Enhancement of thermophilic dark fermentative hydrogen production and the use of molecular biology methods for bioprocess monitoring
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Enhancement of thermophilic dark fermentative hydrogen production and the use of molecular biology methods for bioprocess monitoring

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Abstract

Dark fermentation of organic waste materials such as organic fraction of municipal waste and agricultural wastes is a promising technology to produce renewable hydrogen. However, further research and development is required to improve the efficiency and stability of the process. The aim of this thesis was to enhance thermophilic dark fermentative hydrogen production by using microbial strategies (bioaugmentation and synthetic co-cultures) and by increasing the understanding on the microbial community dynamics especially during stress conditions such as fluctuating temperatures and elevated substrate concentrations.

To study the effects of sudden short-term temperature fluctuations, batch cultures initially incubated at 55°C (control) were subjected to downward (from 55°C to 35°C or 45°C) or upward (from 55°C to 65°C or 75°C) temperature shifts for 48 h after which they were incubated again at 55°C for two consecutive batch cycles. The results showed that sudden, temporal upward and downward temperature fluctuations had a direct impact on the hydrogen yield as well as the microbial community structure. Cultures exposed to downward temperature fluctuation recovered more rapidly enabling almost similar hydrogen yield (92-96%) as the control culture kept at 55 °C. On the contrary, upward temperature shifts from 55 to 65 or 75 °C had more significant negative effect on dark fermentative hydrogen production as the yield remained significantly lower (54-79%) for the exposed cultures compared to the control culture.

To improve the stability of hydrogen production during temperature fluctuations and to speed up the recovery, mixed microbial consortium undergoing a period of either downward or upward temperature fluctuation was augmented with a synthetic mix culture containing well-known hydrogen producers (Thermotoga, Thermoanaerobacter, Thermoanaerobacterium, Caldicellulosiruptor and Thermocellum spp.) The addition of new species into the native consortium significantly improved hydrogen production both during and after the fluctuations. However, when the bioaugmentation was applied during the temperature fluctuation, hydrogen production was enhanced.

This study also investigated the dynamics between pure cultures and co-cultures of highly specialized hydrogen producers, Caldicellulosiruptor saccharolyticus and Thermotoga neapolitana. The highest hydrogen yield (2.8 ± 0.1 mol H₂ mol⁻¹ glucose) was obtained with a synthetic co-culture which resulted in a 3.3 or 12% increase in hydrogen yield when compared to pure cultures of C. saccharolyticus or T. neapolitana, respectively. Furthermore, quantitative
polymerase chain reaction (qPCR) based method for monitoring the growth and contribution of *T. neapolitana* in synthetic co-cultures was developed. With this method, it was verified that *T. neapolitana* was an active member of the synthetic co-culture.

The effect of different feed glucose concentrations (from 5.6 to 111.0 mmol L$^{-1}$) on hydrogen production was investigated with and without augmenting the culture with *T. neapolitana*. Compared to the control (without *T. neapolitana*), bioaugmentated culture resulted in higher hydrogen yields in almost all the concentrations studied even though hydrogen yield decreased the feed glucose concentration was increased. The presence of *T. neapolitana* also had a significant impact on the metabolite distribution when compared to the control. The number gene copies of *T. neapolitana* measured with qPCR was higher at the highest initial glucose concentrations. Thus, the results demonstrated that the use of a single strain with the required properties needed in a biological system can be sufficient for improving dark fermentative hydrogen production.

