High rate continuous biohydrogen production by hyperthermophilic *Thermotoga neapolitana*

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Abstract

This study focused on continuous-flow hydrogen production by *Thermotoga neapolitana* at a hydraulic retention time (HRT) decreasing from 24 to 5 h. At each HRT reduction, the hydrogen yield (HY) immediately dropped, but recovered during prolonged cultivation at constant HRT. The final HY in each operating period decreased from 3.4 (± 0.1) to 2.0 (± 0.0) mol H_2/mol glucose when reducing the HRT from 24 to 7 h. Simultaneously, the hydrogen production rate (HPR) and the liquid phase hydrogen concentration (H_{2aq}) increased from 82 (± 1) to 192 (± 4) mL/L/h and from 9.1 (± 0.3) to 15.6 (± 0.7) mL/L, respectively. Additionally, the effluent glucose concentration increased from 2.1 (± 0.1) to above 10 mM. Recirculating H_2-rich biogas prevented the supersaturation of H_{2aq} reaching a value of 9.3 (± 0.7) mL/L, resulting in complete glucose consumption and the highest HPR of 277 mL/L/h at an HRT of 5 h.

Key words: *Thermotoga neapolitana*, hydrogen, continuous-flow dark fermentation, acetic acid, hydraulic retention time, gas recirculation

Abbreviations

AA	Acetic acid
BMY	Biomass yield
CDW	Cell dry weight
CSTR	Continuously stirred tank reactor
GaR	Biogas recirculation
HPR	Hydrogen production rate
HY	Hydrogen yield
LA	Lactic acid

1 Introduction

Dark fermentation is a sustainable process capable of converting organic matter to the clean and environmentally friendly energy carrier hydrogen (Lee et al., 2011; Ntaikou et al., 2010; Sivagurunathan et al., 2016). While being considered the most promising amongst the biological processes due to the independence from light and the simple reactor operation (Arimi et al., 2015; Balachandar et al., 2013), dark fermentation still faces major limitations. Amongst others, low hydrogen production rates (HPR) and hydrogen yields (HY) are two of the most fundamental drawbacks in order to obtain an economically viable process (de Vrije et al., 2007; Gupta et al., 2013; Lee et al., 2011).

In dark fermentation, the HY is closely connected to the culture used (Balachandar et al., 2013; Ghimire et al., 2015), with high yields being achieved by selecting a suitable production organism (O-Thong et al., 2008). Thermophilic strains are advantageous over mesophilic strains providing the highest HYs (Elsharnouby et al., 2013; Gupta et

al., 2016; Lee et al., 2011). Moreover, most other non-H_2 producing microorganisms competing for substrate or consuming the produced hydrogen are inhibited by elevated temperatures (Hawkes et al., 2007; Yasin et al., 2013).

Thermotoga neapolitana (briefly *T. neapolitana*) is a hyperthermophilic bacterium which has been extensively studied for the production of hydrogen (Pawar and van Niel, 2013; Pradhan et al., 2015). Besides achieving exceptional yields approaching the theoretical value of 4 mol H_2/mol glucose (d'Ippolito et al., 2010; Munro et al., 2009), this bacterium is capable to simultaneously metabolize (Eriksen et al., 2008) a wide range of substrates (Huber and Hannig, 2006; Pradhan et al., 2015). So far, *T. neapolitana* has exclusively been investigated in batch and fed batch operation (Pradhan et al., 2015). However, continuous-flow conditions are generally preferred for an industrial application (Kumar et al., 2014; Ntaikou et al., 2010) due to the more energy efficient reactor operation (Lin et al., 2012; Show et al., 2011). Furthermore, continuous mode allows the culture to reach an acclimatized steady state which has shown to provide better process stability and higher hydrogen yields (Elsharnouby et al., 2013; Hawkes et al., 2007).

In continuous operation, the hydraulic retention time (HRT) is a major factor affecting the reactor performance of dark fermentation (Arimi et al., 2015; Sivagurunathan et al., 2016). At constant reactor volume and substrate removal efficiency, a decrease of the HRT represents an acceleration of the process. Consequently, the same amount of substrate can be metabolized in a shorter period of time, which considerably reduces the bioreactor size and capital costs (Hawkes et al., 2007). Furthermore, decreasing the

HRT has shown to increase the HPR (Palomo-Briones et al., 2017; Whang et al., 2011; Zhang et al., 2013), additionally improving the economic viability of the process. Low HRTs are also advantageous as they selectively wash out from the system unwanted microorganisms such as hydrogen consumers, which exhibit lower growth rates compared to the hydrogen producing bacteria (Ghimire et al., 2015; Hawkes et al., 2007). The minimum accomplishable HRT is thereby determined by the growth rate of the slower desired culture. An excessive shortening of the HRT generally leads to an incomplete substrate consumption or the complete washout of the culture (Ghimire et al., 2015; Lin et al., 2012). Hence, the optimization of the HRT, i.e. the proper bioreactor sizing, is essential for the establishment of a continuous production process.

Another crucial factor in dark fermentation is end product inhibition. *T. neapolitana* metabolism results in the production of mainly acetic acid (through the H_2- producing pathway) and lactic acid (through the competing pathway) (Pradhan et al., 2015), which can be inhibitory at high concentrations (Dreschke et al., 2019c). Furthermore, also the accumulation of hydrogen in the system hampers the efficiency of the process (Balachandar et al., 2013; Verhaart et al., 2010). Verhaart et al. (2010) explain in detail how high H_2 concentrations negatively affect the thermodynamics of hydrogen production in dark fermentation. To determine the effect of H_2 on the process, the relevant parameter which directly acts on the microbial culture is the concentration of liquid phase hydrogen (H_{2aq}), which is often wrongly considered to be in equilibrium with the easily measurable hydrogen partial pressure in the gas phase (Ghimire et al., 2015; Ntaikou et al., 2010). However, an increasing amount of studies have reported the supersaturation of H_{2aq} and demonstrated its considerable impact on dark

fermentation (Gupta et al., 2016; Kraemer and Bagley, 2006; Ljunggren et al., 2011). Especially, the positive correlation between H_{2aq} and HPR (Dreschke et al., 2019a; Dreschke et al., 2019b; Pauss et al., 1990) highlights the importance to prevent H_{2aq} accumulation in order to achieve high H_2 productivities in dark fermentation.

