Monitoring quantitative dynamics of *Thermotoga neapolitana* in synthetic co-cultures using quantitative real-time PCR

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ABSTRACT

This study demonstrates the potential for biohydrogen production in a co-culture of two ecologically distant species, *T. neapolitana* and *C. saccharolyticus*, and the development of a quantitative real-time PCR (qPCR) method for quantifying the hyperthermophilic bacterium of the genus *Thermotoga.* Substrate utilization and H_2 production performance was compared to those of their individual cultures. The highest H_2 yields obtained were 2.7 (±0.05), 2.5 (±0.07) and 2.8 (± 0.09) mol H_2/mol of glucose for *C. saccharolyticus*, *T. neapolitana* and the co-culture, respectively. Statistical analysis comparing the H_2 production rate of the co-culture to either *C. saccahrolyticus* or *T. neapolitana* pure cultures indicated a significant difference in the H_2 production rate ($p<0.05$: t-test), with the maximal rate of H_2 production (36.02 mL L^{-1} h^{-1}) observed from the co-culture fermentations. In order to monitor the presence of *T. neapolitana* in the bioprocess, we developed a qPCR method using 16S rDNA and hydrogenase (hydA) gene targets. The qPCR data using *hydA* primers specific to *T. neapolitana*

showed an increase in *hydA* gene copies from 3.32×10^{7} to 4.4×10^{8} *hydA* gene copies per mL confirming the influence of *T. neapolitana* in the synthetic consortium.

Key words: Thermotoga neapolitana; *hydA*; 16S rDNA gene; Real-time quantitative polymerase chain reaction; Hydrogen production; Primer design.

1. INTRODUCTION

The global trends of fossil fuel depletion and impact on climate change due to over exploitation of natural resources has led to a search for alternative measures to produce renewable energy [1,2]. Today, hydrogen ($H_{2)}$ is used in the chemical industry as a fundamental building block e.g. for the production of ammonia-fertilizers and methanol used for manufacturing of many polymers [3]. Hydrogen is presently produced from natural gas, heavy oils, naphtha and coal [4,5] which are not sustainable feedstocks. Hence, there is a need for alternative hydrogen production routes. One of the means that have been highly considered for sustainable energy is biological hydrogen production [6–9].

Research on biological H_2 production has increased over the years leading to several reports on methods such as: direct and indirect photolysis, water-gas shift reaction, photofermentation, biocatalysed electrolysis and dark fermentation [10,11]. Dark fermentation has garnered interest due to the ability to utilize a wide variety of waste streams and energy crops as substrate for hydrogen production, and high hydrogen production rates ($10–15 \times 10^{3}$ ml H_2/l/h) [7,12]. Dark fermentative H_2

production can occur under mesophilic (typically between 30-45 °C), thermophilic (50-60 °C) or hyperthermophilic conditions (from 60 °C upwards). Compared to mesophilic conditions, higher temperatures favor H_2 production [13,14], because the temperature at which the reaction takes place affects the thermodynamic process according to $\Delta G^0 = \Delta H - T\Delta S^0$ and increases the kinetics of chemical reactions thereby speeding up the reactions [15,16].

Several bacterial species have been identified for their ability to produce high volumes of H_2 at hyperthermophilic conditions. An example is the bacterium *Thermotoga neapolitana.* In recent years, there has been a significant increase in the studies on *T. neapolitana*, as it has enabled one of the best H_2 yields (3.8 mol H_2/mol glucose) that have been reported in literature [20, 21]. Combined with its ability to produce high H_2 yields at elevated temperatures (55 - 85 °C), *T. neapolitana*, is capable of metabolizing a wide range of simple and complex carbohydrates such as hexoses, pentoses, disaccharides, glucan and amorphous cellulose [24–27]. Previous studies on *T. neapolitana* have focused in optimizing growth and H_2 production conditions for pure cultures of members of *Thermotoga* sp. [18,19]. However, molecular methods are still needed to better understand and answer questions relating to *T. neapolitana*'s physiological, ecological and metabolic features. A deeper understanding of the bacterium will allows for its metabolic engineering and use in biotechnological applications. Developing molecular methods for monitoring the activity of *T. neapolitana* in various systems require a more accurate representation that would take into account their dynamics and interactions in a mixed consortium while carrying out individual metabolic processes.

Reports on biohydrogen production from hyperthermophilic microorganisms have mostly utilized conventional methods for monitoring the growth of the microbial population such as dry cell weight, optical density (biased in the case of floc formers like *T. neapolitana*) or microscopy [17–23]. Some of these methods are only suitable for monocultures and fail to differentiate between different species. Furthermore, members of the genus *Thermotoga* undergo floc formation which often causes ambiguities in cell enumeration [28].

In the present work, co-cultivation of two ecologically distant organisms for improved H_2 evolution and the development of a quantitative PCR assay for genus and species-level monitoring of *Thermatoga* was carried out. The 16S rDNA method was designed to target eight members of the group *Themotoga*. Given the high degree of similarity and absence of correlation that may occur between 16S rDNA and H_2 producing activity in *T. neapolitana*, we further developed a qPCR approach targeting the hydrogenase A (*hydA*) gene for a more comprehensive evaluation of *T neapolitana* in a synthetic culture. To our knowledge, studies targeting the hydrogenase gene for specie-level monitoring of hyperthermophiles have not been previously published.

