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Title: Effect of feed glucose and acetic acid on continuous biohydrogen production by Thermotoga neapolitana

Article Type: Original research paper

Keywords: Thermotoga neapolitana; Hydrogen; Continuous-flow dark fermentation; Acetic acid; Feed concentration; Inhibition

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Abstract: This study focused on the effect of feed glucose and acetic acid on biohydrogen production by Thermotoga neapolitana under continuous-flow conditions. Increasing the feed glucose concentration from 11.1 to 41.6 mM decreased the hydrogen yield from 3.6 (± 0.1) to 1.4 (± 0.1) mol H2/mol glucose. The hydrogen production rate concomitantly increased until 27.8 mM of feed glucose but remained unaffected when feed glucose was further raised to 41.6 mM. Increasing the acetic acid concentration from 0 to 240 mM hampered dark fermentation in batch bioassays, diminishing the cumulative hydrogen production by 45% and the hydrogen production rate by 57%, but induced no negative effect during continuous operation. Indeed, throughout the continuous flow operation the process performance improved considerably, as indicated by the 47% increase of hydrogen yield up to 3.1 (± 0.1) mol H2/mol glucose on day 110 at 27.8 mM feed glucose.

Prof. Ashok Pandey
Editor-in-Chief
Bioresource Technology

Cassino, November 8th, 2018

Dear Prof. Pandey.

On behalf of the co-authors, I would like to thank you for the opportunity to resubmit our manuscript to your reputable journal. The reviewers' comments and suggestions helped in producing a research contribution of improved quality. We have carefully revised the manuscript and hope that this version meets the criteria for publication in Bioresource Technology.

Sincerely,
Gilbert Dreschke MSc
On behalf of the co-authors

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Dear Prof. Ashok Pandey,
Editor-in-Chief, Bioresource Technology

On behalf of the co-authors, I would like to thank the reviewers who have helped us greatly to improve the quality of our research contribution. We have carefully revised the manuscript according to their comments and suggestions. The responses to the reviewers' comments and questions were addressed as described below.

**Reviewer #1:**
The study focused on the influence of feed glucose and acetic acid on biohydrogen by Thermotoga neapolitana under continuous-flow condition. This study is meaningful for the practical application of biohydrogen and the quality of the article also meet BT level.

Specific comments:
1. P7L16: The concentration of inoculation should be added.

***RESPONSE:*** *The biomass concentration of the inoculum was approximately 0.4 g CDW/L. However, we decided not to report this information in the text of the manuscript, as the actual biomass concentration in the continuous-flow reactor was the result of an equilibrium between the growth and washout rates of the biomass and, therefore, independent from the initial concentration. Indeed, the biomass concentration in the reactor was continuously monitored and described throughout the continuous-flow operation.*

2. P16L50: It can be known from Fig.1, 11.1 mM is the favorable glucose concentration, but author investigated the effect of acetic acid on biohydrogen using about 20 Mm glucose. please explain the reason?

***RESPONSE:*** *27.8 mM which equals 5 g/L is the most common glucose concentration used with Thermotoga neapolitana in literature and was chosen to allow a comparison to other studies. Furthermore, the final concentration used to investigate the effect of feed glucose without addition of AA was also 27.8 mM which revealed the considerable improvement of the process compared to the beginning of the experiment due to an acclimatization of the culture. Hence, we decided to continue with this glucose concentration, which allowed us to simultaneously investigate the further acclimatization of the culture and the effect of AA addition in the feed.*

3. P18 3.4 Mechanisms for end product inhibition. I think the mechanism is not the result from the present experiment. This content can be considered as discussion section.

***RESPONSE:*** *We agree with the reviewer but, since the manuscript was written with a combined results and discussion section, as encouraged by the Guide for Authors of Bioresource Technology, a separation was not possible. Nonetheless, the section*

*"Mechanisms for end product inhibition" has been numbered as 3.5 in the revised version of the manuscript, in order to first discuss the acclimatization of the culture under the different operating conditions (i.e. section 3.4 "Improved culture performance due to acclimatization at prolonged cultivation" according to the new numbering) and then the possible mechanisms of inhibition by acetate.*

**Reviewer #2:**

Here are my comments and suggestions:

1. Line 53 Referring to experimental design, I can't see any proper experimental design, it would be advisable to include your experimental matrix as well as a brif explanation of the employed analysis. For instance, it would be important to have a clear perspective on how you decided about glucose levels. In the graphical abstracts you claim for optimization is not possible to make an optimization without a proper statistical analysis.

   ***RESPONSE:*** *The employed experimental design was based on a "one-factor-at-a-time" method. Indeed, at each experimental condition we varied only one parameter (e.g. feed glucose or feed acetic acid concentration) per time. A new sentence was added to the revised manuscript (Lines 123 – 124): "The effect of glucose and acetic acid concentration on dark fermentation by T. neapolitana was investigated by using a "one-factor-at-a-time" method".*

   *We started with a feed glucose concentration of 27.8 mM (i.e. 5 g/L) because it was already used in most other scientific articles on T. neapolitana. In this regard, a reference to the study of Pradhan et al. (2015) was added to the manuscript (Lines 126 - 128). Then, we decided to decrease or increase the feed glucose concentration to study its effect on the performance of the microbial species and the continuous-flow reactor.*

   *Furthermore, we applied an unpaired t-test to our data to demonstrate that the improvement of the HY caused by the prolonged cultivation in the continuous process was statistically significant (Lines 366 and 370). Additionally, we used this statistical to confirm that an increase from 27.8 to 41.6 mM of feed glucose concentration did not significantly affect the HPR (Lines 198 – 200).*

2. Line 48 it is stated that glucose concentration was maintained constant at 27.8 mM and AA was increased from 30 to 240 mM, how you decided to kept 27.8 mM glucose?

   ***RESPONSE:*** *As addressed based on the 2$^{nd}$ comment of reviewer 1, we decided to keep 27.8 mM of feed glucose as it is the most common glucose concentration used with T. neapolitana in previous studies. Furthermore, the final concentration used to investigate the effect of feed glucose without addition of AA was also 27.8 mM which revealed the considerable improvement of the process compared to the beginning of the experiment due to an acclimatization of the culture. Maintaining this feed glucose while increasing the AA concentration in the feed allowed us to both investigate the further acclimatization of the culture and the effect of AA addition in the feed.*

3. Line 53 it is reported that glucose and AA fed into the bioreactor was changed after steady state and this was determined by the hydrogen production, but it is not clear for me how this was related. I mean, hoy you decided how much to vary, upon what?

***RESPONSE:*** *Based on the results of the batch bioassays at increasing feed AA concentrations, we assumed that the AA concentration was the inhibiting factor that prevented an increasing hydrogen production when increasing the feed glucose concentration. We expected that adding AA in the feed would have led to a considerable shift towards the LA pathway and, thus, a reduced hydrogen production. The increase of feed AA concentration was based on the AA concentration (i.e. approximately 30 mM) obtained in the broth when only glucose was fed at 27.8 mM. As we did not observe an impairment of the hydrogen production, we continued to stepwise increase AA by 30 mM till reaching 240 mM.*

4. Until this is not clear I can't have a real picture of your results. It looks like you previosuly knew the inflexion points in the fermentation and then acted, but as readears we don't know what you did before. The results looks like many parallel experiments and I can't see clearly what is the main focus or the sequence in your results, I understand your are explaining the graphs you included, but I think that it would be important to guide the reader a little thru your results. I recommend a little more structured section of results to be easier to understand what you are intending to do. Again, I don't see an optimization approach but a sequence of actions which in experimental desing theory is called: experiment one-factor at a time.

   ***RESPONSE:*** *Substrate and end product inhibition is a very common phenomenon in fermentation processes. There were merely 2 sets of experiments. One continuous-flow experiment, consecutively investigating the effect of feed glucose (days 0-82) and acetic acid (days 83-110) concentration as described in section 2.2.1. The batch experiments described in 2.2.2 were run parallelly to the continuous to determine whether a different impact of feed AA occurred on T. neapolitana compared to what observed in the continuous-flow experiment. We obtained different results and, thus, we concluded that an optimization of the process was achieved under continuous-flow conditions rather than batch assays, mainly due to an acclimatization of the microbial culture at increasing stressing factors.*

**Reviewer #3:**

The manuscript focuses on a very important, interesting and up to date subject. It is well written, clear, detailed, well supported on literature discussion and well organized. It has quality to be published.