In summary, this study showed that thermophilic dark fermentative hydrogen production can be enhanced by using synthetic co-cultures or bioaugmentation. The highest hydrogen yield in this study was obtained with the synthetic co-culture, although it should be considered that the incubation conditions differed from those used for the mixed cultures in this study. The use of molecular methods such as qPCR and high-throughput sequencing also helped to understand the role of certain species in the microbial consortia and improved the understanding of the microbial community dynamics during stress conditions. The use of molecular methods is thus important as it helps to create a link between the microbial community structure and observed hydrogen production.
Tiivistelmä


Lyhytaikaisten lämpötilavaihtelujen vaikutusta vedentytuotantoon ja mikrobiyhteisön koostumukseen tutkittiin panoskokeissa, joissa 55 °C:ssä kasvatetun sekaviljemän kasvatuslämpötilaa muutettiin äkillisesti joko kylmemmäksi (35 tai 45 °C) tai lämpimämmäksi (65 tai 75 °C) 48 tunnin ajaksi. Tämän jälkeen kaikki viljelmät palautettiin 55 °C:een ja niitä kasvatettiin vielä kahden peräkkäisen 48 tunnin panosyvykin ajana. Tulokset osoittivat, että kaikki tutkitut äkilliset, lyhytaikaiset lämpötilamuutokset pienensivät vetysaantoja ja aiheuttivat muutoksia mikrobiyhteisön koostumuksessa verrattuna kontrolliviljelmään, jota kasvatettiin koko ajan panosviljelmänä 55 °C:ssa. Sekaviljemissä, jotka altistettiin alhaisemmille lämpötiloille (35 ja 45 °C), palautuivat lämpötilamuutoksesta suhteellisen hyvin ja näiden viljelmien tuottama vetysaanto oli 55 °C:een palauttamisen jälkeen lähes yhtä korkea (92-96 %) kuin vakiolämpötilassa kasvatetun kontrolliviljelmän keskimääräinen vetysaanto. Altistuminen korkeammiille lämpötiloille (65 ja 75 °C) vaikutti matalaa lämpötiloja merkittävämmin vetysaantoihin, sillä näiden viljelmien tuottama vetysaanto oli 55 °C:een palauttamisen jälkeen vain 54-79% kontrolliviljelmän keskimääräisestä vetysaannosta.

Koska kasvatuslämpötilan äkillisen nousun sovitteita johtavat mikrobiyhteisön monimuotoisuuden vähennemiseen, tutkimuksen seuraavassa vaiheessa pyrittiin vedentytuottoa tehostamaan lämpötilavaihtelujen aikana ja nopeuttamaan vedentytuotannon palautumista lämpötilamuutosten jälkeen bioaugmentoinnin avulla. Sekaviljemii, joita altistettiin lämpötilavaihteluihin, lisättiin tunnettuja vedentyttöjä mikrobsejä (Thermotoga, Thermoanaerobacter, Thermoanaerobacterium, Caldicellulosiruptor ja Thermocellum spp.) joko lämpötilamuutoksen aikana tai sen jälkeen. Näiden mikrobioiden lisääminen sekaviljelmän tehosti vedentytuotantoa niin lämpötilamuutosten aikana kuin niiden jälkeenkin. Bioaugmentointi lämpötilamuutoksen aikana
kuitenkin altisti myös bioaugmentoinnissa hyödynnetyt mikrobit lämpötilamuutoksille, mikä hiukan vähensi bioaugmentoinnin vedantuottoa tehostavaa vaikutustaa suhteessa lämpötilavaihtelun jälkeen tehtyyn bioaugmentointiin.

Seuraavissa panoskokeissa tutkittiin vedantuottoa hyödyntäen kahta erilaista vedantuottajamikrobia, *Caldicellulosiruptor saccharolyticus* ja *Thermotoga neapolitana*, sekä niiden yhteisviljelmällä saavutettiin suurin vetyisaanto (2,8 ± 0,1 mol-H₂/mol-glukoosia), joka oli 3,3 % korkeampi kuin *C. saccharolyticus* puhdasviljelmän vetyisaanto ja 12 % korkeampi kuin *T. neapolitana* puhdasviljelmän vetyisaanto. Lisäksi tämän kokeen yhteydessä kehitettiin kvantitatiiviseen polymeraasiketjureaktioon (qPCR) perustuva menetelmä *T. neapolitana:n kasvun seuraamiseksi sekaviljelmissä. Menetelmän avulla varmistettiin, että *T. neapolitana oli metabolisesti aktiivinen synteettisessä yhteisviljelmässä.*