In the present study, *T. neapolitana* was used in a continuous-flow biohydrogen production process. We investigated the effect of a decreasing HRT on the dark fermentation performance and H_{2aq} build-up. Furthermore, the use of H_2-rich biogas recirculation was tested for its potential to counteract the supersaturation of H_{2aq} at the lowest HRTs. This study represents a preliminary study, aiming to gain a broader understanding of the hyperthermophilic, pure culture, continuous dark fermentation under controlled process conditions with the goal to establish a technology, which is capable of treating a real carbohydrate rich waste and efficiently converting it to hydrogen.

2 Material and methods

2.1 Bacterial culture and medium

A pure culture of *T. neapolitana* was obtained from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany). The medium composition was based on a modified ATCC 1977 medium described by Dreschke et al. (2018) containing the following components (in g/L): 10 NaCl; 5 glucose (equals 27.8 mM); 2 yeast extract; 2 tryptone; 1 cysteine; 1 NH_4Cl; 0.3 K_2HPO_4; 0.3 KH_2PO_4; 0.2 $MgCl_2 \cdot 6H_2O$; 0.1 KCl; 0.1 $CaCl_2 \cdot 2H_2O$; 0.001 resazurin dissolved in distilled water, supplemented with 10 mL/L of vitamin and 10 mL/L of trace element solutions (DSM

medium 141). The pH adjusted medium (pH 7.5) was prepared in 10 L Schott Duran bottles before autoclaving at 110°C for 5 min. Subsequently, the headspace of the Schott Duran bottles was sparged with N_2 for 10 min to remove oxygen and subsequently stored anaerobically at 4 °C.

2.2 Experimental conditions

The experiment was conducted in a 3-L fully controlled continuously stirred tank reactor (CSTR) (Applikon Biotechnology, the Netherlands) with a working volume of 2 L. The reactor was kept at a constant temperature of 80 °C and maintained at pH 7 by automatic addition of 5M NaOH, while a 500 rpm stirring was applied. The produced biogas was continuously released from the headspace of the reactor to prevent pressure build-up. To grow and acclimatize *T. neapolitana*, the reactor was operated in batch mode for approximately 16 h after the inoculation with 6% (v/v) of storage culture. Subsequently, the feeding was initiated at a flow rate of 83.3 mL/h resulting in an HRT of 24 h. The working volume was controlled using a level probe.

To investigate the effect of the HRT on dark fermentation by *T. neapolitana*, different operating conditions were used as described in Table 1. The HRT was gradually decreased from 24 to 5 h, whereas H_2-rich biogas recirculation (GaR) was added at the lowest HRTs (i.e. 7 and 5 h) to evaluate the impact of H_{2aq} on the process performance. GaR refers to the recirculation of the produced biogas from the headspace to a distribution device at the base of the reactor at a flow-rate of 350 mL/h via a peristaltic pump (Watson-Marlow, United Kingdom).

2.3 Sampling and analytical methods

To determine the concentration of glucose, acetic acid (AA) and lactic acid (LA), 2 mL of liquid sample was taken twice a day. Furthermore, 20 mL samples were withdrawn from the reactor for the determination of H_{2aq}, while 200 mL of effluent was used for the analysis of cell dry weight (CDW) as described by Dreschke et al. (2019b). The biogas production was quantified by measuring the time to fill a 500 mL water displacement system. The procedures for liquid sample processing (glucose, AA and LA concentration) and the determination of the hydrogen concentration in the biogas were as described previously (Dreschke et al., 2019b). Glucose, LA and AA were determined via HPLC (Prominence LC-20A Series, Shimadzu, Japan), whereas the concentration of hydrogen in the biogas was analyzed via GC (Varian 3400, USA). The conversion from volumetric to molar hydrogen production was performed by applying the ideal gas law (O-Thong et al., 2008).

3 Results and Discussion

3.1 Response of *T. neapolitana* to the HRT decrease

Fig. 1 shows the reactor performance at a decreasing HRT from 24 to 7 h. In 6 days of operation at an HRT of 24 h, we obtained an HY of 3.4 (± 0.1) mol H_2/mol glucose, a biomass yield (BMY) of 28.6 (± 0.7) mg CDW/mol glucose and an HPR of 82 (± 1) mL/L/h which induced a H_{2aq} of 9.1 (± 0.3) mL/L (Fig. 2A and B). Besides H_2, glucose was metabolized to AA (i.e. 44.0 (± 0.8) mM) and LA (i.e. 5.6 (± 0.8) mM) at an HRT of 24 h. A residual glucose concentration of 2.1 (± 0.1) mM remained in the effluent.

The reduction of HRT from 24 to 20 h induced an immediate decrease of the HY from approximately 3.4 mol H_2/mol glucose on day 6 to 2.0 mol H_2/mol glucose on day 7 (Table 1). A concomitant shift of the end product formation from AA to LA and a temporary increase of the residual glucose concentration to 5.2 mM (Fig. 1A) were observed. At the same time, the HPR declined from approximately 82 to 70 mL/L/h (Table 1), while the BMY remained relatively unaffected reaching 30.0 (± 1.4) mg CDW/mol glucose (Fig. 2A). Subsequent to the change of HRT from 24 to 20 h, the process recovered from day 7 to 21, as depicted by the HY increasing to approximately 2.8 mol H_2/mol glucose (Fig. 1A, Table 1), the shift of end products back from LA to AA (Fig. 1A) and the increase of HPR to 96 mL/L/h (Fig. 1B, Table 1). A complete glucose consumption was observed from day 13 onwards (Fig. 1A).

A similar response to a decreasing HRT was observed by Kim et al. (2012) using anaerobic digester sludge as inoculum in a CSTR at a constant organic loading rate of 40 g glucose/L/day. Decreasing the HRT from 24 to 12 h temporarily decreased the glucose consumption and HY from approximately 95 to 40% and from 0.8 to 0.5 mol H_2/mol glucose, respectively. After 5 and 7 days of cultivation, the process recovered reaching 90% of glucose consumption and an HY of 1.2 mol H_2/mol glucose. Peintner et al. (2010) investigated the use of a pure *Caldicellulosiruptor owensensis* culture in a trickling bed bioreactor. They observed a drastic shift from AA to LA formation and cessation of hydrogen production in the first day after reducing the HRT from 7.5 to 5 h. In the subsequent days, the process recovered resulting in a stable hydrogen production and an increase of the AA/LA ratio.

