

**Enhancement of hydrogen production rate by high biomass concentrations  
of *Thermotoga neapolitana***

G. Dreschke<sup>a, b, \*</sup>, G. d'Ippolito<sup>b</sup>, A. Panico<sup>c</sup>, P. Lens<sup>d</sup>, G. Esposito<sup>a</sup>, A. Fontana<sup>b</sup>

<sup>a</sup>Department of Civil and Mechanical Engineering, University of Cassino and Southern Lazio, Via Di Biasio, 43, 03043 Cassino, FR, Italy. (E-mail: giovanni.esposito@unicas.it)

<sup>b</sup>Bio-Organic Chemistry Unit, Institute of Biomolecular Chemistry, Italian National Council of Research, Via Campi Flegrei 34, 80078 Pozzuoli, Napoli, Italy. (E-mails: gdippolito@icb.cnr.it and afontana@icb.cnr.it)

<sup>c</sup>Telematic University Pegaso, Piazza Trieste e Trento, 48, 80132, Naples, Italy. (E-mail: antonio.panico@unipegaso.it)

<sup>d</sup>UNESCO - IHE Institute for Water Education, Westvest 7, 2611-AX Delft, The Netherlands. (E-mail: p.lens@unesco-ihe.org)

\*Corresponding author

Gilbert Dreschke MSc

Present address: Department of Civil and Mechanical Engineering, University of Cassino and Southern Lazio, Via Di Biasio, 43, 03043 Cassino, FR, Italy

Email: gilbert.dreschke@icb.cnr.it

## Abstract

The objective of this study was to enhance the hydrogen production rate of dark fermentation in batch processes. The hyperthermophilic pure culture of *Thermotoga neapolitana* cf. *capnolactica* was carried out in elevated cell concentrations from 0.46 to 1.74 g cell dry weight/L. This increased the volumetric hydrogen production rate from 323 ( $\pm$  11) to 654 ( $\pm$  30) mL/L/h, as well as led to a general acceleration of the fermentation process with enhancement of the biomass growth and glucose consumption rate. The overall effect was the decrease of both fermentation time and lag phase to 3 h and 0.4 h, respectively. The hydrogen yield of 2.45 ( $\pm$  0.09) mol H<sub>2</sub>/mol glucose, the hydrogen concentration of 68% in the produced gas and the composition of the end products in the digestate (62.3 ( $\pm$  2.5)% acetic acid, 23.5 ( $\pm$  2.9)% lactic acid and 2.3 ( $\pm$  0.1)% alanine) remained unaffected in the studied range of biomass concentrations.

## Keywords

Hydrogen; *Thermotoga neapolitana*; Biomass concentration; Dark fermentation; Hyperthermophilic;

## Abbreviations

CDW Cell dry weight [g/L]

HPR Hydrogen production rate

AA Acetic acid

LA Lactic acid

**Highlights:**

- An increase of biomass concentration from 0.46 to 1.74 g/L reduced the fermentation time from 7 to 3 h.
- The hydrogen production rate reached up to 654 ( $\pm 30$ ) mL/L/h at a biomass concentration of 1.74 g/L.
- The specific hydrogen production rate decreased with increasing biomass concentration.
- Hydrogen yield and end product composition were not affected by the biomass concentration.
- The concentration of hydrogen in the produced gas was 67.6 ( $\pm 2.4$ )%.

## 1. Introduction

Hydrogen (H<sub>2</sub>) is a non-polluting and clean fuel of the future with a wide range of applications [1,2]. The demand for hydrogen is continuously increasing and expected to contribute 8–10% to the energy market by 2025 [1]. Biotechnological hydrogen production processes have advanced in recent years and revealed promising results for an environmentally friendly production route [1,3]. Dark fermentation is thereby considered as the most attractive process due to its simplicity, independence of light and the potential to use substrates from renewable sources. However, the low H<sub>2</sub> production rate observed in dark fermentation still remains a fundamental challenge [4–6]. The hydrogen production rate (HPR) is crucial for the production at industrial scale [7] and a considerable increase is required for the establishment of an economically viable process [8,9].

*Thermotoga neapolitana* is a hyperthermophilic organism with a great potential for hydrogen production through dark fermentation [10]. This bacterium grows on a wide range of substrates including glucose, fructose, xylose, maltose, starch, glycogen, glycerol, molasses, cheese whey, algal biomass and carrot pulp [11–15]. The microorganism has fast growth kinetics [16], oxygen tolerance [14] and low contamination risks due to the extreme culture conditions at 80°C [17]. Previous studies using *Thermotoga neapolitana* have primarily focused on the optimization of the hydrogen yield in batch fermentation [14,18–20]. Yields approaching the theoretical 4 mol H<sub>2</sub>/mol glucose have been reached when applying 5 – 10% of inoculum (v/v) [2,14,21]. Promising results of *Thermotoga neapolitana* in attached growth [22–24] further emphasize the potential of the organism, indicating towards a possible use in a biofilm reactor system. However, low HPRs between 23 and 50 mL/L/h observed in simple batch cultivation using *Thermotoga neapolitana* [14] demonstrate the need for further development.

Despite the lower H<sub>2</sub> yields, the highest HPRs are currently reached under mesophilic conditions due to their capability to grow in high biomass concentrations [25]. A positive correlation was demonstrated between the HPR and the biomass concentration in the reactor in continuous dark fermentations [6]. Hyperthermophilic cultures reach much higher hydrogen yields [26], but they commonly grow in low cell densities [5] leading to low H<sub>2</sub> production rates. A substantial improvement of the HPR is expected if hyperthermophilic cultures are cultivated at high biomass concentrations [25,27]. In batch cultivation of suspended cells, high cell concentrations are generally implemented inexpensively by recycling of biomass and enable a faster and more robust process [27].

In the present study, a suspended culture of pure *Thermotoga neapolitana* biomass was used at different concentrations ranging from 0.46 – 1.74 g/L to mimic a sequential batch fermentation described by Basso et al. [28]. The main objective was to study the effect on the fermentation process and the correlation with HPR. Simultaneously, we also evaluated the consequence on bacterial growth and on volumetric and specific glucose consumption.

## **2. Material and Methods**

### **2.1. Culture medium**

A modified ATCC 1977 culture medium as described by d'Ippolito et al. [19] was used for the cultivation of *Thermotoga neapolitana* containing the following components (in g/L): 10 NaCl; 5 glucose; 2 yeast extract; 2 tryptone; 1 cysteine; 1 NH<sub>4</sub>Cl; 0.3 K<sub>2</sub>HPO<sub>4</sub>; 0.3 KH<sub>2</sub>PO<sub>4</sub>; 0.2 MgCl<sub>2</sub>·6 H<sub>2</sub>O; 0.1 KCl; 0.1 CaCl<sub>2</sub>·2 H<sub>2</sub>O;

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).

