Quantitative Real-time PCR Monitoring Dynamics Of Thermotoga Neapolitana In Synthetic Co-Culture For Biohydrogen Production

Citation

Year
2018

Version
Early version (pre-print)

Link to publication
TUTCRIS Portal (http://www.tut.fi/tutcris)

Published in
International Journal of Hydrogen Energy

DOI

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Hydrogen production and quantitative dynamics of Thermotoga neapolitana in synthetic mixed cultures using 16S rDNA and hydrogenase gene

Onyinye Okonkwo*, Aino-Maija Lakaniemi, Ville Santala, Matti Karp, Rahul Mangayil

Department of Chemistry and Bioengineering, Tampere University of Technology, P.O. Box 541, FI-33101 Tampere, Finland

ABSTRACT

This study demonstrates the potential to use ecologically distant and ideal hydrogen producing organisms as co-culture for enhanced hydrogen production. In this study, we developed a quantitative real-time PCR method for quantifying the hyperthermophilic bacterium, Thermotoga. In the co-culture, glucose was rapidly consumed compared to the pure cultures. The maximum H₂ yield obtained was 2.7 (±0.05), 2.5 (±0.07) and 2.8 (± 0.09) mol H₂ per mol of glucose for C. saccharolyticus, T. neapolitana and the co-culture respectively. Statistical analysis to compare the H₂ production rate of the co-culture to either C. saccharolyticus or T. neapolitana pure cultures indicated a significant difference in the hydrogen production rate (p<0.05: t-test), with the maximal rate of hydrogen production observed from co-culture fermentations. QPCR data using hydA primers specific to T. neapolitana showed an increase in hydA gene copies from 3.32×10⁷ to 4.4×10⁸ hydA gene copies per mL confirming the influence of T. neapolitana in the consortium.
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 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 hydrogen 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 hydrogen 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 hydrogen production [13,14], because the
temperature at which the reaction takes place affects the thermodynamic process according to \( \Delta G^0 = \Delta H - T \Delta S^0 \), 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 hydrogen at hyperthermophilic conditions. An example of these is the bacterium *Thermotoga neapolitana*. In recent years, there has been a significant increase in the studies on *T. neapolitana* with one of the best hydrogen yields that have been reported in literature [17–23]. Combined with its ability to produce high 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 for hydrogen production [24–27]. Previous studies on *T. neapolitana* have focused in optimizing growth and hydrogen production conditions for pure cultures of members of *Thermotoga* sp. [17–23]. However, molecular methods are still needed to better understand and answer questions related to the *T. neapolitana*’s physiological, ecological and metabolic features. A deeper understanding will ultimately lead to metabolic engineering of the bacteria for robust applications. Developing molecular methods for monitoring the activity of *T. neapolitana* in various systems will require a more accurate representation that would take into account their dynamics and interactions in a mixed consortium while carrying out individual metabolic processes.

Thus far, the existing methods such as cell dry weight, microscopic and optical density measurements [17–23] 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 enumeration [28].
Reports on biohydrogen production from hyperthermophilic microorganisms have mostly utilized conventional methods for monitoring the activity of the microbial population such as dry cell weight (which often require large volume of samples), optical density (which often creates bias in the case of floc formers like T. neapolitana) or microscopy [17–23].

The aim of this study was to develop and validate a quantitative PCR assay for the genus Thermotoga and specifically for T. neapolitana as an effective method to monitor the dynamics in a synthetic mixed culture producing hydrogen at hyperthermophilic conditions. The 16S rDNA method was designed to target eight members of the group Thermotoga. Given the high degree of similarity and absence of correlation that may occur between 16S rDNA and hydrogen 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

Pure bacterial strains used for this study were T. neapolitana DSM-4359, T. maritima DSM-3109 and Caldicellulosiruptor saccharolyticus DSM-8903, were purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), Braunschweig, Germany. 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.
T. neapolitana contained the following components (g/L): NH$_4$Cl, 1.0; K$_2$HPO$_4$, 0.3; KH$_2$PO$_4$, 0.3; MgCl$_2$ x 6 H$_2$O, 0.2; CaCl$_2$ x 2H$_2$O, 0.1; NaCl, 5.0; KCl, 0.1; cysteine-HCl, 1.0; yeast extract, 2.0; 10.0 ml/L of vitamin and trace element (DSMZ 141, Germany) solution. C. saccharolyticus was maintained in DSMZ 641 medium. The medium used for T. neapolitana. The initial medium pH was adjusted to 7.5 (20 °C) for all cultures with 5 M NaOH Thirty millilitres of 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 growth medium were inoculated with 10 % (v/v) inoculum under anoxic conditions. Triplicate cultivations of T. neapolitana and C. saccharolyticus as a pure culture (previously grown at 75 °C and a synthetic co-culture. Cultivations were operated at 150 rpm.

