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Enhancing thermophilic dark fermentative hydrogen production at high glucose concentrations via bioaugmentation with *Thermotoga neapolitana*

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ABSTRACT

The aim of the present study was to investigate the effect of gradually increasing glucose concentrations (from 5.6 to 111 mmol L^{-1}) on the fermentative H_2 production with and without bioaugmentation. A stirred tank reactor was operated at 70 °C and inoculated with a hyperthermophilic mixed culture or a hyperthermophilic mixed culture bioaugmented with *Thermotoga neapolitana*. With both the unaugmented (control) and augmented cultures, the H_2 production rate was improved when the initial glucose concentration was increased. In contrast, the highest H_2 yield (1.68 mol H_2 mol^{-1} glucose consumed) was obtained with the augmented culture at the lowest glucose concentration of 5.6 mmol L^{-1} and was 37.5% higher than that obtained with the unaugmented culture at the same feed glucose concentration. Overall, H_2 production rates and yields were higher in the bioaugmented cultures than in the unaugmented cultures whatever the glucose concentration. Quantitative polymerase chain reaction targeting *T. neapolitana hydA* gene and MiSeq sequencing proved that *Thermotoga*

was not only present in the augmented cultures but also the most abundant at the highest glucose concentrations.

Keywords: Biohydrogen, Dark fermentation, Metabolic pathways, Microbial dynamics.

INTRODUCTION

Recent developments in dark fermentation by anaerobic bacteria have consistently shown this process to be promising for the transformation of carbohydrate-rich substrates to hydrogen. The theoretical stoichiometric yield for fermentative hydrogen production is 4 mol H_2 mol^{-1} glucose consumed. However, this is exclusively possible when acetate is the sole volatile fermentative product [1,2]. Invariably, the hydrogen yield is lowered by the production of more reduced molecules. Fermentative hydrogen (H_2) production is possible with both pure and mixed microbial cultures originated from natural or engineered environments [3–5]. Typically, the use of mixed cultures is the only option for H_2 generation from non-sterile organic waste and biomass residues unless selective conditions, such as extremely high temperatures, are used during the bioprocess [6–11]. In addition to the microbial culture, the H_2 production performance depends on the operating conditions such as temperature, pH, substrate type and concentration, as well as hydraulic retention time [3,12–15].

Substrate concentration can have a significant effect on the rate, yield and stability of H_2 production. Increasing substrate concentrations have been shown to result in higher production rates but lower H_2 yields with, for example, a mesophilic or thermophilic H_2-producing mixed cultures [16,17]. Increasing the substrate concentration and, thus, the organic load of the system allows to save the energy required for heating the H_2-producing bioreactors, as high substrate concentrations

lead to increased microbial activity and heat generation by microbial metabolism [18]. However, the use of extremely high substrate concentrations can cause substrate and/or product inhibition and result in sub-optimal pH for the H_2-producers due to volatile fatty acid accumulation [19,20]. In addition, the low H_2 yields observed at increasing organic loads can be due to a shift in metabolic flux towards solventogenesis (e.g. formation of butanol, acetone and ethanol) and other reduced end-products, the generation of which is not accompanied by H_2 production [21].

Bioaugmentation has been proposed in several studies as a potential strategy for enhancing dark fermentation under stress conditions [16,22,23]. Bioaugmentation can be defined as the addition of pre-grown highly specialized microorganisms or populations of several microorganisms to improve the capacity of a treatment or production system [24–26]. Bioaugmentation is an emerging strategy for industrial wastewater treatment [27] and has been used to shorten the lag phase and improve the chemical oxygen demand (COD) removal during dark fermentation of the organic fraction of municipal solid waste [28]. It has also been used to enhance thermophilic H_2 production from corn stover hydrolysate [29] and beverage wastewater [30]. Okonkwo et al. [29] applied bioaugmentation with a synthetic co-culture to enhance the H_2 production during or after temporal temperature fluctuation. Given the success of bioaugmentation strategy in several previous studies, bioaugmentation might be a useful tool for enhancing H_2 production also at high substrate concentrations [31]. However, one of the most difficult issues in bioaugmentation is to ensure the survival of the microorganisms introduced in the established mixed culture as the number of exogenous microorganisms has been reported to shortly decrease after inoculation either as a result of abiotic or biotic influence [32]. Some studies used strategies such as repeated bioaugmentation to promote the persistence of the added bacterium in

the system [33,34]. This strategy might be effective for a transient system recovery but might not ensure long-term process enhancement, if the added bacterium or bacteria are not able to compete with the existing microbial consortium. Furthermore, sudden process disturbances such as increased operation temperature can lead to reduced microbial diversity in the mixed culture and lead to a lower process efficiency, requiring bioaugmentation with bacteria that can stably coexist with the existing microbial consortium.