## **2.2. Bacterial strain – Cultivation and storage**

A pure culture of *Thermotoga neapolitana* cf. *capnolactica* [29] (hereafter briefly *Thermotoga neapolitana*) was used in all experiments. For the conservation of the culture, 120 mL serum bottles containing 25 mL of culture medium were prepared. Prior to the inoculation, the medium was heated to remove excess oxygen until losing the characteristic resazurin color, sealed immediately with butyl rubber stoppers and sterilized by autoclaving for 5 min at 110°C [19]. The medium was inoculated using 6% (v/v) of stored cell cultures. After the cultivation at 80°C without agitation overnight, the grown culture was stored at 4°C [30].

## **2.3. Preparation of inoculum**

The inoculum was produced in two 3 L continuously stirred tank reactors (Applikon Biotechnology, The Netherlands) each containing 2000 mL of culture medium. The medium was heated to 80°C and sparged with CO<sub>2</sub> for 5 min to remove the dissolved oxygen. Subsequently, the pH was adjusted to 7 by addition of 1 M NaOH and the reactors were inoculated using 6% v/v of stored culture (described in 2.2). The cultivation was performed at 80°C and 200 rpm for 14.5 h to obtain a culture at the end of the stationary phase where the amount of active biomass reaches its maximum. The biomass was harvested by centrifugation at 3750 rpm for 15 min and resuspended in an isotonic solution (10 g/L NaCl in distilled water) to produce a highly concentrated inoculum (1 mL of inoculum contains biomass of 50 mL of grown culture). The biomass concentration in g CDW/L of each sampling point was estimated from the optical

density at 540 nm ( $OD_{540}$ ) using the relation  $CDW [g/L] = 0.269 * OD_{540} - 0.061$  ( $R^2 = 0,98$ ) (relationship is valid in the exponential growth phase) [20,31].

## 2.4. Experimental design

Eight 250 mL Schott flasks, each containing 200 mL of culture medium were used to investigate the hydrogen production from a *Thermotoga neapolitana* culture at increasing biomass concentrations. Prior to inoculation, the medium was heated to 80°C, sparged with CO<sub>2</sub> for 5 min to remove oxygen and the pH adjusted to 7 by addition of 1 M NaOH. The ratio volume of inoculum to volume of culture medium (v/v) is commonly used to describe the amount of inoculum applied. Considering this ratio, the concentrated inoculum (described in 2.3) was used to inoculate the reactors between 100 and 400% v/v (100% corresponding to biomass harvested from 200 ml of grown culture in 200 ml of fresh medium): C1 - 100% (= 0.46 g CDW/L), C2 - 200% (= 0.91 g CDW/L), C3 - 300% (= 1.33 g CDW/L) and C4 - 400% (= 1.74 g CDW/L). The medium was maintained at 80°C applying 300 rpm agitation by magnetic stirring (STIRRING DRYBATH 15-250, 2mag AG, Germany). 1.5 mL of liquid samples were taken at 1 h (C3, C4) and 30 min (C1, C2) intervals. The pH was manually adjusted to 7 by after each sampling. The produced gas was released continuously and quantified with 500 mL water displacement systems. The fermentation was completed when the gas production stopped, and the pH remained constant. Each experimental condition was conducted in duplicate.

## 2.5. Analytical Methods

Liquid samples were centrifuged at 10000 rpm for 5 min to collect the supernatant for the determination of glucose, acetic acid (AA), lactic acid (LA) and alanine concentrations. The glucose concentration was measured by the dinitrosalicylic acid method calibrated on a standard solution of 1 g/L [32]. AA, LA and alanine were quantified by  $^1\text{H}$  Nuclear Magnetic Resonance (NMR) on a 600 MHz spectrometer (Bruker Avance 400) equipped with a Cryoprobe using 3.8 mM trimethylamine hydrochloride (TMA) as internal standard [33]. The biomass concentration was determined by measuring optical density at 540 nm ( $\text{OD}_{540}$ ) (UV/Vis spectrophotometer DU 730, Beckman Coulter Inc, Brea, USA) of the liquid samples and CDW via lyophilization after the completion of the fermentation. For this purpose, 200 mL of culture broth was centrifuged at 3750 rpm for 20 min. The pellet was subsequently resuspended in 25 mL of 10 g/L NaCl in distilled water and centrifuged at 6000 rpm for 20 min to remove remaining media components. The pellet was stored at  $-20^\circ\text{C}$  and lyophilized overnight.

Produced gas was quantified by water displacement using 500 mL glass containers. At the end of each experiment, the  $\text{H}_2$ -containing gas was sampled and analyzed by gas chromatography as described by Dipasquale et al. [13]. The molar  $\text{H}_2$  production was calculated using the ideal gas law [7]. The hydrogen concentration in the produced gas was calculated considering a dilution of the measured gas with the  $\text{CO}_2$  initially in the headspace of the reactor.

## **2.6. Kinetic study of glucose consumption, biomass growth and biohydrogen production**

To evaluate and compare glucose consumption, biomass growth and hydrogen production at the different biomass concentrations investigated, models based on the Gompertz equation [14,34] were applied to fit the experimental data and calculate the kinetic rates and lag phases. To validate the suitability of the



modified Gompertz model, the models of glucose consumption, biomass growth and hydrogen production were plotted against the experimental data. The quality of the fitting was determined by calculating the coefficients of determination ( $R^2$ ). Specific rates were calculated referring volumetric rates to initial biomass concentration of each experimental condition.

For glucose consumption, Eq. (1) was applied, where  $G$  [g/L] is the glucose concentration at fermentation time  $t$  [h];  $G_0$  [g/L] is the glucose concentration at time 0 h;  $G_m$  [g/L] is the glucose consumed throughout the fermentation;  $R_G$  is the volumetric glucose consumption rate [g/L/h];  $\lambda_G$  is the lag phase of glucose consumption [h]; and  $e$  is the Euler's number, i.e. 2.7183.

For biomass growth, Eq. (2) was applied, where  $B$  [g CDW/L] is the biomass concentration at fermentation time  $t$  [h];  $B_0$  [g CDW/L] is the biomass concentration at time 0 h;  $G_m$  [g CDW/L] is the gain of biomass concentration throughout the fermentation;  $R_B$  is the volumetric growth rate [g CDW/L/h]; and  $\lambda_B$  is the lag phase of biomass growth [h].