2.2. Analytical techniques

The cell concentration in each sample was determined by measuring the absorbance spectrometrically with an Ultraspec 200 Pro spectrophotometer (Amersham Biosciences, Munich, Germany) at 600nm. The cumulative hydrogen production was obtained by calculating the average of triplicate samples. 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 according to [29]. 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.
(TCD). 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[30,31]:

$$V_{H,i} = V_{H,i-1} + C_{H,i}(V_{G,i} - V_{G,i-1}) + V(C_{H,i} - C_{H,i-1})$$ (1)

Where $V_{H,i}$ and $V_{H,i-1}$ are the current and previous time intervals respectively for cumulative hydrogen gas produced, $V_{G(i)}$ and $V_{G(i-1)}$ are the current and next time intervals of the total gas production, $C_{H,i}$ and $C_{H,i-1}$ current and next time interval of the hydrogen gas fraction in the headspace of the culture bottle measured using gas chromatography. $V_{H}$ 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, DGU-20A5 prominence degasser and a CBM-20A prominence communication bus module (Shimadzu, Kyoto, Japan). The mobile phase used was 0.01 N H$_2$SO$_4$ at a flow rate of 0.6 mL/min. At the end of 100 h period of incubation, the samples were harvested 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 and obtain higher yield.
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/maa/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 primer design 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’-TACCCCATAGCTCCATCAA-3’; Rev, 5’-CGTTACCACCCAACCTAC-3’) and T. neapolitana hydA (hydA_F_5GTACACCGCCATGAAGGAGA; hydA_R_CGCAGAACAACCTATCCAC-3’). The applicability and specificity of the developed primers was tested using hydrogen producing cultures of T. neapolitana, the synthetic co-cultures and mixed cultures (see Section 2.1. for more specific information). The 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.

2.5. Real-time quantitative PCR

Optimization of primer annealing temperatures for 16S rDNA and *hydA* was 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 Scientific, 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 in triplicates 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.
and the correlation between the number of hydA copies and hydrogen production was evaluated using Pearson’s correlation coefficient.

\[
\text{Gene copies} = \text{DNA}_{\mu g/\mu L} \times \frac{1g}{1000\text{ng}} \times \frac{1 \text{ mol bp DNA}}{660 \text{ g DNA}} \times \frac{6.023 \times 10^{23} \text{bp}}{\text{mol bp}} \times \frac{1 \text{ copy}}{\text{genome size bp}} \times \text{vol. DNA used}_{\mu L}
\]  

(2)

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

(3)

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

(4)

3. RESULTS

3.1. Specificity of in silico and experimental qPCR assays

In this study, quantitative monitoring of Thermotoga sp. in pure and mixed cultures were performed using primers targeting the variable regions in Thermotoga 16S rDNA and hydA genes. The hydA has been used for quantifying Clostridium butyricum which is known for producing hydrogen at mesophilic conditions [33,35]. 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. 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 1).
Table 1—16S rDNA gene primer specificity towards *Thermotoga* sp. identified from *in silico* PCR using arb-silva database.

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

The overall coverage by the primer obtained for the *in silico* PCR was 79.3 %.

