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

(Paper I)

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Abstract

The objective of this study was to enhance the hydrogen production rate of dark fermentation in batch operation. For the first time, the hyperthermophilic pure culture of *Thermotoga neapolitana cf. capnolactica* was applied at elevated biomass concentrations. The increase of the initial biomass concentration from 0.46 to 1.74 g cell dry weight/L led to a general acceleration of the fermentation process, reducing the fermentation time of 5 g glucose/L down to 3 h with a lag phase of 0.4 h. The volumetric hydrogen production rate increased from 323 (± 11) to 654 (± 30) mL/L/h with a concomitant enhancement of the biomass growth and glucose consumption rate. The hydrogen yield of 2.45 (± 0.09) mol H_2/mol glucose, the hydrogen concentration of 68% in the produced gas and the composition of the end products in the digestate, i.e. 62.3 (± 2.5)% acetic acid, 23.5 (± 2.9)% lactic acid and 2.3 (± 0.1)% alanine, remained unaffected at increasing 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

3.1 Introduction

Hydrogen (H_2) 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_2 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_2/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_2 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_2 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 cell, high biomass concentrations are generally implemented by the recycling of biomass, which enables 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 induce an acceleration of the dark fermentation process, in particular the HPR. Simultaneously, the effect of elevated biomass concentrations on the efficiency of the process and the kinetics of hydrogen production, glucose consumption and biomass growth were evaluated.

3.2 Material and Methods

3.2.1 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_4Cl; 0.3 K_2HPO_4; 0.3 KH_2PO_4; 0.2 $MgCl_2 \cdot 6\ H_2O$; 0.1 KCl; 0.1 $CaCl_2 \cdot 2\ H_2O$; 0.001 resazurin dissolved in distilled water, supplemented with 10 mL/L of vitamin and 10 mL/L of trace element solutions (DSM medium 141).

3.2.2 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].

3.2.3 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_2 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] = 0.27 * OD_{540} – 0.06 (R^2 = 0.98) [20,31].

3.2.4 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_2 for 5 min to remove oxygen and the pH adjusted to 7 by addition of 1 M NaOH, being the optimum pH for hydrogen production by *Thermotoga neapolitana* [32]. A volumetric ratio of inoculum to 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.

3.2.5 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 [33]. AA, LA and alanine were quantified by ^{1}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 [34]. The biomass concentration was determined by measuring optical density at 540 nm (OD_{540}) (UV/Vis spectrophotometer DU 730, Beckman Coulter Inc, Brea, USA) of the liquid samples and the 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°C and lyophilized overnight.

Produced gas was quantified by water displacement using 500 mL glass containers. At the end of each experiment, the H_2-containing gas was sampled and analyzed by gas chromatography as described by Dipasquale et al. [13]. The molar 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 CO_2 initially in the headspace of the reactor.

3.2.6 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 [14,35] 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). The specific rates were calculated referring the volumetric rates to the initial biomass concentration of each experimental condition.

For glucose consumption, Eq. 3.1 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]; λ_G is the lag phase of glucose consumption [h]; and *e* is the Euler's number, i.e. 2.72.

For biomass growth, Eq. 3.2 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; B_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 λ_B is the lag phase of biomass growth [h].

Eq. 3.3 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 λ_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.

$$G = G_0 - G_m \exp\left\{-\exp\left[\frac{R_G\, e}{G_m}(\lambda_G - t) + 1\right]\right\} \quad \text{(Eq. 3.1)}$$

$$B = B_0 + B_m\, exp\left\{-exp\left[\frac{R_B\, e}{B_m}(\lambda_B - t) + 1\right]\right\} \quad \text{(Eq. 3.2)}$$

$$H = H_m\, exp\left\{-exp\left[\frac{R_H\, e}{H_m}(\lambda_H - t) + 1\right]\right\} \quad \text{(Eq. 3.3)}$$

Table 3.1: 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_2/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