A few comments to the authors are:

1. Page 9 - Equations (1) and (2) - what is the difference between "exp" and the Euler's number "e"? The position / place / site of the "e" in both equations is not clear!

   ***RESPONSE:*** *“exp” stands for exponential function, i.e. “e to the power of …”, whereas “e” is the Euler´s number with a value of 2.7183 (approximated). The format of the equation was changed in the revised manuscript to assure a better understanding.*

2. Page 15 - line 16 - the value 0.27 OD540 /h is not marked / shown in Fig 4A (it is missing).

   ***RESPONSE:*** *Fig. 4A was corrected in the revised version of the manuscript by adding the missing data point.*

3. Page 16 - line 58 - the values 77.9 mL/L/h and 694 mg CDW /L, associated to Table 3, are not presented in Table 3 - at least they could be associated to Table 2, but they are not shown in Table 2 as well!

   ***RESPONSE:*** *The values of each individual operating condition are indicated in Table 2. The reference was corrected, and this section rephrased as follows for a better understanding: "The elevated concentrations of AA had no negative impact on the HPR and the biomass concentration, which varied only slightly between 75.3 (± 2.9) and 83.8 (± 2.6) mL/(L h) and 621 (± 19) and 710 (± 26) mg CDW/L, respectively (Table 2). Similarly, the $H_2$ percentage in the produced biogas remained unaffected by AA reaching a value of 69 (± 1)% (data not shown)."*

4. Also the use of the "/" twice is not correct - it would be better to write 77.9 mL L-1 h-1 or 77.9 mL/(L h).

   ***RESPONSE:*** *The unit was changed to mL/(L h) in the revised version of the manuscript.*

5. Page 19 - line 34 - (Fig 1A and 4A) - the reference to Fig 4A is not correct - maybe Fig 5A but, anyway, the increase of HY by 47% at the day 110 is not evident in Fig 5A. … In fact, I realise now that it is OK but it is missing to say somewhere that Fig 5 is the follow up of Fig 1, both of them associated to Table 1.

   ***RESPONSE:*** *We thank the reviewer for this and, in fact, the correct reference is Fig. 5A. Additionally to correcting this error, we added a reference to Table 1 in the captions of Fig. 4 and 5. The fact that only one continuous-flow reactor was run and the conditions were changed sequentially, is described in chapter 2.2.1 and indicated by the continuing numbering of the X-Axis of Fig. 1 and 5.*

**Editor's note:**

1. "Abbreviations" is not usual for the journal, except in the case of intensive modelling, which is not the case here. So, please remove this section and make sure each abbreviation is defined at its first appearance in the text

   ***RESPONSE:*** *The "Abbreviations" section was deleted in the revised manuscript.*

2. The overall document is not in BITE format (http://www.elsevier.com/journals/bioresource-technology/0960-8524/guide-for-authors). Please check all the document

   ***RESPONSE:*** *The format of the document was changed to A4 with wide margins (3 cm).*

3. References list: number them, it is an editorial requirement. Please ensure that you follow the maximum limit of references allowed.

   ***RESPONSE:*** *The manuscript contains 41 references which is below the maximum limit of 50.*

4. Journal names should be abbreviated according to the List of title word abbreviations: http://www.issn.org/2-22661-LTWA-online.php. Check carefully all references, several errors are detected.

***RESPONSE:*** *We carefully checked the abbreviations of journal names in the reference list and revised where needed.*

5. Revise the format of equations
   ***RESPONSE:*** *The format of the equations was revised.*

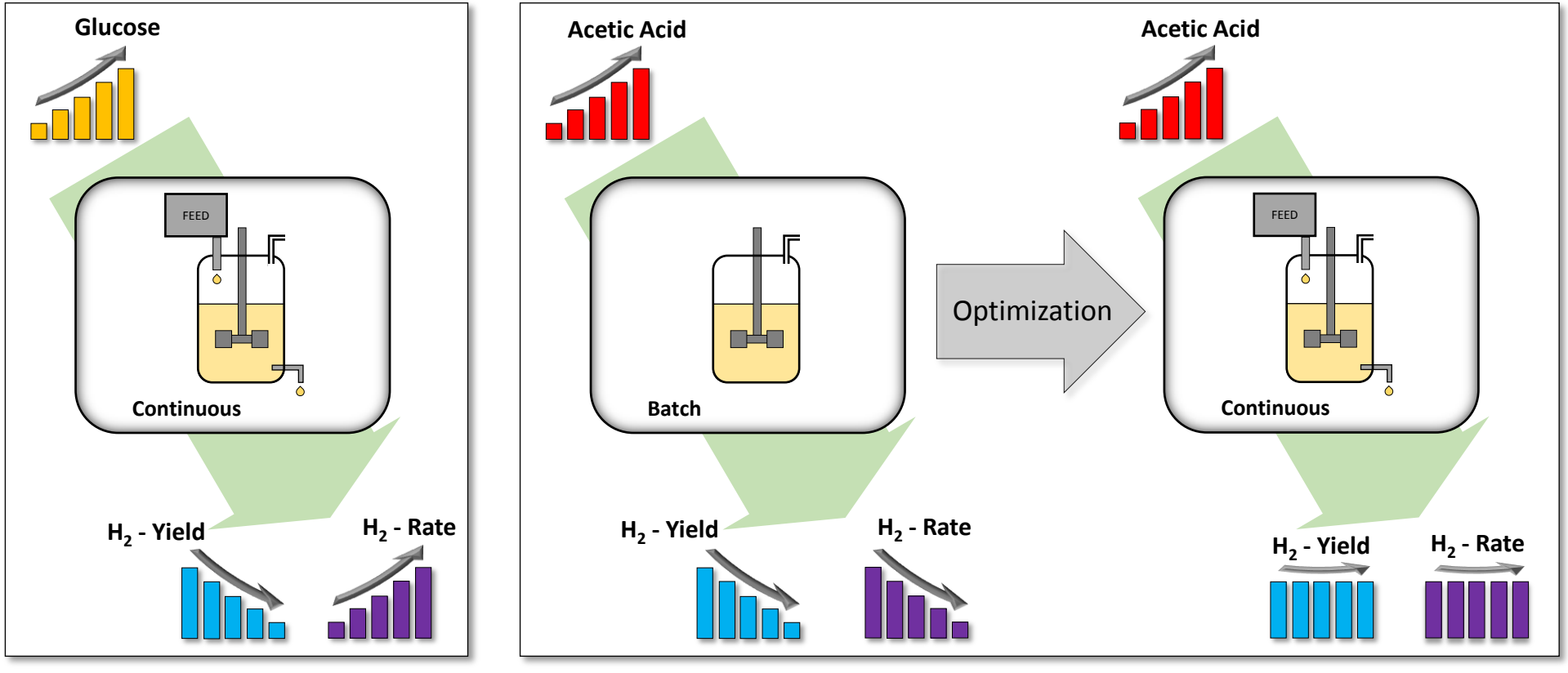
6. Format line numbers
   ***RESPONSE:*** *The line numbers present in the previous submission were added automatically by the online submission portal of BITE. In the revised manuscript continuous line numbering was added in the word file.*

Hoping that the revised version meets the criteria for publication in Bioresource Technology, I would like to thank you again for the opportunity to revise and resubmit the manuscript.

Sincerely,

Gilbert Dreschke
on behalf of all the co-authors

**Graphical Abstract (for review)**



## Highlights

- Hydrogen production rate increased with increasing feed glucose until 27.8 mM
- Hydrogen yield was negatively correlated with feed glucose concentration
- Process performance was unaffected by continuously-fed acetic acid up to 240 mM
- Acetic acid reduced the hydrogen yield and production rate in batch bioassays
- Hydrogen yield increased by 47% in 110 d of continuous operation

*Manuscript
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# Effect of feed glucose and acetic acid on continuous biohydrogen production by *Thermotoga neapolitana*

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**Abstract**

This study focused on the effect of feed glucose and acetic acid on biohydrogen production by *Thermotoga neapolitana* under continuous-flow conditions. Increasing the feed glucose concentration from 11.1 to 41.6 mM decreased the hydrogen yield from 3.6 (± 0.1) to 1.4 (± 0.1) mol $H_2$/mol glucose. The hydrogen production rate concomitantly increased until 27.8 mM of feed glucose but remained unaffected when feed glucose was further raised to 41.6 mM. Increasing the acetic acid concentration from 0 to 240 mM hampered dark fermentation in batch bioassays, diminishing the cumulative hydrogen production by 45% and the hydrogen production rate by 57%, but induced no negative effect during continuous operation. Indeed, throughout the continuous flow operation the process performance improved considerably, as indicated by the 47% increase of hydrogen yield up to 3.1 (± 0.1) mol $H_2$/mol glucose on day 110 at 27.8 mM feed glucose.