Experimental evaluations for primer specificities were performed by a real-time quantitative PCR with melt curve analysis using target and non-target bacterial species (*T. neapolitana*, *T. maritima* and *C. saccharolyticus*), co-cultures of *T. neapolitana* and *C. saccharolyticus*, fluidized bed bioreactor samples and fluidized bed bioreactor samples amended with *T. neapolitana*). Since the 16S rDNA amplicons had different lengths in 16S rDNA, the melt curve analysis for *T. neapolitana* and *T. maritima* showed a slightly different melting temperature (Tm) as similarly predicted from the *in silico* analysis (Table 1). 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 as shown in Figure 1. These together with the melt curve (Figure 2) indicated the primer’s specificity towards *T. neapolitana*.
Figure 1– qPCR profile of *Thermotoga* sp. 16S rDNA and *T. neapolitana* *hydA* amplicons in 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.  
- **c**, Negative control without template.
Figure 2– The melt curves obtained from real-time quantification standard curve for *T. neapolitana* A.) 16S rDNA and B.) *hydA*. The melt curves showed no evidence of primer dimer formation. The different color representations on the graph show the different serial dilutions used (10\(^{-1}\): red, 10\(^{-2}\): gold, 10\(^{-3}\): lime, 10\(^{-4}\): light green, 10\(^{-5}\): turquoise, negative: pink).

### 3.2. 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 [36,37]. The regression coefficients showed strong linear correlations for 16S rDNA and *hydA* with an R\(^2\) value of 0.99 for both gene targets (Figure 3).
Fig. 3– Standard curve for *Thermotoga* 16S rDNA and *hydA*. Shown are: a) *Thermotoga* sp. specific 16S rDNA and b) *T. neapolitana hydA* with *T. neapolitana* genomic DNA as the template.

The amplification efficiencies of the designed real time qPCR primers were evaluated using DNA extracted from *T. neapolitana* genomic DNA. The reproducibility of both primer pairs were determined with *T. neapolitana* and synthetic (*T. neapolitana* and *C. saccharolyticus*) cultivations. The standard curve from *T. neapolitana* pure culture indicated amplifications efficiencies of 75% – 85% for 16S rDNA and 99% for *hydA*, for each qPCR assay. Similar results on the primer specificities and efficiencies were obtained when standards were run together with synthetic co-cultures. The amplification efficiencies obtained from the standard curve was calculated as 78% and 99% for 16SrDNA and *hydA* respectively. In general, both primers showed a similar amplification pattern, confirming the specificity of the primers (Figure 4a). However, on comparison with 16S rDNA primers, *hydA* primers demonstrated a higher amplification efficiency and sensitivity towards the target gene. Figure 4b representing the percentage composition of detected 16S rDNA and *hydA* from qPCR assay denotes that after 18hrs
of amplification, the *T. neapolitana* gene pool was predominated with *hydA* gene (~80% of targeted gene pool).

Fig. 4—Comparison of quantification results obtained from 16S rDNA and *hydA* based approaches of *T. neapolitana* in a synthetic co-culture with *C. saccharolyticus*. A) Quantification results were obtained by 16S rDNA- (circle) and *hydA* - (square) qPCR approach. B) Percentage composition of each target detected in the qPCR assay at the different time points between 16S rDNA (black) and *hydA* (grey).

3.3. 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 co-culture. For the pure culture of *C. saccharolyticus*, a lag phase of 9 hours (h) was observed and a maximum of 50.8 (± 1.0) mmol H₂ L⁻¹ was obtained during the cultivation. The growth obtained from OD measurements reached a final OD₆₀₀nm of 0.7 ±0.03 (Figure 5b). A decrease in the OD was observed after glucose was fully depleted at 100 h suggesting that the cultures entered death phase upon depletion of the carbon source. The maximum H₂
content obtained from the cultivation of *C. saccharolyticus* was 66% and the average percentage of $H_2$ was 65.5% while the maximum $H_2$ yield obtained was 2.7 (±0.05) mol $H_2$/mol of glucose. The major metabolite produced was acetate (Figure 5b) but trace amounts of ethanol were also observed during the cultivation. In the pure cultures of *C. saccharolyticus*, the absence of lactate was likely as a result of a low partial $H_2$ pressure ($P_{H_2}$) during the cultivations since a high $P_{H_2}$ during dark fermentation is known to cause a shift in the metabolic pathway and *C. saccharolyticus* has been reported to be relatively insensitive to $P_{H_2}$ [38], formation of lactate was reported from xylose and cellobiose at 59 ± 14 kPa and 46 ± 9 kPa respectively [39].