Tässä työssä korkein vetyisaanto saavutettiin synteettisellä yhteisviljelmällä (*Caldicellulosiruptor saccharolyticus ja Thermotoga neapolitana*). On kuitenkin syytä huomioida, että kasvatusolosuhteet tämän työn eri pansokokeissa poikkesivat toisistaan, eivätkä tulokset siis ole täysin vertailtavissa. Yhteenvetona voidaan kuitenkin todeta, että tämän työn perusteella synteettiset yhteisviljelmät ja bioaugmentointi ovat lupavia tapoja tehostaa termofiilista pimeäfermentatiivista vedantuottoa. Molekyylibiologiset menetelmät kuten qPCR ja suurimmittakaavainen DNA-luenta myös lisäisivät ymmärrystä eri mikrobilajien roolista sekaviljelmissä ja mikrobiyhteisöjen muutoksista simuloiduissa häiriötilanteissa. Molekyylibiologisten menetelmien hyödyntämien bioprosessien seurannassa onkin tärkeää, koska ne auttavat luomaan yhteyden mikrobiyhteisöjen koostumukseen ja mitatun vedantuoton välille.
Résumé

La fermentation à l'obscurité de déchets organismes comme la fraction organique des déchets municipaux et agricoles est une technologie prometteuse pour produire de l'hydrogène renouvelable. Toutefois, il faut poursuivre la recherche et le développement pour améliorer l'efficacité et la stabilité du processus. Le but de cette thèse était d'améliorer la production thermophile d'hydrogène noir fermenté en utilisant des stratégies microbiennes (bioaugmentation et co-cultures synthétiques) et en améliorant la compréhension de la dynamique de la communauté microbienne, particulièrement dans des conditions de stress telles que des températures fluctuantes et des concentrations élevées du substrat.

Afin d'étudier les effets des fluctuations soudaines et à court terme de la température, des cultures de lots incubées initialement à 55 °C (témoin) ont été soumises à des variations de température vers le bas (de 55 °C à 35 °C ou 45 °C) ou vers le haut (de 55 °C à 65 °C ou 75 °C) pendant 48 heures, puis à nouveau incubées à 55 °C pendant deux cycles consécutifs. Les résultats ont montré que des fluctuations soudaines et temporelles de la température à la hausse et à la baisse avaient un impact direct sur le rendement en hydrogène ainsi que sur la structure de la communauté microbienne. Les cultures exposées à des fluctuations de température à la baisse se sont rétablies plus rapidement, ce qui a permis d'obtenir un rendement en hydrogène presque similaire (92-96 %) puisque la culture témoin a été maintenue à 55 °C. Au contraire, les changements de température à la hausse de 55 à 65 ou 75 °C ont eu un effet négatif plus important sur la production d'hydrogène fermenté foncé car le rendement est resté significativement plus faible (54-79 %) pour les cultures exposées que pour la culture témoin.

Afin d'améliorer la stabilité de la production d'hydrogène pendant les fluctuations de température et d'accélérer la récupération, le consortium microbien mixte subissant une période de fluctuation de température à la baisse ou à la hausse a été complété par une culture de mélange synthétique contenant des producteurs d'hydrogène bien connus (Thermotoga, Thermoanaerobacter, Thermoanaerobacterium, Thermoanérobacterium, Caldicellulosiruptor et Thermocellum spp.) L'introduction de nouvelles espèces au consortium naturel a considérablement amélioré la production d'hydrogène tant pendant les variations qu'après celles-ci. Cependant, lorsque la bioaugmentation a été appliquée pendant la fluctuation de température, les micro-organismes utilisés pour l'augmentation ont été exposés à un stress thermique, ce qui a augmenté la capacité de production d'hydrogène.
Cette étude a également étudié la dynamique entre les cultures pures et les co-cultures de producteurs d'hydrogène hautement spécialisés, *Caldicellulosiruptor saccharolyticus* et *Thermotoga neapolitana*. Le rendement en hydrogène le plus élevé (2,8 ± 0,1 mol H₂ mol-1 glucose) a été obtenu avec une co-culture synthétique constituée de *Caldicellulosiruptor saccharolyticus* et *Thermotoga neapolitana*, qui a entraîné une augmentation de 3,3 ou 12% du rendement en hydrogène par rapport aux cultures pures respectivement de *C. saccharolyticus* et *T. neapolitana*. En outre, une méthode quantitative basée sur l'amplification en chaîne par polymérase (qPCR) a été mise au point pour surveiller la croissance et l'apport de *T. neapolitana* dans les co-cultures synthétiques. Avec cette méthode, il a été vérifié que *T. neapolitana* était un membre actif de la co-culture synthétique.