The aim of this study was to study the dynamics of *Thermotoga neapolitana* in a mixed microbial consortium after a period of pre-adaptation as a strategy to make *T. neapolitana* a stable member of the native microbial community. This study further examined the effects of different feed glucose concentrations on H_2 production in a thermophilic mixed culture with and without *T. neapolitana*, which is a hyperthermophilic bacterium capable of utilizing a wide range of organic substrates as carbon source and able to produce high hydrogen yields [35,36]. Previous reports showed that *Thermotoga neapolitana* is capable of producing up to 3.8 mol H_2 mol^{-1} glucose, which is close to the theoretical limit of 4 mol H_2 mol^{-1} glucose, and producing acetate, lactate and CO_2 as other major metabolic end products [35,37]. This makes *T. neapolitana* ideal for bioaugmentation purposes. To the best of our knowledge, this is the first study to use pre-adaptation as a strategy for allowing *T. neapolitana* to be a stable member of a native H_2-producing microbial community and for enhancing H_2 production.

MATERIALS AND METHODS

Experimental Design

The medium used for the cultivation consisted of the following components (g L^{-1}): 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 solution (DSMZ 141, Germany). Nitrogen gas was used to sparge the fermentation medium and create an anaerobic environment. Dark fermentation experiments were carried out in batch mode in a double jacketed glass stirred tank reactor (STR) with a working volume of 2 L (Figure 1). The reactor temperature was kept constant at 70 °C using a heated water bath. The reactor was equipped with a pH electrode and temperature probe connected to a programmable controller (Bluelab pH Controller, New Zealand) to maintain the pH of the cultures at 6.5 by automatic dosing of potassium hydroxide (2 molar). The fermentation broth inside the reactor was mixed by a magnetic stirrer (Argolab, Italy) at 150 rpm.

