growth, hydrogen production and glucose consumption were 431 (± 9) mg CDW/h/g CDW, 1016 (± 22) mL H_2/h/g CDW and 12.7 (± 0.2) mmol glucose/h/g CDW (Fig. 2, Table 3), respectively. The HY reached 3.3 (± 0.1) mol H_2/mol glucose (Table 4) coupled with a LA/AA ratio of 0.13 (Table 5). The fermentation time and lag phase of hydrogen production remained at 4 h and 0.5 (± 0.1) h (Table 3), respectively, with an approximate BMY of 27.7 g CDW/mol glucose.

The highest volumetric HPRs of 850 (± 71) and 813 (± 18) mL/h/L were obtained when GaR was applied at 300 and 500 rpm, respectively (Table 3). The hydrogen concentration in the biogas produced was not affected by the use of GaR or the agitation speed and remained constant at 65 (± 2)% (data not shown) under all studied conditions. In all bioassays, glucose was consumed up to 82 - 89 % (Table 4) with AA and LA as the main fermentation end products in the digestate and alanine constituting a minor proportion of 2% (Table 5). A mass balance based on the stoichiometric equations 1 and 2 was performed to validate the experimental results. The dark fermentation model suggests the formation of two moles of

fermentation end products (AA and LA) per mole of glucose with the concomitant production of 2 moles of hydrogen per mole of AA. Under all operating conditions, the sum of fermentation products in the digestate reached 92% or more of the theoretical value calculated from the glucose consumption and 2.08 (± 0.01) moles H_2 per mole of AA were averagely produced.

$$C_6H_{12}O_6 + 4\,ADP + 4\,H_2PO_4^- \leftrightarrow 2\,CH_3CO_2H + 2\,CO_2 + 4\,ATP + 4\,H_2 + 2\,H_2O \quad (1)$$

$$C_6H_{12}O_6 + 2\,ADP + 2\,H_2PO_4^- \leftrightarrow 2\,CH_3CH(OH)CO_2H + 2\,ATP + 2\,H_2O \quad (2)$$

3.2. Effect of GaR and agitation speed on H_{2aq} and HPR (assay 2)

3.2.1. Assay 2A – Use of different agitation speeds

Assay 2A assessed the effect of the agitation speed on H_{2aq} and HPR. After the initial 15 h at 100 rpm, H_{2aq} accumulated up to 30.1 (± 4.4) mL/L (Fig. 3B). Then, using 500 rpm + GaR for 5 min before each operating condition reduced H_{2aq} to an average of 7.5 (± 1.0) mL/L (Fig. 4A). The subsequent agitation of the culture at 300 and 500 rpm without GaR for 2 h led to a H_{2aq} of 17.0 (± 2.3) and 7.4 (± 0.7) mL/L, respectively (Fig. 3B).

To better demonstrate the effect of hydrogen retention in the liquid phase on the HPR, the 2 h duration of each operating condition was subdivided in 3 phases and the HPR was calculated for each individual phase: 1 – removal phase, i.e. period from 0 to 5 min after changing the operating condition when additional GaR was applied (“HPR GaR”); 2 – retention phase, i.e. period from 5 to 30 min after changing the operating condition (“HPR post GaR”); 3 – equilibrium phase, i.e. period from 30 to 120 min after changing the operating condition (“HPR”) (Fig. 3A). At 300 rpm, a significantly higher “HPR GaR” of 316 (± 54) mL/L/h (Fig. 3A) was observed than “HPR” of 54 (± 13) mL/L/h (p-value: 0.0002) and

252 “HPR post GaR” of 19 (± 17) mL/L/h (p-value: 0.0017), respectively. In contrast, at 500 rpm,
253 the HPR remained similar before, during, and after GaR. The “HPR” reached 112 (± 22)
254 mL/L/h, which was not significantly different from “HPR post GaR” (100 (± 16) mL/L/h, p-
255 value: 0.397) and “HPR GaR” (120 (± 11) mL/L/h, p-value: 0.614) (Fig. 3A). Changing the
256 agitation from 300 to 500 rpm roughly doubled the “HPR”, and concomitantly increased the
257 glucose consumption rate (GCR) from 3.6 (± 0.4) to 4.7 ± (0.3) mmol/L/h (p-value: 0.016)
258 (data not shown).