Eq. (3) was applied for hydrogen production, with  $H$  [mL] being the cumulative hydrogen at time  $t$  [h];  $H_m$  [mL] the hydrogen produced throughout the fermentation;  $R_H$  [mL/L/h] the volumetric HPR; and  $\lambda_H$  the lag phase of hydrogen production [h]. For the calculation of the HPR, the gas remaining in the headspace of the reactor at the end of the fermentation was equally distributed throughout the length of the batch experiment.

$$G = G_0 - G_m \exp \left\{ -\exp \left[ \frac{R_G e}{G_m} (\lambda_G - t) + 1 \right] \right\} \quad (1)$$

$$B = B_0 + B_m \exp \left\{ -\exp \left[ \frac{R_B e}{B_m} (\lambda_B - t) + 1 \right] \right\} \quad (2)$$

$$H = H_m \exp \left\{ -\exp \left[ \frac{R_H e}{H_m} (\lambda_H - t) + 1 \right] \right\} \quad (3)$$

### 3. Results and Discussion

#### 3.1. Hydrogen yield and production rate

A change of initial biomass concentration between 0.46 and 1.74 g/L did not affect the hydrogen yield i.e. 2.45 ( $\pm 0.09$ ) mol H<sub>2</sub>/mol glucose (Table 1) or the composition of the biogas that maintained a constant level of hydrogen at 67.6 ( $\pm 2.4$ )%. The HPR observed at the lowest initial biomass concentration of 0.46 g CDW/L (C1) reached 323 ( $\pm 11$ ) mL/L/h (Table 2). Increment of the biomass concentration to 0.91 (C2), 1.33 (C3) and 1.74 g CDW/L (C4) linearly enhanced the volumetric HPR to 448 ( $\pm 18$ ), 608 ( $\pm 18$ ) and 654 ( $\pm 30$ ) mL/L/h (Fig. 1 B; Table 2). On the whole, a fourfold expansion of the biomass concentration caused an approximately twofold increase of the volumetric production rate. On the whole, a fourfold expansion of the biomass concentration caused an approximately twofold increase of the volumetric production rate. This is consistent with previous studies on anaerobic fermentation by sludge in serum bottles where a general increase of HPR is obtained by rise of biomass concentrations [36,37]. On the other hand, Ngo and coworkers have reported a reverse correlation between the two parameters in *T. neapolitana*. In a fed batch process (4 cycles), these authors observed that the initial increase of the biomass from  $1.3 \pm 0.1$  to  $2.4 \pm 0.1$  g/L yielded a mild reduction of HPR from 114 to 106 mL/L/h [35]. The negative effect was explained on the basis of the accumulation of acetic acid and lactic acid in the reactor up to  $123 \pm 7$  and  $28 \pm 1$  mM, respectively.

While hyperthermophilic cultures are capable to achieve even higher hydrogen yields (Table 3) the volumetric HPR observed in this work exceeds those achieved in similar studies using different hyperthermophilic cultures with low inoculum size (Table 3). The highest production rate of 654 ( $\pm 30$ ) mL/L/h (C4) (Table 2) depicts a roughly 13-fold increase to the maximum production rate of 50 mL/L/h

achieved in similar experiments using *Thermotoga neapolitana* in batch cultures with 6% (v/v) of inoculum and 5 g/L of glucose as a substrate [19]. Only two studies achieved HPRs in a similar range. Mars et al. [31] and de Vrije et al. [11] reached production rates of 269 and 304 mL/L/h, respectively, by continuously flushing the reactor headspace with N<sub>2</sub> gas at 7 L/h both using 10% (v/v) of preculture as inoculum and 10 g glucose/L as a substrate. While gas sparging is a common method to counteract hydrogen inhibition [25,38], it is an unsatisfying solution due to a manifold dilution of the produced hydrogen gas. The resulting need to separate the sparging gas from the hydrogen creates additional operating cost [39].

### **3.2. Volumetric and specific rates of glucose consumption, biomass growth and hydrogen production**

Volumetric (per L of working volume) and specific (per g CDW) glucose consumption, biomass growth and HPRs (Fig. 1, Table 2) were calculated via the Gompertz model (described in 2.6) (Fig. 2). The increase of the volumetric HPR with increasing biomass concentrations was coupled to a general acceleration of the process, indicated by an increase of the volumetric biomass growth and glucose consumption rate (Table 2). The calculation of the Pearson correlation coefficient, confirmed a positive linear correlation of volumetric glucose consumption rate and volumetric growth rate ( $r=0.99$ ) as well as volumetric HPR ( $r=0.99$ ). In contrast, all specific rates (Fig. 1, Table 2) exhibited a decreasing trend with increasing biomass concentrations. The decrease in specific rates was particularly distinct between C1 and C2. The specific glucose consumption rate (g glucose/h/g CDW) dropped by 34% from 2.33 ( $\pm 0.08$ ) of C1 to 1.55 ( $\pm 0$ ) of C2 and by 9% to 1.35 ( $\pm 0.04$ ) of C4 (Table 2). The specific growth rate (mg CDW/h/g CDW) decreased by 38% from 412 ( $\pm 0$ ) of C1 to 254 ( $\pm 0$ ) of C2 and merely by 6% to 229 ( $\pm 16$ ) of C4 (Table 2). The specific

HPR (mL/h/g CDW) decreased by 29% from 699 ( $\pm 23$ ) of C1 to 494 ( $\pm 20$ ) of C2 and by 17% to 375 ( $\pm 17$ ) of C4 (Table 2). A similar trend of decreasing specific HPR was observed in previous studies when the initial biomass was increased with mixed cultures [36,37]. The specific HPR dropped from 48 to approximately 3 mL/g/h when the biomass concentration was increased from 0.48 to 2.88 g/L biomass concentration [37]. This suggests that the overall process was considerably accelerated, while the individual cells were partially repressed at increased biomass concentration. Substrate limitation caused by flock formation [37] and hydrogen consuming homo-acetogenic bacteria [36] were presumed to be causing the inhibitory effect on specific HPR at higher initial biomass concentrations.

In the present study, no hydrogen consuming bacteria were present and flock formation was not observed. However, an increase of HPR induces an increased concentration of hydrogen in the liquid phase which mainly depends on the HPR and the mass transfer rate of the system [40]. The concentration of liquid phase hydrogen can reach multiple fold the equilibrium concentration suggested by Henry's Law even in hyperthermophilic stirred reactor systems [40,41], acting as a potent inhibitor of hydrogen production by dark fermentation [38,42]. According to Ljunggren et al. [40], cultures of *Caldicellulosiruptor saccharolyticus* respond to increasing concentrations of liquid phase hydrogen by adjusting the specific growth rate to reduce the HPR and prevent hydrogen from reaching inhibitory concentrations. Similarly, a reduction of specific HPRs was observed in the presented study when the volumetric production rates increased. This is supported by the results obtained in dark fermentation by *Thermotoga neapolitana*, achieving the highest hydrogen yields and production rates, when hydrogen was removed through headspace sparging with N<sub>2</sub> continuously [11,31] or in regular intervals [19].