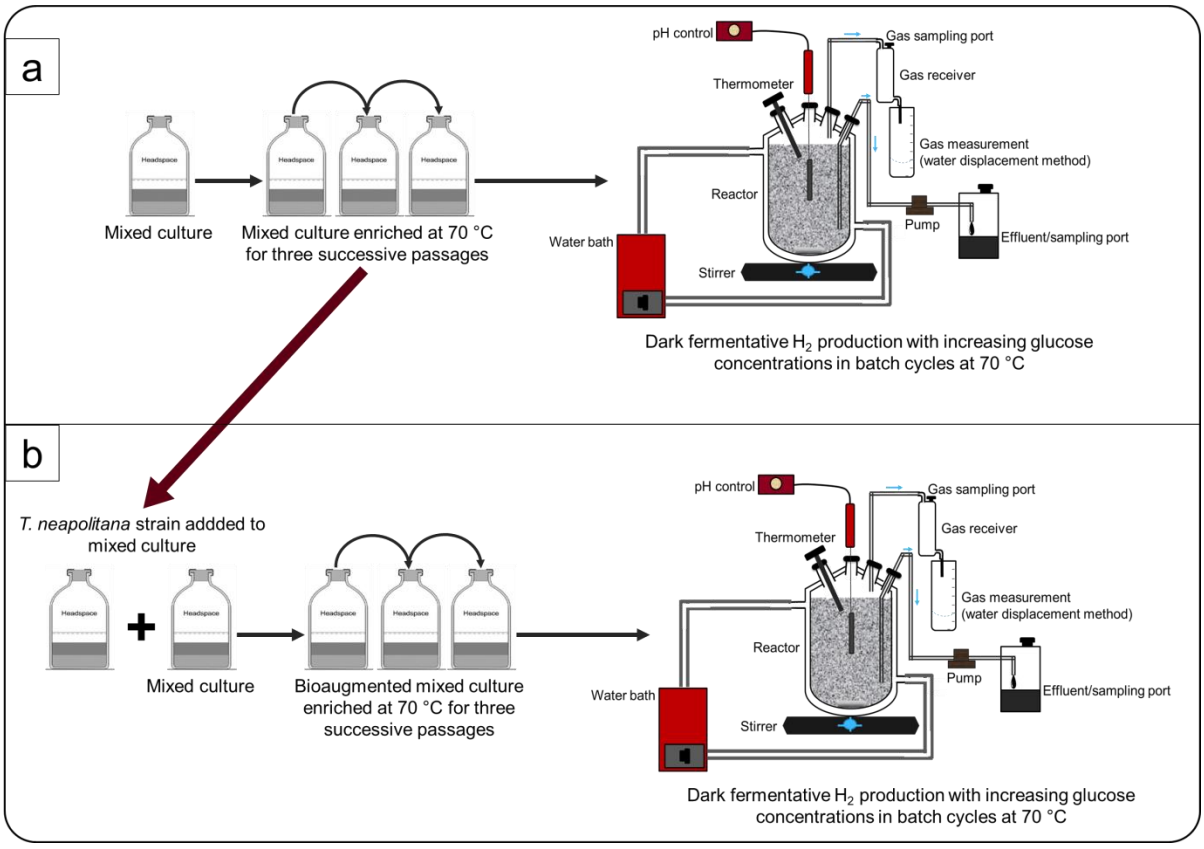


Figure 1. Experimental design to study the effects of bioaugmentation at various feed glucose concentrations during dark fermentation with a thermophilic mixed culture without augmentation (a) and augmented with *Thermotoga neapolitana* (b).

The seed source used in this study was a mixed culture obtained from a laboratory scale continuously stirred tank bioreactor producing H_2 from glucose and xylose at 55 °C [22]. Instead, *T. neapolitana* was purchased from DSMZ, Braunschweig, Germany.

The cultivation of the mixed culture was initiated at 70 °C in 250 mL batch bottles with a working volume of 200 mL at an initial pH of 6.5 with 27.8 mmol L^{-1} glucose as substrate. Twenty milliliters of the inoculum (10% v/v) was transferred to 180 mL of the culture medium (mg L^{-1}). The cultivation was carried out in batch for three transfers prior to the start of the experiment to acclimatize the culture to the higher incubation temperature (Figure 1a).

To determine the influence of bioaugmentation at increasing substrate concentrations, *T. neapolitana* DSM 4359 (DSMZ, Germany) was added to the mixed culture in a 1:1 ratio (based on optical density measurements, OD_{600}). The bioaugmented culture was then cultivated with glucose in batch mode in 250 mL anaerobic serum bottles with a working volume of 200 mL for three successive transfers at 70 °C (Figure 1b) to adapt *T. neapolitana* to growing alongside the native microbial community. For each successive transfer, 20 mL of the inoculum (10% v/v) was transferred to 180 mL of the culture medium (mg L^{-1}) to a final volume of 200 mL.

H_2 production with the unaugmented and the bioaugmented mixed culture was separately investigated in batch mode in the STR described in section 2.1 and each experiment lasted for a period of 48 h. The initial glucose concentration was stepwise increased from 5.6 to 27.8, 55.5 and 111.0 mmol L^{-1} in order to determine the impact of increasing substrate concentration on H_2 production, biomass concentration and metabolic patterns.

Analytical methods and calculation procedures

The gas produced in the STR was quantified using a water displacement method with 500 mL glass containers. The H_2 containing gas produced was sampled from the gas sampling port using a gas-tight syringe (Hamilton, USA) and the H_2 concentration of the biogas was measured using a 3400 gas chromatograph (GC) (Varian, USA) equipped with a thermal conductivity detector (TCD) and a Restek packed column using argon as the carrier gas. The total volume of the produced H_2 at each time point was calculated using Equation 1 [38]:

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

where $V_{H_2,t}$ is the cumulative H_2 produced at time t, $V_{H_2,t-1}$ is the cumulative H_2 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 H_2 fraction in the headspace at time t, $C_{H_2,t-1}$ is the H_2 fraction in the headspace at time t-1 and V_H is the total headspace volume in the bioreactor.

H_2 production was converted into moles on the basis that one mole of an ideal gas occupies a volume of 22.4 L at standard temperature and pressure according to the ideal gas law. Therefore, the volume of H_2 gas produced was divided by 22.4 L in order to obtain H_2 produced in moles. The H_2 yield and productivity were calculated using Equations 2 and 3, respectively.

$$H_2\ yield = \frac{mol\ H_2}{mol\ glucose\ consumed} \quad (2)$$

$$H_2\ productivity = \frac{mmol\ H_2}{reaction\ volume \times fermentation\ time\ (hour)} \quad (3)$$

Microbial analyses

Genomic DNA was extracted using the PowerSoil™ DNA Isolation Kit (MoBio Laboratories, Inc., Carlsbad, CA, USA) according to the manufacturer's instructions.