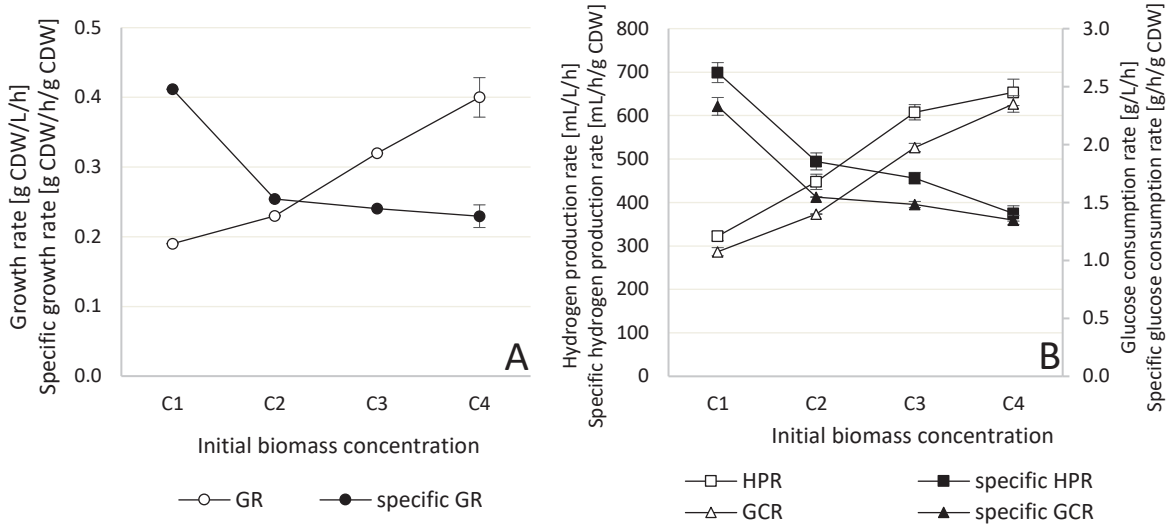

Fig. 3.1: A - Volumetric and specific growth rate; B - Volumetric as well as specific glucose consumption and hydrogen production rate at different initial biomass concentrations (C1 = 0.46; C2 = 0.91; C3 = 1.33; C4 = 1.74 g CDW/L) of *Thermotoga neapolitana* fermenting 5 g/L of glucose. Specific rates were calculated per g initial cell dry weight. (GR – Growth rate; HPR – Hydrogen production rate; GCR – Glucose consumption rate). Error bars depict the standard deviation.

3.3 Results and Discussion

3.3.1 Hydrogen yield and production rate

A change of the initial biomass concentration between 0.46 and 1.74 g/L did not affect the hydrogen yield i.e. 2.45 (± 0.09) mol H_2/mol glucose (Table 3.1) or the composition of the biogas that maintained a constant level of hydrogen at 67.6 (± 2.4)% (data not shown). The HPR observed at the lowest initial biomass concentration of 0.46 g CDW/L (C1) reached 323 (± 11) mL/L/h (Table 3.2). A further increment of the biomass concentration to 0.91 (C2), 1.33 (C3) and 1.74 g CDW/L (C4) increasingly enhanced the volumetric HPR to 448 (± 18), 608 (± 18) and 654 (± 30) mL/L/h (Fig. 3.1B; Table 3.2), respectively. 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 dark fermentation by anaerobic sludge in closed serum bottles, where a general increase of HPR was obtained by raising the biomass concentrations [36,37]. On the other hand, Ngo and coworkers reported a reverse correlation between the two parameters in a fed batch process using *T. neapolitana*. In 4 feeding cycles, the authors observed an increase of the initial biomass from

1.3 ± 0.1 to 2.4 ± 0.1 g/L yielding a mild reduction of HPR from 114 to 106 mL/L/h [32]. Simultaneously, acetic acid and lactic acid accumulated in the fermentation broth up to 123 ± 7 and 28 ± 1 mM, respectively, which could potentially have caused an inhibitory effect.

While hyperthermophilic cultures are capable to achieve even higher hydrogen yields (Table 3.3) the volumetric HPR observed in this work exceeds those achieved in similar studies using pure hyperthermophilic cultures at low biomass concentrations (Table 3.3).