The above described response, i.e. a drop of process efficiency and the ensuing recovery of the process performance, was subsequently observed at each stepwise HRT reduction (Fig. 1A). To allow a more detailed analysis, the recovery at each individual operating condition was described using a linear regression (Fig. 1A and B, Fig. 2A and B). This allowed the calculation of the initial and end value of the HY and HPR at each HRT, as reported in Table 1. The stoichiometric sum of LA, AA and residual glucose in the effluent constituted for 95 (± 5)% of the initial glucose feed throughout the entire experiment (Fig. 1A). The hydrogen concentration, i.e. 67.2 (± 2.4)%, in the produced biogas remained constant along the 129 days of operation (data not shown) and, hence, unaffected by the change of operating condition.

3.2 *T. neapolitana* metabolism at decreasing HRT

The decrease of HY described in section 3.1 strongly indicates that the reduction of the HRT induced a shock response. The glucose degradation by *T. neapolitana* is dominated by 2 pathways defined by their final products, either AA or LA (Pradhan et al., 2015). Only the AA pathway leads to the formation of hydrogen, as demonstrated by the increase of HY when AA simultaneously increased (Fig. 1A and 3A). The AA pathway also results in an additional energy gain of two moles ATP/mol glucose, although this requires a high redox potential ($E^{o\prime}$ = -414 mV). This is demonstrated by the Gibbs free energy under standard conditions for the reduction of H^+ by the internal electron carrier NADH to LA (ΔG^0 = -25.0 kJ/mol) or to AA and H_2 (ΔG^0 = +18.1 kJ/mol) (Balachandar et al., 2013), rendering the AA pathway energetically more challenging than the LA pathway. Hence, the metabolism of *T. neapolitana* shifts towards the LA pathway as a response to unfavorable or changeable conditions, allowing the organism

to continue the fermentation, however with a lower energy yield. This phenomenon was observed at each decrease of HRT in this study (Fig. 1A) or previously at elevated hydrogen (d'Ippolito et al., 2010; Dreschke et al., 2019b) and AA (Dreschke et al., 2019c) concentrations. The subsequent recovery during each operating phase is assumed to be an acclimatization (i.e. an improvement of the culture metabolic abilities allowing it to tolerate more stressing conditions after a certain operational time Dreschke et al., 2019c) of *T. neapolitana* at stable environmental conditions, driven by the higher energy yield of the AA pathway. Accordingly, Dreschke et al. (2019c) observed a 47% increase of the HY over 130 days of continuous flow cultivation increasing the feed glucose (i.e. 11.1-41.6 mM) and AA (i.e. 0-240 mM) concentrations at a constant HRT. The described change in metabolism implies the synthesis of new enzymes, indicating why acclimatization is a slow process occurring exclusively during a prolonged cultivation at stable conditions.

3.3 Impact of HRT on hydrogen yield and production rate

As mentioned in section 3.1, the efficiency of the process considerably improved throughout each operating phase. For a better comparison of the reactor performance at different HRTs, an average value of HY and HPR in the final 3 days of each operating condition is given in Fig. 2. The HY gradually decreased from 3.4 (± 0.1) to 2.0 (± 0.0) mol H_2/mol glucose when the HRT was reduced from 24 to 7 h (Fig. 2A). At the same time, the HPR increased from 82 (± 1) to 192 (± 4) mL/L/h, despite the decline of the increasing HPR is generally observed when lowering the HRT and considered to be caused by the higher loading rate (Barca et al., 2015).

de Vrije et al. (2007) used *Caldicellulosiruptor saccharolyticus* in a CSTR at 72.5 (± 0.5) °C using 10.7 mM as feed glucose. In their study, lowering the HRT from 11.1 to 3.3 h decreased the HY from 4.0 (± 0.1) to 3.3 (± 0.1) mol H_2/mol glucose while increasing the HPR from 4.0 (± 0.3) to 9.9 (± 0.5) mmol/L/h. Similarly, Xing et al. (2008) reported an increase of HPR and a decrease of HY when reducing the HRT from 10 to 1.7 h using *Ethanoligenens harbinense* YUAN-3 in a CSTR with 1 g/L of feed glucose concentration. Jo et al. (2008) used *Clostridium tyrobutyricum* JM1 in a fixed bed bioreactor at 37°C. Reducing the HRT from 24 to 2 h increased the HPR by approximately 7 times up to a maximum of 7.2 L H_2/L/d with a glucose conversion efficiency of 97%. The further decrease to an HRT of 1 h induced a sharp drop of conversion efficiency to 41% and an HPR of approximately 2.2 L/L/d. The HY of *C. tyrobutyricum* JM1 was not discussed in detail by Jo et al. (2008).

3.4 Correlation of HPR and H_{2aq} at decreasing HRT

Similar to the HPR, also the H_{2aq} increased with decreasing HRT (Fig. 2B). At an HRT of 24 h, the H_{2aq} was 9.1 (± 0.3) mL/L (Fig. 2B), i.e. lower than 9.7 mL/L which is the liquid phase concentration in thermodynamic equilibrium with a gas phase containing 65% H_2 at 80 °C, as suggested by Henry´s law (Dreschke et al., 2019b). The applied 500 rpm agitation provided a sufficient gas-liquid mass transfer to efficiently remove hydrogen from the liquid phase as previously reported (Dreschke et al., 2019b). However, when the HRT was reduced to 7 h and the HPR increased to 192 (± 4) mL/L/h (Fig. 2B), the same agitation could not maintain the gas-liquid equilibrium leading to a supersaturated H_{2aq} of 15.6 (± 0.7) mL/L. The H_{2aq} was directly correlated to the HPR (Fig. 2B) under all operating conditions until day 103, i.e. prior to applying GaR.

The importance of the gas-liquid mass transfer on the process has been demonstrated in previous studies (Beckers et al., 2015; Dreschke et al., 2019b; Kraemer and Bagley, 2006; Pauss et al., 1990). When adequate gas-liquid mass transfer is provided, H_{2aq} remains in equilibrium with the gas phase preventing the supersaturation of H_{2aq} (Dreschke et al., 2019b; Pauss et al., 1990). If, however, the gas-liquid mass transfer is limited, hydrogen accumulates in the liquid phase depending on the HPR as theoretically and experimentally demonstrated by Pauss et al. (1990) using mixed cultures and observed by Dreschke et al. (2019b) using *T. neapolitana*. Hydrogen is a well-known inhibitor of dark fermentation, acting on the yield as well as the dark fermentation rate (Dreschke et al., 2019b). Due to this inhibition of HPR by H_{2aq}, both parameters reciprocally impact each other, resulting in a process performance which is primarily determined by the mass transfer of the system.

