Abstract

Hydrogen is a strong inhibitor of dark fermentation. We aimed at directly correlating the hydrogen production by *Thermotoga neapolitana* with the supersaturation of hydrogen in the liquid phase (H_{2aq}), which is often disregarded. Different agitation speeds, biogas recirculation and bubble induction by AnoxK^{TM} K1 carrier were tested to prevent the supersaturation of H_{2aq}. At 100 rpm agitation, the H_{2aq} was 29.7 (± 1.4) mL/L, which is 3-times higher than 9.7 mL/L, i.e. the equilibrium concentration given by Henry's law. Increasing the agitation speed up to 600 rpm reduced the H_{2aq} until 8.5 (± 0.1) mL/L in 2 h and increased the hydrogen production rate (HPR) from 39 (± 2) mL/L/h at 0 rpm to 198 (± 4) mL/L/h at 600 rpm. Similar to 600 rpm, biogas recirculation and the presence of K1 carrier at 200 rpm maintained the H_{2aq} below the equilibrium concentration. This study demonstrates the reciprocal influence of HPR and H_{2aq} and revealed an inverse nonlinear correlation between the two parameters. Therefore, we conclude that an adequate gas-liquid mass transfer, efficiently provided by biogas recirculation or the presence of solid materials (e.g. a biomass carrier), is essential to remove H_2 from the liquid phase and prevent H_2 supersaturation.

Key Words: *Thermotoga neapolitana*, hyperthermophilic, dark fermentation, gas recirculation, hydrogen inhibition, supersaturation

Abbreviations

AA	acetic acid
BGR	biomass growth rate
GaR	recirculation of H_2-rich biogas
H_{2aq}	liquid phase hydrogen
HPR	hydrogen production rate
HY	hydrogen yield
LA	lactic acid

1. Introduction

The continuing rise of the worldwide energy consumption, the depletion of fossil fuels and climate change due to CO_2 emissions emphasize the need to change the current energy system. Hydrogen is predicted to play a major role in this transition as it is a highly flexible clean energy carrier which can be effectively transformed and used in many applications [1]. However, while the use of hydrogen has major advantages, its current production is primarily based on fossil resources. To successfully introduce hydrogen as the energy carrier of the future, a sustainable production process from renewable sources needs to be established [1].

Amongst the environmentally friendly hydrogen production processes, dark fermentation represents one of the most promising processes [2]. Dark fermentation refers to the conversion of organic material to mainly hydrogen, CO_2 and organic acids [3]. To reach the economic viability of this H_2 production process, low hydrogen yields (HY) and hydrogen production rates (HPR) are major constraints that still need to be overcome [4,5]. In dark fermentation, higher yields are generally achieved under thermophilic compared to mesophilic conditions [6]. *Thermotoga neapolitana* is a hyperthermophilic microorganism which has repeatedly been reported to approach the theoretical yield of 4 mol H_2/mol of glucose [7–9]. In addition, the potential of this culture for hydrogen production is emphasized by an efficient hydrolysis of a wide range of substrates combined with fast growth kinetics [8].

The HPR primarily depends on the biomass concentration in the reactor [10,11]. However, the HPR is strongly inhibited by the presence of hydrogen in the liquid phase (H_{2aq}) [12]. H_{2aq} is often wrongly considered in equilibrium with the gas phase [2,13], as it has been

94 demonstrated to supersaturate up to multiple times the equilibrium concentration
95 suggested by Henry´s law [14,15]. H_{2aq} has been identified as the crucial parameter for
96 hydrogen inhibition as this product inhibition directly acts on the bioconversion capacity of
97 microbial cultures [3].
98 According to Andre et al. [12], H_{2aq} supersaturates if HPR exceeds the gas-liquid mass
99 transfer potential of the system. High levels of H_{2aq} thermodynamically constrain the further
100 production of hydrogen [16,17] and hamper the HPR and the HY of the system until an
101 equilibrium between the HPR and the H_{2aq} concentration is reached [12]. As a result, high
102 HPRs can only be achieved if an adequate gas-liquid mass transfer is provided. In this regard,
103 various techniques have been applied to counteract the inhibition by hydrogen [13,16],
104 including headspace sparging with N_2 [15] or CO_2 [18], reduction of the total pressure [19],
105 increase of agitation [20], increase of the surface area [14] and recirculation of the H_2-rich
106 biogas (GaR) [21]. While most of the studies focused on the effect of such techniques on HPR
107 and HY, only a few included a direct measurement of H_{2aq} [14,15,22,23], which allows the
108 direct correlation between H_{2aq} and the inhibition of dark fermentation.