259 ### 3.2.2. Assay 2B – Gas sparging

260 Assay 2B was run to assess the effect of GaR on H_{2aq} and HPR at a constant agitation at 200
261 rpm. Similar to assay 2A, the highest H_{2aq} of 26.8 (± 3.6) mL/L (Fig. 4B and C) was obtained
262 after 15 h of operation at 100 rpm. Applying 200 rpm + GaR rapidly decreased the H_{2aq} to 9.0
263 (± 1.8) mL/L in 5 min (Fig. 4B and C) reaching a final value of 7.2 (± 1.2) mL/L after 2 h (Fig.
264 4B). The 200-rpm agitation without GaR caused an accumulation of H_{2aq} up to a
265 concentration of 16.4 (± 0.9) mL/L in 30 min, which was 91% of 18.0 (± 0.9) mL/L reached
266 after 2 h (Fig. 4B and C). The “HPR” at 200 rpm + GaR of 91 (± 20) mL/L/h was significantly
267 higher than 47 (± 8) mL/L/h (p-value: 0.017) obtained at 200 rpm without GaR (Fig. 3A).
268 Concomitantly, the GCR reached values of 4.2 (± 0.1) and 3.1 (± 0.8) mmol/L/h (p-value:
269 0.077), at 200 rpm + GaR and 200 rpm without GaR, respectively (data of GCR not shown).
270 When agitation was increased from 100 to 200 rpm after 15 h in the absence of GaR, H_{2aq}
271 decreased from 29.4 to 24.8 mL/L in 2 h (Fig. 4C), which was higher than 18.7 (± 4.6) mL/L
272 obtained at 200 rpm agitation at 21 h after applying GaR between 17 and 19 h.

273 ## 4. Discussion

274 ### 4.1. Effect of agitation speed and gas recirculation on H_{2aq} concentration

In this study, GaR and agitation speed were examined as strategies to lower H_{2aq} and, thus, stimulate H_2 production by *T. neapolitana*. Agitating at 100 rpm led to the highest H_{2aq}, i.e.30.1 (± 4.4) mL/L, which was approximately 3 times higher than the theoretical dissolved hydrogen concentration (i.e. 9.7 mL/L) in equilibrium with a gas phase containing 65% H_2 at 80 °C according to Henry's Law (Eq. 3):

$$H(T) = H^{\Theta} * \exp\left(\frac{d\ln(H)}{d(1/T)} * \left(\frac{1}{T} - \frac{1}{T^{\Theta}}\right)\right) \quad (3)$$

where H(T) and H^{Θ} are Henry's constants expressed in mol/m^3/Pa at temperature T and T^{Θ} (= 298.15 K), respectively; H^{Θ} equals 7.7 x 10^{-6} [38]; *d*ln (*H*)/*d*(1/T) equals 500 K [38] and is the temperature dependence factor of Henry's constant; and T is the temperature in K.

Increasing the agitation speed to 500 rpm effectively improved the gas-liquid mass transfer, decreasing the H_{2aq} concentration (Fig. 4 and 3B) until reaching values below the theoretical equilibrium concentration. Similarly, GaR maintained the hydrogen partial pressure of gas and liquid phase in equilibrium (Fig. 3B).

The extent of H_{2aq} accumulation is determined by the HPR and the mass transfer from liquid to gas phase (Eq. 4) [17,32].

$$\frac{dH_{aq}}{dt} = HPR - k_L a\,(H_{aq} - H_{dis}) \quad (4)$$

with H_{2aq} being the hydrogen concentration in the liquid phase [mL/L]; H_{dis} the concentration of dissolved hydrogen at the thermodynamic equilibrium; HPR the volumetric production of hydrogen in the liquid phase [mL/L/h]; k_La the global mass transfer coefficient [1/h] made up of two terms, i.e. k_L (the "film" coefficient) and *a* (the specific interfacial area per unit of liquid volume in the reactor).

When the HPR exceeds the gas-liquid mass transfer of the system, H_{2aq} supersaturates. However, elevated H_{2aq} concentrations inhibit the HPR, which again reduce the degree of

298 H_{2aq} supersaturation (Eq. 4) until HPR and H_{2aq} reach an equilibrium. Because of this
299 feedback inhibition, the HPR of a production system is in many cases primarily determined
300 by an insufficient gas-liquid mass transfer instead of the kinetic potential of the culture.

301 The evolution of H_{2aq} accumulation and its dependence on the HPR and the mass transfer
302 was clearly shown in the 3 phases of assay 2A at 300 rpm (Fig. 4A). In the retention phase, a
303 low "HPR post GaR" of 19 (± 17) mL/L/h (Fig. 3A) was obtained as HPR was strongly affected
304 by the fast retention of hydrogen in the liquid phase (Fig. 4A). The "HPR" during the
305 equilibrium phase was instead higher, reaching 54 (± 13) mL/L/h (Fig. 3A). Contrary to the
306 "HPR post GaR", "HPR" was no longer affected by hydrogen retention but rather by the
307 prevalent H_{2aq} of 17.0 (± 2.3) mL/L, which was determined by the mass transfer of the
308 system (Fig. 3B). The subsequent application of GaR after 2 h removed the accumulated
309 hydrogen from the liquid phase, leading to a high "HPR GaR" of 316 (± 54) mL/L/h and a
310 decrease of H_{2aq} to 7.5 (± 1.0) mL/L (Fig. 3A and B). The length of the retention phase was
311 based on the results of assay 2B, where a cultivation at 200 rpm after GaR increased the H_{2aq}
312 to 16.4 (± 0.9) mL/L in 30 min, i.e. 91% of 18.0 (± 0.9) mL/L measured after 2 h (Fig. 4B and
313 C). The accumulation of H_{2aq} was, therefore, considered completed at the end of the
314 retention phase and the HPR in equilibrium with H_{2aq} as explained in section 4.1 (Eq. 4). At
315 500 rpm, hydrogen did not accumulate in the liquid phase as demonstrated by the low H_{2aq}
316 of 7.4 (± 0.7) mL/L after 2 h (Fig. 3B) and the statistically similar values of "HPR post GaR",
317 HPR" and "HPR GaR", i.e. 110 (± 18) mL/L/h (Fig. 3A).
318 In conclusion, at low cell concentrations the sole agitation at 500 rpm as well as GaR
319 provided an adequate mass transfer to remove the produced hydrogen from the liquid to
320 the gas phase and prevent hydrogen supersaturation.