L'effet de différentes concentrations de glucose alimentaire (de 5,6 à 111,0 mmol L-1) sur la production d'hydrogène a été étudié avec et sans augmentation de la culture avec *T. neapolitana*. Par rapport au témoin (sans *T. neapolitana*), la culture bioaugmentée a donné des rendements en hydrogène plus élevés dans presque toutes les concentrations étudiées, même si le rendement en hydrogène a diminué la concentration de glucose alimentaire a augmenté. La présence de *T. neapolitana* a également eu un impact significatif sur la distribution des métabolites par rapport au contrôle. Le nombre de copies du gène de *T. neapolitana* mesuré avec qPCR était plus élevé aux concentrations initiales les plus élevées de glucose. Ainsi, les résultats ont démontré que l'utilisation d'une seule souche ayant les propriétés requises dans un système biologique peut être suffisante pour améliorer la production d'hydrogène fermenté foncé.

En résumé, cette étude a montré que la production thermophile d'hydrogène foncé fermenté peut être améliorée en utilisant des co-cultures synthétiques ou la bioaugmentation. Le rendement en hydrogène le plus élevé dans cette étude a été obtenu avec la co-culture synthétique, bien qu'il faille considérer que les conditions d'incubation diffèrent de celles utilisées pour les cultures mixtes dans cette étude. L'utilisation de méthodes moléculaires telles que le qPCR et le séquençage à haut débit a également aidé à comprendre le rôle de certaines espèces dans les consortiums microbiens et a amélioré la compréhension de la dynamique des communautés microbiennes en situation de stress. L'utilisation de méthodes moléculaires est donc importante car elle permet de créer un lien entre la structure de la communauté microbienne et la production d'hydrogène observée.
Samenvatting

Donkere gisting van organische afvalstoffen zoals de organische fractie van stedelijk afval en landbouwafval is een veelbelovende technologie voor de productie van hernieuwbare waterstof. Er is echter verder onderzoek en ontwikkeling nodig om de efficiëntie en stabiliteit van het proces te verbeteren. Het doel van dit proefschrift was om de thermofiele donkere fermentatieve waterstofproductie te verbeteren door microbiële strategieën (bioaugmentatie en synthetische co-culturen) te gebruiken en door het inzicht in de microbiële gemeenschapsdynamiek te vergroten, met name tijdens stressomstandigheden zoals fluctuerende temperatuur en verhoogde substraatconcentratie.

Om de effecten van plotselinge temperatuurschommelingen op korte termijn te bestuderen, werden batches, eerst geïncubeerd bij 55 °C (controle), gedurende 48 uur onderworpen aan neerwaartse (van 55 °C tot 35 °C of 45 °C) of stijgende (van 55 °C tot 65 °C of 75 °C) temperatuurverschuivingen waarna ze opnieuw werden geïncubeerd bij 55 °C gedurende twee opeenvolgende batchcycli. De resultaten toonden aan dat plotselinge, tijdelijke stijgende en neerwaartse temperatuurschommelingen een directe invloed hadden op de waterstofopbrengst en de microbiële gemeenschapsstructuur. Culturen die werden blootgesteld aan temperatuurschommelingen herstelden zich sneller waardoor een vrijwel vergelijkbare waterstofopbrengst (92-96%) mogelijk was als de controelcultuur op 55 °C werd gehouden. Integendeel, stijgende temperatuurverschuivingen van 55 naar 65 of 75 °C hadden meer significant negatief effect op de donkere fermentatieve waterstofproductie omdat de opbrengst aanzienlijk lager (54-79%) bleef voor de blootgestelde kweken in vergelijking met de controle incubatie.