109 The present study, therefore, focused on the effect of hydrogen accumulation in the liquid
110 phase on dark fermentation by *Thermotoga neapolitana*. The main objective was to directly
111 correlate the H_{2aq} concentration to HPR and HY. Different agitation speeds between 0 and
112 600 rpm were investigated, as well as the recirculation of the H_2-rich biogas and the addition
113 of AnoxK^{TM} K1 carriers as potential counteracting measures against hydrogen
114 supersaturation.
115

2. Materials and Methods

2.1. Bacterial culture and medium

A pure culture of *Thermotoga neapolitana* (briefly *T. neapolitana*) purchased from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany) was used in all experiments. The protocol for cultivation and storage of the culture as well as the composition and preparation of the culture medium (modified ATCC 1977) with a glucose concentration of 27.8 mmol/L are described in detail in [10].

2.2. Experimental design

All batch experiments were conducted in a 3 L fully controlled continuously stirred tank reactor (Applikon Biotechnology, the Netherlands) with a working volume of 2 L. The medium was heated to 80 °C for 30 min and sparged with N_2 for 5 min to remove dissolved oxygen before the inoculation with 20 mL of storage culture (1% v/v). Throughout the experiments, the temperature was kept at 80 °C and the pH was automatically controlled to 7 by adding 1 M NaOH.

The culture was maintained at 100 rpm of agitation for 15 h (phase 1) to be acclimatized prior to applying the following operating conditions (phase 2): 0, 200, 400 and 600 rpm agitation as well as biogas recirculation without agitation (0 + GaR). GaR was applied by continuously pumping the produced biogas with a 323D peristaltic pump (Watson-Marlow, UK) from the headspace to a gas dispersion device at the base of the reactor at a flow rate of 350 mL/min. Furthermore, the presence of AnoxK^{TM} K1 carriers (1 L packed volume, density 0.95 g/cm³, diameter 9.1 mm, height 7.2 mm and a specific biofilm surface area of 500 m²/m³, Veolia Water Technologies, Italy) in the fermentative solution was investigated for their ability to enhance the gas-liquid mass transfer by inducing bubble formation. Due to

the anaerobic conditions in the gastight reactor, the K1 carriers were added before the inoculation and agitated at 100 rpm for 15 h in phase 1 and at 200 rpm in phase 2 (200 + K1).

The produced biogas was continuously released into 500 mL water displacement systems to avoid pressure build-up and quantified after 15 h and subsequently every hour. Liquid samples of 2 mL were withdrawn every hour for the analysis of biomass concentration and metabolite composition, i.e. glucose, acetic acid (AA) and lactic acid (LA). At selected time points (i.e. after 15, 17, 20 and 23 h), 20 mL of liquid sample was taken for the determination of H_{2aq}. The fermentation was considered completed when a reactor ceased to produce biogas. Each operating condition was investigated in duplicate.

2.3. Analytical methods

Cell growth was determined by measuring spectrophotometrically the optical density (OD_{540}) of the liquid samples at 540 nm. Liquid samples were centrifuged at 10000 rpm for 5 min to collect the supernatant for the determination of the glucose, AA and LA concentration. The 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, using the method described by Mancini et al. [24] with 0.0065 M 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. The conversion from volumetric to molar H_2 production was performed using the ideal gas law.