2. MATERIALS AND METHODS

2.1. Bacterial strains and growth conditions

Bacterial strains *Thermotoga neapolitana* DSM-4359, *Thermotoga maritima* DSM-3109 and *Caldicellulosiruptor saccharolyticus* DSM-8903 (DSMZ, Germany) were used in this study. *T. neapolitana* was cultivated either as a pure culture or with *C. saccharolyticus* as a synthetic co-culture. The medium used for cultivation of *T. neapolitana* contained

the following components (g/L): NH_4Cl, 1.0; K_2HPO_4, 0.3; KH_2PO_4, 0.3; $MgCl_2$ x 6 H_2O, 0.2; $CaCl_2$ x $2H_2O$, 0.1; NaCl, 5.0; KCl, 0.1; cysteine-HCl, 1.0; yeast extract, 2.0; 10.0 ml L^{-1} of vitamin and trace element (DSMZ 141, Germany) solution. *C. saccharolyticus* pure culture was initially maintained in DSMZ 641 medium. The initial medium pH was adjusted to 7.5 (20 °C) for all cultures with 5 M NaOH. Thirty milliliter of the prepared media was dispensed anaerobically into 120 ml serum bottles. The bottles were sealed with butyl rubber stoppers, capped with aluminum crimps and autoclaved for 15 min at 121 °C. Unless otherwise mentioned, glucose (20mM) was added after sterilization and the bottles containing the growth medium were inoculated with 10 % (v/v) inoculum under anoxic conditions. Triplicate cultivations of *T. neapolitana* and *C. saccharolyticus* as a pure culture and as a synthetic co-culture were carried out. Cultures were incubated at 75°C and 150 rpm for 1-100 hours. To validate the specificity of the primers developed in this study, microbial community from a H_2 producing fluidized bed bioreactor without *Thermotoga* was used [29] as well as pure cultures of *T. neapolitana* and *C. saccharolyticus*.

2.2. Analytical techniques

The cell concentration of the culture suspension containing bacterial cells was determined by measuring the absorbance spectrometrically with an Ultraspec 200 Pro spectrophotometer (Amersham Biosciences, Munich, Germany) at 600 nm. Hydrogen and carbon dioxide levels in the gas phase were measured at regular intervals to monitor the activity of the microbial consortium. The total gas volume was first measured by releasing the pressure in the culture using a syringe [30]. The headspace of each culture bottle was sampled using a gas tight syringe with 0.2 mL injection

volume. The gas composition was then analyzed with a Shimadzu gas chromatograph GC–2014 equipped with a Porapak N column (80/100 mesh) and a thermal conductivity detector. Nitrogen was used as the carrier gas and the injector, column and detector temperatures were 110 °C, 80 °C and 110 °C respectively. The total volume of the gas produced at each time point was calculated using equation 1 [29,31]:

$$V_{H_2,t} = V_{H_2,t-1} + C_{H_2,t}(V_{G,t} - V_{G,t-1}) + V_H(C_{H_2,t} - C_{H_2,t-1}) \quad (1)$$

Where $V_{H_2,t}$ is the cumulative hydrogen gas produced at time t, $V_{H_2,t-1}$ is the cumulative hydrogen gas produced at time t-1, $V_{G,t}$ is the total gas volume at time t, $V_{G,t-1}$ is the total gas volume at time t-1, $C_{H_2,t}$ is the hydrogen gas fraction in the headspace at time t, $C_{H_2,t-1}$ is the hydrogen gas fraction in the headspace at time t-1 and V_H is the total headspace volume in the culture bottle. Glucose, lactate and acetate and ethanol were measured using a high performance liquid chromatograph (HPLC) equipped with a Rezex RHM-monosaccharide H+ (8%) column (Phenomenex, USA) and a refractive index detector (Shimadzu, Kyoto, Japan). The mobile phase used was 0.01 N H_2SO_4 at a flow rate of 0.6 mL/min. At the end of 100 h period of incubation, samples from the co-cultures were harvested, centrifuged and stored at -20 ºC for genomic isolation and molecular analyses.

2.3. Genomic DNA isolation

Genomic DNA of both pure (*T. neapolitana*, *T. maritima* and *C. saccharolyticus*) and synthetic co-cultures of *T. neapolitana* and *C. saccharolyticus* were isolated with Blood and tissue genomic DNA extraction miniprep system (Viogen, USA) according to

the manufacturer's instructions. The protocol was optimized by including three freeze and thaw cycles to enhance genomic DNA recovery.

2.4. 16S rDNA and *hydA* primer design

In this study 16s rDNA and *hydA* were targeted for genus and species level monitoring of *Thermotoga* sp. The 16S rDNA primers for the members of *Thermotoga* sp. was designed with Clustal Omega software (http://www.ebi.ac.uk/Tools/msa/clustalo/). The multiple sequence alignments were designed to contain four different bacterial species from three genera: *T. neapolitana*, *T. maritima*, *C. saccharolyticus, Thermoanaerobacterium thermosaccharolyticum.* The region, conserved only for *Thermotoga* sp. were selected and employed in the primer designing using the Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). Primer specificities towards 16S rDNA of *Thermotoga* sp. were evaluated using arb-silva (https://www.arb-silva.de/search/testprime/), an *in silico* PCR analysis tool which uses 16S/18S rDNA non-redundant reference dataset, SSURef 108 NR [32].