**Key words:** *Thermotoga neapolitana*; Hydrogen; Continuous-flow dark fermentation; Acetic acid; Feed concentration; Inhibition

## 1. Introduction

In the past decades, our society has been primarily fueled by fossil resources, which are non-sustainable and polluting energy sources, releasing green-house gases and other toxic compounds upon combustion (Elbeshbishy et al., 2017). The resulting aggravation of air pollution, global warming and extreme weather phenomena in recent years have indicated that a continuing excessive use of fossil fuels will have devastating results on the climate, sea levels and the quality of living for a large part of the population worldwide. To counteract this negative trend, an increasing amount of research is being dedicated to find and establish sustainable sources for clean energy.

Hydrogen has been identified as a highly versatile energy carrier, providing high energy density, a good conversion efficiency, without creating further pollution upon combustion (Baykara, 2018). In this regard, biological processes have gained increased attention, representing a green and sustainable alternative to produce hydrogen. Among these, dark fermentation is considered the most promising (Arimi et al., 2015), resulting in a high productivity with a flexible and simple operation (Sivagurunathan et al., 2016), while allowing the use of waste streams as a substrate.

*Thermotoga neapolitana* is a hyperthermophilic bacterium which has a high potential for dark fermentative hydrogen production (Chou et al., 2008; Pradhan et al., 2015). Hydrogen yields (HY) approaching the theoretical value of 4 mol $H_2$/mol hexose, fast growth kinetics and a large range of potential substrates are its main advantages (Pradhan et al., 2015). Up to now, *T. neapolitana* has been exclusively studied in batch or semi batch operation with the aim of identifying the optimal range of operating

parameters such as temperature, pH and mixing (Munro et al., 2009; Nguyen et al., 2008; Pradhan et al., 2015).

However, in large scale applications, a continuous-flow process is generally preferred (Balachandar et al., 2013; Kumar et al., 2014). Besides the hydraulic retention time (HRT), one of the most important parameters in a continuous process is the organic loading rate (OLR), which is defined by the ratio between the influent substrate concentration and the HRT (Arimi et al., 2015; Elbeshbishy et al., 2017; Sivagurunathan et al., 2016). The increase of the OLR within a certain range allows a more energy-efficient operation (Jung et al., 2011) and has shown to enhance $H_2$ production in dark fermentation (Arimi et al., 2015; Elbeshbishy et al., 2017; Hawkes et al., 2007; Lin et al., 2012; Sivagurunathan et al., 2016).

High feed substrate concentrations do not only lead to an increased hydrogen production, but also to higher concentrations of fermentation end products, e.g. volatile fatty acids (VFAs) and alcohols. When these products exceed a certain threshold level, which is specific to the microbial culture and the particular substrate used (Lin et al., 2012), inhibition of dark fermentation can occur (Lin et al., 2012; Sivagurunathan et al., 2016) resulting in changes of the $H_2$ producing pathways as well as the microbial activity (Ciranna et al., 2014; Jung et al., 2011). Feedback inhibition, which acts on the HY as well as the hydrogen production rate (HPR) (Tang et al., 2012), is considered one of the main challenges in dark fermentation (Boodhun et al., 2017). Therefore, it is essential for a dark fermentative hydrogen production process to find the substrate concentrations that allow for the highest HPR and efficiency, while minimizing the effect of inhibitory compounds.

In the present study, we established for the first time a continuous hydrogen production process using a pure culture of *T. neapolitana*. The main goal of this study was to initially determine the impact of different feed glucose concentrations on the process yields and rates. Secondly, the inhibition by acetic acid (AA), i.e. the main fermentation end product, on $H_2$ production and biomass growth was investigated in both batch and continuous experiments.

## 2. Material and Methods

### 2.1. Bacterial culture and medium

A pure culture of *Thermotoga neapolitana* purchased from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany) was cultivated and stored according to Dreschke et al. (2018) and subsequently used in all experiments. The medium was based on a modified ATCC 1977 medium (Dreschke et al., 2018), in which glucose and AA concentrations were varied as specified in section 2.2. The medium was autoclaved at 110 °C for 5 min, pH-adjusted to 7 and sparged with $N_2$ for 10 min to establish anaerobic conditions.

### 2.2. Experimental design

The continuous and batch experiments were run using a working volume of 2 L, a constant temperature of 80 °C and a pH of 7, automatically adjusted by adding 5M NaOH in a 3-L fully controlled, continuously stirred tank reactor (CSTR) (Applikon Biotechnology, the Netherlands). To avoid pressure build-up, the produced biogas was continuously released from the headspace of the reactor.

### 2.2.1. Continuous process

After the inoculation with 6% (v/v) of storage culture, the reactor was run in batch mode for 16 h to allow the culture to grow and acclimatize. After this initial phase, the feeding was started in continuous mode at a flow rate of 83.3 mL/h to maintain an HRT of 24 h. The feed medium was stored at 4 °C after autoclaving and removing the oxygen by sparging the headspace of the container with $N_2$ for 10 min. The working volume was controlled using a level probe. Twice a day, liquid samples were drawn for the determination of turbidity ($OD_{540}$), glucose, AA and lactic acid (LA) concentrations. Furthermore, 200 mL of effluent was taken to determine the cell dry weight (CDW). The biogas production rate was measured by measuring the time to fill a 500 mL water displacement system.

The effect of glucose and acetic acid concentration on dark fermentation by *T. neapolitana* was investigated by using a "one-factor-at-a-time" method. Initially, the reactor was operated to investigate the effect of the OLR on dark fermentation by varying only the glucose concentration in the feed as reported in Table 1. The range of the feed glucose concentration was based on previous studies using *T. neapolitana* (Pradhan et al., 2015). From day 83 onwards, the feed glucose was maintained constant at 27.8 mM, while only the AA concentration was gradually increased from 30 to 240 mM (Table 1) to evaluate the effect of increasing AA concentrations on the process. The feed glucose and AA concentrations were changed when a steady state was reached, determined by a variation of the hydrogen production by less than 10%.

### 2.2.2. Batch bioassays

Simultaneously to the continuous-flow operation, batch bioassays were run in order to assess the effect of the AA concentration (i.e. 0, 30, 60, 90, 120, 180 and 240 mM) on dark fermentation by *T. neapolitana*. Prior to investigating each condition, the reactor was inoculated with 1% (v/v) of storage culture and stirred at 100 rpm for 15 h to allow the culture to grow and acclimatize. After 15 h, the agitation speed was increased to 500 rpm to accelerate the process. The produced biogas was captured in a 500 mL water displacement system and quantified every hour. Liquid samples of 2 mL were drawn every hour to measure turbidity as well as the glucose, AA and LA concentrations. The fermentation was terminated after 23 h or previously, when the reactor ceased to produce further biogas. Duplicates were used for each operating condition.

### 2.3. Analytical methods

The biomass concentration of batch bioassays was quantified by measuring the optical density ($OD_{540}$) at 540 nm (8453 UV-Visible Spectrophotometer, Agilent Technologies, USA), whereas in the continuous experiment 200 mL of effluent was dried at 105 °C until constant weight to determine the CDW. Subsequently, the samples were centrifuged (10,000 rpm at 5 min) and the supernatant was used to measure the concentrations of glucose, AA and LA applying the method described by Mancini et al. (Mancini et al., 2018) with an HPLC (Prominence LC-20A Series, Shimadzu, Japan), equipped with UV/Vis (SPD-20A, Shimadzu Japan) and refractive index (RID-20A, Shimadzu, Japan) detectors and 0.0065 M of sulfuric acid as the mobile phase. The hydrogen concentration of the biogas was measured 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 hydrogen production was converted from volumetric to molar by using the ideal gas law (O-Thong et al., 2008).