For the determination of H_{2aq}, a modified method of Kraemer and Bagley [15] 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 the fridge to equilibrate the liquid and the gas phases. After approximately 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 hydrogen concentration in the gas phase was measured by the GC described above and referred to the value of the liquid sample.

2.4. Kinetic study of biomass growth and hydrogen production

The rates of biomass growth and hydrogen production during phase 2 were calculated by fitting the experimental data with a modified Gompertz model based on the calculations by Dreschke et al. [10]. For biomass growth, Eq. (1) was applied, where B [OD_{540}] is the biomass concentration at fermentation time t [h]; B_0 [OD_{540}] is the biomass concentration at time 15 h (beginning of phase 2); B_m [OD_{540}] is the gain of biomass concentration throughout phase 2; R_B is the volumetric biomass growth rate (BGR) in phase 2 [OD_{540}/h]; and λ_B is the lag phase of the cell growth [h]. As *T. neapolitana* morphologically adapts to the exhaustion of the substrate by decreasing its size resulting in a lower OD_{540} [10], the fitting of the data with the Gompertz model was performed until the highest biomass concentration was reached.

Eq. (2) was applied for hydrogen production, with H [mL/L] being the cumulative hydrogen concentration at time t [h]; H_0 [mL/L] is the hydrogen produced after 15 h (beginning of phase 2); H_m [mL/L] is the hydrogen produced throughout phase 2; R_H [mL/L/h] is the volumetric HPR in phase 2; and λ_H the lag phase of hydrogen production [h]. The gas-liquid mass transfer of the system determines the removal or retention of hydrogen in the liquid phase and consequently has a high impact on the HPR [12]. To avoid the alteration of the

HPR, the experimental data from 16 h onwards were used for the Gompertz model of hydrogen production (Fig. 1B).

$$B = B_0 + B_m \exp\left\{-\exp\left[\frac{R_B\, e}{B_m}(\lambda_B - t) + 1\right]\right\} \quad (1)$$

$$H = H_0 + H_m \exp\left\{-\exp\left[\frac{R_H\, e}{H_m}(\lambda_H - t) + 1\right]\right\} \quad (2)$$

2.5. Statistical analysis

The statistical analysis of the experimental data was performed using Microsoft Excel 2016 (Microsoft Corporation, USA). To determine significance, we applied a paired t-test for the decrease of H_{2aq} due to a change of the operating condition and an unpaired t-test with Benjamini Hochberg correction for the differences at varying operating conditions.

3. Results

3.1. Effect of agitation speed on H_{2aq} accumulation and dark fermentation

3.1.1. Phase 1: Culture growth and acclimatization

In phase 1, excluding the bioassay with the K1 carriers, a similar performance was obtained in all experiments (Fig. 1 and 2). Approximately 33% of glucose consumption, a cumulative hydrogen production of 552 (± 36) mL/L (Fig. 1B), a biomass concentration of 1.20 (± 0.13) (OD_{540}) (Fig. 1 A) and a H_{2aq} of 29.7 (± 1.4) mL/L (Fig. 1C) were obtained after 15 h. In contrast, when K1 carriers were used, approximately 80% of the glucose was consumed in the first 15 h resulting in a hydrogen production of 1307 (± 84) mL/L, a H_{2aq} of 14.5 (± 0.9) mL/L and a biomass concentration (OD_{540}) of 1.86 (± 0.02) (Fig. 2).

3.1.2. Phase 2: Application of different operating conditions

From 15 h onwards, increasing the agitation speed to 200, 400 and 600 rpm rapidly decreased the H_{2aq} concentration in 0.5 h (Fig. 1C). This decrease continued linearly but more slowly, reaching a significant difference compared to 15 h and values of 21.9 (± 2.2) (p-value: 0.031), 12.0 (± 2.9) (p-value: 0.041) and 8.5 (± 0.1) (p-value: 0.024) mL/L at 17 h (Fig. 1C). At the end of the experiment (i.e. after 23 h), agitation at 200, 400 and 600 rpm led to a H_{2aq} of 17.6 (± 1.1), 8.8 (± 5.3) and 6.0 (± 0.1) mL/L, respectively (Fig. 1C). When the agitation was omitted (i.e. 0 rpm) in phase 2, the H_{2aq} concentration increased to a maximum of 40.6 (± 0.6) mL/L at 17 h before decreasing again to 30.1 (± 1.3) mL/L after 23 h incubation (Fig. 1C).