### 3.3. Fermentation Time and lag phase

The lag phase was determined via the Gompertz model, while the fermentation time was estimated from the moment of inoculation to the completion of the fermentation. An inoculum size of 0.46 g CDW /L (C1) induced a lag phase of 1.50 ( $\pm$  0.07) h (Table 2) and the completion of the fermentation within approximately 7 h (Fig. 2). In previous studies, the duration of the batch fermentation with *Thermotoga neapolitana* was longer than 18 h [11,23,31] when applying a 5-10% (v/v) inoculum. By increasing the initial biomass concentration to 1.74 g CDW /L (C4), the fermentation time and the lag phase were reduced to approximately 3 h (Fig. 2) and 0.4 ( $\pm$  0.02) h (Table 2), respectively. This is in agreement with observations made in bioethanol production plants [28], where yeast cultures were recycled at high densities in sequential batch fermentations resulting in the reduction of the fermentation time and the unproductive lag phase [27]. In all conditions, independent from the initial biomass concentration a substrate consumption of 88.8 ( $\pm$  0.9)% (data not shown) was reached at the end of the fermentation.

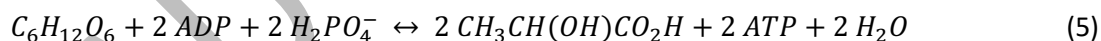
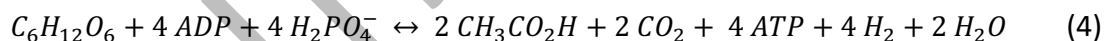
### 3.4. Biomass production

The biomass concentration at the end of a batch fermentation is defined by the initial biomass concentration, the biomass yield (g CDW/g glucose) and the amount of glucose converted. A similar formation of biomass was observed using initial biomass concentrations between 0.46 (C1) and 1.33 (C3), with a biomass yield ranging between 0.12 - 0.14 g CDW/g glucose (Table 1). In contrast, at the highest biomass concentration of 1.74 g CDW/L (C4) the biomass production was lower, corresponding to a biomass yield of 0.08 g CDW/g glucose (Table 1), similar to what observed in previous studies with lower inoculum concentrations of *Thermotoga neapolitana*. De Vrije et al. [11] and Mars et al. [31] reported biomass yields of 81.6 and 87.1 mg CDW/ g glucose, respectively, using 10% inoculum (v/v) with additional

headspace sparging. Ngo et al. [23] obtained 0.71 ( $\pm$  0.04) g CDW/ L with 10 g/L pure glycerol as main substrate and 10% of inoculum (v/v). Van Niel et al. [43] reached 0.89 g CDW/L by fermenting 10 g/L glucose with 10% inoculum of *Thermotoga elfi*. At the end of each fermentation *Thermotoga neapolitana* is morphologically adapting to the nutrient limitation causing the OD<sub>540</sub> to decrease (Fig. 2) [44], while the CDW remains high (Table 1). Consequently, the curve fitting to the Gompertz model for biomass growth was done exclusively until the highest value of turbidity was reached (Fig. 2).

### 3.5. Production of fermentation products

The distribution of end products obtained in the digestate (62.3 ( $\pm$  2.5)% AA; 23.5 ( $\pm$  2.9)% LA; 2.3 ( $\pm$  0.1)% alanine) was similar in the range of biomass concentrations investigated (Table 4), corresponding to yields of 1.33 ( $\pm$  0.05) mol/mol glucose for AA, 0.50 ( $\pm$  0.06) mol/mol glucose for LA and 0.05 ( $\pm$  0.001) mol/mol glucose for alanine (Table 4). As observed in previous studies [14], *Thermotoga neapolitana* primarily ferments glucose via the hydrogen producing acetic acid (Eq. 4) or the lactic acid pathway (Eq. 5).



Considering a production of 2 mol of end product per mol of glucose (Eq. 4 and 5), an average of 95 ( $\pm$  1)% (data not shown) of the initial substrate could be accounted for in the fermentation broth (Table 4). Similar results were obtained by Mars et al. [31] and de Vrije et al. [11] when low inoculum concentrations were used, reporting AA yields of 1.6 and 1.4 mol/ mol glucose and LA yields of < 0.1 and 0.03 mol/ mol glucose,

respectively. The highest initial biomass concentration (C4) showed a slightly higher LA/ AA ratio (Table 4) coupled with a slightly lower hydrogen yield (Table 1). This is in accordance to the dark fermentation model suggesting a negative correlation between hydrogen yield and LA/AA ratio [42]. Additionally, an impact of capnophilic lactic acid fermentation caused by sparging with CO<sub>2</sub> needs to be considered resulting in elevated amounts of LA without significant loss in hydrogen yield [30,33]. Dipasquale et al. [30] observed LA/AA ratio of 0.28 by N<sub>2</sub> sparging and 0.56 by CO<sub>2</sub> sparging twice throughout the batch experiment. LA/AA ratios between 0.32 and 0.47 observed in this study suggest a similar influence of capnophilic lactic acid fermentation.

The use of high initial biomass concentrations of *Thermotoga neapolitana* has been identified a suitable method to increase the HPR in batch fermentation. Further research is needed to understand the role of hydrogen in the liquid phase as an inhibitor of the process and establish counteracting measures if required. Additionally, more suitable substrates like organic wastes need to be tested for hydrogen production by *Thermotoga neapolitana* at high biomass concentrations as glucose is neither economical nor environmentally friendly in large-scale applications.