In this study, the response of *T. neapolitana* at each stepwise HRT decrease might have been induced by a rapid increase of H_{2aq}, caused by the increase of the HPR. We assume that *T. neapolitana* reduced the hydrogen yield to prevent high H_{2aq} concentrations. This hypothesis is supported by the low impact of an HRT change on HPR which is directly correlated to H_{2aq}.

3.5 Application of GaR

At low HRTs, the glucose consumption efficiency was impaired. In particular, the residual glucose concentration remained above 5 mM and 10 mM for approximately 6 and 10 days when the HRT was reduced from 13 to 10 h and from 10 to 7 h (Fig. 1A), respectively. An incomplete substrate consumption is commonly observed when decreasing the HRT below a certain threshold value (Kumar et al., 2014; Palomo-

Briones et al., 2017; Whang et al., 2011). At an HRT of 7 h, glucose consumption improved from day 98 onwards. The additionally consumed fraction of glucose was primarily metabolized via the non-hydrogen-producing LA pathway, as demonstrated by the sharp LA increase in the reactor (Fig. 1A). The higher H_{2aq} concentrations observed at an HRT of 7 h (Fig. 3B) likely hampered the dark fermentation yield and rate.

Therefore, GaR was initiated on day 104 to improve the gas-liquid mass transfer and discern whether the reduced performance was due to the inhibition by accumulated H_{2aq}, or a kinetic limitation of the culture. The use of GaR immediately decreased the H_{2aq}, maintaining it at 9.3 (± 0.7) mL/L independent from the HPR (Fig. 3B). GaR initially induced a slight decrease of HY and HPR from approximately 2.1 to 1.7 mol H_2/mol glucose and 207 to 158 mL/L/h, respectively (Table 1). This is assumed to be caused by the response of *T. neapolitana* to the change of environmental conditions discussed in section 3.2. As previously observed, the process recovered, reaching an HY of 2.3 mol/mol glucose and a HPR of 216 mL/L/h (Table 1) after 13 days of operation, i.e. 7% higher than the values obtained at an HRT of 7 h in the absence of GaR. Furthermore, glucose was completely consumed throughout the operating period, while the AA concentration increased from approximately 28 to 33 mM and the LA concentration decreased from approximately 24 to 19 mM from day 104 to 117 (Fig. 3A).

To confirm that this higher process performance was only due to a low H_{2aq} at HRT 7 h, the GaR was stopped on day 118. The cessation of GaR drastically decreased the HY from 1.9 to 0.2 mol H_2/mol glucose, simultaneously shifting from AA to LA production (Fig. 3A) and reducing the HPR from 184 to 15 mL/L/h (Table 1). It is not entirely clear

why returning to an HRT of 7 h in the absence of GaR induced such a substantial difference in the process performance. The primary difference of the two phases was the velocity at which the environmental conditions were changed. Until day 103, *T. neapolitana* slowly acclimatized to increasing levels of H_{2aq}, whereas the deactivation of GaR immediately changed the gas-liquid mass transfer after cultivation at low H_{2aq} for 13 days. We assume that the considerable reduction of HPR was a shock response by *T. neapolitana* triggered by elevated levels of H_{2aq}, which subsequently decreased again to 11.9 mL/L on day 118 when H_{2aq} was first measured after the GaR stop (Fig. 3B). Despite the absence of GaR, H_{2aq} declined even further until day 121 (Fig. 3B and C), due to the collapse of the hydrogen production with the HPR decreasing to 15 ml/L/h in this phase (Table 1).

On day 121, GaR was applied again to continue investigating the impact of the HRT on *T. neapolitana* at low H_{2aq} concentrations at an HRT of 7 h. The process immediately recovered, as depicted by the increase of HPR and HY (Fig. 3A and C, Table 1). On day 124, the HRT was reduced to 5 h in the presence of GaR to determine whether a low H_{2aq} would permit a further increase of the process velocity. In contrast to the previous HRT reductions to 10 and 7 h, glucose continued to be completely degraded to 2.1 (± 0.6) mM in the presence of GaR (Fig. 3A) and the process continued to recover with the HY increasing from 1.8 to 2.0 mol H_2/mol glucose in 5 days of cultivation (Table 1). This resulted in an HPR of 277 mL/L/h at the end of the operating period (Table 1), i.e. the highest obtained under all the process conditions tested.

We demonstrate that the increase of HPR leads to an increase of H_{2aq} and inevitably inhibition. GaR is a successful technique preventing H_{2aq} supersaturation, allowing high

glucose consumption and HPR even at an HRT of 5 h. However, the mechanisms acting on the culture are not entirely clear, as the initiation and stopping of GaR seem to induce a response of *T. neapolitana* similar to that observed at changes of H_{2aq} (Dreschke et al., 2019b) or HRT (Fig. 1). Further long-term investigations using real waste in non-axenic conditions are necessary to determine the real potential of this technique. Such investigations would also allow the much-needed evaluation, whether the thermophilic process is energetically justified and the advantages (e.g. higher yields and process rates, waste treatment, and facilitated control due to lower contamination) outweigh the additional heating expenses. The presented process is especially suited for one of the many industrial processes, which simultaneously produce waste heat together with organic waste thereby eliminating or reducing the costs for heating.

3.6 Effect of HRT on biomass yield, concentration and agglomeration

Contrary to the HY, the biomass concentration was not negatively affected by the HRT decrease, but gradually increased throughout the initial 101 days of operation from 0.67 (± 0.02) to 0.89 (± 0.05) g CDW/L (Fig. 1B). Interestingly, the biomass concentration remained in the same range (Fig. 1B), despite the considerably lower glucose consumption when decreasing the HRT from 10 to 7 h (Fig. 1A). This explains the steady increase of BMY from 28.6 (± 0.7) to 39.7 (± 2.9) mg CDW/mol glucose between the HRT 24 and 10 h, followed by the sharp increase to 57.6 (± 5.1) mg CDW/mol glucose at an HRT of 7 h (Fig. 2A). The results suggest that the biomass concentrations in a *T. neapolitana* cultivation is only marginally influenced by the HRT or the glucose consumption but increases slightly with acclimatization. Contrary to a

change in HRT, the shock applied by the deactivation of GaR on day 118 induced a notable decrease of the biomass concentration, however exhibiting a considerably lower impact than that observed on the HY and the HPR.

A restrained growth by *T. neapolitana* to approximately 0.7 g CDW/L has previously been observed, when the biomass concentration remained unaffected by an increase of feed glucose concentration from 22.2 to 41.6 mM in continuous operation at an HRT of 24 h (Dreschke et al., 2019c).