Om de stabiliteit van de waterstofproductie tijdens temperatuurschommelingen te verbeteren en het herstel te versnellen, werd een gemengd microbiële consortium dat een periode van neerwaartse of stijgende temperatuurschommelingen onderging, uitgebreid met een synthetische mengcultuur met bekende waterstofproducenten (Thermotoga, Thermoanaerobacter, Thermoanaerobacterium, Caldicellulosiruptor en Thermocellum spp.) De toevoeging van nieuwe soorten aan het oorspronkelijke consortium verbeterde de waterstofproductie aanzienlijk, zowel tijdens als na de fluctuaties. Toen de bio-analyse werd toegepast tijdens de temperatuurfluctuatie, werd de waterstofproductie echter verbeterd.
Deze studie onderzocht ook de dynamiek tussen rein- en co-culturen van de gespecialiseerde waterstofproducenent *Caldicellulosiruptor saccharolyticus* en *Thermotoga neapolitana*. De hoogste waterstofopbrengst (2.8 ± 0.1 mol H₂ mol⁻¹ glucose) werd verkregen met een synthetische co-cultuur die resulteerde in een toename van de waterstofopbrengst met 3.3 of 12% in vergelijking met reinculturen van, respectievelijk, *C. saccharolyticus* of *T. neapolitana*. Verder werd een op kwantitatieve polymerasekettingreactie (qPCR) gebaseerde methode ontwikkeld voor het volgen van de groei en bijdrage van *T. neapolitana* in synthetische co-culturen. Met deze methode werd geverifieerd of *T. neapolitana* een actief lid was van de synthetische co-cultuur.

Het effect van verschillende glucose concentraties (van 5.6 tot 111.0 mmol L⁻¹) op de waterstofproductie werd onderzocht met en zonder *T. neapolitana* aan de cultuur toe te voegen. Vergeleken met de controle (zonder *T. neapolitana*) resulteerde de gebioaugmenteerde cultuur in hogere waterstofopbrengsten in bijna alle bestudeerde concentraties, hoewel de waterstofopbrengst daalde als de glucose concentratie in de voeding werd verhoogd. De aanwezigheid van *T. neapolitana* had ook een significante invloed op de metabolieterverdeling in vergelijking met de controle. Het aantal genkopieën van *T. neapolitana* gemeten met qPCR was hoger bij de hoogste initiële glucose concentraties. Aldus toonden de resultaten aan dat het gebruik van een enkele stam met de vereiste eigenschappen die nodig zijn in een biologisch systeem voldoende kan zijn om de productie van donkere fermentatieve waterstof te verbeteren.

Samenvattend heeft deze studie aangetoond dat de productie van thermofiele donkere fermentatieve waterstof kan worden verbeterd door synthetische co-culturen of bio-analyse te gebruiken. De hoogste waterstofopbrengst in deze studie werd verkregen met de synthetische co-cultuur, hoewel er rekening mee moet worden gehouden dat in deze studie de incubatieomstandigheden verschillen van die gebruikt voor de gemengde culturen. Het gebruik van moleculaire methoden zoals qPCR en high-throughput sequencing hielp ook bij het begrijpen van de rol van bepaalde soorten in de microbiële consortia en verbeterde het begrip van de dynamiek van de microbiële gemeenschap tijdens stressomstandigheden. Het gebruik van moleculaire methoden is dus belangrijk omdat het helpt een verband te leggen tussen de structuur van de microbiële gemeenschap en de waargenomen waterstofproductie.
La fermentazione di rifiuti organici, tra i quali la frazione organica dei rifiuti solidi urbani e i rifiuti della filiera agro-industriale, è una tecnologia promettente per la produzione di idrogeno come fonte di energia rinnovabile. Tuttavia, ulteriori sforzi di ricerca sono necessari per migliorare l’efficienza e la stabilità del processo. Lo scopo di questo lavoro di tesi è stato quello di massimizzare la produzione di idrogeno in condizioni termofile usando differenti strategie (quali la bioaugmentation e co-colture sintetiche) e valutando l’evoluzione delle comunità microbiche in condizioni di stress, quali la variazione di temperatura e l’uso di elevate concentrazioni di substrato.