#### 4. Conclusion

The results of this study show a considerable acceleration of hydrogen production by *Thermotoga neapolitana* cf. *capnolactica* by using elevated biomass concentrations (from 0.46 to 1.74 g cell dry weight/L). At the highest biomass concentration, the hydrogen production rate reached a maximum of 654 (± 30) mL/L/h, consuming 5 g/L of glucose within 3 h. The increase of the biomass concentration did not affect the yield (2.45 (± 0.09) mol H<sub>2</sub>/mol glucose), the concentration of hydrogen in the produced gas

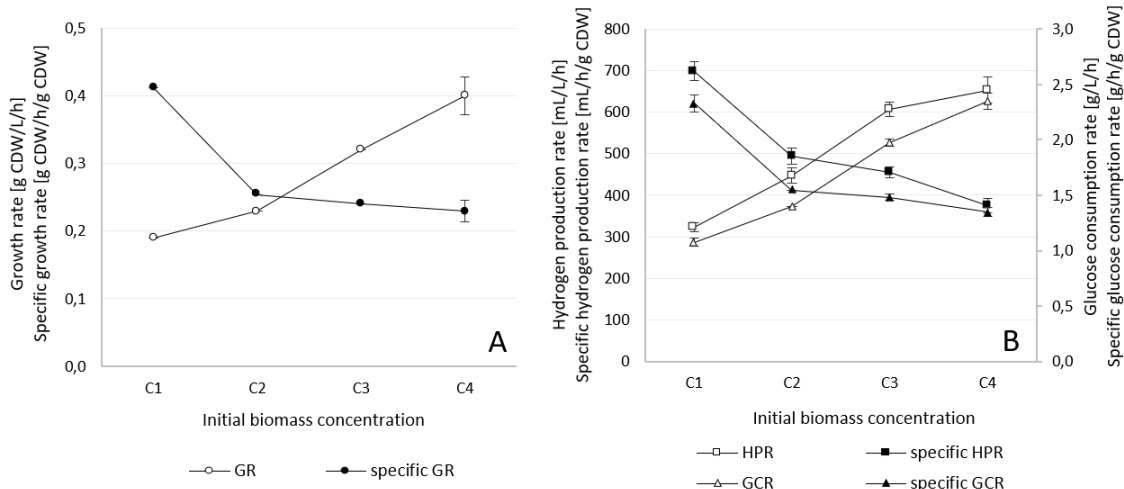
(68%) or the composition of fermentation end products (i.e. 62.3 ( $\pm$  2.5)% AA, 23.5 ( $\pm$  2.9)% LA and 2.3 ( $\pm$  0.1)% alanine).

## **Acknowledgements**

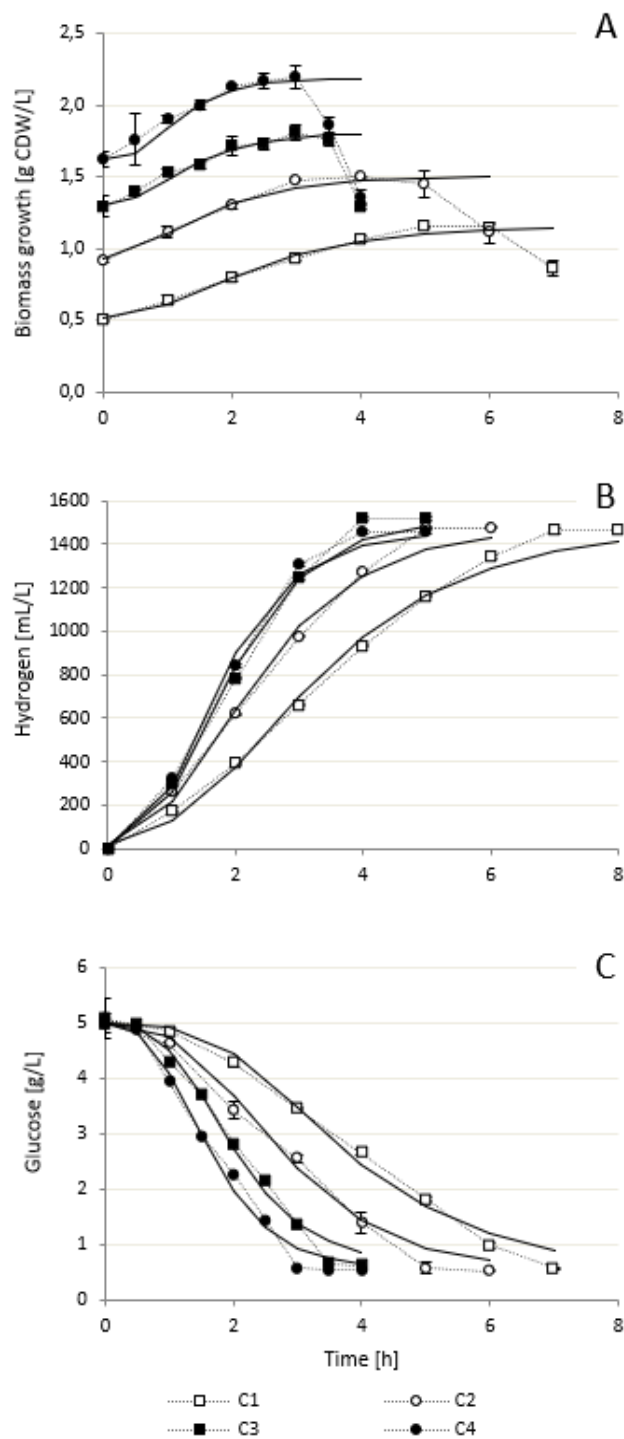
This work was supported by the Marie Skłodowska-Curie European Joint Doctorate (EJD) in Advanced Biological Waste-To-Energy Technologies (ABWET) funded from Horizon 2020 under grant agreement no. 643071. AF and GdI thank the support from the EU Horizon 2020 Research and Innovation Programme under the Grant Agreement No. 760431 (BioRECO2VER). The authors also express their appreciation to the staff of CNR-ICB for the consistent help throughout this study.

Conflicts of interest: none.





**Fig. 1.:** A - Volumetric and specific growth rate; B - Volumetric as well as specific glucose consumption and hydrogen production rate at different initial biomass concentrations (C1 = 0.46; C2 = 0.91; C3 = 1.33; C4 = 1.74 g CDW/L) of *Thermotoga neapolitana* fermenting 5 g/L of glucose. Specific rates were calculated per g initial cell dry weight. (GR – Growth rate; HPR – Hydrogen production rate; GCR – Glucose consumption rate)



**Fig. 2.:** A – Biomass growth; B – Cumulative hydrogen and C – Glucose consumption production at different initial biomass concentrations (C1 = 0.46; C2 = 0.91; C3 = 1.33; C4 = 1.74 g CDW/L) of *Thermotoga neapolitana* fermenting 5 g/L of glucose. The solid line represents the fitting to the Gompertz model.

**Table 1.** Hydrogen yield, cumulative hydrogen, final biomass and biomass yield fermenting 5 g/L glucose at different initial biomass concentrations of *Thermotoga neapolitana*.