The primers were designed to have an amplicon length of 100 to 150 bp and primer melting temperature of 55 °C to 60 °C. The G+C content was between 50 - 60% with not more than three consecutive G or C bases in the primer sequence. The specificity of the primers was manually verified using nucleotide BLAST. Based on the nucleotide sequences, a primer set was designed to amplify the 16S rDNA gene of *Thermotoga* sp. (Fwd, 5'-TACCCCATACGCTCCATCAA-3'; Rev, 5'-CCGTTACCCCACCAACTAC-3') and *T. neapolitana hydA* (*hydA*_F_AGTACACGGCATGAAGGAGA; *hydA*_R_CGCAGAACACAACTATCCAC-3'). The applicability and specificity of the developed primers was tested using the hydrogen

producing cultures of *T. neapolitana*, *T. maritima, C. saccharolyticus* and mixed cultures from fluidized bed bioreactor, known to contain members of the genus *Thermoanaerobacterium* [29]. Melt curve analysis was conducted simultaneously with the real-time quantitative PCR (qPCR) and the success of the assays (qPCR products) was evaluated on agarose gel electrophoresis (1 % agarose, 0.2 V for 20 min).

2.5. Real-time quantitative PCR

Optimization of primer annealing temperatures for 16S rDNA and *hydA* genes were performed by testing six annealing temperatures for 16S rDNA and *hydA* amplifications (48 °C, 53 °C, 55 °C, 58 °C, 60 °C and 65 °C) separately for both primer pairs. *T. neapolitana* genomic DNA was used as the template to generate the standard curve and the assays were conducted using 10-fold serial dilutions of the template DNA. Genomic standards were subsequently run simultaneously with unknown samples to determine the gene copy number (gene copies per mL) and the copy number of *T. neapolitana* was calculated based on the genome size (1.88 Mb). The amplifications were carried out in triplicates with the Applied biosystems StepOnePlus real-time PCR (ThermoFisher Scientfic, USA). Each 20 µL qPCR reaction mixture contained 10 µL of Maxima SybrGreen/ROX qPCR master mix (Thermo Scientific, USA), 0.4 µL each of 10 µM forward and reverse primers, 7.2 µL of nuclease free water and 2 µL of genomic DNA as template. The qPCR conditions were as follows: 10 min at 95 °C followed by an extension step of 40 cycles of 15s at 95 °C and 1 min at 60 °C. To determine primer specificity, melt curve analysis was done under the following conditions: 15 s at 95 °C, 1 min at 65 °C and 15 s at 95 °C. The slope and y-intercept of the standard curves were evaluated using a linear regression analysis [33]. Gene copies per ng of DNA extracted

and the gene copies per sample were evaluated according to equation 2 and 3, respectively [34]. The amplification efficiencies were calculated using equation 4.

$$\text{Gene copies} = \text{DNA}_{[\text{ng}/\mu\text{L}]} \times \frac{1\text{g}}{1000^3\text{ng}} \times \frac{1\text{ mol bp DNA}}{660\text{ g DNA}} \times \frac{6.023\times10^{23}\text{bp}}{\text{mol bp}} \times \frac{1\text{ copy}}{\text{genome size}_{\text{bp}}} \times \text{vol. DNA used}_{\mu\text{L}} \quad (2)$$

$$Gene\ copies\ per\ sample\ volume = \frac{(\text{gene copies per rxn mix})\times(\text{volume of DNA in }\mu\text{L})}{(\text{volume of DNA template per rxn mix})\times(\text{volume of sample used})} \quad (3)$$

$$Efficiency = [-1 + 10^{(-\frac{1}{slope})}] \quad (4)$$

3. RESULTS AND DISCUSSION

3.1. Comparing hydrogen production between individual cultures and co-cultures of *T. neapolitana* and *C. saccharolyticus* monocultures

Hydrogen production by the hyperthermophiles *T. neapolitana* and *C. saccharolyticus* was evaluated both as pure cultures and as a synthetic co-culture. For the pure culture of *C. saccharolyticus,* a lag phase of 9 h was observed. The maximum H_2 production rate obtained was 24.65 mL L^{-1} h^{-1}. The growth obtained from OD measurements reached a maximum OD_{600nm} of 0.66 (±0.03) (Figure 1). A decrease in the OD was observed after glucose was completely consumed at 100 h suggesting that the cultures entered death phase upon depletion of the carbon source. The H_2 yield obtained was 2.7 (±0.05) mol H_2/mol glucose. Glucose consumption resulted in acetate production with the absence of lactate formation (Figure 1).The absence of lactate in the pure culture of *C. saccharolyticus* was likely as a result of a low partial H_2 pressure (P_{H2})

during the cultivations. A high P_{H2} during dark fermentation is known to cause a shift in the metabolic pathway.