321 **4.2. Enhancement of dark fermentation kinetics**

322 Independent of the technique applied, the reduction of H_{2aq} in assay 2 resulted in a
323 significant increase of the HPR. Adding GaR increased the "HPR" by 94% from 47 (± 8) to 91
324 (± 20) mL/L/h at 200 rpm, whereas increasing the agitation from 300 to 500 rpm enhanced
325 the "HPR" by 107% from 54 (± 13) to 112 (± 22) mL/L/h (Fig. 3A), both accompanied by a
326 simultaneous increase of the GCR.
327 In assay 1, we accelerated the dark fermentation process by using a higher cell concentration
328 (0.79 g CDW/L). At the lowest agitation of 300 rpm, we observed a specific HPR of 294 (± 44)
329 mL H_2/h/g CDW. Increasing the agitation to 500 rpm accelerated the dark fermentation
330 process, enhancing the specific HPR by 136% to 695 (± 46) mL H_2/h/g CDW. As observed in
331 assay 2, the higher HPR obtained at 500 rpm was likely due to a decreased H_{2aq}
332 concentration. However, the addition of GaR was capable to further increase the HPR to
333 1090 (± 91) and 1016 (± 22) mL H_2/h/g CDW at 300 + GaR and 500 + GaR, respectively, i.e.
334 271 and 246% higher than the HPR achieved at 300 rpm in the absence of GaR. This indicates
335 that, in contrast to what was observed at low cell concentrations, 500 rpm agitating was
336 likely insufficient to completely prevent hydrogen supersaturation at high cell
337 concentrations. In contrast, GaR effectively enhanced the gas-liquid mass transfer (by
338 increasing the specific interfacial area *a* in Eq. 4) and maintained the H_{2aq} low, leading to the
339 highest values of specific HPR. The similar reactor performance at 300 + GaR and 500 + GaR
340 furthermore shows that hydrogen supersaturation was the crucial factor limiting the process
341 as, once it was prevented by GaR, the agitation speed had no influence on the process.
342 The present results are in line with those obtained by Beckers et al. [29], who observed an
343 enhancement of the HPR from 0.14 to 0.26 L/h when agitation was increased from 0 to 400
344 rpm in 2-L reactors using *Clostridium butyricum* at 30°C and glucose as a substrate at a feed

concentration of 27.8 mM. However, in that study the H_{2aq} was observed to remain high at approximately 4 times the equilibrium concentration despite 400 rpm agitation.

In our experiments, the specific rates of glucose consumption and biomass growth were closely linked to the HPR (Table 3), showing a similar enhancement when the different measures to prevent H_{2aq} supersaturation were applied. The specific GCR and biomass growth rate (BGR) at high cell concentrations increased by 202% and 479% from 4.4 (± 1.3) to 13.3 (± 0.8) mmol glucose/h/g CDW and from 73 (± 11) to 423 (± 9) mg CDW/h/g CDW at 300 rpm and 300 rpm + GaR, respectively. This acceleration of the process led to a reduction of the total fermentation time from 11 and 6 h with the sole agitation at 300 and 500 rpm, respectively, to 4 h when GaR was applied independent from the agitation speed.

Ljunggren et al. [17] successfully increased the HPR by decreasing H_{2aq} through N_2 headspace sparging using *Caldicellulosiruptor saccharolyticus*. The authors stated that the culture self-regulates the growth rate as a response to high H_{2aq} to slow down the process, decrease the HPR and consequently prevent the H_{2aq} from reaching inhibitory levels (Eq. 4). However, in our study the effect on the specific HPR, BGR and GCR was simultaneous (Fig. 2), indicating that H_{2aq} directly acts on all rates of dark fermentation.

4.3. Hydrogen and biomass yields under different operating conditions and H_{2aq} concentrations

Despite the inhibition by H_{2aq} discussed in section 4.2, the HY remained high at 3.0 (± 0.0) mol H_2/mol glucose (Table 4) when applying 300 rpm agitation without GaR in assay 1. The HY increased even further to 3.5 mol H_2/mol glucose coupled with a decrease of the LA/AA ratio (Table 4) when GaR maintained the H_{2aq} in equilibrium with the gas phase.

T. neapolitana predominately ferments glucose via the AA and the LA pathways, with the former leading to hydrogen production and one additional mole of ATP [7].