	Initial biomass [g CDW /L]	Hydrogen yield [mol H <sub>2</sub> /mol glucose]	Cumulative hydrogen [mL/L]	Final biomass [g CDW/L]	Biomass yield [g CDW/g glucose]
C1	0.46	2.39	1462 (± 12)	1.10 (± 0.03)	0.14
C2	0.91	2.44	1477 (± 3)	1.43 (± 0.09)	0.12
C3	1.33	2.58	1516 (± 10)	1.89 (± 0.06)	0.13
C4	1.74	2.37	1456 (± 5)	2.10 (± 0.15)	0.08

**Table 2.** *Thermotoga neapolitana* cultivated on 5 g/L of glucose as a main substrate using different initial biomass concentrations. Rates and lag phase determined through curve fitting to a modified Gompertz model. Fit quality illustrated through coefficient of determination ( $R^2$ ).

	Initial biomass	Volumetric rate	Specific rate	Lag phase	$R^2$
<b>Hydrogen production</b>					
	[g CDW/L]	[mL/L/h]	[mL/h/g initial CDW]	[h]	
C1	0.46	323 ( $\pm 11$ )	699 ( $\pm 23$ )	1.50 ( $\pm 0.07$ )	0.99
C2	0.91	448 ( $\pm 18$ )	494 ( $\pm 20$ )	0.79 ( $\pm 0.01$ )	0.99
C3	1.33	608 ( $\pm 18$ )	456 ( $\pm 13$ )	0.52 ( $\pm 0.01$ )	0.99
C4	1.74	654 ( $\pm 30$ )	375 ( $\pm 17$ )	0.40 ( $\pm 0.02$ )	0.99
<b>Cell growth</b>					
	[g CDW/L]	[mg CDW/L/h]	[mg CDW/h/g initial CDW]		
C1	0.46	190 ( $\pm 0$ )	412 ( $\pm 0$ )		0.99
C2	0.91	230 ( $\pm 0$ )	254 ( $\pm 0$ )		0.98
C3	1.33	320 ( $\pm 0$ )	240 ( $\pm 0$ )		0.96
C4	1.74	400 ( $\pm 28$ )	229 ( $\pm 16$ )		0.98
<b>Glucose consumption</b>					
	[g CDW/L]	[g Glucose/L/h]	[g Glucose/h/g initial CDW]		
C1	0.46	1.08 ( $\pm 0.04$ )	2.33 ( $\pm 0.08$ )		0.99
C2	0.91	1.40 ( $\pm 0.00$ )	1.55 ( $\pm 0.00$ )		0.98
C3	1.33	1.98 ( $\pm 0.04$ )	1.48 ( $\pm 0.03$ )		0.98
C4	1.74	2.35 ( $\pm 0.07$ )	1.35 ( $\pm 0.04$ )		0.99

**Table 3:** Hydrogen production rate and hydrogen yield of selected studies of batch fermentations by various hyperthermophilic pure cultures using glucose as a substrate.

Microorganism	Hydrogen production rate	Hydrogen yield [mol H <sub>2</sub> /mol glucose]	Reference
<i>Caldicellulosiruptor saccharolyticus</i>	264	2.5	[45]
	296	3.4	[31]
	277	3.2	[11]
<i>Thermotoga elfi</i>	200	3.3	[43]
<i>Thermotoga maritima</i>	170	4	[46]
<i>Thermotoga neapolitana</i>	304	2.9	[31]
	269	3.5	[11]
	21	3.9	[18]
	252	1.8	[20]
	50	3.9	[19]
<i>Thermoanaerobacter mathranii</i> A3N	100	2.6	[47]
<i>Thermoanaerobacterium thermosaccharophilum</i>	287	2.4	[48]
	2		
<i>Thermoanaerobacterium thermosaccharophilum</i>	309	2.4	[49]
<i>Thermotoga neapolitana</i>	654	2.5	Present study

**Table 4.** Composition of broth after completed fermentation of 5 g/L glucose by different initial biomass concentrations (C1 = 0.46; C2 = 0.91; C3 = 1.33; C4 = 1.74 g cell dry weight/L) of *Thermotoga neapolitana*.

	AA [mM] {yield [mol/mol glu]}	LA [mM] {yield [mol/mol glu]}	Ratio LA / AA	Alanine [ $\mu$ M] {yield [mol/mol glu]}	Glucose [mM]
C1	34.3 ( $\pm$ 0.6) {1.39}	10.9 ( $\pm$ 0.4) {0.44}	0.32	1190 ( $\pm$ 27) {0.05}	3.2 ( $\pm$ 0.04)
C2	32.9 ( $\pm$ 0.8) {1.32}	12.2 ( $\pm$ 0.8) {0.49}	0.37	1260 ( $\pm$ 1) {0.05}	2.9 ( $\pm$ 0.06)
C3	32.3 ( $\pm$ 0.2) {1.33}	11.5 ( $\pm$ 0.5) {0.47}	0.36	1220 ( $\pm$ 18) {0.05}	3.4 ( $\pm$ 0.01)
C4	31.4 ( $\pm$ 1.1) {1.27}	14.7 ( $\pm$ 0.7) {0.59}	0.47	1260 ( $\pm$ 43) {0.05}	3.0 ( $\pm$ 0.04)

AA - acetic acid; LA - lactic acid; glu – glucose;