The total production of hydrogen and biomass increased with agitation speed. The hydrogen produced reached 868 (± 15), 1083 (± 62), 1421 (± 118) and 1557 (± 18) mL/L with a maximum biomass concentration (OD_{540}) of 1.31 (± 0.01), 1.59 (± 0.01), 1.65 (± 0.01) and 2.41 (± 0.19) at 0, 200, 400 and 600 rpm (Fig. 1A and B) at 23 h, respectively. Concomitantly, the HPR and the BGR were 39 (± 2), 67 (± 1), 131 (± 16) and 198 (± 4) mL/L/h and 0.03, 0.11, 0.29 and 0.39 OD_{540}/h at 0, 200, 400 and 600 rpm, respectively (Fig. 3). Similarly, the glucose consumption accelerated at increasing agitation speeds and was complete at 400 and 600 rpm (Fig. 2A).

3.2. Use of H_2-rich biogas recirculation and addition of K1 carriers

The effect of GaR on H_{2aq} was similar to that observed with agitation at 600 rpm, with the H_{2aq} concentration dropping significantly from 28.1 (± 2.4) to 10.6 (± 1.2) mL/L (p-value: 0.032) in 0.5 h (Fig. 1C). Glucose was completely consumed in 23 h and led to a hydrogen production of 1345 (± 23) mL/L and a biomass concentration (OD_{540}) of 2.39 (± 0.03) (Fig. 1).

231 The BGR and HPR reached approximately 0.32 OD_{540}/h and 113 (± 3.5) mL/L/h (Fig. 3),
232 respectively, which was significantly different to the HPR at 0 rpm (p-value: 0.0015).

233 When K1 carriers were used, increasing the agitation to 200 rpm from 15 h onwards (phase
234 2) decreased H_{2aq} to 10.5 (± 0.1) mL/L in 0.5 h and led to the completion of the fermentation
235 in about 19 h. The final values of H_{2aq}, hydrogen production and biomass concentration
236 reached 5.8 (± 0.5) mL/L, 1630 (± 38) mL/L and 1.87 OD_{540}, respectively.

237 ### 3.3. Gas composition and biomass and hydrogen production yields

238 Independent from the operating conditions used, the concentration of hydrogen in the
239 produced biogas reached 65 – 70%, the hydrogen yield was between 2.0 and 2.5 mol H_2/mol
240 glucose and an average production of 1.95 (± 0.5) mol H_2/mol AA was obtained (Table 1).
241 The sum of AA and LA at the end of the fermentation accounted for 92 (± 2)% of the glucose
242 consumed in all conditions, except for 200 + K1 where it reached 85% (Fig. 2C). The biomass
243 yields ranged from 0.065 and 0.093 OD_{540}/mol glucose (Table 1).

244 ## 4. Discussion

245 ### 4.1. Techniques to enhance hydrogen gas-liquid mass transfer

246 This study showed the importance of an efficient gas-liquid mass transfer to prevent the
247 supersaturation of H_{2aq} and its inhibitory effect on dark fermentation, which consequently
248 increased the HPR and BGR considerably (Fig. 3). Despite often disregarding the
249 supersaturation of H_{2aq}, many studies have investigated the inhibition of dark fermentation
250 by hydrogen and proposed a variety of counteracting measures [13,16]. While most of the
251 applied measures successfully diminished the inhibitory effect of hydrogen at lab scale, only
252 few have the potential to be applied at a larger scale. More specifically, headspace sparging