The highest production rate of 654 (± 30) mL/L/h (C4) (Table 3.2) 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_2 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 [25,44], 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 [45].

Table 3.2: *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
Hydrogen production					
	[g CDW/L]	[mL/L/h]	[mL/h/g initial CDW]	[h]	
C1	0.46	323 (± 11)	699 (± 23)	1.50 (± 0.07)	0.99
C2	0.91	448 (± 18)	494 (± 20)	0.79 (± 0.01)	0.99
C3	1.33	608 (± 18)	456 (± 13)	0.52 (± 0.01)	0.99
C4	1.74	654 (± 30)	375 (± 17)	0.40 (± 0.02)	0.99
Biomass growth					
	[g CDW/L]	[mg CDW/L/h]	[mg CDW/h/g initial CDW]		
C1	0.46	190 (± 0)	412 (± 0)		0.99
C2	0.91	230 (± 0)	254 (± 0)		0.98
C3	1.33	320 (± 0)	240 (± 0)		0.96
C4	1.74	400 (± 28)	229 (± 16)		0.98
Glucose consumption					
	[g CDW/L]	[g Glucose/L/h]	[g Glucose/h/g initial CDW]		
C1	0.46	1.08 (± 0.04)	2.33 (± 0.08)		0.99
C2	0.91	1.40 (± 0.00)	1.55 (± 0.00)		0.98
C3	1.33	1.98 (± 0.04)	1.48 (± 0.03)		0.98
C4	1.74	2.35 (± 0.07)	1.35 (± 0.04)		0.99

Table 3.3: 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 [mL/L/h]	Hydrogen yield [mol H_2/mol glucose]	Reference
Caldicellulosiruptor saccharolyticus	264	2.5	[38]
	296	3.4	[31]
	277	3.2	[11]
Thermotoga elfi	200	3.3	[39]
Thermotoga maritima	170	4	[40]
Thermotoga neapolitana	304	2.9	[31]
	269	3.5	[11]
	21	3.9	[18]
	252	1.8	[20]
	50	3.9	[19]
Thermoanaerobacter mathranii A3N	100	2.6	[41]
Thermoanaerobacterium thermosaccharolyticum PSU-2	287	2.4	[42]
Thermoanaerobacterium thermosaccharolyticum W16	309	2.4	[43]
Thermotoga neapolitana	654	2.5	Present study