Primers 515_532U and 909_928U [39] including their respective linkers were used to amplify the V4_V5 region of the 16S rRNA gene. The resulting products were purified and loaded onto Illumina MiSeq cartridge for sequencing. Sequencing and library preparation were performed at the Genotoul Lifescience Network Genome and Transcriptome Core Facility in Toulouse, France (get.genotoul.fr). The sequence analysis was done as described by [40]. Real-time quantitative polymerase chain reaction (qPCR) monitoring of *T. neapolitana* was carried out using *HydA* primers following the method described by [41]. The 16S rRNA sequences used to support the findings of this study have been deposited in the NCBI Sequence Read Archive under project file SUB6057042: MN203737 - MN203763.

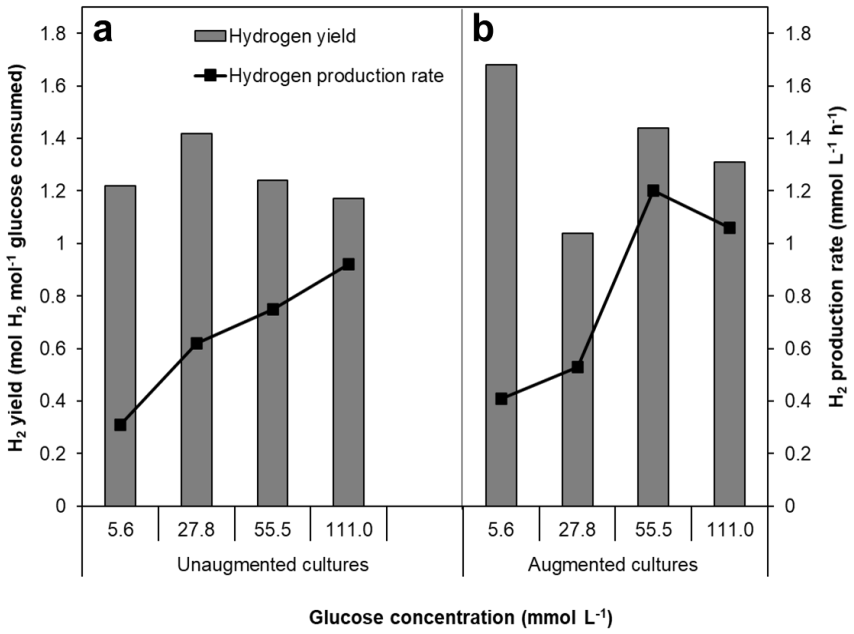
RESULTS AND DISCUSSION

H_2 production rates and yields at increasing glucose concentrations

In the unaugmented cultures, the highest H_2 yield was 1.42 mol H_2 mol^{-1} of glucose consumed at an initial concentration of 27.8 mmol L^{-1} of glucose. The H_2 yield dropped to 1.17 mol H_2 mol^{-1} of glucose consumed at 111 mmol L^{-1} of feed glucose concentration (Figure 2a). The H_2 yield obtained in the augmented cultures was higher than that obtained in the unaugmented cultures. Nonetheless, similar to the unaugmented cultures, the H_2 yield decreased by increasing the substrate concentration. With bioaugmentation, H_2 yield increased by 37, 16 and 12% at 5.6, 55.5 and 111 mmol L^{-1} of feed glucose, respectively, compared to the unaugmented cultures. The highest H_2 yield (1.68 mol H_2 per mol of consumed glucose) was obtained at the feed glucose concentration of 5.6 mmol L^{-1}. Qiu et al. [42] studied the effect of xylose concentrations (ranging from 16.7 to 100.0 mmol L^{-1}) on dark fermentative H_2 production by an extreme thermophilic culture, and reported that the fermentation reached the highest H_2 yield of 1.29 mol H_2 mol^{-1} xylose consumed at

initial pH 7.0 and 50.0 mmol L^{-1} of feed xylose. However, based on other literature reports, it seems that the optimal initial substrate concentration depends on the inoculum, substrate type, reactor configuration, temperature and pH range [43,44].