The AA pathway is thereby energetically more challenging [1], because the formation of hydrogen requires a high free Gibbs energy change as protons are poor electron acceptors [39]. The reaction to gaseous hydrogen is favored by low hydrogen concentrations and high temperatures as explained in detail by Verhaart et al. [39]. This indicates that the temperature was most likely responsible for the high HYs at sole agitation of 300 rpm. The additional decrease of H_{2aq} by GaR caused an additional energetical advantage and allowed the further shift towards the AA pathway leading to the increase of the HY.

A similar effect on the HY was obtained by Beckers et al. [29], measuring H_{2aq} in 200 mL serum bottles inoculated with *Clostridium butyricum* at 30°C. Unstirred cultures experienced a supersaturation up to 7 times the equilibrium concentration resulting in a reduced HY of 1.16 mol H_2/mol glucose, in comparison to 1.52 mol H_2/mol glucose obtained when agitating at 120 rpm. The inhibition by hydrogen has also been demonstrated previously in *T. neapolitana* cultivation using closed 120 mL serum bottles. d'Ippolito et al. [12] were able to increase the HY by reducing the culture/headspace ratio leading to a lower hydrogen partial pressure in the gas phase.

When GaR was applied at high cell concentrations, a lower LA/AA ratio indicates that more energy was gained by the culture per unit of substrate. Concomitantly, a higher BMY was observed, which suggests that biomass production did not, as often assumed, compete with the final product formation, but rather increased simultaneously when the environmental conditions were optimized. The decrease of the biomass concentration at 500 rpm depicted in Fig. 1C was caused by a change of *T. neapolitana* cell morphology in response to nutrient limitations. This phenomenon interferes with the optical density measurement as described in more detail by Dreschke et al. [34], whereas the actual biomass concentration is not affected. In our study, the variation of HY and BMY was much lower than that of HPR under

different operating conditions, indicating that hydrogen inhibition primarily acts on the process rates. We assume that a reduction of the dark fermentation rate by the culture at high H_{2aq} concentrations aims to prevent the inhibition of the AA pathway which allows the higher energy yield.

4.4. Biogas recirculation as a strategy to prevent hydrogen supersaturation

A large number of studies used headspace sparging with gases such as N_2 or CO_2 (Table 1) to counteract hydrogen inhibition by lowering H_{dis} (Eq. 4). For instance, Sonnleitner et al. [26] observed an increase of HPR from 25 to 119 mL/L/h when sparging the headspace of a *Caldicellulosiruptor saccharolyticus* culture with N_2 at 1 L/L/h. However, while sparging with external gases is generally successful to improve the process performance (Table 1), it inevitably leads to an undesired dilution of the produced hydrogen and consequently an increase of costs for gas purification. In contrast, the concentration of hydrogen in the produced gas remains high when GaR is applied.

To our knowledge, only two studies have so far focused on the recirculation of the H_2-rich biogas in dark fermentation. Kim et al. [22] used GaR at flow rates ranging from 100 to 400 mL/min in a CSTR with a mixed culture at 35°C. They obtained similar HPRs and HYs, i.e. 0.77–0.86 and 0.77 mol H_2/ mol hexose, respectively, with and without GaR. This suggests that at the prevalent reactor conditions, hydrogen did not supersaturate in the liquid phase which rendered GaR ineffective. Bakonyi et al. [23] recirculated the internal biogas at two different H_2 concentrations into a CSTR using a mixed culture at 35°C. The recirculation of the less concentrated gas (50% H_2) resulted in a HPR of 8.9 – 9.2, whereas the use of a more concentrated gas (66% H_2) reduced the HPR to 2.7 – 3.0 L H_2/L/d, compared to 7.4 L H_2/L/d obtained without recirculation. Unfortunately, a direct measurement of H_{2aq} was not included in any of the two studies, making the correlation of the reactor performance to H_{2aq}

417 impossible. In our study, we directly demonstrate the impact of stirring speed and GaR on
418 H_{2aq} and correlate it to the process performance. GaR has proven highly effective to provide
419 an adequate gas-liquid mass transfer, that can be readily adjusted by adapting the
420 recirculation flow to meet the requirements of the system. Furthermore, the results confirm
421 that maintaining H_{2aq} in equilibrium with the gas phase is sufficient to reach high HY up to
422 3.5 mol H_2/mol glucose (Table 4) and a high specific HPR of 1090 ± 91 mL/h/g CDW (Table 3).

423 **Conclusions**

424 This study confirms that hydrogen supersaturates in the liquid phase and strongly inhibits
425 dark fermentation by *T. neapolitana cf. capnolactica*. GaR and the sole agitation at 500 rpm
426 efficiently reduced the H_{2aq} (i.e. 30.1 ± 4.4 mL/L) observed at low cell concentration and
427 agitation of 100 rpm to below the equilibrium value suggested by Henry´s law (i.e. 9.7 mL/L).
428 At high cell concentrations (i.e. 0.79 g CDW/L), 500 rpm agitation did not provide sufficient
429 gas-liquid mass transfer to prevent H_{2aq} supersaturation, which was instead counteracted by
430 GaR. High H_{2aq} concentrations led to thermodynamic constraints and the reciprocal influence
431 of H_{2aq} and HPR, which directly hampered the dark fermentation rates. In this line, we
432 observed the specific HPR increasing by 271% when adding GaR at 300 rpm, as well as a HY
433 increase from 3.0 to 3.5 mol H_2/mol glucose. Hence, we conclude that recirculation of H_2-
434 rich biogas is an efficient method to prevent hydrogen supersaturation and allow high
435 production rates and yields without negatively effecting the hydrogen content of the
436 produced biogas.