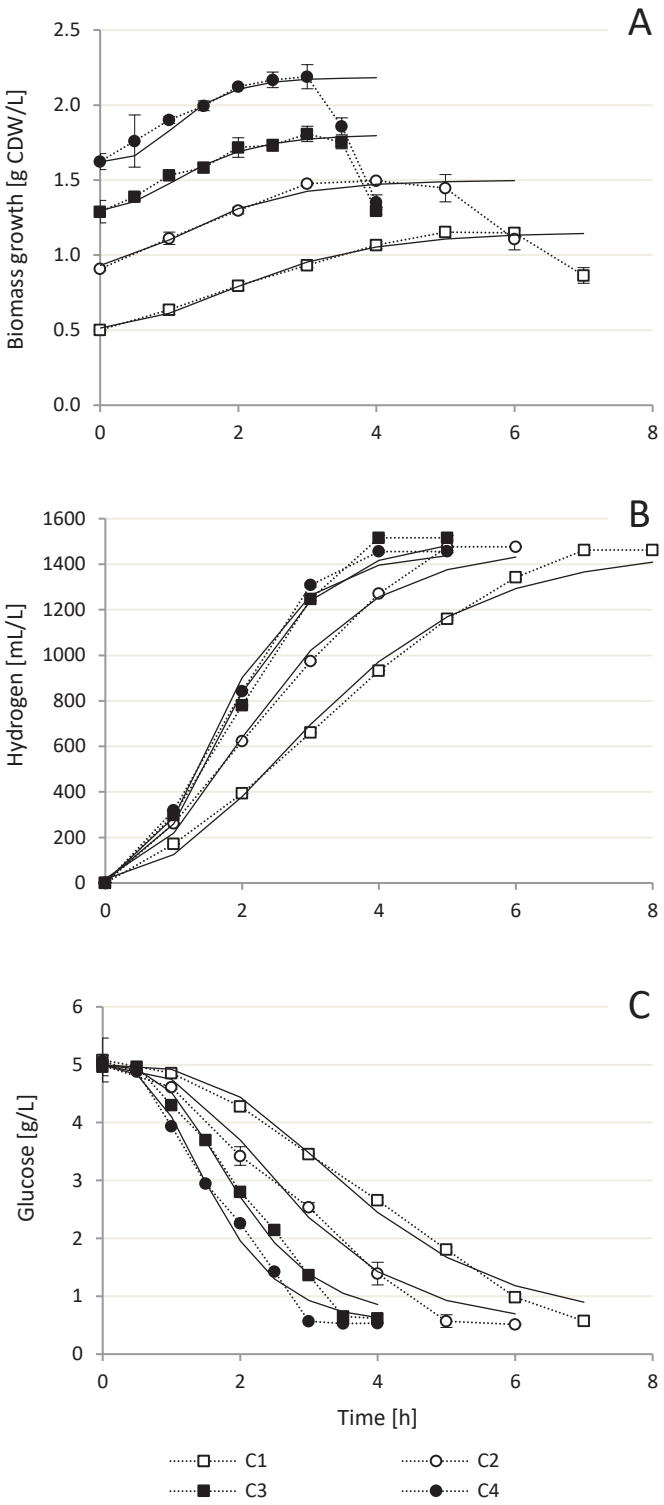

Fig. 3.2: A – Biomass growth; B – Cumulative hydrogen and C – Glucose consumption production at different initial biomass concentrations (C1 = 0.46; C2 = 0.91; C3 = 1.33; C4 = 1.74 g CDW/L) of *Thermotoga neapolitana* fermenting 5 g/L of glucose. The solid line represents the fitting to the Gompertz model. Error bars depict the standard deviation.

3.3.2 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. 3.1, Table 3.2) were calculated via the Gompertz model (described in 2.6)(Fig. 3.2). 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 3.2). 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. 3.1, Table 3.2) exhibited a decreasing trend with increasing biomass concentrations. This suggests that the overall process was considerably accelerated, while the individual cells were partially repressed at increased biomass concentration. 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 (± 0.08) of C1 to 1.55 (± 0) of C2 and by 9% to 1.35 (± 0.04) of C4 (Table 3.2). The specific growth rate (mg CDW/h/g CDW) decreased by 38% from 412 (± 0) of C1 to 254 (± 0) of C2 and merely by 6% to 229 (± 16) of C4 (Table 3.2). The specific HPR (mL/h/g CDW) decreased by 29% from 699 (± 23) of C1 to 494 (± 20) of C2 and by 17% to 375 (± 17) of C4 (Table 3.2). A similar trend of decreasing specific HPR was observed in previous studies when the initial biomass was increased using mixed cultures [36,37]. Kargi et al. [37] observed a drop of the specific HPR from 48 to approximately 3 mL/h/g when the biomass concentration was increased from 0.48 to 2.88 g/L. Substrate limitation caused by flock formation [37] and hydrogen consuming homo-acetogenic bacteria [36] were presumed to be responsible for the decrease of specific HPR at higher initial biomass concentrations.

In the present study, the formation of flocks was not observed, and hydrogen was not consumed, indicated by the ratio of hydrogen to acetic acid being consistent with the dark fermentation model Eq. 3.4. However, an increase of HPR induces the accumulation of hydrogen in the liquid phase which mainly depends on the HPR and the mass transfer rate of the system [46]. The concentration of liquid phase hydrogen can reach multiple fold the equilibrium concentration suggested by Henry´s Law even in hyperthermophilic stirred reactor systems [46,47], acting as a potent inhibitor of hydrogen production by dark fermentation [44,48]. In the present study, the accumulation of hydrogen was observed at

the highest biomass concentration (C4) where the highest HPR was obtained. After 3 h of fermentation, the glucose consumption of C4 was completed (Fig. 3.2C), while hydrogen continued to be produced by the reactor (Fig. 3.2B), due to the transport of accumulated hydrogen from the liquid to the gas phase. According to Ljunggren et al. [46], 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_2 continuously [11,31] or in regular intervals [19].