253 with N_2 or CO_2 leads to an undesired dilution of the produced biogas [25], while the
254 reduction of the total reactor pressure and vigorous stirring are extremely challenging in
255 industrial applications and entail an inevitable increase of the reactor operation costs.
256 Recirculation of biogas [21,26] has proven promising as it increases the area for gas-liquid
257 mass transfer similar to inert gas sparging, without dilution of the final biogas. In addition,
258 the application of bubble inducing objects in the reactor has revealed a high potential. The
259 underlying principle of this technique is that the energy required for the formation of
260 bubbles in a supersaturated liquid is much lower on a surface (heterogeneous nucleation)
261 compared to bubble formation in a homogenous solution (homogeneous nucleation) and
262 can consequently occur at lower levels of supersaturation [27]. As a result, the bubble
263 formation is facilitated by providing a high surface area in the reactor, which increases the
264 gas-liquid mass transfer without the need to change other operating conditions [28].

265 #### 4.1.1. Enhancement of H_2 production at increasing agitation speeds

266 In the absence of K1 carriers, agitation at 100 rpm during the first 15 h (phase 1) led to an
267 accumulation of H_{2aq} to 29.7 (± 1.4) mL/L (Fig. 1C), i.e. approximately 3-fold the theoretically
268 calculated value of 9.7 mL/L in equilibrium with the gas phase containing 65% H_2 at 80 °C as
269 suggested by Henry´s law [29]. Increasing the agitation from 15 h onwards (phase 2) induced
270 a higher turbulence and improved the gas-liquid mass transfer of hydrogen, causing the
271 decrease of H_{2aq} (Fig. 1C) and a significant increase of HPR (Fig. 3) in proportion to the
272 agitation speed. In particular, the agitation at 600 rpm resulted in a H_{2aq} of 8.5 (± 0.1) mL/L
273 after 17 h (Fig. 1C), demonstrating that the required mass transfer for an equilibrium
274 between the gas and liquid phase was provided.

No agitation (0 rpm) from 15 h onwards resulted in a further accumulation of H_{2aq} up to 40.6 (± 0.6) mL/L at 17 h (Fig. 1C). Between 15 and 17 h, approximately 70 mL H_2/L/h was released to the gas phase despite the retention of hydrogen in the liquid phase. With the continuation of the process at 0 rpm, the H_{2aq} declined back to 32.8 (± 1.3) and 30.1 (± 1.3) mL/L after 20 and 23 h (Fig. 1C), respectively, indicating a net removal of hydrogen from the liquid phase. Regardless, the HPR decreased from 17 h onwards to approximately 30 mL H_2/L/h, due to the self-regulatory bilateral interaction of H_{2aq} and HPR [12]. The time required by the culture to adapt to the change in mass transfer and reach the equilibrium between H_{2aq} and HPR was about 2 h.

4.1.2. Biogas recirculation to effectively reduce H2aq

Similar to the highest agitation speed investigated (i.e. 600 rpm), the use of GaR decreased the H_{2aq} concentration below the theoretical equilibrium concentration. Concomitantly, dark fermentation was significantly accelerated, indicated by an increase of the HPR and BGR by 192% and 870%, respectively, in comparison to the condition without GaR (0 rpm) (Fig. 3), while the total cumulative hydrogen increased to 1345 (± 23) mL/L and more biomass (i.e. 2.36 (± 0.03) OD_{540}) was produced (Fig. 1A and B).

Similarly, Frigon et al. [26] studied H_{2aq} in an anaerobic upflow sludge bed and filter reactor. They reported the reduction of the H_{2aq} concentration from 1.1 µM without recirculation to 0.4 µM when biogas was recirculated at a flow of 85 L/L/d resulting in a higher gas production rate from approximately 4.5 to 12.0 L/L/d and an increase of COD removal. In line, Bakonyi et al. [21] reported an increase of HPR from 7.4 to 8.9 – 9.2 L H_2/L/d by recirculating internal biogas with 50% H_2 in a continuously stirred tank reactor using a mixed culture.