In this study, the H_2 production rate increased with increased feed glucose concentration and reached the highest value of 0.92 mmol-$L^{-1}h^{-1}$ at 111 mmol L^{-1} of feed glucose in the unaugmented culture. In the augmented culture, the H_2 production rate increased from 0.41 mmol L^{-1} h^{-1} at feed glucose concentration of 5.6 mmol L^{-1} up to 1.44 mmol L^{-1} h^{-1} at 55.5 mmol L^{-1} and then decreased to 1.13 mmol L^{-1} h^{-1} at 111 mmol L^{-1} of feed glucose (Figure 2b). Higher H_2 production rates than observed in this study have been observed with mixed cultures under different operating conditions [45,46]. The obtained H_2 production rate and yield was generally higher in the culture augmented with *T. neapolitana* than in the unaugmented culture at the various glucose concentrations studied. This indicates that *T. neapolitana* was able to survive alongside the native microbial communities.



211

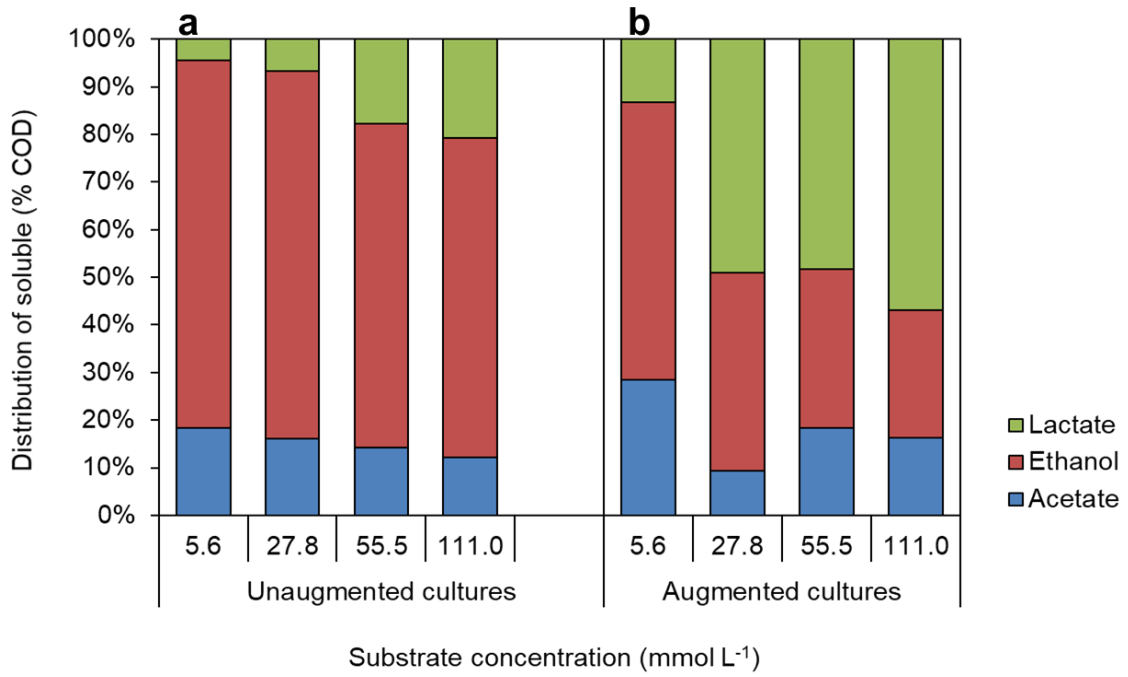
212 Figure 2. H_2 yield and production rate obtained with the unaugmented (a) and
213 augmented (b) cultures at different initial glucose concentrations.

214

215 **Effect of glucose concentration on the composition of soluble metabolites**

216 The main soluble microbial products associated with glucose degradation were
217 ethanol, acetate and lactate (Figure 3). In the unaugmented cultures, ethanol was the
218 main soluble metabolite produced and corresponded to 67–77% of the total soluble
219 metabolites produced as COD equivalents. The share of acetate decreased with
220 increasing glucose concentration (from 18 to 12%), while that of lactate increased from
221 4.5 to 18% (Figure 3a). In the augmented cultures, the share of ethanol decreased
222 with increasing glucose concentration (from 58% at 5.6 mmol L^{-1} glucose to 27% at
223 111 mmol L^{-1} glucose). Thus, the share of ethanol was lower in the augmented culture
224 compared to the unaugmented culture at all studied glucose concentrations. The
225 percentage of acetate decreased in the augmented culture from 29% at 5.6 mmol L^{-1}
226 of feed glucose to 16% at 111 mmol L^{-1} of feed glucose, while the share of lactate