### 2.4. Kinetic study of biohydrogen production and biomass growth

The rates of biomass growth and hydrogen production were determined by fitting the batch experimental data with the Gompertz model as described by Dreschke et al. (2018). Equations 1 and 2 were applied for biomass growth and hydrogen production, respectively:

$$B = B_0 + B_m * \exp\{-\exp[R_B * e * (\lambda_B - t)/B_m + 1]\} \quad (1)$$

$$H = H_m * \exp\{-\exp[R_H * e * (\lambda_H - t)/H_m + 1]\} \quad (2)$$

where $B$ [$OD_{540}$] is the biomass concentration at fermentation time t [h]; $B_0$ [$OD_{540}$] is the biomass concentration at time 0 h; $B_m$ [$OD_{540}$] is the gain of biomass concentration throughout the fermentation; $R_B$ is the volumetric biomass growth rate (BGR) [$OD_{540}$/h]; and $\lambda_B$ is the lag phase of biomass growth [h]; $H$ [mL] is the cumulative hydrogen at time t [h]; $H_m$ [mL] is the hydrogen produced throughout the fermentation; $R_H$ [mL/(L h)] is the volumetric hydrogen production rate; and $\lambda_H$ is the lag phase of hydrogen production [h]; and $e$ is the Euler's number, i.e. 2.7183.

### 2.5. Microbial community analysis

On day 82 and 102 of the continuous operation, 3 mL of liquid sample were extracted for the determination of the microbial community. DNA was extracted, stored and sequenced as explained by Kostrytsia et al. (2018). Quality filtering, sequence clustering, chimera removal and taxonomy assignment using the Silva (v.128) database (Glöckner et al., 2017; Pruesse et al., 2007) was applied on the raw sequence data

before generating an operational taxonomic unit (OTU) table via the Quantitative Insight into Microbial Ecology (QIIME v1.9) pipeline (Caporaso et al., 2010). A threshold of 0.1% was employed to distinguish rare from abundant taxa.

### 2.6. Statistical analysis

An unpaired t-test using Microsoft Excel 2016 (Microsoft Corporation, USA) was performed to compare the experimental data obtained under the varying operating conditions during the continuous-flow experimentation.

## 3. Results and Discussion

### 3.1. Effect of glucose concentration in a continuous system

#### 3.1.1. Limit of feed glucose concentration

Fig. 1 shows the evolution of dark fermentation in continuous operation at different feed glucose concentrations. During phase G1, a feed glucose concentration of 27.8 mM led to a stable process. Within 1 day, a HPR of 55.2 (± 4.7) mL/(L h) (Table 2), a HY of 2.1 (± 0.2) mol $H_2$/mol glucose and a biomass yield of 26.9 (± 1.2) g CDW/mol glucose were reached (Fig. 1A) and the produced biogas contained 70 (± 4)% of $H_2$ (data not shown). The process remained stable for the subsequent 10 days of operation producing 30.9 (± 0.7) mM of AA and 17.6 (± 1.1) mM of LA (Fig. 1B), with an AA/LA ratio of 1.8 (± 0.2) (Fig. 2A) and a residual glucose concentration of 2.1 (± 0.1) mM (Fig. 1B).

Increasing the feed glucose concentration to 41.6 mM in phase G2 did not significantly improve the reactor performance exhibiting an HPR of 53.7 (± 4.0) mL/(L h) (p-value: 0.35), similar to that observed in phase G1 (Table 2). The glucose concentration in the

effluent increased to 4.2 (± 0.6) mM, while the AA concentration remained unaffected at 28.2 (± 1.1) mM and the LA concentration significantly increased to 43.2 (± 2.3) mM (p- value: $1.7 * 10^{-13}$) (Fig. 1B), entailing an AA/LA ratio of 0.7 (± 0.1) (Fig. 2A). In *T. neapolitana*, only 2 pathways are involved to a relevant extent in the dark fermentation of glucose, i.e. the AA pathway yielding 4 moles of hydrogen and 4 moles of ATP per mole of glucose and the energetically less challenging LA pathway producing no hydrogen but 2 moles of ATP (Balachandar et al., 2013; Pradhan et al., 2015). The AA/LA ratio is, thus, tightly linked to the HY as shown in Fig. 2A and represents another indicator for the conversion efficiency to hydrogen. Consequently, the decrease of the AA/LA ratio in phase G2 was accompanied by the reduction of the HY to 1.4 (± 0.1) mol $H_2$/mol glucose (Fig. 1A and Fig. 2A).

The lower $H_2$ production efficiency at 41.6 mM of feed glucose strongly suggests a substrate overload of *T. neapolitana*, which is commonly observed in dark fermentation (Akutsu et al., 2009; Hafez et al., 2010). For instance, Zhang et al. (2013) raised the feed glucose concentration from 5 to 15 g/L in a CSTR at an HRT of 6 h using *Clostridium bifermentans 3AT-ma*. Similar to our results, this induced a decrease of the HY and AA concentration from 1.1 to 0.7 mol $H_2$/mol glucose and from 10.0 to 6.8 mM, respectively, with a sharp increase of the LA and butyric acid concentrations. Zhang et al. (2013) assumed the VFA accumulation responsible for the HY decrease. Due to the low residual glucose concentration, i.e. between 2.2 (± 0.0) and 4.2 (± 0.6) mM in the effluent (Fig. 1B), we assume a similar effect (Elbeshbishy et al., 2017; Zhang et al., 2013) prevented the further AA formation and subsequently limited the hydrogen production.

### 3.1.2. Effect of feed glucose concentration on the hydrogen and biomass yield

To better study the effect of the glucose concentration on dark fermentation, lower feed glucose concentrations (i.e. 16.7, 11.1 and 22.2 mM) were used in phases G4, G5 and G6, respectively. The reduction of the feed concentration increased the HY to 3.3 (± 0.2), 3.6 (± 0.1) and 2.9 (± 0.2) mol $H_2$/mol glucose and biomass yield to 39.3 (± 1.8), 47.2 (± 2.3) and 31.6 (± 1.4) g CDW/mol glucose, in phases G4, G5 and G6, respectively (Fig. 1A). Concomitantly, the AA/LA ratio increased to 4.6 (± 1.1), 6.0 (± 0.7) and 3.8 (± 0.9) (Fig. 2A). This revealed an almost linear negative correlation between the HY and feed glucose concentration (Fig. 2A), with a maximum yield of 3.6 (± 0.1) mol $H_2$/mol glucose at the lowest feed (i.e. 11.1 mM) and the concomitant shift from AA to LA at increasing feed glucose concentrations (Fig. 1B). A similar correlation was observed for the biomass yield (Fig. 2A). The biomass concentration increased with the feed glucose concentration until reaching a plateau at 687 (± 21) mg CDW/L above 22.2 mM of feed glucose (Table 2). It is unclear why the biomass concentration did not increase further at higher glucose concentrations.

Up to now, the effect of substrate concentration on *T. neapolitana* activity has exclusively been studied in batch operation, most commonly in 120 mL closed serum bottles without pH control (Ngo et al., 2012; Nguyen et al., 2010; Nguyen et al., 2008). While reporting a notable increase of the HY with increasing glucose (Nguyen et al., 2010; Nguyen et al., 2008) or xylose (Ngo et al., 2012) concentrations up to a certain threshold level, the results obtained under these conditions are generally highly affected by the decrease of pH (Brynjarsdottir et al., 2013) and the build-up of the

hydrogen partial pressure (Ngo et al., 2012). Mars et al. (2010) used *T. neapolitana* in a pH-controlled reactor using headspace sparging. When increasing the glucose concentration from 10 to 27 g/L, the HY and HPR remained similar at 2.9 and 3.0 mol $H_2$/mol glucose as well as 12.3 and 12.4 mmol/(L h), respectively. However, the fermentation time increased from 20 to over 71 h. Similarly, the general HPR increased and HY decreased when increasing the substrate concentration of a continuous-flow reactor with mixed cultures, using glucose (van Ginkel and Logan, 2005b) or organic-containing wastewater (Lin et al., 2012) as substrates.

### 3.2. Impact of the initial AA concentration in batch bioassays

#### 3.2.1. Effect on hydrogen production and yield

The batch fermentation of 27.8 mM of glucose by *T. neapolitana* without AA addition resulted in a HY of 2.8 (± 0.0) mol $H_2$/mol glucose and an AA/LA ratio of 2.3 (Table 3). Increasing the initial AA concentration from 0 to 240 mM gradually reduced the total hydrogen production by 45% from 1739 (± 12) to 950 (± 29) mL/L (Table 3). Up to 120 mM of AA, glucose was completely consumed within 23 h with a 30% decrease of the HY to 2.0 (± 0.0) mol $H_2$/mol glucose, accompanied by a decline of the AA/LA ratio (Table 3).