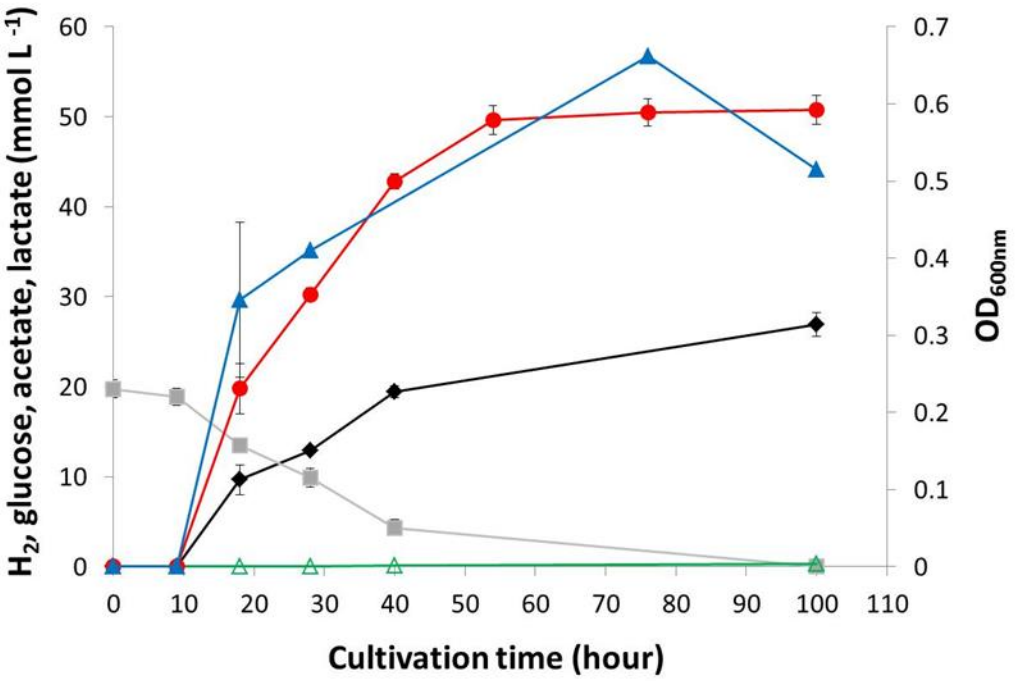

Figure 1– Hydrogen production, glucose consumption, optical density (OD) of the culture suspensions and soluble metabolites produced by *C. saccharolyticus* (●) H_2 production, (■) Glucose consumption, (Δ) Lactate, (◆) Acetate and (▲) OD. The error bars represent the standard deviation from triplicate cultures. In some cases, the error bars are smaller than the symbol.

Compared to *C. saccharolyticus*, *T. neapolitana* pure cultures had a longer lag phase and the maximum H_2 production rate obtained was lower (15.51 mL L^{-1} h^{-1}) than in the synthetic co-culture or pure culture of *C. saccharolyticus*. The H_2 yield achieved was 2.5 (±0.07) mol H_2/mol of glucose and compared to *C. saccharolyticus*, the

consumption of glucose was slower (Figure 2). Similar to *C. saccharolyticus* however, the major metabolite formed was acetate. Lactate was also produced between incubation time 60 h and 100 h. The OD values obtained for *T. neapolitana* were low (0.44 ± 0.05) but this was expected due to the floc formation by the bacterium [28]. Prior to OD measurements, vigorously shaking of the culture to disentangle the floc had little impact on the cell density measurements. Though the growth pattern as measured by the OD was similar to that obtained in *C. saccharolyticus*, the OD obtained was not accurate enough to be considered in this study. Hence, an alternative molecular biology based method was developed for accurate enumeration of *T. neapolitana.*

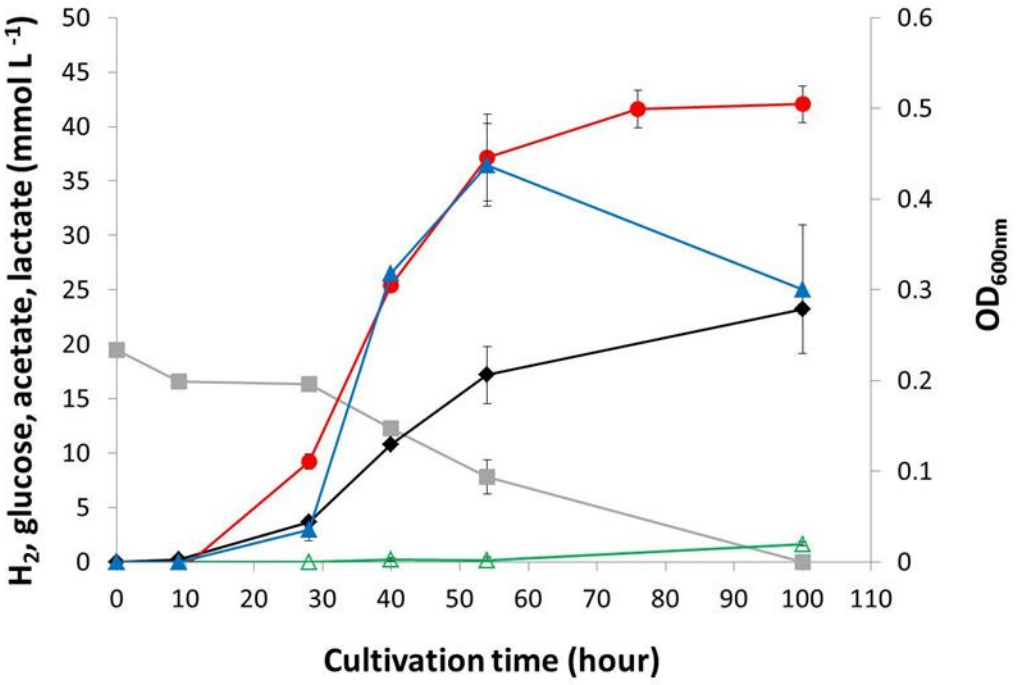

Figure 2– Hydrogen production, glucose consumption, optical density (OD) of the culture suspensions and soluble metabolites produced by *T. neapolitana* (●) H_2 production, (■) Glucose consumption, (Δ) Lactate, (◆) Acetate and (▲) OD. The error

bars represent the standard deviation from triplicate cultures. In some cases, the error bars are smaller than the symbol.