227 significantly increased from 13% at initial concentration of 5.6 mmol L^{-1} glucose to 57%
228 at 111 mmol L^{-1} of feed glucose (Figure 3b).
229



230

231 Figure 3. The distribution of soluble metabolites as chemical oxygen demand (COD)
232 equivalents at the endpoint of fermentation at the different initial glucose
233 concentrations with the unaugmented (a) and augmented (b) cultures.
234

235 In many previous studies, dark fermentation of glucose has resulted in the
236 production of mainly butyrate and acetate as soluble metabolites under mesophilic,
237 thermophilic and hyperthermophilic conditions [47–51]. However, it seems that
238 ethanol-based fermentation was the major pathway leading to H_2 production in this
239 study due to the high ethanol yields obtained especially with the unaugmented
240 cultures. The ethanol-type fermentation (Equation 4) has a theoretical maximum of 2
241 mol of H_2 per mol of glucose and has been reported to occur under mesophilic
242 conditions [52,53] but not for mixed cultures at temperatures as high as 70 °C.

$$C_6H_{12}O_6 + H_2O \rightarrow C_2H_5OH + CH_3COOH + 2CO_2 + 2H_2 \quad (4)$$

Previous studies have reported yields of 1.8 mol ethanol mol^{-1} glucose with pure culture of *T. ethanolicus* [54] and 1.5 mol ethanol mol^{-1} glucose from *T. hydrosulfuricus* [55] at 72 and 69 °C respectively. The highest ethanol yield obtained with the unaugmented culture in this study was 1.4 mol H_2 mol^{-1} of glucose. Meanwhile, the highest ethanol yield in the augmented culture was 1.2 mol ethanol mol^{-1} of glucose and was obtained at initial glucose concentration of 27.8 mmol L^{-1}.

In addition to the increased H_2 yield obtained by bioaugmentation, a shift in the metabolic networks was observed with the *T. neapolitana*-augmented culture when compared to the unaugmented culture. Indeed, the *T. neapolitana*-augmented culture produced a lower share of ethanol and a higher share of acetate and lactate to the fermentation broth (Figure 3). Thus, the bioaugmentation with *T. neapolitana* directed the metabolic pathway towards acetate and lactate production. Previous reports on pure cultures of *T. neapolitana* have shown that acetate, lactate and alanine are the major soluble metabolites produced by *T. neapolitana* [41,56,57]. The direction of the metabolic pathway towards acetate production allows producing more H_2 and seemed to be the case with the bioaugmented culture in this study. Nonetheless, as *T. neapolitana* is also capable of producing high concentrations of lactate at increased substrate concentrations [17], the increase in the share of lactate observed in the augmented culture was at least partly attributed to presence of *T. neapolitana*. Lactate as an electron sink takes a large amount of reducing power away from H_2 production thereby reducing the H_2 yield [58,59].

Quantification of *Thermotoga neapolitana* within the mixed microbial communities

The qPCR method applied in this study to quantify and confirm the presence of *T. neapolitana* in the bioaugmented mixed cultures has previously been successfully used in quantitation of *T. neapolitana* from pure and mixed cultures [41]. The quantitative analysis of *T. neapolitana hydA* gene from the bioaugmented cultures showed an increase of the *hydA* gene copies per mL of culture as the initial glucose concentration was increased (Figure 4). Thus, the qPCR results indicated that after bioaugmentation, *T. neapolitana* became an active member of the microbial consortium and likely responsible for the shift in the soluble metabolites and enhancement of H_2 production compared to the unaugmented culture. The qPCR carried out on the unaugmented culture confirmed that *T. neapolitana* was not present in the culture.