437

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Fig. 1: Glucose consumption (A), cumulative hydrogen production (B) and biomass growth (C) throughout the dark fermentation of 27.8 mM of glucose by *T. neapolitana cf. capnolactica* using 300 and 500 rpm agitation speed, excluding or including recirculation of the produced gas (GaR).

Fig. 2: Specific rates of biomass growth (BGR), glucose consumption (GCR) and hydrogen production (HPR) obtained during the dark fermentation of 27.8 mM of glucose by 0.79 (± 0.03) g CDW /L of *T. neapolitana cf. capnolactica* using 300 and 500 rpm agitation speed, excluding or including recirculation of the produced gas (GaR). Specific rates were calculated based on the initial biomass concentration in the reactor expressed as cell dry weight (CDW).

Fig. 3: Hydrogen production rate (HPR) (A) and concentration of hydrogen in the liquid phase (H_{2aq}) (B) at different operating conditions using 100, 200, 300 and 500 rpm as well as 200 rpm and 500 rpm with gas recirculation (GaR) for 2 h during the dark fermentation of 27.8 mM of glucose by *T. neapolitana cf. capnolactica*. ("HPR post GaR" – retention phase, i.e. 25 min after applying GaR; "HPR" – equilibrium phase, i.e. 90 min before the subsequent GaR; "HPR GaR" – removal phase, i.e. when applying GaR for 5 min). The dashed line (----) represents the H_{2aq} in equilibrium with the gas phase at 65% H_2 at 80 °C, i.e. 9.7 mL/L.

Fig. 4: Cumulative hydrogen production (•) and H_{2aq} evolution (□) during the dark fermentation of 27.8 mM of glucose by *T. neapolitana cf. capnolactica* under different operating conditions: (A) 100, 300 and 500 rpm agitation speed with 5 min of gas recirculation (GaR) between each condition; (B) and (C) 100, 200 and 200 rpm + GaR. The dashed line (----) represents the H_{2aq} in equilibrium with the gas phase at 65% H_2 at 80 °C, i.e. 9.7 mL/L.

Table 1: Different techniques applied to decrease the H_{2aq} and reduce its inhibition on dark fermentation. A "x" was used to indicate if H_{2aq} was directly measured in the referenced study or an effect on H_2 yield and production rate was observed.

Table 2: Operating conditions during assay 2. 100, 200, 300 and 500 represent the agitation speed in rpm.

Table 3: Hydrogen production, biomass growth and glucose consumption rates during the dark fermentation of 27.8 mM of glucose by *T. neapolitana cf. capnolactica* at 300 and 500 rpm, excluding or including recirculation of the produced gas (GaR). The rates and lag phase were determined through data fitting with a modified Gompertz model. The adequateness of the fit is illustrated by the coefficient of determination (R^2). Specific rates are calculated based on the initial biomass concentration in the reactor.

Table 4: Hydrogen yield (HY), biomass yield (BMY) and glucose consumption obtained in a 2 L batch reactor inoculated with 0.79 (± 0.03) g CDW/L of *T. neapolitana cf. capnolactica*, performing the dark fermentation of 27.8 mM of glucose at 300 and 500 rpm agitation speed, in the presence or absence of recirculation of the produced gas (GaR).

Table 5: Composition of the digestate at the end of the fermentation of 27.8 mM of glucose by *T. neapolitana cf. capnolactica* using 300 and 500 rpm agitation speed with or without the recirculation of the produced gas (GaR).

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Table 1

Table 1: Different techniques applied to decrease the H_{2aq} and reduce its inhibition on dark fermentation. A "x" was used to indicate if H_{2aq} was directly measured in the referenced study or an effect on H_2 yield and production rate was observed.

Counteracting measures	H_{2aq} measurement	Effect on yield	Effect on rate	Reference
Headspace sparging with N_2	x	x		[16–18]
Headspace sparging with CO_2		x	x	[19]
Headspace sparging with N_2/H_2, stirring		x		[20]
Sparging with biogas (CH_4)		x	x	[21]
CO_2/N_2 and internal gas injection		x		[22]
Internal CO_2 enriched gas injection		x	x	[23]
N_2 gas injection	x	x	x	[24]
Control of reactor pressure		x		[12]
Control of reactor pressure		x	x	[25,26]
Stirring and organic loading rate	x	x	x	[27]
Bubble induction		x	x	[28]
Stirring and surface enlargement	x	x	x	[29]
Recycling of degassed effluent		x	x	[30]

Table 2: Operating conditions during assay 2. 100, 200, 300 and 500 represent the agitation speed in rpm. Grey cells represent 5 min of agitation at 500 rpm in the presence of GaR.