Contrary to $H_2$ production by *C. saccharolyticus*, *T. neapolitana* had a lag phase of 24 h and lower $H_2$ production of 42.1 (±1.7) mmol $H_2$ L$^{-1}$ and $H_2$ yield of 2.5 (±0.07) mol $H_2$/mol of glucose. Compared to *C. saccharolyticus*, the consumption of glucose was slower (Figure 5a). The average $H_2$ content obtained in the pure was 63.4%. Similar to *C. saccharolyticus*, the major metabolite formed was acetate. However, lactate was formed between time 60 h and 100 h. The OD measurements obtained for *T. neapolitana* were low (0.44 ± 0.05) but this was expected due to the floc formation by the bacterium which causes ambiguities in OD measurement, thereby affecting the accuracy of the results [28]. Similar observations on such enumeration ambiguities have been reported for bacteria in the genus *T. maritima* [28]. Prior to OD measurements, vigorously shaking of the culture prior 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* and the co-culture, the OD obtained was not
accurate enough to be considered in this study. Hence, an alternative method was essential to enumerate *T. neapolitana*.

Fig. 5— Hydrogen production in monocultures and co-cultures from, glucose consumption and metabolic products (a) *T. neapolitana* (b) *C. saccharolyticus* and (c) Co-culture (○) H₂ production, (■) Glucose consumption, (▲) Lactate, (●) Acetate and (△) OD. The error bars represent the standard deviation from triplicate measurements.

Results obtained for co-cultures of *T. neapolitana* and *C. saccharolyticus* showed that glucose was more rapidly depleted compared to the pure cultures (Figure 5f). The rapid depletion occurred simultaneously with fast hydrogen production. Like in the pure cultures of *C. saccharolyticus*, there was a lag phase of 9 h. A maximum OD₆₀₀nm of 0.9
(±0.04) was obtained during the cultivation. The cumulative H$_2$ production was 53.7 ±0.8 mmol H$_2$ L$^{-1}$ with an average H$_2$ content of 67% and H$_2$ yield of 2.8 ± 0.09 mol H$_2$/mol of glucose. Compared to the performance of the pure cultures, the co-cultures showed a significant increase in the H$_2$ production rate (4.36 ± 0.05 mmol-H$_2$ h$^{-1}$) compared to the pure cultures. Statistical analysis to compare the H$_2$ production rate of the co-culture with either of the pure cultures gave a probability value of 0.02 with C. saccharolyticus and T. neapolitana with 0.014. This showed that there was a significant difference in the hydrogen production rate (p<0.05: t-test).

4. DISCUSSION

Several co-culture methods have been accessed for biohydrogen production and even though the use of co-cultures for hydrogen production is not common, they have been shown to be promising strategy for hydrogen production [40–42]. Extreme thermophilic bacteria such as, Thermotoga maritima and C. saccharolyticus have a great ability to hydrolyze polysaccharides and are also able to utilize the reducing equivalents formed during the glycolytic process for hydrogen production [39,43]. Additionally, C. saccharolyticus has been recognized as being somewhat insensitive to hydrogen partial pressure [38]. The results obtained here when T. neapolitana cultivated with C. saccharolyticus or in a mixed culture from fluidized bed bioreactor showed improvements in hydrogen production suggesting a synergistic effect in both systems. Results obtained for co-cultures of T. neapolitana and C. saccharolyticus showed that glucose was more rapidly depleted compared to the pure cultures (Figure 5). The rapid depletion occurred simultaneously with fast hydrogen production. Like in the pure
cultures of *C. saccharolyticus*, there was a lag phase of 9 h. A maximum OD$_{600nm}$ of 0.9 (±0.04) was obtained during the cultivation. The cumulative H$_2$ production was 53.7 ±0.8 mmol H$_2$ L$^{-1}$ with an average H$_2$ content of 67% and H$_2$ yield of 2.8 ± 0.09 mol H$_2$/mol of glucose. Compared to the performance of the pure cultures, the co-cultures showed a significant increase in the H$_2$ production rate (4.36 ± 0.05 mmol-H$_2$ h$^{-1}$) compared to the pure cultures. Statistical analysis to compare the H$_2$ production rate of the co-culture with either of the pure cultures gave a probability value of 0.02 with *C. saccharolyticus* and *T. neapolitana* with 0.014. This showed that there was a significant difference in the hydrogen production rate (p<0.05: t-test). The results obtained confirmed previous reports on improvement of hydrogen production using synthetic cultures [39,41,44–49].