Such growth limitation is common for hyperthermophilic suspended cultures (Lee et al., 2011) and considered a major obstacle for their application in large scale hydrogen production (Gupta et al., 2016). However, in the present study, we noticed the formation of biomass agglomerates attached to the stainless-steel baffles inside the reactor. After 111 days of cultivation, the whitish agglomerates were approximately 2-4 mm in diameter, protruding roughly 1 mm from the surface of attachment. *T. neapolitana* has previously been reported to form aggregates in batch (Eriksen et al., 2011) or grow attached to solid surfaces in repeated fed-batch (Basile et al., 2012) cultivation. Furthermore, based on the hydrogen yield and acid production it can be assumed that despite the nonsterile conditions no relevant contamination occurred as it has been demonstrated in previous experiments after 110 d of continuous operation (Dreschke et al., 2019c). This strongly suggests the application of *T. neapolitana* in an advanced bioreactor system exploiting self-aggregation or biofilm formation to counteract low biomass concentrations. Such systems not only increase the biomass concentration, but generally allow lower HRTs resulting in higher HPRs (Cheng et al.,

2010; Ghimire et al., 2015; Show and Lee, 2013), while being considered more stable and resistant against unfavorable environmental conditions (Cheng et al., 2010).

Conclusion

- HY decreased from 3.4 (± 0.1) to 2.0 (± 0.0) mol H_2/mol glucose when decreasing the HRT from 24 to 7 h. In contrast, the HPR increased, reaching a maximum of 277 mL/L/h at an HRT of 5 h including GaR.
- Each HRT reduction induced a shift from the AA to the LA pathway, a drop of the HY and an impaired glucose consumption at an HRT of 10 and 7 h. However, a prolonged cultivation at constant HRT allowed *T. neapolitana* to acclimatize, as indicated by an increase of the HY.
- The H_{2aq} positively correlated with the HPR reaching 15.6 (± 0.7) mL/L at 192 (± 4) mL/L/h.
- The use of GaR effectively prevented the supersaturation of H_{2aq}, allowing a complete glucose consumption by *T. neapolitana* at an HRT as low as 5 h.

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Fig. 1: Continuous dark fermentation of glucose (feed concentration 27.8 mM) by *T. neapolitana* at decreasing HRT from 24 to 7 h. Hydrogen yield (HY) and cumulative composition of the liquid phase, i.e. residual glucose (Glu), acetic acid (AA) and lactic acid (LA) (A) as well as biomass concentration and hydrogen production rate (HPR) (B).

Fig. 2: Mean values of the 3 final days of each operational phase of the hydrogen yield (HY) and the biomass yield (BMY) (A) and hydrogen concentration in the liquid phase (H_{2aq}) and hydrogen production rate (HPR) (B) at decreasing HRT from 24 to 7 h during the continuous dark fermentation of 27.8 mM Glu by *T. neapolitana*.

Fig. 3: Continuous dark fermentation of 27.8 mM of glucose (Glu) by *T. neapolitana* at an HRT of 7 and 5 h, including or excluding recirculation of the H_2-rich biogas (GaR). Hydrogen yield and composition of the digestate, i.e. residual Glu, acetic acid (AA) and lactic acid (LA) concentration (A), concentration of hydrogen in the liquid phase (H_{2aq}) (B) as well as hydrogen production rate (HPR) and biomass concentration (C).

References

1 Arimi, M.M., Knodel, J., Kiprop, A., Namango, S.S., Zhang, Y., Geißen, S.-U., 2015. Strategies for improvement of biohydrogen production from organic-rich wastewater: A review. Biom Bioenergy 75, 101–118.

2 Balachandar, G., Khanna, N., Das, D., 2013. Biohydrogen production from organic wastes by dark fermentation, in: Biohydrogen. Elsevier, pp. 103–144.

3 Barca, C., Soric, A., Ranava, D., Giudici-Orticoni, M., Ferrasse, J., 2015. Anaerobic biofilm reactors for dark fermentative hydrogen production from wastewater: A review. Bioresour Technol 185, 386–398.

4 Basile, M.A., Carfagna, C., Cerruti, P., Gomez d'Ayala, G., Fontana, A., Gambacorta, A., Malinconico, M., Dipasquale, L., 2012. Continuous hydrogen production by immobilized cultures of *Thermotoga neapolitana* on an acrylic hydrogel with pH-buffering properties. RSC Adv. 2 (9), 3611.

5 Beckers, L., Masset, J., Hamilton, C., Delvigne, F., Toye, D., Crine, M., Thonart, P., Hiligsmann, S., 2015. Investigation of the links between mass transfer conditions, dissolved hydrogen concentration and biohydrogen production by the pure strain *Clostridium butyricum* CWBI1009. Biochem Eng J 98, 18–28.

6 Cheng, K.-C., Demirci, A., Catchmark, J.M., 2010. Advances in biofilm reactors for production of value-added products. Appl Microbiol Biotechnol 87 (2), 445–456.

7 de Vrije, T., Mars, A.E., Budde, M.A.W., Lai, M.H., Dijkema, C., de Waard, P., Claassen, P.A.M., 2007. Glycolytic pathway and hydrogen yield studies of the extreme thermophile *Caldicellulosiruptor saccharolyticus*. Appl Microbiol Biotechnol 74 (6), 1358–1367.

8 d'Ippolito, G., Dipasquale, L., Vella, F.M., Romano, I., Gambacorta, A., Cutignano, A., Fontana, A., 2010. Hydrogen metabolism in the extreme thermophile *Thermotoga neapolitana*. Int J Hydrogen Energy 35 (6), 2290–2295.

9 Dreschke, G., d'Ippolito, G., Panico, A., Lens, P.N.L., Esposito, G., Fontana, A., 2018. Enhancement of hydrogen production rate by high biomass concentrations of *Thermotoga neapolitana*. Int J Hydrogen Energy.

10 Dreschke, G., Papirio, S., d'Ippolito, G., Panico, A., Lens, P.N., Esposito, G., Fontana, A., 2019a. H2-rich biogas recirculation prevents hydrogen supersaturation and enhances hydrogen production by Thermotoga neapolitana cf. capnolactica. Int J Hydrogen Energy.

11 Dreschke, G., Papirio, S., Lens, P.N., Esposito, G., 2019b. Influence of liquid-phase hydrogen on dark fermentation by Thermotoga neapolitana. Renew Energy 140, 354–360.