Results obtained for the synthetic co-cultures of *T. neapolitana* and *C. saccharolyticus* showed that glucose was rapidly consumed compared to the pure cultures (Figure 3). Similar to the pure cultures of *C. saccharolyticus,* a lag phase of 9 h was observed and a maximum OD_{600nm} of 0.9 (±0.04) was obtained during the cultivation. The H_2 yield was 2.8 (±0.09) mol H_2/mol of glucose. Compared to the performance of the pure cultures, the synthetic co-cultures showed a clear improvement in the H_2 production (Figure 3). The maximum H_2 production rate obtained was 36.02 mL L^{-1} h^{-1}. Statistical analysis to compare the H_2 production rate of the synthetic co-culture with either of the pure cultures gave a probability value of 0.15 ($p<0.05$: t-test) with *C. saccahrolyticus* and 0.014 ($p<0.05$: t-test) with *T. neapolitana* showing a significant difference in the H_2 production rate.

Overall, the maximum hydrogen content in the pure cultures and the synthetic co-culture was between 63% and 67%. Though *T. neapolitana* is able to form biofilm as was observed in the pure culture, there was no biofilm formation observed when it was cultivated with *C. saccharolyticus* as a synthetic co-culture. Cultivation of both organisms also did not induce a shift in metabolic pathway as the major metabolite produced was acetate. This therefore did not give a prediction on the interspecies relationship that exists between both organisms. However, a more comprehensive study to determine the relationship between both organisms will take into account the differences in protein expression of the pure cultures and synthetic co-cultures.

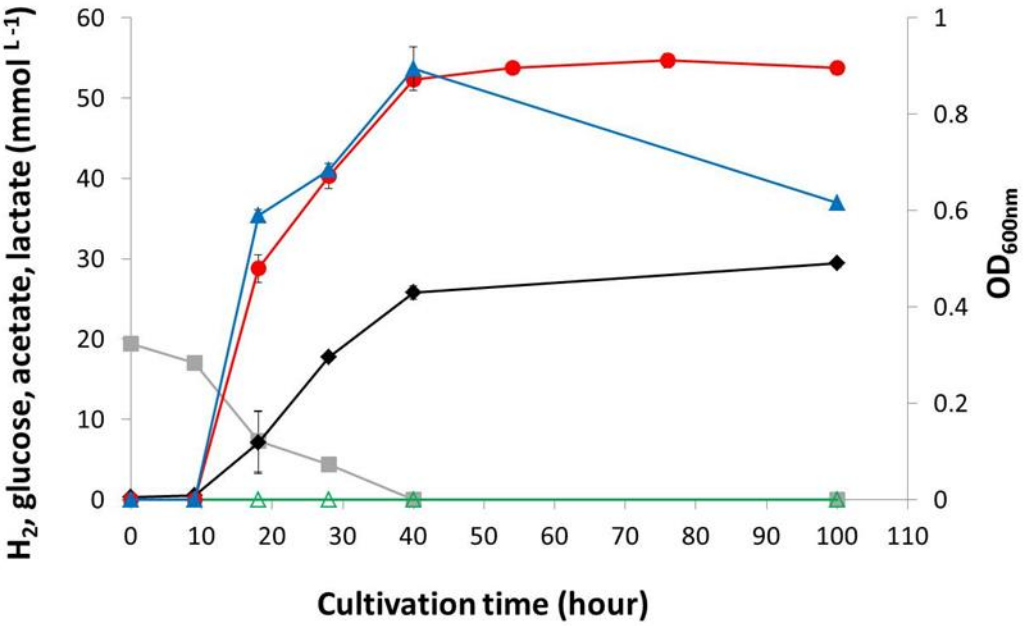

Figure 3– Hydrogen production by co-culture of *T. neapolitana* and *C. saccharolyticus* (●) H_2 production, (■) Glucose consumption, (Δ) Lactate, (◆) Acetate and (▲) OD. The error bars represent the standard deviation from triplicate cultures. In some cases, the error bars are smaller than the symbol.

Several co-culture methods have been accessed for biohydrogen production under hyperthermophilic conditions and have been shown to be a promising strategy for improved H_2 production [35, 36]. Some extreme thermophilic bacteria such as, *Thermotoga maritima* and *C. saccharolyticus* are capable to hydrolyze polysaccharides and utilize the reducing equivalents formed during the glycolytic process for H_2 production [38,39]. Additionally, *C. saccharolyticus* has been recognized as being relatively insensitive to H_2 partial pressure [40]. In this study, the results obtained with the synthetic co-culture showed improvements in H_2 production, suggesting a

synergistic effect between *T. neapolitana* and *C. saccharolyticus*. Compared to the pure strains, the co-cultures demonstrated a rapid glucose utilization and higher H_2 production rate. The results obtained from this study is in line with the literature reporting enhanced H_2 production using synthetic co-cultures [36,38,41–46].