298 The use of GaR to prevent the supersaturation of H_{2aq} is a technique rarely applied in dark
299 fermentation [21,30]. Compared to inert gas sparging, the lower limit of H_{2aq} is defined by
300 the equilibrium concentration with the gas phase when the hydrogen-rich biogas is
301 recirculated. Therefore, an improvement by GaR can only be expected when hydrogen
302 supersaturation occurs hampering the hydrogen production in the control condition. This
303 might explain why GaR has sometimes been reported ineffective when H_{2aq} was not directly
304 monitored in the experiments [30].

305 **4.1.3. K1 carriers to enhance hydrogen bubble formation**

306 The addition of the K1 carriers at the beginning of the fermentation induced a better process
307 performance in phase 1. Despite the same agitation speed of 100 rpm, the hydrogen
308 production, glucose consumption and biomass growth were significantly higher (Fig. 1 and 2)
309 compared to the other operating conditions. Several studies reported a faster process under
310 batch conditions when different solid materials were placed inside the reactor [31,32]. Ngo
311 et al. [32] ascribed the better performance to a higher efficiency of hydrogen production by
312 immobilized compared to suspended cells. In our study, the attachment of cells onto the
313 carrier was unlikely due to the short cultivation time, and the higher fermentation rates
314 were rather induced by the approximately 50% lower H_{2aq} at 15 h (Fig. 1C). The lower H_{2aq}
315 concentration was more likely caused by an enhanced bubble formation due to
316 heterogeneous nucleation of the supersaturated hydrogen at the surface of the carrier
317 material. Similarly, Fritsch et al. [28] obtained a higher production rate when placing a static
318 monolith column inside a continuous dark fermentation system, ascribing the enhanced
319 nucleation at the high surface area of the column as the responsible factor for the improved
320 reactor performance. Also, Wu et al. [33] could achieve an HPR as high as 15.1 (± 0.3) L/L/h
321 with a yield of 3.2 (± 0.1) mol H_2/mol glucose, when applying silicone gel particles inside the

reactor that promoted heterogeneous nucleation and improved the gas-liquid mass transfer. Unfortunately, Wu et al. [33] did not directly measure the H_{2aq} concentration in their study.

When the agitation speed was increased to 200 rpm after 15 h, the H_{2aq} concentration further decreased to 10.5 (± 0.1) mL/L in 0.5 h reaching a final value of 5.8 (± 0.5) mL/L after 19 h (Fig. 1C), which was similar to the value observed at 600 rpm in the absence of K1 carriers. This indicates that a similar gas-liquid mass transfer can be obtained at a 67% lower agitation speed by inducing bubble formation through heterogeneous nucleation, which would entail a considerable reduction of energy consumption and operating costs.

4.2. Impact of the H_{2aq} concentration on the hydrogen yield and rate of the process

When the release of H_2 from the fermentation broth is lower than the potential HPR of the system, hydrogen accumulates in the liquid phase. Elevated concentrations of H_{2aq} restrain the HPR by product inhibition [16,17], which consequently results in a lower H_{2aq} concentration until an equilibrium is reached, primarily determined by the gas-liquid mass transfer of the system [12]. Ljunggren et al. [22] attributed this to a self-regulatory mechanism within microbial cultures, which decrease the growth rate and, consequently, the HPR to avoid hydrogen supersaturation.

Fig. 3 shows the H_{2aq} after 17 h of cultivation, which was considered to evaluate the impact on the cultures performance, revealing an inverse nonlinear correlation between the H_{2aq} and the HPR as well as the BGR. In the studied range of H_{2aq}, the HPR and the BGR increased by 413 and 1082%, respectively, when the H_{2aq} concentration was decreased from 40.6 to 8.5 mL/L. This correlation confirms that elevated H_{2aq} concentrations act as a strong inhibitor on the dark fermentation rates. Furthermore, the variation of H_{2aq} at low (i.e. between 8.5

346 and 21.9 mL/L) H_{2aq} concentrations had a greater impact on the process compared to a
347 change at high (i.e. between 21.9 and 40.6 mL/L) H_{2aq} concentrations.