Time [h]	0 – 15	15 – 17		17 – 19		19 – 21		21 – 23	
Assay 2A	100	GaR	300	GaR	500	GaR	300	GaR	500
2A (duplicate)	100	GaR	500	GaR	300	GaR	500	GaR	300
Assay 2B	100		200		200 + GaR		200		200 + GaR
2B (duplicate)	100		200 + GaR		200		200 + GaR		200

GaR = recirculation of the produced biogas

Table 3

Table 3: Hydrogen production, biomass growth and glucose consumption rates during the dark fermentation of 27.8 mM of glucose by *T. neapolitana cf. capnolactica* at 300 and 500 rpm, excluding or including recirculation of the produced gas (GaR). The rates and lag phase were determined through data fitting with a modified Gompertz model. The adequateness of the fit is illustrated by the coefficient of determination (R^2). Specific rates are calculated based on the initial biomass concentration in the reactor.

	Specific rate	**Volumetric rate**	**Lag phase**	**R^2**
Hydrogen production	[mL H_2/h/g CDW]	[mL H_2/L/h]	[h]	
300 rpm	294 ± 44	235 ± 35	2.9 ± 0.2	0.982
500 rpm	695 ± 46	535 ± 35	1.3 ± 0.1	0.991
300 rpm + GaR	1090 ± 91	850 ± 71	0.4 ± 0.0	0.994
500 rpm + GaR	1016 ± 22	813 ± 18	0.5 ± 0.1	0.991
Biomass growth	[mg CDW/h/g CDW]			
300 rpm	73 ± 11			0.981
500 rpm	266 ± 9			0.996
300 rpm + GaR	423 ± 9			0.996
500 rpm + GaR	431 ± 9			0.996
Glucose consumption	[mmol glucose/h/g CDW]			
300 rpm	4.4 ± 1.3			0.985
500 rpm	10.1 ± 0.6			0.990
300 rpm + GaR	13.3 ± 0.8			0.991
500 rpm + GaR	12.7 ± 0.2			0.985

Table 4

Table 4: Hydrogen yield (HY), biomass yield (BMY) and glucose consumption obtained in a 2 L batch reactor inoculated with 0.79 (± 0.03) g CDW/L of *T. neapolitana cf. capnolactica*, performing the dark fermentation of 27.8 mM of glucose at 300 and 500 rpm agitation speed, in the presence or absence of recirculation of the produced gas (GaR).

Operating condition	HY [mol H_2/mol glucose]	BMY [g CDW/mol glucose]	Glucose consumption [%]
300 rpm	3.0 ± 0.0	16.7	82
500 rpm	3.2 ± 0.1	21.9	89
300 rpm + GaR	3.5 ± 0.2	24.8	88
500 rpm + GaR	3.3 ± 0.1	27.7	89

Table 5: Composition of the digestate at the end of the fermentation of 27.8 mM of glucose by *T. neapolitana cf. capnolactica* using 300 and 500 rpm agitation speed with or without the recirculation of the produced gas (GaR).

Operating condition	AA [mM] (yield [mol/mol glu])	LA [mM] (yield [mol/mol glu])	Alanine [mM]	Ratio LA/AA	Residual glucose [mM]	End product balance [%][1]
300 rpm	32.3 ± 4.3 (1.4)	10.0 ± 1.0 (0.44)	1.1 ± 0.1	0.31	4.9 ± 2.7	96
500 rpm	37.7 ± 2.7 (1.5)	8.1 ± 0.2 (0.33)	1.0 ± 0.1	0.21	3.0 ± 0.4	95
300 rpm + GaR	39.2 ± 1.2 (1.6)	4.4 ± 0.1 (0.18)	0.9 ± 0.0	0.11	3.3 ± 0.2	92
500 rpm + GaR	38.7 ± 2.2 (1.6)	5.1 ± 0.5 (0.21)	0.8 ± 0.0	0.13	3.1 ± 0.4	92

[1] End product balance was calculated by summing AA, LA, alanine and residual glucose and referring it to the theoretical end product formation associated with the initial glucose concentration.