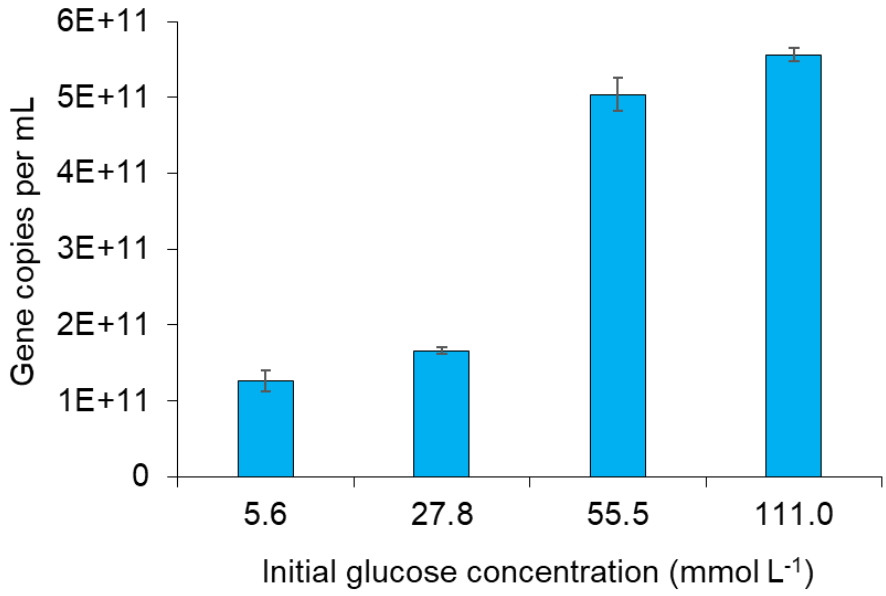


Figure 4. Real-time qPCR monitoring of hydA gene copy numbers of *T. neapolitana* in the augmented culture at different initial glucose concentrations.

Microbial community profiles at different glucose concentrations

The MiSeq data obtained from the cultures assessed in this study covered over 33000 effective sequences with the lowest number of sequences being 24967. The number of operational taxonomic units (OTUs) was relatively low, indicating that the microbial communities in both the unaugmented and the augmented culture were rather simple because high temperature environments are extremely selective [60]. The unaugmented culture was dominated by *Thermoanaerobacter* spp. at all initial glucose concentrations. The share of *Thermoanaerobacter* in the microbial community was 99.9% at all other glucose concentrations than 55.5 mol L^{-1}, when *Bacillus* was detected at an abundance of 8%.

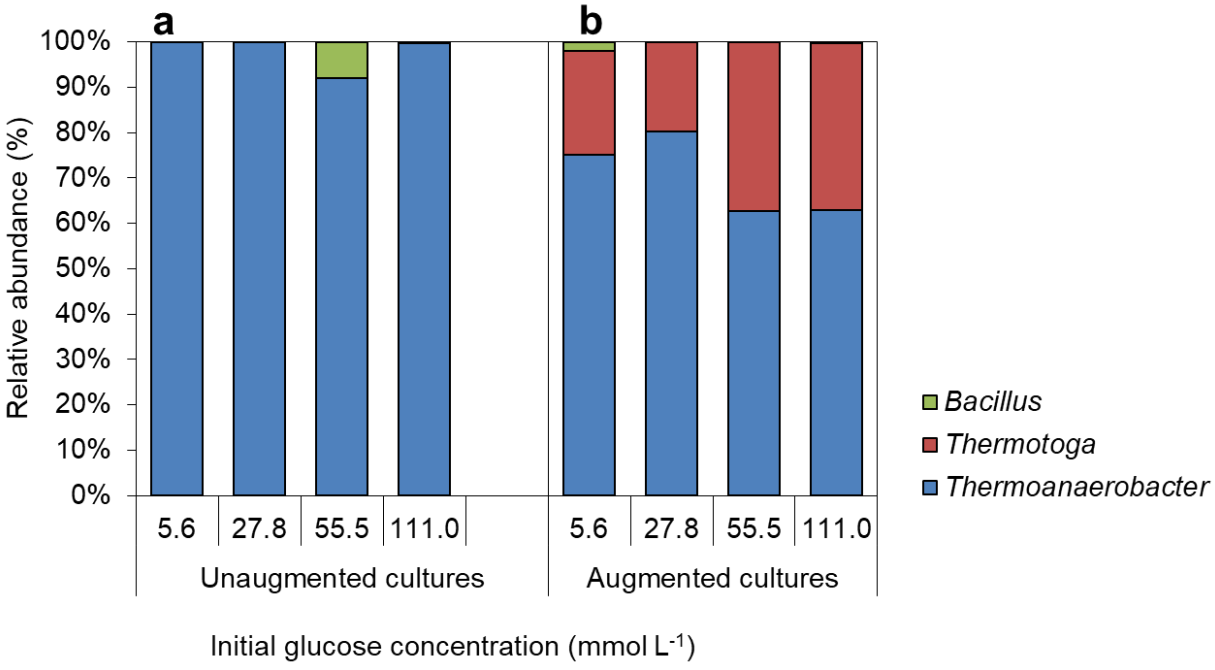


Figure 5. Microbial community composition and relative abundance of genera identified at different feed glucose concentrations in the unaugmented (a) and augmented (b) culture.