Per studiare l’effetto di improvvisi e brevi fluttuazioni di temperatura, esperimenti in batch sono stati condotti con colture incubate a partire da 55°C (come controllo) e soggetti a decrementi (da 55 a 35 o 45°C) e incrementi (da 55 a 65 e 75°C) per 48 ore prima di essere nuovamente mantenuti a 55°C per altre 48 ore. I risultati hanno mostrato che queste improvvisi e brevi variazioni di temperatura hanno influenzato direttamente le rese di produzione di idrogeno così come le strutture delle comunità microbiche. I batteri sottoposti a temperature decrescenti hanno mostrato capacità di recupero migliori, arrivando a mantenere rese di produzione di idrogeno pari a circa il 92-96% rispetto al valore ottenuto a 55°C. Invece, una netta riduzione in termini di resa di H2, nel range 54-79%, si è avuta quando i microrganismi sono stati sottoposti a temperature crescenti.

Per migliorare la stabilità del processo di produzione di idrogeno durante le fluttuazioni improvvisi di temperatura e velocizzare il recupero delle colture microbiche, al consorzio di microrganismi precedentemente sottoposti a shock di temperatura decrescente e crescente è stato addizionato una coltura mista sintetica a base di risaputi microrganismi produttori di idrogeno (Thermotoga, Thermoanaerobacter, Thermoanaerobacterium, Cricellaruptor e Thermocellum spp.). L’aggiunta delle nuove specie nel consorzio nativo ha migliorato la produzione di idrogeno sia durante che dopo le fluttuazioni di temperatura indotte. Tuttavia, quanto la strategia di bioaugmentation è stata utilizzata durante la variazione della temperatura, i microrganismi utilizzati hanno dimostrato un più elevato potenziale di produzione di idrogeno.

Questo studio ha permesso anche di valutare le dinamiche tra colture pure e co-colture di microrganismi altamente specializzati nel produrre idrogeno, quali Cricella ruptor saccharolyticus e Thermotoga neapolitana. Il più elevato rendimento di idrogeno (pari a 2.8 ± 0.1
mol H₂ mol⁻¹ glucosio) è stato ottenuto con una co-coltura sintetica di *Caldicellulosiruptor saccharolyticus* e *Thermotoga neapolitana*, ed è risultato del 3,3 e 12%, rispettivamente, maggiore rispetto ai valori ottenuti con le due specie utilizzate singolarmente. Inoltre, l’analisi effettuata attraverso la tecnica qPCR ha confermato che il *Thermotoga neapolitana* è risultato essere un microrganismo attivo nella co-coltura sintetica.

L’effetto di differenti concentrazioni di glucosio (da 5,6 a 111,0 mmol L⁻¹) nella soluzione di partenza sulla produzione di idrogeno è stato, altresì, valutato con e senza utilizzare l’*augmentation* di *T. neapolitana*. In presenza del *T. neapolitana*, è stata ottenuta una migliore resa di produzione di idrogeno con tutti i valori di glucosio iniziali, sebbene si sia osservato una generale diminuzione delle rese di H₂ all’aumentare della concentrazione iniziale di glucosio. Inoltre, la presenza del *T. neapolitana* ha anche avuto un impatto notevole sulla composizione dei prodotti nel fermentato e il numero di copie di geni del *T. neapolitana*, misurato con la qPCR, è risultato maggiore alla concentrazione più elevata di glucosio. Pertanto, è stato possibile dimostrare che l’uso di un solo ceppo microbico con le giuste caratteristiche “bioaugmentato” in un sistema biologico riesce a migliorare considerevolmente la produzione di idrogeno in un processo di dark fermentation.
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List of publications