Table 1. Comparison of biohydrogen production in hyperthermophilic co-cultures from different feedstocks and inoculum

Strain/co-culture	Substrate	Temperature (°C)	Maximum H_2 yield (mol H_2/mol of glucose)	Reference
C. saccharolyticus and *T. maritima*	Xylose Cellobiose	70	2.7 ± 0.1	[38]
C.Thermocellum JN4 *and T. thermosaccharolyticum* GD17	Microcrystalline cellulose, cellobiose or filter paper	60	1.8 ± 0.09	[35]
C. saccharolyticus and *T. neapolitana*	Glucose	75	2.81 ± 0.09	This study

3.2. Specificity of *in silico* and experimental qPCR assays

In this study, quantitative monitoring of *Thermotoga* sp. was performed using real-time PCR and primers targeting the variable regions in *Thermotoga* 16S rDNA and *hydA* genes. The *hydA* gene has been used in quantitative monitoring of *Clostridium butyricum* in mesophilic hydrogen producing bioprocess systems [33,47]. The specificities of the newly designed primers were evaluated by *in silico* PCR of 16S rDNA primer and Primer-BLAST for both 16S rDNA and *hydA* primers. The *in silico* PCR analysis was conducted to include coverage of 613789 sequences that gave an output specific for members of the genus *Thermotoga* (Table 2).

Table 2– 16S rDNA gene primer specificity towards *Thermotoga* sp. identified from *in silico* PCR using arb-silva database.

Primary accession	Organism name	Start position	Stop position	Specificity (%)
CP000702	*Thermotoga petrophila*	753682	755234	100
CP000916	*Thermotoga neapolitana*	505958	507506	100
CP000969	*Thermotoga sp*. RQ2	788689	790242	100
CP001839	*Thermotoga naphthophila*	839610	841157	100
CP003408	*Thermotoga sp.* 2812B	793453	795015	100
CP003409	*Thermotoga sp.* Cell2	738805	740367	100
CP007633	*Thermotoga sp*. RQ7	783074	784640	100
CP010967	*Thermotoga maritima*	188967	190530	100

The overall sequence coverage by the primer obtained for the *in silico* PCR was 79.3%. Experimental evaluations for primer specificities were performed by qPCR with melt curve analysis using target and non-target bacterial species as reference (*T. neapolitana*, *T. maritima* and *C. saccharolyticus*, co-cultures of *T. neapolitana* and *C. saccharolyticus* and mixed cultures from fluidized bed bioreactor). Since the 16S rDNA amplicons had different lengths, the melt curve analysis for *T. neapolitana* and *T. maritima* showed a slightly different melting temperature (Tm). However, the 16S rDNA primers were specific towards *Thermotoga* sp. corroborating with the results predicted by the *in silico* PCR. The *hydA* primers did not show any amplification with the reference strains except with *T. neapolitana* which was the target organism (Figure 4). This indicated the *hydA* primer's specificity towards *T. neapolitana*.

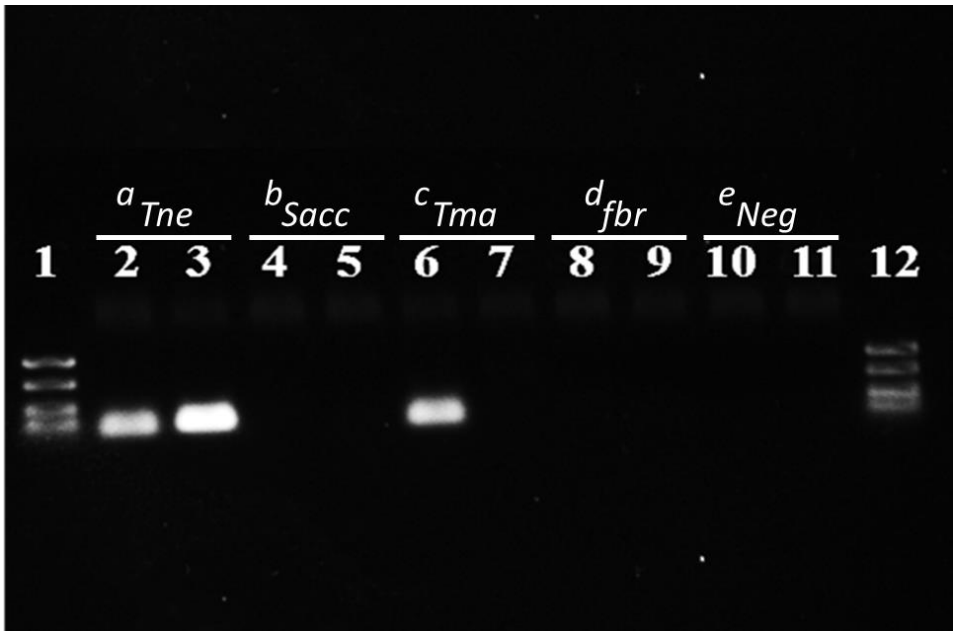

Figure 4– qPCR profile of *Thermotoga* sp. 16S rDNA and *T. neapolitana hydA* amplicons on agarose gel. (Lanes: 1 and 12= 1 kb GeneRuler DNA Ladder (Thermo Scientific, USA), lanes 2,4,6,8, and 10 were amplicons from 16S rDNA qPCR assay and lanes 3,5,7,9 and 11 were amplicons from *hydA* qPCR assay). *a, T. neapolitana. b, C. saccharolyticus. c, T. maritima. d,* fluidized bed bioreactor sample. *e*, Negative control without template.