348 Contrary to the HPR, the HY at different H_{2aq} concentrations only ranged between 2.0 and
349 2.5 mol H_2/mol glucose with the highest value observed when the K1 carriers were used
350 (Table 1). A slightly negative correlation between HY and H_{2aq} was obtained when applying
351 agitation between 200 and 600 rpm (Table 1). On the other hand, the HY at 0 rpm was 2.3 (±
352 0.1) mol/mol glucose (Table 1), but it needs to be considered that only about 50% of the
353 glucose was consumed at the end of the experiment (Fig. 2).

354 The H_{2aq} concentration had a more severe impact on the HPR compared to the HY. According
355 to the rate law, changing the concentration of a reactant (i.e. H_{2aq}) would directly affect the
356 rate of the chemical reaction. Hence, we assume that the direct impact on the
357 thermodynamics of the hydrogen producing reaction [16,17] is responsible for the
358 immediate response of the HPR to changes of the H_{2aq} concentration. In contrast, a change
359 in the yield of the process implies the culture to shift its metabolism to another pathway,
360 requiring more energy and time. In agreement, Ljunggren et al. [22] reported the
361 importance of the culture growth history for the performance of dark fermentation. We
362 hypothesize that a prolonged cultivation at low H_{2aq} would have a positive effect on the
363 hydrogen yield. This is supported by the highest total yield achieved when the H_{2aq}
364 concentration was reduced in phase 1 by the use of K1 carriers.

365 Independent from the operating condition used, an average of 1.95 (± 0.5) mol H_2/mol AA
366 (Table 1) was produced. This is in accordance with the theoretical value of 2 mol H_2/mol AA
367 suggested by the dark fermentation model. Furthermore, dark fermentation predicts the
368 formation of 2 moles of fermentation end products per mole of glucose consumed. The sum

of AA and LA at the end of the fermentation accounted for 92 (± 2)% in the absence of K1 carriers and 85% at 200 + K1 (Fig. 2C). This discrepancy might be due to the production of alanine, an end product which is often produced to a minor fraction without formation of hydrogen [8].

4.3. Outlook

This study showed the importance of an efficient mass transfer of hydrogen in dark fermentation. Agitation at 600 rpm effectively kept H_{2aq} low, reaching high HPR and HY. However, accomplishing the required turbulence by agitation at larger scale is challenging and creates an elevated economic burden. Furthermore, based on the proportionate increase of mass transfer with agitation speed, 600 rpm might be insufficient to prevent hydrogen supersaturation if the HPR would further increase, e.g. by higher biomass concentrations. Biogas recirculation is a promising alternative to agitation by stirring and has shown to effectively enhance the gas-liquid mass transfer [21,26]. The potential of GaR is further emphasized by the possibility to adjust the recirculation flow rates or apply intermittent GaR to meet the required mass transfer without unnecessary energy consumption. Further research is required to compare the operating costs for GaR and stirring at a larger scale and investigate the slightly lower hydrogen yields and rates despite a similar H_{2aq} concentration compared to 600 rpm.

The application of a solid material mixed in the liquid solution has also revealed potential to maintain a high gas-liquid mass transfer through bubble formation by heterogeneous nucleation at a much reduced agitation speed. The bubble formation largely depends on the area and the nature of the provided surface, whereas bubble formation is favored by a rougher surface [27]. Therefore, K1 carrier might not be the best option, especially

392 considering the high capital costs for their application at a larger scale. Different, cheaper
393 materials should be further investigated for their bubble formation capacity. Commonly,
394 solid materials are used in biological processes to promote biofilm growth and consequently
395 high biomass concentrations in the reactor, leading to an acceleration and a better stability
396 of the process. The ability of *T. neapolitana* to form agglomerates has recently been
397 observed in pure culture continuous cultivation [34]. The combined positive effect of
398 biomass retention and improved mass transfer strongly suggests the use of immobilized cell
399 reactors for dark fermentation. However, many aspects of such a process, including the
400 effect of biofilm formation on bubble formation and the influence of attached biomass on
401 the required agitation speed, still need to be investigated. Only then, the savings in energy
402 consumption and operating costs indicated by the lab scale experiments in the present work
403 can be confirmed.