The reduction of the HY at elevated concentrations of fermentation end products is commonly observed in dark fermentation (Jones et al., 2017; Tang et al., 2012; van Ginkel and Logan, 2005a; Wang et al., 2008). For example, Ciranna et al. (2014) reported the decrease of HY from 3.0 (± 0.2) to 0.6 (± 0.4) mol $H_2$/mol glucose when

increasing the AA concentration from 40 to 225 mM in a batch fermentation of 55 mM glucose using *Caloramator celer* in 120 mL closed serum bottles.

In this study, a further increase of the feed AA to 180 and 240 mM led to an elevated residual glucose concentration, i.e. 7.8 (± 1.2) and 11.0 (± 1.6) mM, in the effluent after 23 h and a slight increase of the HY to 2.1 (± 0.0) and 2.3 (± 0.0) mol $H_2$/mol glucose, respectively (Table 3). A more detailed analysis revealed that the AA/LA ratio was 161 (± 84) % higher from 15 to 18 h than from 18 to 22 h in all batch bioassays. This indicates that the HY was higher in the early stages of the experiment. A possible cause for this effect is the higher initial glucose concentration. Nguyen et al. (2010) reported a considerable increase of the HY when increasing the initial glucose concentration from 1 to 4 g/L in batch fermentation by *T. neapolitana*. Therefore, we assume that the slightly higher HY at 180 and 240 mM AA was not linked to the AA concentration, but rather caused by the evolution of the process.

### 3.2.2. Dark fermentation kinetics

In a continuous-flow operation, the process rate is strongly determined by the substrate feeding rate. Therefore, batch bioassays were performed to study the effect of AA on the biomass growth and hydrogen production kinetics of *T. neapolitana*.

As indicated by the incomplete glucose consumption at 180 and 240 mM AA, an elevated initial AA concentration notably decreased the dark fermentation rate. To better compare the results obtained at different AA feed concentrations, a modified Gompertz model was used to fit the data of hydrogen production and biomass growth (Fig. 3A and B). Under all operating conditions, the quality of the fit was confirmed by

an $R^2$ of 0.99 (± 0.00) for hydrogen production and 0.98 (± 0.01) for biomass growth. Without AA in the medium, the HPR and BGR reached 265 (± 7) mL/(L h) and 0.27 (± 0.03) $OD_{540}$/h, respectively (Fig. 4A). The low BGR obtained at 0 mM AA was presumably caused by the high biomass growth already achieved after 15 h (Fig. 3B), leading to a distorted value from the Gompertz model.

In the range between 30 - 240 mM, the initial AA concentration was found to be negatively correlated to the BGR and the HPR, which decreased from 0.42 (± 0.06) to 0.24 (± 0.04) $OD_{540}$/h and from 230 (± 7) to 115 (± 0) mL/(L h), respectively (Fig. 4A). This corresponds to a deceleration of hydrogen production and biomass growth by 50 and 43%, respectively. The slowdown of the fermentation was furthermore confirmed by an increase of the lag phase from 14.0 (± 0.3) to 15.7 (± 0.4) h for biomass growth and from 15.7 (± 0.1) to 18.0 (± 0.1) h for hydrogen production (Fig. 4B). Both lag phases were similarly affected by the AA concentration increase by approximately 2.5 h in the studied AA concentration range (i.e. 0 - 240 mM) (Fig. 4B). However, the hydrogen production initiated about 2.2 h after the biomass growth according to the Gompertz analysis.

Mars et al. (2010) previously investigated the effect of increased AA concentrations on the performance of *T. neapolitana* using closed 120 mL serum bottles without pH control. They reported biomass growth at up to 300 mM AA with a decreasing amount of total VFAs produced with increasing initial AA concentration. However, they did not present detailed information on the production of AA and hydrogen or the evolution of the fermentation.

The effect of AA on dark fermentation of pure cultures was studied in more detail by Van Niel et al. (2003) and Ciranna et al. (2014) in 120 mL closed serum bottles without pH control. Van Niel et al. (2003) added sodium acetate in the exponential growth phase of the extreme thermophile *Caldicellulosiruptor saccharolyticus* obtaining concentrations from 0 to 300 mM AA, while Ciranna et al. (2014) investigated the effect of an initial AA concentration up to 350 mM on *Caloramator celer*. Both studies observed a drastic decrease of the process rate, revealed by a reduction of the HPR and the BGR. Concomitantly, they reported no further biomass growth above 200 mM AA for *Caldicellulosiruptor saccharolyticus* (van Niel et al., 2003) and approximately 150 mM AA for *Caloramator celer* (Ciranna et al., 2014). In both studies, increasing the undissociated AA fraction in the fermentation broth due to a pH decrease did not alter the inhibitory effect. Furthermore, Van Niel et al. (2003) found the inhibition of sodium chloride and sodium acetate to be identical. Therefore, both studies concluded that ionic strength was responsible for the inhibition of dark fermentation rather than the free AA.

### 3.3. Effect of acetic acid concentration in a continuous system

In the second stage of the continuous process, the feed AA concentration was gradually increased from 0 to 240 mM. The elevated concentrations of AA had no negative impact on the HPR and the biomass concentration, which varied only slightly between 75.3 (± 2.9) and 83.8 (± 2.6) mL/(L h) and 621 (± 19) and 710 (± 26) mg CDW/L, respectively (Table 2). Similarly, the $H_2$ percentage in the produced biogas remained unaffected by AA reaching a value of 69 (± 1)% (data not shown). Glucose was completely consumed under all operating conditions (Fig. 5B), entailing that also

the hydrogen and biomass yields remained constant at 3.0 (± 0.2) mol $H_2$/mol glucose and 27.1 (± 1.6) g CDW/mol glucose, respectively (Fig. 2B and 5A). Hence, contrary to the results obtained in the batch bioassays, the increase of the feed AA concentration had no effect on the continuous-flow dark fermentation. In particular, the HY of the continuous process increased by approximately 6% at 0 mM AA and 48% at 120 mM AA compared to the batch experiments. We assume that the prolonged cultivation at high AA concentrations allows *T. neapolitana* to adapt its metabolism and continue to ferment via the energetically more challenging AA pathway, which results in a higher yield of ATP (Pradhan et al., 2015).

Van Ginkel (van Ginkel and Logan, 2005a) studied the effect of undissociated VFAs on continuous dark fermentation of a mixed culture operated at 30 °C, an HRT of 10 h and a stable pH of 5.5 with glucose (10 – 50 g/L) as a substrate. A total AA concentration of approximately 10, 100 and 165 mM, resulting in an undissociated AA concentration of 2, 15 and 25 mM, induced HYs of about 2.5, 2.4 and 2.0 mol $H_2$/mol glucose and HPRs of 0.29, 0.30 and 0.20 L/h, indicating a little effect of non-dissociated AA on $H_2$ yields. Jones et al. (2017) ran a continuous hydrogen production reactor with a mixed culture (35 °C; pH 5.5; HRT 48 h and 40 g sucrose/L as a substrate) applying electrodialysis to remove VFAs from the liquid phase. When the AA concentration was decreased from 3.08 to 1.77 g/L, the HY increased from 0.24 to 0.90 mol $H_2$/mol hexose and the carbohydrate consumption from 12 to 25%, indicating a higher dark fermentation rate.

### 3.4. Improved culture performance due to acclimatization at prolonged cultivation

Within the course of the continuous-flow operation, an initial glucose concentration of 27.8 mM was repeatedly used in phases G1, G3 and G7 to confirm the reproducibility and investigate the acclimatization of the culture. After 26 days of cultivation, the reactor performance in phase G3 remained comparable to that previously observed in phase G1, as demonstrated by the similar values of hydrogen and biomass yield (Fig. 1A) as well as fermentation end products (Fig. 1B). However, when using 27.8 mM as feed glucose again in phase G7 after 65 days of operation, an HY of 2.7 (± 0.1) mol $H_2$/mol glucose (Fig. 1A) was obtained, which was significantly higher (i.e. by approximately 29%; p- value: $2.0 * 10^{-13}$) than that observed in phase G1. Concomitantly, the AA/LA ratio increased to 3.5 (± 0.8) (Fig. 2A), while the biomass yield remained constant at 27.8 (± 1.0) g CDW/mol glucose (Fig. 1A). The same trend continued when raising the feed AA concentration from 0 to 240 mM with the HY increasing by a further 12% from 2.8 (± 0.2) to 3.1 (± 0.1) mol $H_2$/mol glucose (p- value: $9.5 * 10^{-7}$) (Fig. 2B and 5A) and a simultaneous reduction of the LA concentration from 11.1 (± 2.0) to 3.4 (± 0.2) mM (Fig. 5B). Hence, the HY increased by a total of 47% throughout the 110 days of continuous-flow operation (Fig. 1A and 5A). We presume that this substantial improvement of the process efficiency was directly correlated to an acclimatization of *T. neapolitana*.