## References

- [1] Gupta SK, Kumari S, Reddy K, Bux F. Trends in biohydrogen production: major challenges and state-of-the-art developments. *Environ Technol* 2013;34(13-16):1653–70. doi:10.1080/09593330.2013.822022.
- [2] Elsharnouby O, Hafez H, Nakhla G, El Nagggar MH. A critical literature review on biohydrogen production by pure cultures. *Int J Hydrogen Energ* 2013;38(12):4945–66. doi:10.1016/j.ijhydene.2013.02.032.
- [3] Yasin NHM, Mumtaz T, Hassan MA, Abd Rahman N. Food waste and food processing waste for biohydrogen production: a review. *J Environ Manage* 2013;130:375–85. doi:10.1016/j.jenvman.2013.09.009.
- [4] Balachandar G, Khanna N, Das D. Biohydrogen production from organic wastes by dark fermentation. In: *Biohydrogen*. Elsevier; 2013, p. 103–144. doi:10.1016/B978-0-444-59555-3.00006-4.
- [5] Lee D-J, Show K-Y, Su A. Dark fermentation on biohydrogen production: Pure culture. *Bioresour Technol* 2011;102(18):8393–402. doi:10.1016/j.biortech.2011.03.041.
- [6] Show KY, Lee DJ, Tay JH, Lin CY, Chang JS. Biohydrogen production: Current perspectives and the way forward. *Int J Hydrogen Energ* 2012;37(20):15616–31. doi:10.1016/j.ijhydene.2012.04.109.
- [7] O-Thong S, Prasertsan P, Karakashev D, Angelidaki I. High-rate continuous hydrogen production by *Thermoanaerobacterium thermosaccharolyticum* PSU-2 immobilized on heat-pretreated methanogenic granules. *Int J Hydrogen Energ* 2008;33(22):6498–508. doi:10.1016/j.ijhydene.2008.07.060.
- [8] Argun H, Kargi F, Kapdan IK. Effects of the substrate and cell concentration on bio-hydrogen production from ground wheat by combined dark and photo-fermentation. *Int J Hydrogen Energ* 2009;34(15):6181–8. doi:10.1016/j.ijhydene.2009.05.130.
- [9] Davila-Vazquez G, Cota-Navarro CB, Rosales-Colunga LM, León-Rodríguez A de, Razo-Flores E. Continuous biohydrogen production using cheese whey: Improving the hydrogen production rate. *Int J Hydrogen Energ* 2009;34(10):4296–304. doi:10.1016/j.ijhydene.2009.02.063.
- [10] Pawar SS, Van Niel EWJ. Thermophilic biohydrogen production: how far are we? *Appl Microbiol Biotechnol* 2013;97(18):7999–8009. doi:10.1007/s00253-013-5141-1.
- [11] de Vrije T, Budde MA, Lips SJ, Bakker RR, Mars AE, Claassen PA. Hydrogen production from carrot pulp by the extreme thermophiles *Caldicellulosiruptor saccharolyticus* and *Thermotoga neapolitana*. *Int J Hydrogen Energ* 2010;35(24):13206–13. doi:10.1016/j.ijhydene.2010.09.014.
- [12] Cappelletti M, Bucchi G, Sousa Mendes J de, Alberini A, Fedi S, Bertin L et al. Biohydrogen production from glucose, molasses and cheese whey by suspended and attached cells of four hyperthermophilic *Thermotoga* strains. *Journal of Chemical Technology & Biotechnology* 2012;87(9):1291–301. doi:10.1002/jctb.3782.
- [13] Dipasquale L, d'Ippolito G, Gallo C, Vella FM, Gambacorta A, Picariello G et al. Hydrogen production by the thermophilic eubacterium *Thermotoga neapolitana* from storage polysaccharides of the CO<sub>2</sub>-fixing diatom *Thalassiosira weissflogii*. *Int J Hydrogen Energ* 2012;37(17):12250–7. doi:10.1016/j.ijhydene.2012.05.160.

- [14] Pradhan N, Dipasquale L, d'Ippolito G, Panico A, Lens PNL, Esposito G et al. Hydrogen production by the thermophilic bacterium *Thermotoga neapolitana*. *Int J Mol Sci* 2015;16(6):12578–600. doi:10.3390/ijms160612578.
- [15] Huber R, Hannig M. Thermotogales. In: Dworkin M, Falkow S, Rosenberg E, Schleifer K-H, Stackebrandt E, editors. *The Prokaryotes*. New York, NY: Springer New York; 2006, p. 899–922. doi:10.1007/0-387-30747-8\_38.
- [16] Pradhan N, Dipasquale L, d'Ippolito G, Fontana A, Panico A, Lens PN et al. Kinetic modeling of fermentative hydrogen production by *Thermotoga neapolitana*. *Int J Hydrogen Energ* 2016;41(9):4931–40. doi:10.1016/j.ijhydene.2016.01.107.
- [17] Nguyen T-AD, Han SJ, Kim JP, Kim MS, Sim SJ. Hydrogen production of the hyperthermophilic eubacterium, *Thermotoga neapolitana* under N<sub>2</sub> sparging condition. *Bioresour Technol* 2010;101 Suppl 1:41. doi:10.1016/j.biortech.2009.03.041.
- [18] Munro SA, Zinder SH, Walker LP. The fermentation stoichiometry of *Thermotoga neapolitana* and influence of temperature, oxygen, and pH on hydrogen production. *Biotechnol Prog* 2009;25(4):1035–42. doi:10.1002/btpr.201.
- [19] d'Ippolito G, Dipasquale L, Vella FM, Romano I, Gambacorta A, Cutignano A et al. Hydrogen metabolism in the extreme thermophile *Thermotoga neapolitana*. *Int J Hydrogen Energ* 2010;35(6):2290–5. doi:10.1016/j.ijhydene.2009.12.044.
- [20] Nguyen T-AD, Kim JP, Kim MS, Oh YK, Sim SJ. Optimization of hydrogen production by hyperthermophilic eubacteria, *Thermotoga maritima* and *Thermotoga neapolitana* in batch fermentation. *Int J Hydrogen Energ* 2008;33(5):1483–8. doi:10.1016/j.ijhydene.2007.09.033.
- [21] Thauer RK, Jungermann K, Decker K. Energy conservation in chemotrophic anaerobic bacteria. *Bacteriological Reviews* 1977;41(1):100–80.
- [22] Basile MA, Carfagna C, Cerruti P, Gomez d'Ayala G, Fontana A, Gambacorta A et al. Continuous hydrogen production by immobilized cultures of *Thermotoga neapolitana* on an acrylic hydrogel with pH-buffering properties. *RSC Adv*. 2012;2(9):3611. doi:10.1039/c2ra01025a.
- [23] Ngo TA, Bui HTV. Biohydrogen production using immobilized cells of hyperthermophilic eubacterium *Thermotoga neapolitana* on porous glass beads. *Journal of Technology Innovations in Renewable Energy* 2013:231–8.
- [24] Frascari D, Cappelletti M, Mendes JDS, Alberini A, Scimonelli F, Manfreda C et al. A kinetic study of biohydrogen production from glucose, molasses and cheese whey by suspended and attached cells of *Thermotoga neapolitana*. *Bioresour Technol* 2013;147:553–61. doi:10.1016/j.biortech.2013.08.047.
- [25] Gupta N, Pal M, Sachdeva M, Yadav M, Tiwari A. Thermophilic biohydrogen production for commercial application: The whole picture. *Int. J. Energy Res*. 2016;40(2):127–45. doi:10.1002/er.3438.
- [26] Chou C-J, Jenney FE JR, Adams MWW, Kelly RM. Hydrogenesis in hyperthermophilic microorganisms: implications for biofuels. *Metab Eng* 2008;10(6):394–404. doi:10.1016/j.ymben.2008.06.007.
- [27] Westman JO, Franzen CJ. Current progress in high cell density yeast bioprocesses for bioethanol production. *Biotechnol J* 2015;10(8):1185–95. doi:10.1002/biot.201400581.