404 **Conclusion**

405 This study has shown the importance of the hydrogen gas-liquid mass transfer in dark
406 fermentation. Hydrogen accumulated up to 29.7 (± 1.4) mL/L at 100 rpm, resulting in a 3-fold
407 supersaturation compared to the H_{2aq} equilibrium concentration of 9.7 mL/L suggested by
408 Henry ’s law. Stopping the agitation further increased the H_{2aq} to 40.6 (± 0.6) mL/L after 17 h,
409 which decreased back to 30.1 (± 1.3) mL/L at 23 h due to the equilibrium reached between
410 H_{2aq} and HPR. The inhibitory effect on HPR decreased nonlinearly with decreasing H_{2aq}.
411 Hence, the low H_{2aq} concentration of 8.5 (± 0.1) mL/L at 600 rpm led to a 413% increase of
412 HPR compared to that obtained in the absence of agitation. The use of GaR at 0 rpm and the
413 addition of the K1 carriers at 200 rpm similarly reduced the H_{2aq} to 8.5 (± 0.4) and 9.3 (± 3.2)
414 mL/L in 2 h, respectively. In particular, the use of a solid carrier indicates a high potential to
415 keep the H_{2aq} concentration low by increasing the gas-liquid mass transfer through

heterogeneous nucleation of the supersaturated hydrogen, without requiring additional energy for mixing or recirculating the gas. Not requiring additional energy for mixing or recirculating the biogas, the addition of K1 carrier is a promising H_{2aq} mitigation strategy, which can be further explored for the cultivation of *T. Neapolitana* biofilms.

Funding: This work was supported by the Marie Skłodowska-Curie European Joint Doctorate (EJD) in Advanced Biological Waste-To-Energy Technologies (ABWET) funded by Horizon 2020 under the grant agreement no. 643071.

Declarations of interest: none

Acknowledgements

All authors want to express their gratitude to their colleagues at the University of Cassino and Southern Lazio, especially Gelsomino Monteverde and Désirée M.G. Sisinni for their invaluable help and assistance throughout this project. Furthermore, we would like to thank Veolia Water Technologies for supplying the AnoxK^{TM} K1 carrier.

Fig. 1: *T. neapolitana* fermenting 27.8 mM of glucose in batch operation at 100 rpm and 80 °C for 15 h (phase 1) and subsequent application of different operating conditions: 0 rpm, 200 rpm, 400 rpm, 600 rpm, 0 rpm with gas recirculation (GaR) and 200 rpm with K1 carriers from 15 to 23 h (phase 2). Evolution of biomass growth (A), hydrogen production (B) and hydrogen in the liquid phase – H_{2aq} (C) during phase 2. In A and B, the symbols represent the average of the experimental data, while the lines depict the corresponding Gompertz model.

Fig. 2: Dark fermentation of 27.8 mM of glucose by *T. neapolitana* in batch operation at 100 rpm and 80 °C for 15 h (phase 1) and subsequent application of different operational conditions: 0 rpm, 200 rpm, 400 rpm, 600 rpm, 0 rpm with gas recirculation (GaR) and 200 rpm with K1 carriers from 15 to 23 h (phase 2). Glucose consumption (A) and production of fermentation end products (AA – acetic acid; LA – lactic acid) during phase 1 (white bars) and phase 2 (grey bars).

Fig. 3: Batch dark fermentation by *T. neapolitana* (at 80 °C) using different operational conditions (agitation at 0, 200, 400 and 600 rpm, no agitation with gas recirculation and 200 rpm with K1 carriers) after an acclimatization phase of 15 h at 100 rpm. Hydrogen production rate (full symbols – circles: 0 – 600 rpm; square: 0 + re) and biomass growth rate (hollow symbols – circles: 0 – 600 rpm; square: 0 + re)

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