12 Dreschke, G., Papirio, S., Sisinni, D.M.G., Lens, P.N.L., Esposito, G., 2019c. Effect of feed glucose and acetic acid on continuous biohydrogen production by *Thermotoga neapolitana*. Bioresour Technol 273, 416–424.

13 Elsharnouby, O., Hafez, H., Nakhla, G., El Naggar, M.H., 2013. A critical literature review on biohydrogen production by pure cultures. Int J Hydrogen Energy 38 (12), 4945–4966.

14 Eriksen, N.T., Nielsen, T.M., Iversen, N., 2008. Hydrogen production in anaerobic and microaerobic *Thermotoga neapolitana*. Biotechnol Lett 30 (1), 103–109.

15 Eriksen, N.T., Riis, M.L., Holm, N.K., Iversen, N., 2011. H(2) synthesis from pentoses and biomass in *Thermotoga spp*. Biotechnol Lett 33 (2), 293–300.

16 Ghimire, A., Frunzo, L., Pirozzi, F., Trably, E., Escudie, R., Lens, P.N., Esposito, G., 2015. A review on dark fermentative biohydrogen production from organic biomass: Process parameters and use of by-products. Appl Energy 144, 73–95.

17 Gupta, N., Pal, M., Sachdeva, M., Yadav, M., Tiwari, A., 2016. Thermophilic biohydrogen production for commercial application: The whole picture. Int J Energy Res 40 (2), 127–145.

18 Gupta, S.K., Kumari, S., Reddy, K., Bux, F., 2013. Trends in biohydrogen production: major challenges and state-of-the-art developments. Environ Technol 34 (13-16), 1653–1670.
19 Hawkes, F., Hussy, I., Kyazze, G., Dinsdale, R., Hawkes, D., 2007. Continuous dark fermentative hydrogen production by mesophilic microflora: Principles and progress. Int J Hydrogen Energy 32 (2), 172–184.
20 Huber, R., Hannig, M., 2006. Thermotogales, in: Dworkin, M., Falkow, S., Rosenberg, E., Schleifer, K.-H., Stackebrandt, E. (Eds.), The Prokaryotes. Springer New York, New York, NY, pp. 899–922.
21 Jo, J.H., Lee, D.S., Park, D., Park, J.M., 2008. Biological hydrogen production by immobilized cells of *Clostridium tyrobutyricum* JM1 isolated from a food waste treatment process. Bioresour Technol 99 (14), 6666–6672.
22 Kim, T.-H., Lee, Y., Chang, K.-H., Hwang, S.-J., 2012. Effects of initial lactic acid concentration, HRTs, and OLRs on bio-hydrogen production from lactate-type fermentation. Bioresour Technol 103 (1), 136–141.
23 Kraemer, J.T., Bagley, D.M., 2006. Supersaturation of dissolved H_2 and CO_2 during fermentative hydrogen production with N_2 sparging. Biotechnol Lett 28 (18), 1485–1491.
24 Kumar, G., Park, J.-H., Kim, M.-S., Kim, D.-H., Kim, S.-H., 2014. Hydrogen fermentation of different galactose–glucose compositions during various hydraulic retention times (HRTs). Int J Hydrogen Energy 39 (35), 20625–20631.
25 Lee, D.-J., Show, K.-Y., Su, A., 2011. Dark fermentation on biohydrogen production: Pure culture. Bioresour Technol 102 (18), 8393–8402.
26 Lin, C.-Y., Lay, C.-H., Sen, B., Chu, C.-Y., Kumar, G., Chen, C.-C., Chang, J.-S., 2012. Fermentative hydrogen production from wastewaters: A review and prognosis. Int J Hydrogen Energy 37 (20), 15632–15642.
27 Ljunggren, M., Willquist, K., Zacchi, G., Van Niel, E.W.J., 2011. A kinetic model for quantitative evaluation of the effect of hydrogen and osmolarity on hydrogen production by *Caldicellulosiruptor saccharolyticus*. Biotechnol Biofuels 4 (1), 31.
28 Munro, S.A., Zinder, S.H., Walker, L.P., 2009. The fermentation stoichiometry of *Thermotoga neapolitana* and influence of temperature, oxygen, and pH on hydrogen production. Biotechnol Prog 25 (4), 1035–1042.
29 Ntaikou, I., Antonopoulou, G., Lyberatos, G., 2010. Biohydrogen Production from Biomass and Wastes via Dark Fermentation: A Review. Waste Biomass Valor 1 (1), 21–39.
30 O-Thong, S., Prasertsan, P., Karakashev, D., Angelidaki, I., 2008. High-rate continuous hydrogen production by *Thermoanaerobacterium thermosaccharolyticum* PSU-2 immobilized on heat-pretreated methanogenic granules. Int J Hydrogen Energy 33 (22), 6498–6508.
31 Palomo-Briones, R., Razo-Flores, E., Bernet, N., Trably, E., 2017. Dark-fermentative biohydrogen pathways and microbial networks in continuous stirred tank reactors: Novel insights on their control. Appl Energy 198, 77–87.
32 Pauss, A., Andre, G., Perrier, M., Guiot, S.R., 1990. Liquid-to-gas mass transfer in anaerobic processes: Inevitable transfer limitations of methane and hydrogen in the biomethanation process. Appl Environ Microbiol 56 (6), 1636–1644.
33 Pawar, S.S., van Niel, E.W.J., 2013. Thermophilic biohydrogen production: how far are we? Appl Microbiol Biotechnol 97 (18), 7999–8009.
34 Peintner, C., Zeidan, A.A., Schnitzhofer, W., 2010. Bioreactor systems for thermophilic fermentative hydrogen production: Evaluation and comparison of appropriate systems. J Clean Prod 18, S15-S22.
35 Pradhan, N., Dipasquale, L., d'Ippolito, G., Panico, A., Lens, P.N.L., Esposito, G., Fontana, A., 2015. Hydrogen production by the thermophilic bacterium *Thermotoga neapolitana*. Int J Mol Sci 16 (6), 12578–12600.