The importance of acclimatization has previously been demonstrated for mixed cultures (Haroun et al., 2016), where it is generally considered as a shift in the microbial community structure (Cisneros-Pérez et al., 2017; Dessì et al., 2017). However, this study demonstrates that acclimatization also occurs in pure cultures in terms of a metabolic shift and represents a large potential to enhance the process

performance in continuous fermentation. Acclimatization in pure cultures is still poorly understood and can require long operation times, which complicate the assessment of the culture potential.

### 3.5. Mechanisms for end product inhibition

Several effects have been discussed in the literature to explain the inhibition of high end product concentrations on dark fermentation (Elbeshbishy et al., 2017). The most common is the decrease of the extracellular pH until the normal functions of the cell cannot be kept active (Elbeshbishy et al., 2017; Srikanth and Venkata Mohan, 2014). This mechanism can be excluded in the present study, as the pH was continuously controlled at 7. At acidic pH, inhibition may also occur due to the presence of undissociated organic acids (Elbeshbishy et al., 2017; Srikanth and Venkata Mohan, 2014), which penetrate the cells and disrupt the cell functions by changing the intracellular pH and osmolarity (Akutsu et al., 2009). In this study, a concentration of 270 mM AA (240 mM fed + approximately 30 mM produced) was observed in the reactor at the highest AA feed. At pH 7, this results in an undissociated AA concentration below 2 mM, calculated via the equation presented by Akutsu et al. (2009), and its inhibitory effect on the process was therefore considered negligible. Ciranna et al. (2014) identified the increase of ionic strength to be responsible for the feedback inhibition. However, Pradhan et al. (2017) reported no effect of salinity on HY and biomass growth of *T. neapolitana* up to 855 mM of NaCl. Jones et al. (2017) ascribed dark fermentation inhibition by VFAs to be a thermodynamic limitation.

### 3.6. Microbiological considerations

In the MiSeq, the bacterial community analysis after 102 days of continuous operation revealed 2 genera above 0.1% relative abundance, i.e. *Thermotoga* and *Enterococcus* with 98 and 2%, respectively. As *Enterococci* are mesophilic bacteria growing in the range from 10 to 45 °C (Sherman, 1937), their minor appearance in the community analysis was most likely caused by a contamination during sampling. Further evidence for an exclusive substrate degradation by *T. neapolitana* was provided by the composition of end products in the effluent. Throughout the 110 d of continuous operation the sum of AA and LA, constituted for 95 (± 10)% of the glucose consumed. Nguyen et al. (2010) reported that batch bioassays using *T. neapolitana* remained free of contamination due to the extreme growth temperature of 80 °C. Our results suggest that this finding also applied to a prolonged cultivation of 102 days in non-sterile continuous operation.

## Conclusions

During dark fermentation by *T. neapolitana*, increasing feed glucose concentrations from 11.1 to 27.8 mM simultaneously led to higher HPR and lower HY. When further raised to 41.6 mM, the additional glucose was metabolized to LA without producing extra hydrogen, resulting in stable HPR and AA production. Increasing the feed AA concentration up to 240 mM induced no negative effect, suggesting that biohydrogen production was not hampered by end-product inhibition. Moreover, the HY improved by 47% throughout the 110 days of continuous cultivation, reaching a final value of 3.1 (± 0.1) mol $H_2$/mol glucose at 27.8 mM feed glucose.

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**Fig. 1:** Continuous hydrogen production by *T. neapolitana* at varying feed glucose (Glu) concentrations (11.1 – 41.6 mM) and a HRT of 24 h (Table 1; G1-G7). Yields of biomass (BMY) and hydrogen (HY) (A) as well as residual Glu, acetic acid (AA) and lactic acid (LA) concentrations in the effluent (B).

**Fig. 2:** Hydrogen yield (HY), biomass yield (BMY) and acetic acid to lactic acid ratio (AA/LA) of a continuous dark fermentation by *T. neapolitana* at (A) different feed glucose (11.1 – 41.6 mM) and (B) acetic acid (0 – 240 mM) concentrations.

**Fig. 3:** Evolution of cumulative hydrogen (A) and biomass growth (B) in batch bioassays fermenting 27.8 mM glucose with *T. neapolitana* at different initial AA concentrations (0 – 240 mM). The symbols depict the experimental data while the lines exhibit the Gompertz model.

**Fig. 4:** Effect of different initial acetic acid (AA) concentrations on (A) biomass growth (BGR) and hydrogen production (HPR) rates and (B) the lag phases of hydrogen production (HP) and biomass growth (BG) during the batch dark fermentation of 27.8 mM of glucose as substrate with *T. neapolitana*.

**Fig. 5:** Continuous hydrogen production by *T. neapolitana* at varying feed acetic acid (AA) concentrations (0 – 240 mM) at 27.8 mM of feed glucose (Glu) concentration and an HRT of 24 h (Table 1; AA1-AA6). Yields of biomass (BMY) and hydrogen (HY) (A) as well as AA, lactic acid (LA) and residual Glu concentration in the effluent (B).

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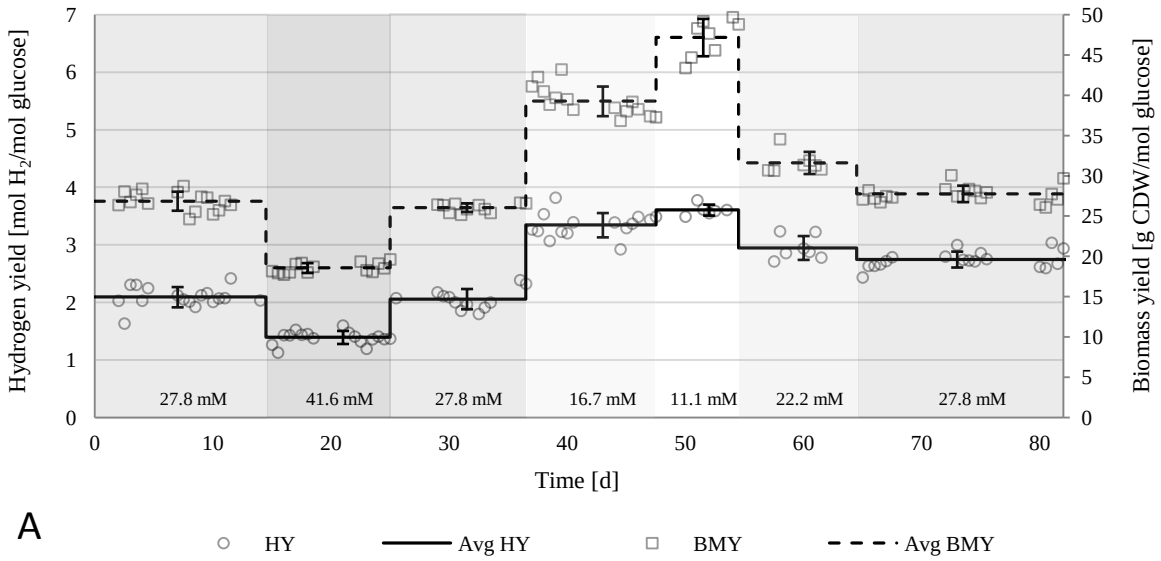