Figure 1

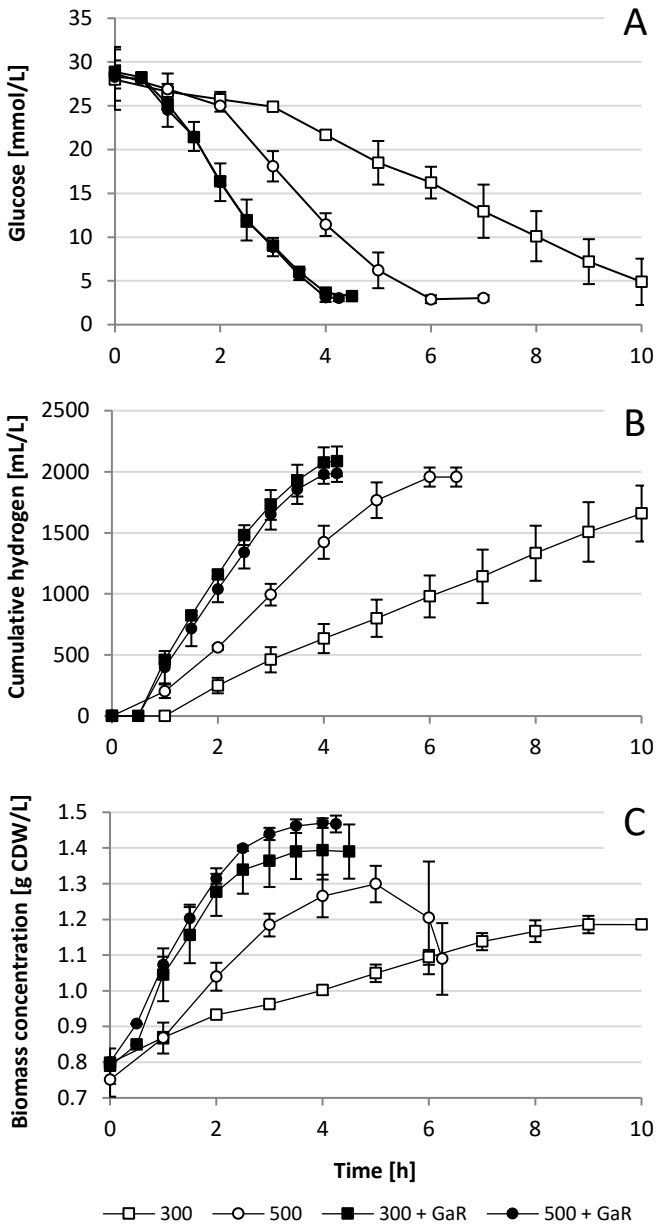

A
Glucose [mmol/L]
35
30
25
20
15
10
5
0
0
2
4
6
8
10
B
Cumulative hydrogen [mL/L]
2500
2000
1500
1000
500
0
C
Biomass concentration [g CDW/L]
1.5
1.4
1.3
1.2
1.1
1.0
0.9
0.8
0.7
Time [h]
300
500
300 + GaR
500 + GaR

Figure 2

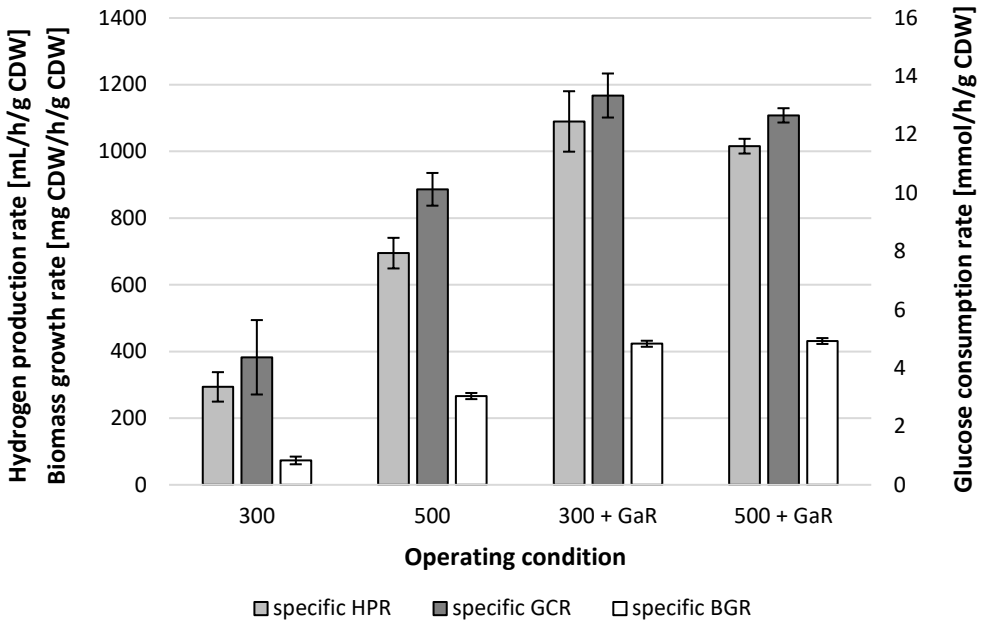

Hydrogen production rate [mL/h/g CDW]
Biomass growth rate [mg CDW/h/g CDW]
Glucose consumption rate [mmol/h/g CDW]
1400
1200
1000
800
600
400
200
0
16
14
12
10
8
6
4
2
0
300
500
300 + GaR
500 + GaR
Operating condition
specific HPR
specific GCR
specific BGR