36 Show, K.-Y., Lee, D.-J., 2013. Bioreactor and Bioprocess Design for Biohydrogen Production, in: Biohydrogen. Elsevier, pp. 317–337.
37 Show, K.-Y., Lee, D.-J., Chang, J.-S., 2011. Bioreactor and process design for biohydrogen production. Bioresour Technol 102 (18), 8524–8533.
38 Sivagurunathan, P., Kumar, G., Bakonyi, P., Kim, S.-H., Kobayashi, T., Xu, K.Q., Lakner, G., Tóth, G., Nemestóthy, N., Bélafi-Bakó, K., 2016. A critical review on issues and overcoming strategies for the enhancement of dark fermentative hydrogen production in continuous systems. Int J Hydrogen Energy 41 (6), 3820–3836.
39 Verhaart, M.R.A., Bielen, A.A.M., van der Oost, J., Stams, A.J.M., Kengen, S.W.M., 2010. Hydrogen production by hyperthermophilic and extremely thermophilic bacteria and archaea: Mechanisms for reductant disposal. Environ Technol 31 (8-9), 993–1003.
40 Whang, L.-M., Lin, C.-A., Liu, I.-C., Wu, C.-W., Cheng, H.-H., 2011. Metabolic and energetic aspects of biohydrogen production of *Clostridium tyrobutyricum*: The effects of hydraulic retention time and peptone addition. Bioresour Technol 102 (18), 8378–8383.
41 Xing, D., Ren, N., Wang, A., LI, Q., Feng, Y., Ma, F., 2008. Continuous hydrogen production of auto-aggregative *Ethanoligenens harbinense* YUAN-3 under non-sterile condition. Int J Hydrogen Energy 33 (5), 1489–1495.
42 Yasin, N.H.M., Mumtaz, T., Hassan, M.A., Abd Rahman, N., 2013. Food waste and food processing waste for biohydrogen production: a review. J Environ Manage 130, 375–385.
43 Zhang, S., Lee, Y., Kim, T.-H., Hwang, S.-J., 2013. Effects of OLRs and HRTs on hydrogen production from high salinity substrate by halophilic hydrogen producing bacterium (HHPB). Bioresour Technol 141, 227–232.

Figure S1

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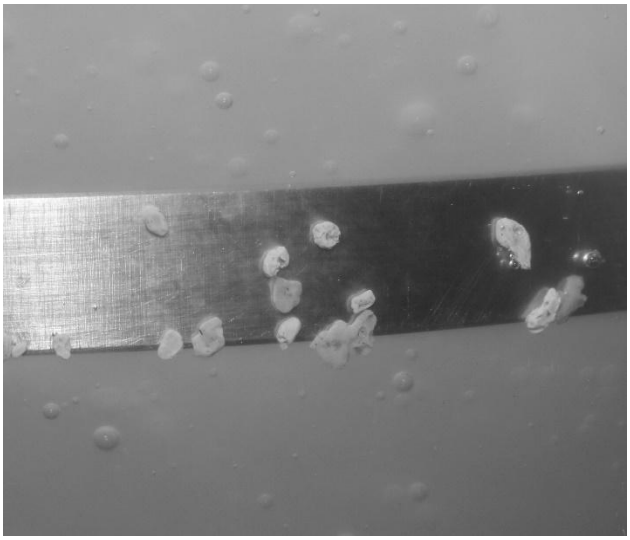

Fig. S1: Photograph of a stainless-steel baffle inside the reactor after 111 days of operation showing the formation of attached biomass agglomerates of up to approximately 4 mm in diameter.

Fig. 1

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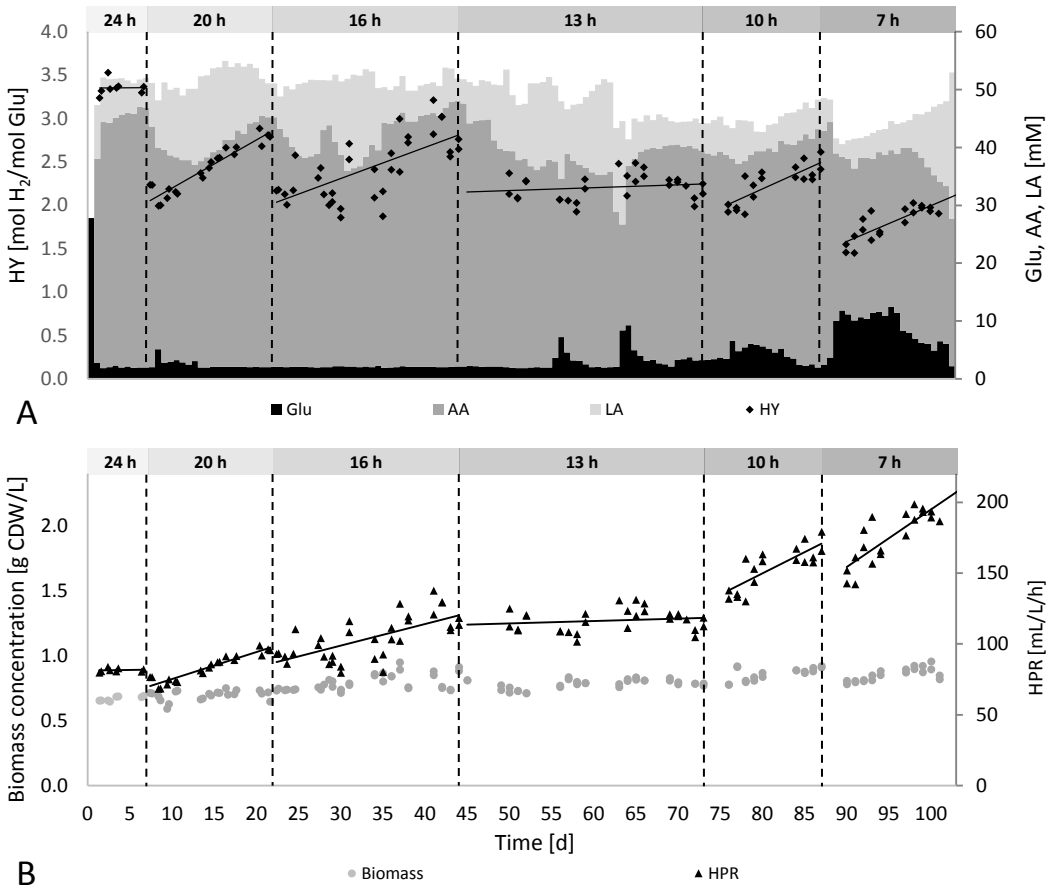