Author’s contribution

Paper I: Onyinye Jeneth Okonkwo was involved in the design of this study and carried out the experimental work, the analyses, data interpretation and the drafting and completion of the manuscript. Aino-Maija Lakaniemi, Rahul Mangayil, Renaud Escudie and Eric Trably were involved in the design of the study, data interpretation, reviewing and completion of the manuscript.

Paper II: Onyinye Jeneth Okonkwo was involved in the design of this study and carried out the experimental work, the analyses, data interpretation and the drafting and completion of the manuscript. Aino-Maija Lakaniemi, Renaud Escudie and Eric Trably were involved in the design of the study and data interpretation. Aino-Maija Lakaniemi, Rahul Mangayil, Renaud Escudie and Eric Trably were involved in the reviewing and completion of the manuscript.

Paper III: Onyinye Jeneth Okonkwo was involved in the design of this study and carried out the experimental work, the analyses, data interpretation and the drafting and completion of the manuscript. Aino-Maija Lakaniemi, Rahul Mangayil, Ville Santala and Matti Karp were involved in the design of the study, data interpretation, reviewing and completion of the manuscript.

Paper IV: Onyinye Jeneth Okonkwo was involved in the design of this study and carried out the experimental work, the analyses, data interpretation and the drafting and completion of the manuscript. Aino-Maija Lakaniemi, Renaud Escudie, Eric Trably, Stefano Papirio and Giovanni Esposito were involved in the design of the study, data interpretation, reviewing and completion of the manuscript.
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<th>Symbol</th>
<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>H\textsubscript{2}</td>
<td>Hydrogen</td>
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<tr>
<td>MEC</td>
<td>Microbial electrolysis cell</td>
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</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
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<tr>
<td>NAD</td>
<td>Nicotinamide adenine dinucleotide</td>
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<tr>
<td>Fd</td>
<td>Ferredoxin</td>
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<tr>
<td>F/M</td>
<td>Food to microorganism ratio</td>
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<tr>
<td>N\textsubscript{2}</td>
<td>Nitrogen</td>
<td></td>
</tr>
<tr>
<td>CH\textsubscript{4}</td>
<td>Methane</td>
<td></td>
</tr>
<tr>
<td>CO\textsubscript{2}</td>
<td>Carbon dioxide</td>
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<tr>
<td>BHP</td>
<td>Biohydrogen production</td>
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<tr>
<td>CSTR</td>
<td>Continuously stirred tank reactor</td>
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</tr>
<tr>
<td>VFAs</td>
<td>Volatile fatty acids</td>
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<tr>
<td>ΔG\textsuperscript{0}</td>
<td>Gibbs free energy</td>
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<tr>
<td>ΔH</td>
<td>Enthalpy</td>
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<tr>
<td>T</td>
<td>temperature</td>
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<tr>
<td>ΔS\textsuperscript{0}</td>
<td>Entropy</td>
<td></td>
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<tr>
<td>Tm</td>
<td>Melting temperature</td>
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<tr>
<td>COD</td>
<td>Chemical oxygen demand</td>
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<tr>
<td>OD</td>
<td>Optical density</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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</tr>
<tr>
<td>qPCR</td>
<td>Quantitative polymerase chain reaction</td>
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<tr>
<td>OTU</td>
<td>Operational taxonomic unit</td>
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CHAPTER 1

General introduction and thesis outline
1.1. Biohydrogen production: need and current state-of-the-art