Figure 3

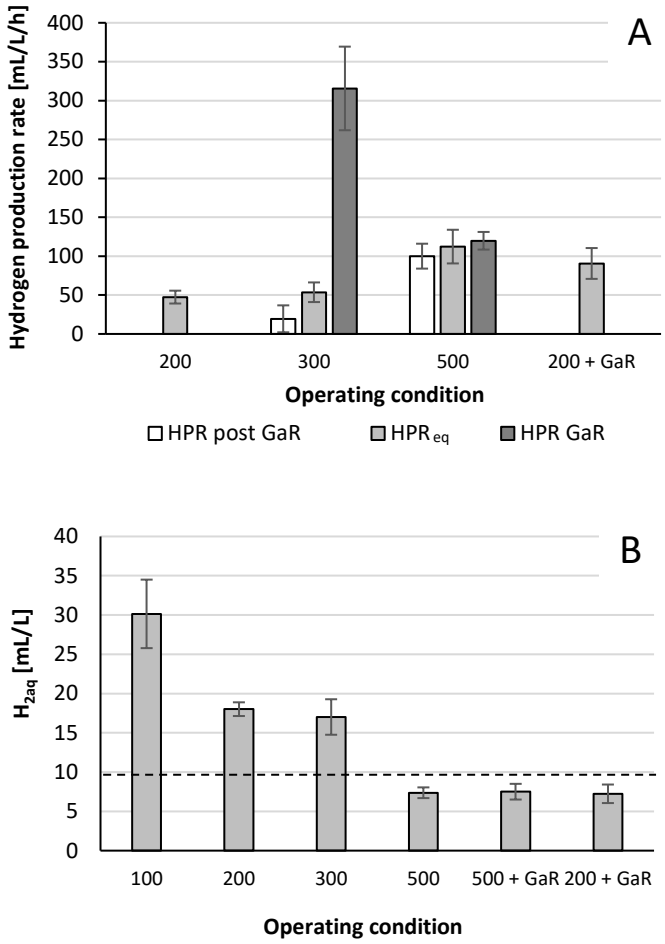
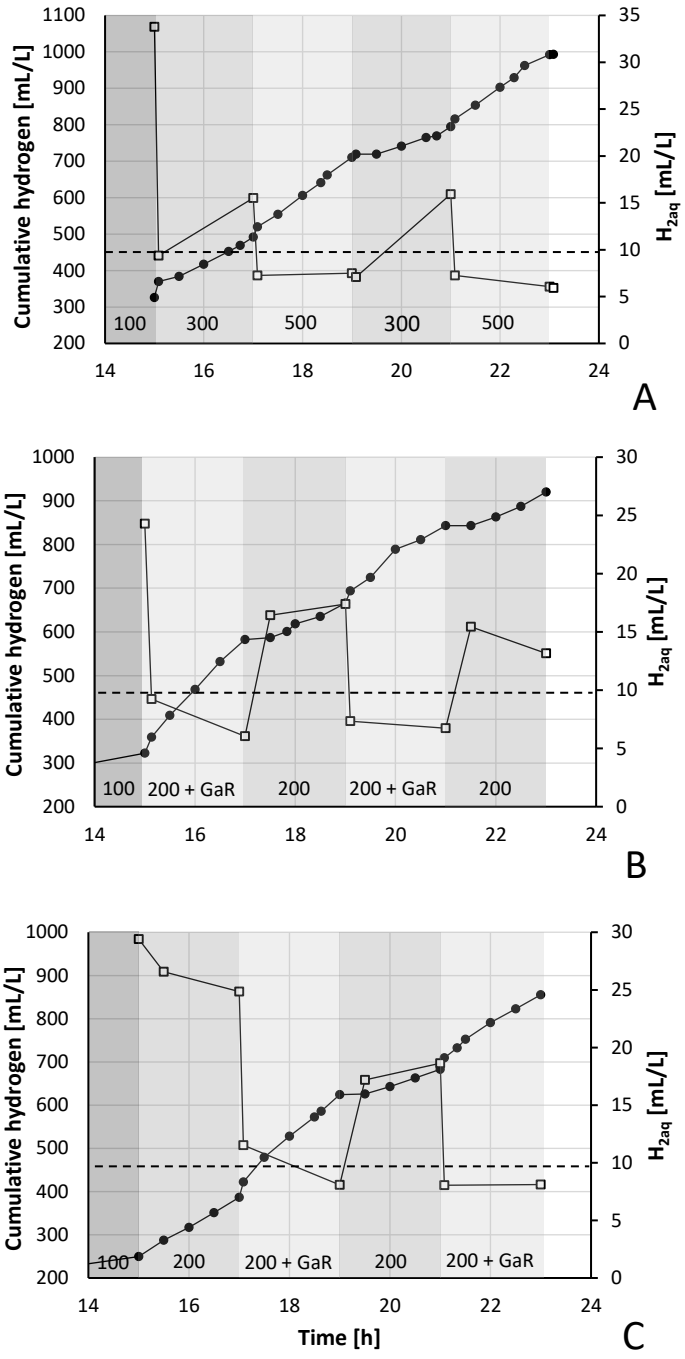

A
Hydrogen production rate [mL/L/h]
400
350
300
250
200
150
100
50
0
200
300
500
200 + GaR
Operating condition
HPR post GaR
HPR eq
HPR GaR
B
H2aq [mL/L]
40
35
30
25
20
15
10
5
0
100
200
300
500
500 + GaR
200 + GaR
Operating condition

Figure 4


Cumulative hydrogen [mL/L]
H_{2aq} [mL/L]
100
300
500
300
500
A
100
200 + GaR
200
200 + GaR
200
B
100
200
200 + GaR
200
200 + GaR
Time [h]
C