3.3. Real-time quantitative PCR

The number of gene copies calculated from the constructed *T. neapolitana* 16S rDNA standard curve did not go beyond the defined value of 10^6 gene copies. The absolute cell number was calculated using equations 2 and 3, assuming that the genes of interest exist as a single copy in the genome [48,49]. The regression coefficients showed strong linear correlations for all targets during qPCR analysis (Figure 5).

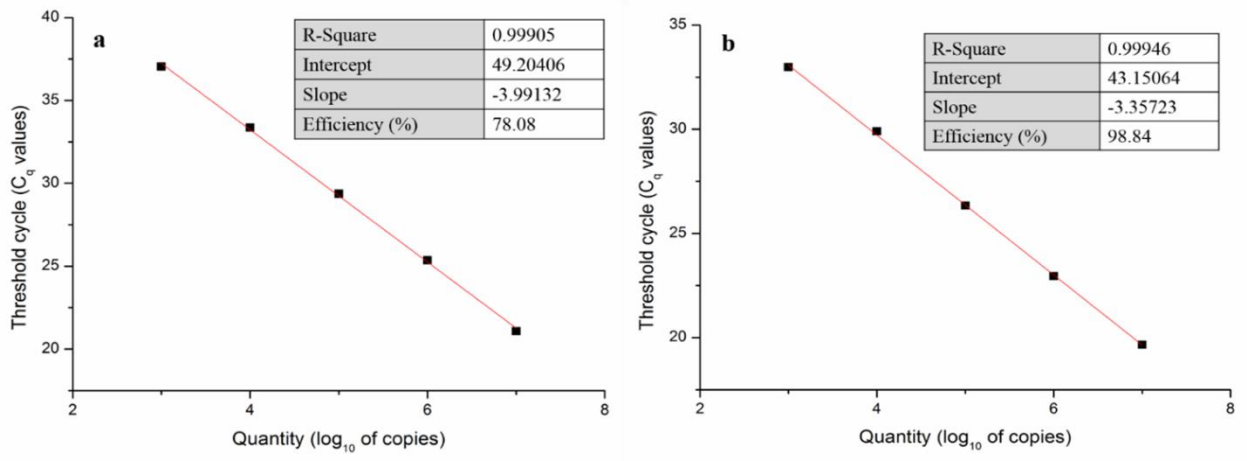

Figure 5– Standard curve for *Thermotoga* 16S rDNA and *hydA* amplicons. Shown are: a) *Thermotoga* sp. specific 16S rDNA amplicons and b) *T. neapolitana hydA* amplicons with *T. neapolitana* genomic DNA as the template.

The amplification efficiencies of the designed real time qPCR primers were evaluated using genomic DNA extracted from *T. neapolitana*. The reproducibility of both primer pairs was determined with cultivation of *T. neapolitana* and the synthetic co-culture (*T. neapolitana* and *C. saccharolyticus*). The standard curve from *T. neapolitana* pure culture indicated amplifications efficiencies of 75% – 85% for 16S rDNA and 99% for *hydA* (Figure 5) for each qPCR assay. Similar results on the primer specificities and efficiencies were obtained when standards were run together with the synthetic co-cultures. The qPCR data from the synthetic co-culture showed an increase in *hydA* gene copies from 3.32×10^{7} to 4.4×10^{8} *hydA* gene copies per mL of sample during cultivation. This result proved that *T. neapolitana* in the synthetic co-culture had an influence in the dark fermentation process. The 16S rDNA qPCR approach with the same samples, under the same conditions showed a much lower number of gene copies per mL of sample (Figure 6). This was suggested to be as a result of non-optimal

thermocycling condition. An *in silico* approach to optimize the 16S rDNA amplification showed that increasing the annealing temperature from 60 °C to 72 °C had a significant effect on the amplification efficiency.