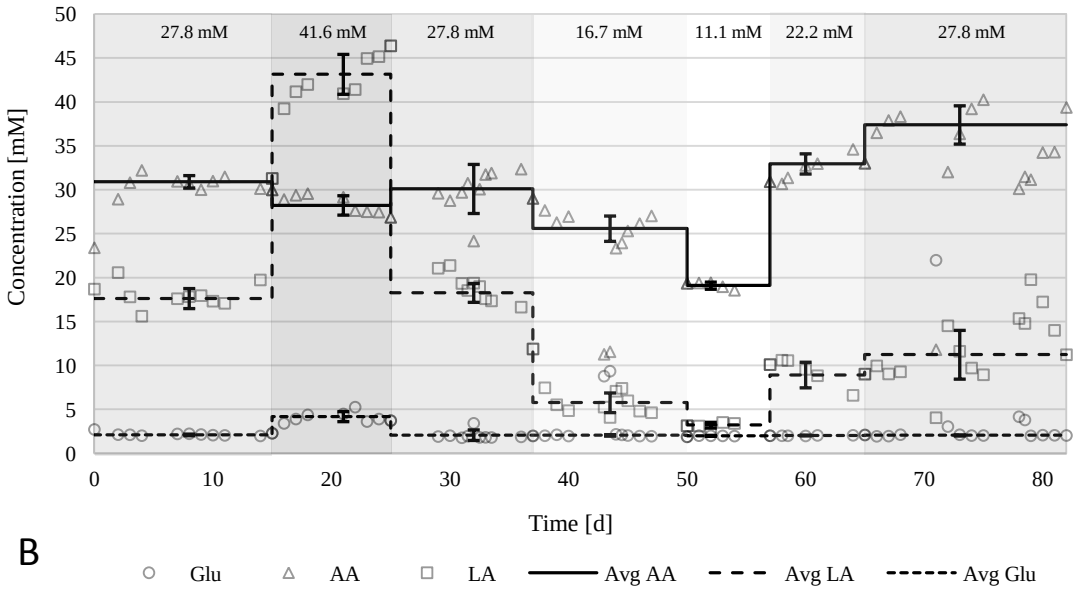

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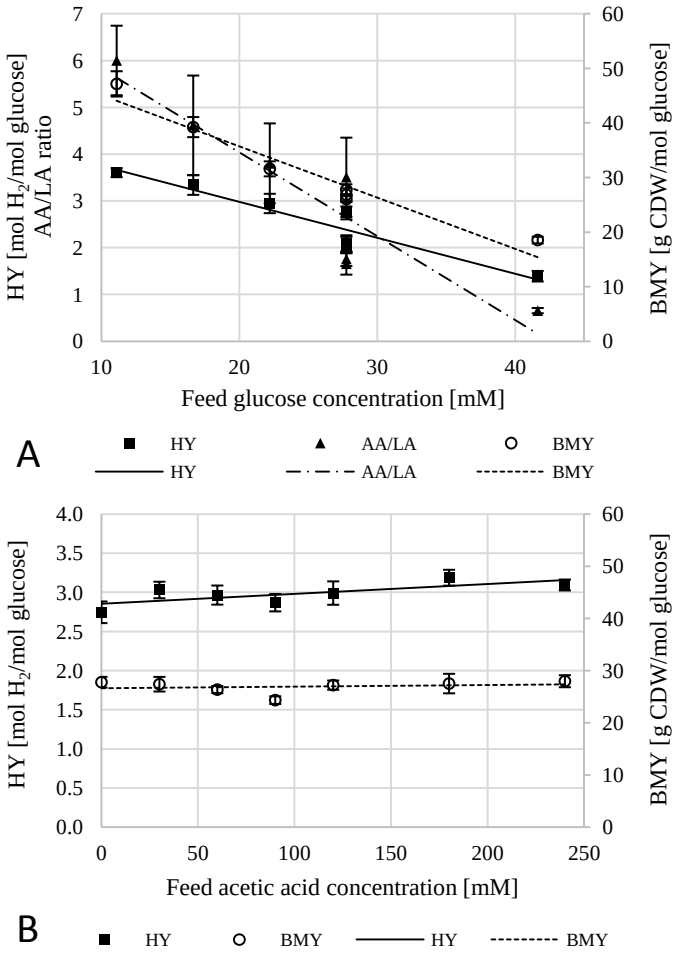

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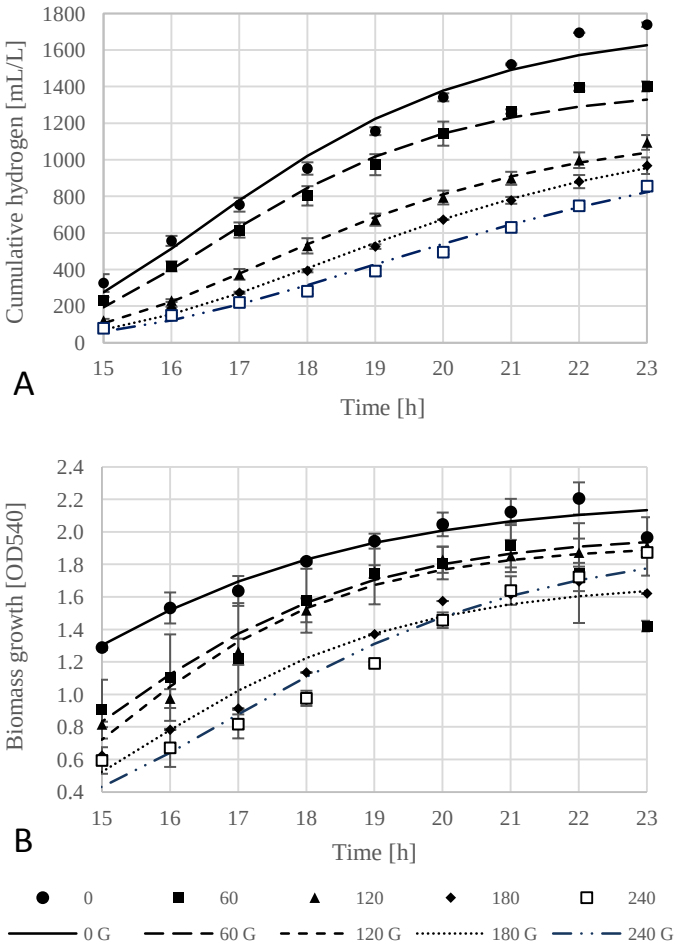

**Figure 4**

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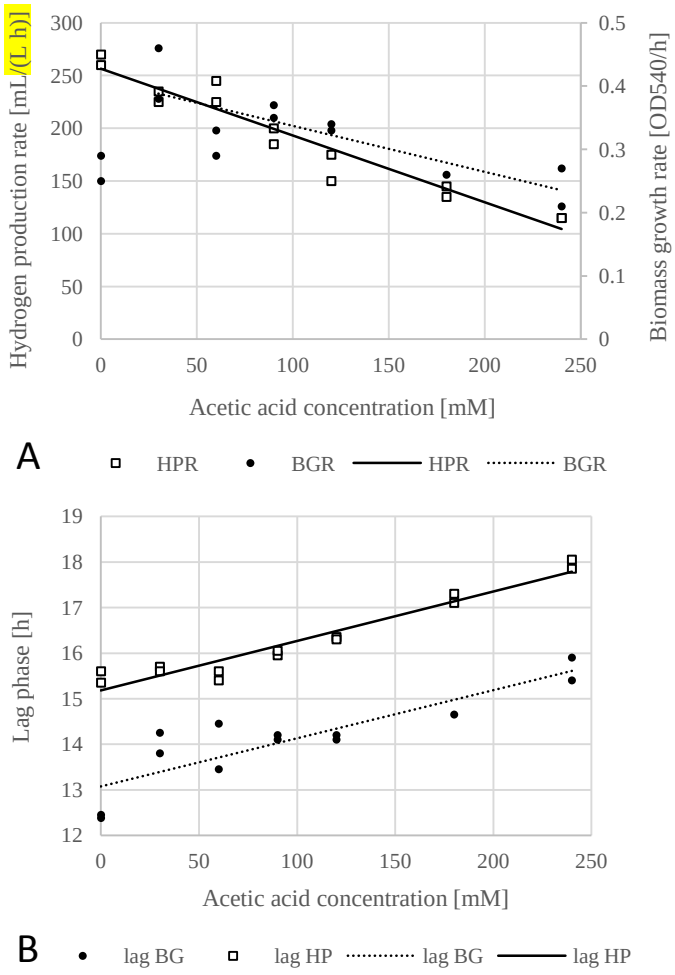