At the lowest initial glucose concentration of 5.6 mmol L^{-1}, the augmented cultures had 75, 23 and 2% abundance of *Thermoanaerobacter, Thermotoga* and *Bacillus*

301 spp., respectively. However, at the higher initial glucose concentrations, *Bacillus* spp.
302 were not detected anymore from the microbial community and the shares of
303 *Thermoanaerobacter* and *Thermotoga* spp. were 62-80% and 20-37%, respectively
304 (Figure 5). The abundance of *Thermotoga* in the community was higher at the two
305 highest initial glucose concentrations, which is accordance with the qPCR results
306 (Figure 4).

307 The bioaugmentation of a *Thermoanaerobacter*-dominated mixed culture with *T.*
308 *neapolitana* improved both the H_2 production yield and rate. *Thermoanaerobacter*
309 species are well known thermophilic bacteria capable of producing H_2, ethanol and
310 acetate [61–63]. Thus, their presence explains also the high ethanol production
311 observed. Bacteria within this genus have also been reported to use the Embden–
312 Meyerhof–Parnas pathway for sugar degradation and produce ethanol, acetate and
313 lactate as major volatile end products [64], which is in accordance with the metabolite
314 profiles observed in this study. In the bioaugmented culture, the presence of *T.*
315 *neapolitana* resulted in lower ethanol production, while the shares of acetate and
316 lactate increased compared to the unugmented cultures. Thus, the differences
317 observed in the abundance of different soluble metabolites in the unaugmented and
318 augmented cultures can be explained with the observed differences in the microbial
319 community composition.

320 The pre-adaptation as a strategy to make *T. neapolitana* a stable member of the
321 native microbial community was successful based on the molecular monitoring
322 methods used this study, as both the *T. neapolitana* hydA gene copy numbers and
323 relative abundance of *Thermotoga* were shown to increase towards the end of the
324 study. The pre-adaptation of a bacteria to a mixed culture prior to its application to a
325 large scale process could thus be beneficial for enhancing microbial activity levels,

treating complex waste materials and driving the metabolic pathway towards the desired products. Bioaugmentation also has the potential to improve the microbial community structure and enhance resistance and resilience in case of unforeseen disturbances [65]. However, pre-adaptation may not be feasible in the case of sudden transient disturbances due to the fact that it is time consuming.

Based on the results obtained from chemical analysis and molecular data, it is evident that *T. neapolitana* contributed to the H_2 production in the mixed culture. Reports from this and previous studies have shown that *T. neapolitana* is able to produce H_2, CO_2, acetate and lactate from mono and polysaccharides as the major products of metabolism. However, its primary role in nature is to reduce sulfur to hydrogen sulfide through the oxidation of organic molecules [66]. Nonetheless, no extensive research exists on the interactions of this organism with other organisms up to now. Except for the switch in the metabolic pathways and an enhanced H_2 production, it is not known what kind of interactions occurred between *T. neapolitana* and the native microbial community. It would be useful to further investigate the characteristics of *T. neapolitana* in the augmented culture at a functional level by studying the protein expression to identify the mechanisms responsible for its adaptation and survival within the native microbial community [67–69], as this could enable a further process optimization.

CONCLUSIONS

The bioaugmentation of a *Thermoanaerobacter*-dominated mixed culture with *Thermotoga neapolitana* improved both the H_2 production yield and rate. Thus, the results of this study indicate that the addition of a single strain with required characteristics can be enough for improving the performance of a biological process. The H_2 production rate of the augmented cultures increased when the initial glucose

concentration was increased from 5.6 to 55.5 mmol L^{-1}, while the highest H_2 production yield, 1.68 mol H_2 per mol of consumed glucose, was obtained at the lowest initial glucose concentration of 5.6 mmol L^{-1}. The pre-adaptation of *T. neapolitana* to the mixed culture during three successive batch incubations prior to the reactor experiments was demonstrated to be a successful strategy to ensure that *T. neapolitana* was able to co-exist within the mixed microbial consortium. However, further experiments utilizing continuously-fed bioreactor systems are recommended to evaluate the long-term effects of the selected bioaugmentation strategy.

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