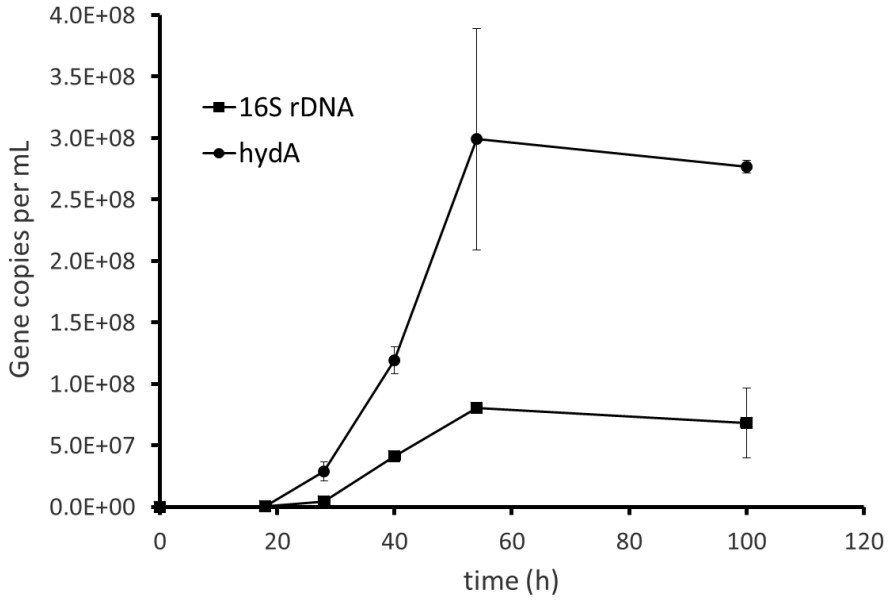

Figure 5– Comparison of quantification results obtained from 16S rDNA and *hydA* based approaches of *T. neapolitana* in synthetic co-culture. The error bars represent the standard deviation from triplicate cultures. In some cases, the error bars are smaller than the symbol.

Though the *hydA* primers demonstrated a considerably higher amplification efficiency and sensitivity towards the target gene, both primers showed a similar amplification pattern, confirming the specificity to their various targets. The molecular method applied to this study facilitated to quantify and confirm the influence of *T. neapolitana* in the H_2 production process. To the best of our knowledge, this is the first report to demonstrate the application of *hydA* gene for species specific quantitative

monitoring of hyperthermophiles. Reports on biohydrogen production from hyperthermophilic microorganisms have mostly utilized conventional methods for monitoring the activity of the microbial population. Therefore, the molecular method developed could be used as an alternative method in the cultivation of *Thermotoga* sp. The quantitative analysis of *T. neapolitana hydA* in the synthetic co-culture showed an increased number of *hydA* gene copies simultaneous to the hydrogen production suggesting the growth of *T. neapolitana* in the co-culture culture. In a similar study, co-cultivations of *C. saccharolyticus* and *T. maritima* [42] improved the hydrogen production performance from simple sugars and complex substrates suggesting a relationship of mutualism or commensalism. However, the kind of relationship that exists in these systems and how beneficial it is for hydrogen production or metabolite formation or the long term cultivation is still unknown and should be studied further.

One of the deterring factors for successful qPCR reaction is often tagged on the efficiency of the designed primers. Studies on primer design, validation and usage in microbial monitoring have used efficiencies ranging between 78% to 100% [33,34,47]. Factors such as amplicon length, melting temperature, annealing temperature and the ΔG of the amplicon should always be taken into account. Using Mfold web server DNA folding form prediction tool (http://unafold.rna.albany.edu/?q=mfold/dna-folding-form), the ΔG value obtained for *Thermotoga* 16S rDNA and *hydA* amplicons at the qPCR annealing temperature (60 °C) used in the experiment was calculated. There was a significant variation in the 16S rDNA and the *hydA* (-14.69 kcal/mol and -0.09 to -0.7 kcal/mol, respectively). Given the values obtained for both target genes, it is probable that the reason for the low efficiency obtained in 16S rDNA amplification was due to the

low ΔG value. The ΔG represents the quantity of energy needed to fully break a secondary DNA structure and the lower the ΔG, the higher the quantity of energy that is required to separate the DNA strands if self-dimers or hetero-dimers are formed. In essence, higher temperatures are needed to break the dimer. When the folding temperature of *Thermotoga* 16S rDNA amplicon sequence was increased to 72 °C *in silico* (using the Mfold web server DNA folding form), a ΔG value of -8.80 kcal/mol was obtained. With this value, the structure formed can be irrelevant in the qPCR reaction, which means the efficiency could be improved by altering the annealing temperature. Hence optimizing the thermocycling conditions can significantly influence the amplification efficiency with the developed 16S rDNA primers. The high R-squared values obtained in the 16S rDNA amplifications confirm that the reactions were consistent with an absence of any non-specific product or primer dimer formation. Overall, 16S rDNA and *hydA* based quantitative methods established were specific for the genus *Thermotoga* and *T. neapolitana* respectively.

CONCLUSIONS

This study demonstrates that ecologically distant hydrogen producing organisms with different cultivation conditions such as, *T. neapolitana* and *C. saccharolyticus*, can be used for improvement of H_2 production yield and rate when cultivated as a synthetic co-culture under optimized conditions. The co-cultures showed synergy such that there was rapid substrate consumption and higher hydrogen production rate compared to the respective monocultures. Additionally, qPCR methods were successfully developed for genus and species specific quantitative monitoring of *Thermotoga* sp. in H_2 producing

systems. The *hydA* provided a promising target to complement with the existing 16S rDNA gene-based methods for accurate monitoring growth and activity of *T. neapolitana.* This study, therefore, offers a new avenue for research on simultaneous utilization of pentose and hexose from various lignocellulosic waste materials for H_2 production by the co-culture. Furthermore, the work presents an alternative quantification method for genus-level monitoring of *Thermotoga* sp. and specie specific monitoring of *T. neapolitana* which can be further explored for its ability to form biofilm and retain biomass for improved hydrogen production under different cultivation conditions.

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