**Figure 5**

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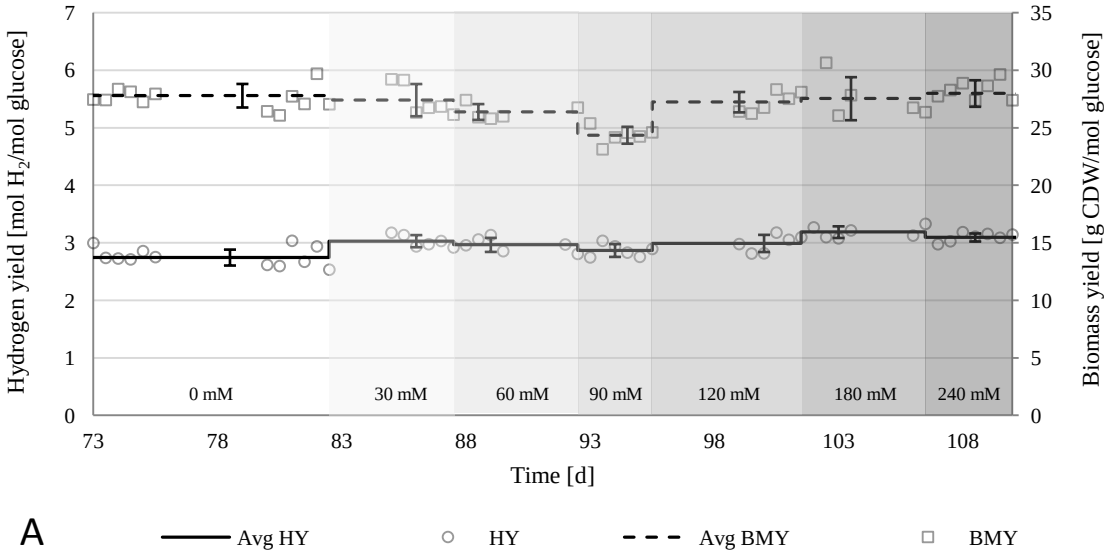


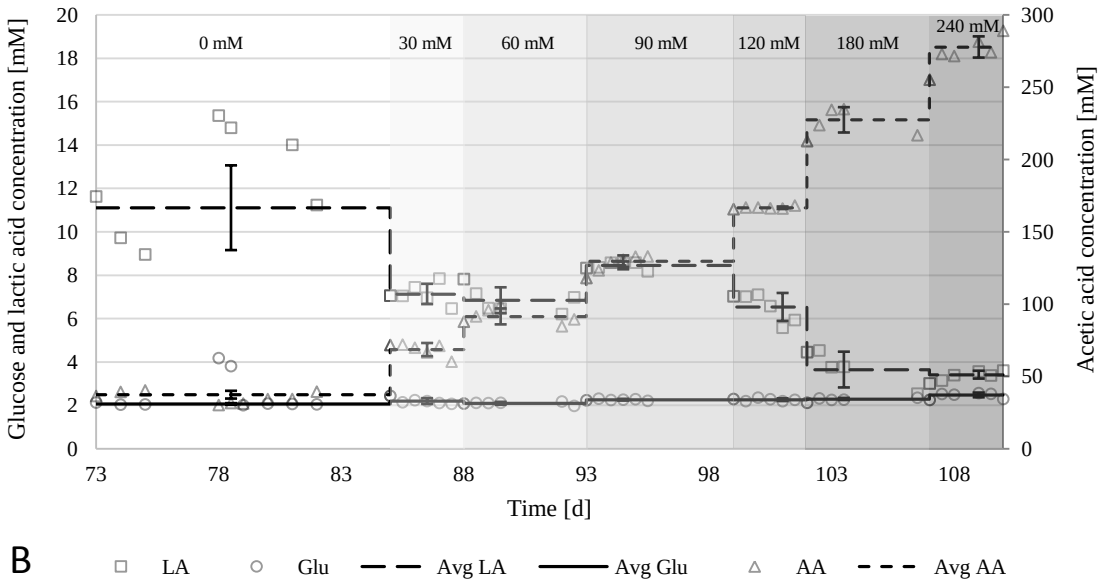

**Table 1**

**Table 1:** Feed glucose (Glu) and acetic acid (AA) concentrations used in the operation of the continuously stirred tank reactor aimed at dark fermentation by *T. neapolitana*. Seven (G1 - G7) and six (AA1 – AA6) experimental phases were used to assess the effect of Glu and AA, respectively. Feed Glu remained at 27.8 mM when increasing the feed AA.

| Phase | Feed Glu [mM] | Operation period [d] |
|---|---|---|
| G1 | 27.8 | 0 - 14 |
| G2 | 41.6 | 15 - 25 |
| G3 | 27.8 | 26 - 36 |
| G4 | 16.7 | 37 - 47 |
| G5 | 11.1 | 48 - 54 |
| G6 | 22.2 | 55 - 64 |
| G7 | 27.8 | 65 - 82 |

| Phase | Feed AA [mM] | Operation period [d] |
|---|---|---|
| AA1 | 30 | 83 - 87 |
| AA2 | 60 | 88 - 92 |
| AA3 | 90 | 93 - 96 |
| AA4 | 120 | 97 - 107 |
| AA5 | 180 | 102 - 106 |
| AA6 | 240 | 107 - 110 |

**Table 2:** Hydrogen production rate (HPR) and biomass concentration (BM) obtained during the continuous dark fermentation by *T. neapolitana* at different feed glucose (phases G1 – G7) and acetic acid (phases AA1 – AA6) concentrations.

| Phase | Feed Glu [mM] | HPR [mL/(L h)] | BM [mg CDW/L] |
|---|---|---|---|
| G1 | 27.8 | 55.2 (± 4.7) | 698 (± 39) |
| G2 | 41.6 | 53.7 (± 4.0) | 703 (± 20) |
| G3 | 27.8 | 54.7 (± 4.9) | 679 (± 21) |
| G4 | 16.7 | 50.3 (± 3.2) | 574 (± 27) |
| G5 | 11.1 | 33.8 (± 0.8) | 440 (± 26) |
| G6 | 22.2 | 61.2 (± 4.3) | 655 (± 54) |
| G7 | 27.8 | 72.8 (± 3.4) | 702 (± 36) |

| Phase | Feed AA [mM] | HPR [mL/(L h)] | BM [mg CDW/L] |
|---|---|---|---|
| AA1 | 30 | 79.7 (± 2.6) | 701 (± 34) |
| AA2 | 60 | 78.3 (± 3.1) | 678 (± 18) |
| AA3 | 90 | 75.3 (± 2.9) | 621 (± 19) |
| AA4 | 120 | 78.5 (± 3.9) | 694 (± 23) |
| AA5 | 180 | 83.8 (± 2.6) | 703 (± 47) |
| AA6 | 240 | 80.8 (± 1.8) | 710 (± 26) |

**Table 3:** Cumulative hydrogen production and hydrogen yield (HY), final concentrations of glucose (Glu), acetic acid (AA) and lactic acid (LA) in the effluent and the molar AA/LA ratio after the batch dark fermentation of 27.8 mM of glucose by *T. neapolitana* at different initial AA concentrations (0 – 240 mM).

| Initial AA [mM] | Cumulative $H_2$ [mL] | HY [mol/mol] | residual Glu [mM] | AA [mM] | LA [mM] | AA/LA ratio |
|---|---|---|---|---|---|---|
| 0 | 1739 (± 12) | 2.8 (± 0.0) | 2.7 (± 0.1) | 33.2 (± 0.2) | 14.6 (± 0.9) | 2.28 |
| 30 | 1474 (± 112) | 2.4 (± 0.2) | 2.7 (± 0.1) | 29.2 (± 0.1) | 17.5 (± 1.6) | 1.67 |
| 60 | 1402 (± 27) | 2.3 (± 0.0) | 3.2 (± 0.2) | 28.6 (± 2.1) | 18.0 (± 2.4) | 1.59 |
| 90 | 1273 (± 54) | 2.1 (± 0.0) | 3.2 (± 0.1) | 27.6 (± 0.6) | 20.7 (± 0.6) | 1.34 |
| 120 | 1167 (± 34) | 2.0 (± 0.0) | 3.8 (± 1.0) | 27.0 (± 1.1) | 21.2 (± 2.3) | 1.28 |
| 180 | 1066 (± 67) | 2.1 (± 0.0) | 7.8 (± 1.2) | 18.4 (± 1.8) | 15.4 (± 1.4) | 1.20 |
| 240 | 950 (± 29) | 2.3 (± 0.0) | 11.0 (± 1.6) | 16.5 (± 1.2) | 11.2 (± 0.2) | 1.48 |