Fig. 2

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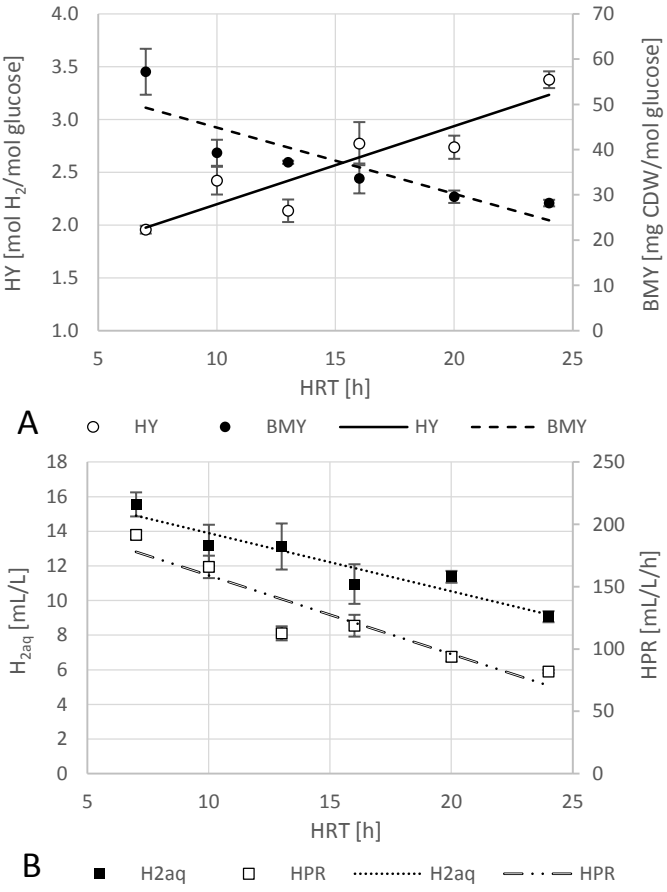

Fig. 3

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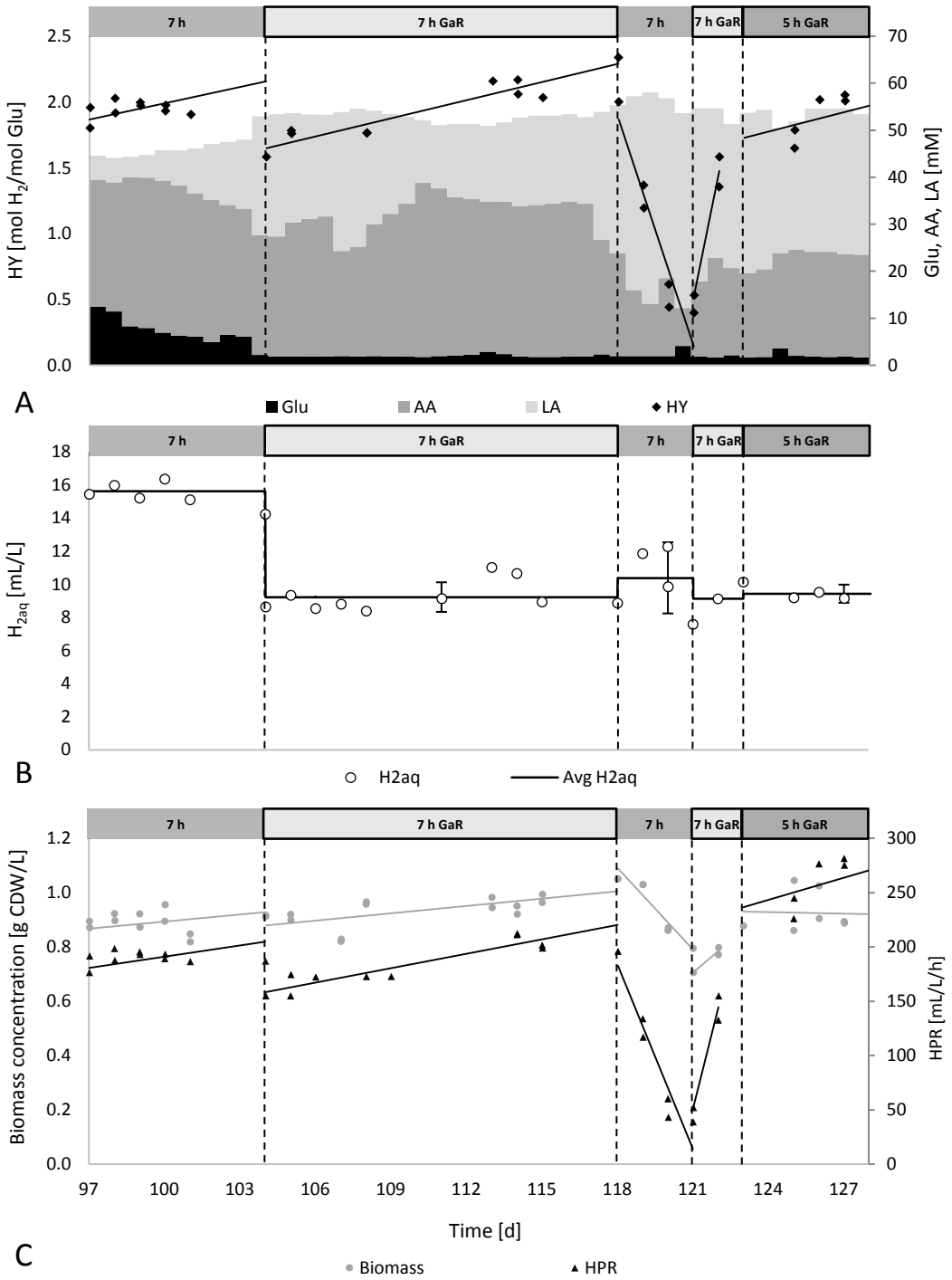

Table 1

Table 1: Biohydrogen production by *T. neapolitana* in continuous dark fermentation of 27.8 mM feed glucose at decreasing HRT excluding or including H_2-rich biogas recirculation (GaR). Hydrogen yield (HY) and hydrogen production rate (HPR) are provided at the start and the end of each operating condition, calculated via the linear regression of each phase, as depicted in Fig. 1A and B as well as Fig. 2A and B.

HRT [h]	GaR	Operating period [d]	HY [mol H_2/mol glucose]		HPR [mL/L/h]	
			start	end	start	end
24	-	0 – 6	3.4	3.4	81	82
20	-	7 – 21	2.0	2.8	70	96
16	-	22 – 44	2.0	2.8	87	120
13	-	45 – 73	2.2	2.3	114	118
10	-	74 – 87	1.9	2.5	132	171
7	-	88 – 103	1.5	2.1	146	207
7	+	104 – 117	1.7	2.3	158	216
7	-	118 – 121	1.9	0.2	184	15
7	+	122 – 123	1.5	2.4	142	235
5	+	124 – 129	1.8	2.0	243	277