3.3.3 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 initial biomass concentration of 0.46 g CDW /L (C1) induced a lag phase of 1.50 (± 0.07) h (Table 3.2) and the completion of the fermentation within approximately 7 h (Fig. 3.2). 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. 3.2) and 0.4 (± 0.02) h (Table 3.2), 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 (± 0.9)% (data not shown) was reached at the end of the fermentation.

3.3.4 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 3.1). 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 3.1), 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 (± 0.04) g CDW/ L with 10 g/L pure glycerol as main substrate and 10% of inoculum (v/v). Van Niel et al. [39] 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_{540} to decrease (Fig. 3.2) [49], while the CDW remains high (Table 3.1). Consequently, the curve fitting to the Gompertz model for biomass growth was done exclusively until the highest value of turbidity was reached (Fig. 3.2).

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

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

3.3.5 Production of fermentation products

The distribution of end products obtained in the digestate (62.3 (± 2.5)% AA; 23.5 (± 2.9)% LA; 2.3 (± 0.1)% alanine) was similar in the range of biomass concentrations investigated (Table 3.4), corresponding to yields of 1.33 (± 0.05) mol/mol glucose for AA, 0.50 (± 0.06) mol/mol glucose for LA and 0.05 (± 0.001) mol/mol glucose for alanine (Table 3.4). As observed in previous studies [14], *Thermotoga neapolitana* primarily ferments glucose via the hydrogen producing acetic acid (Eq. 3.4) or the lactic acid pathway (Eq. 3.5).

$$C_6H_{12}O_6 + 4\,ADP + 4\,H_2PO_4^- \leftrightarrow 2\,CH_3CO_2H + 2\,CO_2 + 4\,ATP + 4\,H_2 + 2\,H_2O \quad \text{(Eq. 3.4)}$$

$$C_6H_{12}O_6 + 2\,ADP + 2\,H_2PO_4^- \leftrightarrow 2\,CH_3CH(OH)CO_2H + 2\,ATP + 2\,H_2O \quad \text{(Eq. 3.5)}$$

Considering a production of 2 mol of end product per mol of glucose (Eq. 3.4 and Eq. 3.5), an average of 95 (± 1)% (data not shown) of the initial substrate could be accounted for in the fermentation broth (Table 3.4). 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 3.4) coupled with a slightly lower hydrogen yield (Table 3.1). This is in accordance to the dark fermentation model suggesting a negative correlation between hydrogen yield and LA/AA ratio [48].

Additionally, an impact of capnophilic lactic acid fermentation caused by sparging with CO_2 needs to be considered resulting in elevated amounts of LA without significant loss in hydrogen yield [30,34]. Dipasquale et al. [30] observed LA/AA ratio of 0.28 by N_2 sparging and 0.56 by CO_2 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.

Conclusion

This study identified the use of high initial biomass concentrations of *Thermotoga neapolitana* (from 0.46 to 1.74 g cell dry weight/L) as a suitable method to accelerate the dark fermentation process and increase the HPR. The four-fold increase in biomass concentration led to the consumption of 5 g/L of glucose within 3 h and accelerated the hydrogen production rate by approximately 50% reaching a maximum of 654 (± 30) mL/L/h. The variation of the biomass concentration had no effect on the yield (2.45 (± 0.09) mol H_2/mol glucose), the concentration of hydrogen in the produced gas (68%) or the composition of fermentation end products (i.e. 62.3 (± 2.5)% AA, 23.5 (± 2.9)% LA and 2.3 (± 0.1)% alanine). To continue optimizing the rate of dark fermentation processes more research is required to understand the role of hydrogen in the liquid phase as an inhibitor at elevated hydrogen production rates. Furthermore, economical and environmentally friendly

substrates like organic waste streams need to be investigated for their suitability in large-scale applications of the proposed process.

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