Lignocellulosic biomass being the most abundant in nature is a suitable alternative and to maximize the energy obtained from through the dark fermentation process will require the use of ideal H$_2$ producers capable of attaining high H$_2$ yields. In a review by Pradhan et al. (2015), the H$_2$ production efficiency achievable with *T. neapolitana* on different feedstocks was found to be between 1.9 to 3.5 mol H$_2$/mol hexose and the byproducts of the degradations are acetate, lactate and ethanol. Several co-culture methods have been developed for biohydrogen production and have been shown to be promising strategy for hydrogen production (Table 2).

<table>
<thead>
<tr>
<th>Strain/co-culture</th>
<th>Substrate</th>
<th>Temperature (°C)</th>
<th>Maximum H$_2$ yield (mol H$_2$/mol glucose)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>C. saccharolyticus</em> and <em>T. maritima</em></td>
<td>Xylose, Cellobiose</td>
<td>70</td>
<td>2.7 ± 0.1</td>
<td>[39]</td>
</tr>
<tr>
<td>Co-culture $^a$</td>
<td>Glucose</td>
<td>37</td>
<td>1.65 ± 0.07</td>
<td>[41]</td>
</tr>
<tr>
<td><em>C. butyricum</em> and <em>E.coli</em></td>
<td>glucose</td>
<td>37</td>
<td>1.65</td>
<td>[49]</td>
</tr>
</tbody>
</table>
A Co-culture (Citrobacter. freundi 01, Enterobacter. aerogens E10 and Rhodopseudomonas. palustris P2).

The molecular method applied to this study helped to confirm the role of T. neapolitana in the H₂ production process. Previous studies on T. neapolitana have focused in optimizing growth and hydrogen production conditions for pure cultures of members of Thermotoga sp. [17–23]. The method developed helped to confirm the influence of T. neapolitana in the co-culture which was inconclusive by using only statistical analysis. Thus far, the existing methods such as cell dry weight, microscopic and optical density measurements [17–23] are only suitable for monocultures and fail to differentiate between different species. Furthermore, members of the genus Thermotoga undergo floc formation which often cause ambiguities in enumeration [28]. Reports on biohydrogen production from hyperthermophilic microorganisms have mostly utilized conventional methods for monitoring the activity of the microbial population such as dry cell weight (which often require large volume of samples), optical density (which often creates bias in the case of floc formers like T. neapolitana) or microscopy [17–23]. Very few have applied qPCR or fluorescence in situ hybridization for detection and quantification. 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 at the initial and final stage of the T. neapolitana-spiked cultivation showed an increased number of hydA gene copies...
simultaneous to the hydrogen production suggesting the growth of *T. neapolitana* in the co-culture culture. 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 of cultivation is still unknown and should be studied further. In a previous study, synthetic culture with *C. saccharolyticus* and *T. maritima* [42] suggested a relationship of mutualism and commensalism. Hence, it is likely that such relationship also existed in the synthetic co-culture and *T. neapolitana*-spiked mixed culture systems of this study.

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 % [33–35]. Factors such as amplicon length, melting temperature, annealing temperature and the $\Delta G$ 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 $\Delta G$ 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 $\Delta G$ value. The $\Delta G$ represents the quantity of energy needed to fully break a secondary DNA structure and the lower the $\Delta G$, 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
(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. 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

In this study, we used qPCR methods for genus and species specific quantitative monitoring of Thermotoga sp. in hydrogen producing systems. The hydA provides a promising target to complement with the existing 16S rDNA gene-based methods for accurate detailing of T. neapolitana. This method therefore, allows for sensitive detection of hydA of T. neapolitana in the dark fermentative hydrogen production process. The use of hydA gene based methods may offer more insight into the performance of T. neapolitana in mixed culture systems as it is the gene which is directly involved for hydrogen metabolism in T. neapolitana. The hydA gene proofs to be a promising target to complement 16S rDNA approach to comprehensively monitor T. neapolitana. It was also demonstrated that ecologically distant and ideal hydrogen producing organisms can be used for improvement of hydrogen production. Though failures haves been reported from co-cultures with distant related organism, the results
showed that there was a synergistic relationship between *T. neapolitana* and *C. saccharolyticus* and this was observed by the significant increase in the H₂ production rate. A further outlook would be the comparative studies for hydrogen production from mixed sugars as well as lignocellulosic biomass using co-cultures of *T. neapolitana* and *C. saccharolyticus*.

**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.

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