- [28] Basso CL, Basso OT, Rocha NS. Ethanol production in brazil: The industrial process and its impact on yeast fermentation. In: Dos Santos Bernardes MA, editor. Biofuel Production-Recent Developments and Prospects. InTech; 2011, p. 85–100. doi:10.5772/17047.
- [29] Pradhan N, Dipasquale L, d'Ippolito G, Panico A, Lens PN, Esposito G et al. Hydrogen and lactic acid synthesis by the wild-type and a laboratory strain of the hyperthermophilic bacterium *Thermotoga neapolitana* DSMZ 4359 T under capnophilic lactic fermentation conditions. Int J Hydrogen Energ2017;42(25):16023–30. doi:10.1016/j.ijhydene.2017.05.052.
- [30] Dipasquale L, d'Ippolito G, Fontana A. Capnophilic lactic fermentation and hydrogen synthesis by *Thermotoga neapolitana*: An unexpected deviation from the dark fermentation model. Int J Hydrogen Energ2014;39(10):4857–62. doi:10.1016/j.ijhydene.2013.12.183.
- [31] Mars AE, Veuskens T, Budde MA, van Doeveren PF, Lips SJ, Bakker RR et al. Biohydrogen production from untreated and hydrolyzed potato steam peels by the extreme thermophiles *Caldicellulosiruptor saccharolyticus* and *Thermotoga neapolitana*. Int J Hydrogen Energ2010;35(15):7730–7. doi:10.1016/j.ijhydene.2010.05.063.
- [32] Bernfeld P. Amylases,  $\alpha$  and  $\beta$ . In: Chance B, editor. Preparation and assay of enzymes. San Diego, Calif.: Acad. Press; 1955, p. 149–158.
- [33] d'Ippolito G, Dipasquale L, Fontana A. Recycling of carbon dioxide and acetate as lactic acid by the hydrogen-producing bacterium *Thermotoga neapolitana*. ChemSusChem 2014;7(9):2678–83. doi:10.1002/cssc.201402155.
- [34] Lay J-J, Li Y-Y, Noike T. Influences of pH and moisture content on the methane production in high-solids sludge digestion. Water Res 1997;31(6):1518–24. doi:10.1016/S0043-1354(96)00413-7.
- [35] Ngo TA, Kim M-S, Sim SJ. Thermophilic hydrogen fermentation using *Thermotoga neapolitana* DSM 4359 by fed-batch culture. Int J Hydrogen Energ2011;36(21):14014–23. doi:10.1016/j.ijhydene.2011.04.058.
- [36] Argun H, Kargi F, Kapdan I, Oztekin R. Batch dark fermentation of powdered wheat starch to hydrogen gas: Effects of the initial substrate and biomass concentrations. Int J Hydrogen Energ2008;33(21):6109–15. doi:10.1016/j.ijhydene.2008.08.004.
- [37] Kargi F, Eren NS, Ozmihci S. Effect of initial bacteria concentration on hydrogen gas production from cheese whey powder solution by thermophilic dark fermentation. Biotechnol Prog 2012;28(4):931–6. doi:10.1002/btpr.1558.
- [38] Beckers L, Masset J, Hamilton C, Delvigne F, Toye D, Crine M et al. Investigation of the links between mass transfer conditions, dissolved hydrogen concentration and biohydrogen production by the pure strain *Clostridium butyricum* CWB1009. Biochemical Engineering Journal 2015;98:18–28. doi:10.1016/j.bej.2015.01.008.
- [39] Sonnleitner A, Peintner C, Wukovits W, Friedl A, Schnitzhofer W. Process investigations of extreme thermophilic fermentations for hydrogen production: effect of bubble induction and reduced pressure. Bioresour Technol 2012;118:170–6. doi:10.1016/j.biortech.2012.05.046.
- [40] Ljunggren M, Willquist K, Zacchi G, Van Niel EWJ. A kinetic model for quantitative evaluation of the effect of hydrogen and osmolarity on hydrogen production by *Caldicellulosiruptor saccharolyticus*. Biotechnol Biofuels 2011;4(1):31. doi:10.1186/1754-6834-4-31.

- [41] Zhang Y, Zhang F, Chen M, Chu P-N, Ding J, Zeng RJ. Hydrogen supersaturation in extreme-thermophilic (70°C) mixed culture fermentation. *Applied Energy* 2013;109:213–9. doi:10.1016/j.apenergy.2013.04.019.
- [42] Verhaart MRA, Bielen AAM, van der Oost J, Stams AJM, Kengen SWM. Hydrogen production by hyperthermophilic and extremely thermophilic bacteria and archaea: Mechanisms for reductant disposal. *Environ Technol* 2010;31(8-9):993–1003. doi:10.1080/09593331003710244.
- [43] Van Niel EWJ, Budde MAW, Haas GG de, van der Wal, F. J., Claassen PAM, Stams AJM. Distinctive properties of high hydrogen producing extreme thermophiles, *Caldicellulosiruptor saccharolyticus* and *Thermotoga elfii*. *Int J Hydrogen Energ* 2002(27):1391–8.
- [44] Belkin S, Wirsén CO, Jannasch HW. A new sulfur-reducing, extremely thermophilic eubacterium from a submarine thermal vent. *Appl Environ Microbiol* 1986:1180–5.
- [45] Kádár Z, Vrije T de, van Noorden GE, Budde MAW, Szengyel Z, Réczey K et al. Yields from Glucose, Xylose, and Paper Sludge Hydrolysate During Hydrogen Production by the Extreme Thermophile *Caldicellulosiruptor saccharolyticus*. *ABAB* 2004;114(1-3):497–508. doi:10.1385/ABAB:114:1-3:497.
- [46] Schröder C, Selig M, Schönheit P. Glucose fermentation to acetate, CO<sub>2</sub> and H<sub>2</sub> in the anaerobic hyperthermophilic eubacterium *Thermotoga maritima*: Involvement of the Embden-Meyerhof pathway. *Arch. Microbiol.* 1994;161(6):460–70. doi:10.1007/BF00307766.
- [47] Jayasinghearachchi HS, Sarma PM, Lal B. Biological hydrogen production by extremely thermophilic novel bacterium *Thermoanaerobacter mathranii* A3N isolated from oil producing well. *Int J Hydrogen Energ* 2012;37(7):5569–78. doi:10.1016/j.ijhydene.2011.12.145.
- [48] O-Thong S, Prasertsan P, Karakashev D, Angelidaki I. Thermophilic fermentative hydrogen production by the newly isolated *Thermoanaerobacterium thermosaccharolyticum* PSU-2. *Int J Hydrogen Energ* 2008;33(4):1204–14. doi:10.1016/j.ijhydene.2007.12.015.
- [49] Ren N, Cao G, Wang A, Lee D, Guo W, Zhu Y. Dark fermentation of xylose and glucose mix using isolated *Thermoanaerobacterium thermosaccharolyticum* W16. *Int J Hydrogen Energ* 2008;33(21):6124–32. doi:10.1016/j.ijhydene.2008.07.107.