The increase in the global population and living standards especially in developing countries has led to an upsurge in the global energy consumption (Dudley, 2019; McKinsey Global Institute, 2019). Currently, approximately three-quarter of the total energy consumption is derived from fossil fuels (crude oil, coal and natural gas) as shown in Figure 1 (International Energy Agency, 2018). This has important impacts on energy security as fossil fuels are depleting non-renewable resources. Furthermore, carbon dioxide (CO₂) emissions from energy production contribute to air pollution. Over the past 150 years, the concentration of greenhouse gases (GHG) in the atmosphere has been on a constant rise following human activities such as industrialization, and the global average temperature has risen by 0.88 °C since the late 19th century (National Oceanic and Atmospheric Administration, 2019). It has been generally agreed that if the global climate system warms up more than 2 °C above the pre-industrial levels (1850–1900), the implications would be severe (Masson-Delmotte et al., 2018; Myles et al., 2018). Thus, there is an increasing need to find renewable energy sources that provide sustainable energy and have lower carbon footprint compared to fossil fuels. Renewable energy (including solar, wind, hydro and bioenergy) is rapidly becoming a preferred solution to the world’s energy challenge. In 2018, 25% of energy consumption was from renewable energy sources (International Energy Agency, 2018), most of which is obtained from bioenergy sources (World Bioenergy Association, 2018). In some places, such as in developing countries, the traditional use of biomass which involves burning of wood, forest residues and agricultural waste biomass for cooking and heating is still prevalent (Bourguignon, 2015). As such, it is usually unsustainable and causes deforestation as well as health problems due to smoke pollution (International Energy Agency, 2018).
Wastes have also become a local and global challenge affecting the environment, wildlife as well as the society (World Bank, 2018). Similar to the consumption of energy, the generation of waste for example in developing countries has been increasing along with the growing population and economic growth (International Solid Waste Association, 2012; McAllister, 2015; Mondal and Sanaul, 2019). Without an effective and efficient waste management program, the wastes generated from various human activities, both industrial and domestic, can result in health and environmental hazards. According to the World Bank report (2012), the amount of municipal wastes of the cities around the world might reach 2.2 billion tons per year by 2025. Meanwhile the rates of waste generation in developing countries might double over the next two decades. In EU, a comprehensive legislation has been built with objectives and targets to improve waste management, as well as to lower the carbon footprint from GHG emissions and other environmental impacts as well as potential adverse health effects (European Commission, 2014; Liobikienė and Butkus, 2017). Developing countries such as Rwanda, Ethiopia and Mauritius have also sketched ambitious plans to decouple industrialization from environmental impacts to promote green economies (Liobikienė and Butkus, 2017). Biomasses and biodegradable wastes have been recognized as having the potential to at least partly replace energy production from fossil fuels (Ben-Iwo et al., 2016; Guo et al., 2015; Srirangan et al., 2012). Waste-to-energy conversion processes, as a source of renewable energy, are expected to play an increasingly important role in sustainable management of wastes at global level (Pandey and Teixeira, 2016). This is driving many industries towards valorizing the biorefinery concept of waste to energy conversion. Nowadays, anaerobic digestion is widely used in biological waste-to-energy

![Figure 1.1 Global energy consumption in 2018. Data obtained from Global Energy and CO₂ Status Report (International Energy Agency, 2018).]
conversion (Angenent et al., 2004; Gunaseelan, 1997; Hejnfelt and Angelidaki, 2009; Jingura and Matengaia, 2009; Parawira et al., 2008; Wilkie et al., 2000). It is a collection of processes by which microorganisms break down biodegradable material in the absence of oxygen to produce methane (CH$_4$) and carbon dioxide (CO$_2$) (Bouallagui et al., 2005; Holm-Nielsen et al., 2009; Khalid et al., 2011). However, due to the global environmental considerations such as GHG CO$_2$ emissions from the combustion of CH$_4$, microbial H$_2$ from renewable organic waste sources is a potentially cleaner source of bioenergy as it has high purity level upon combustion. H$_2$ is a key intermediate in anaerobic reactions which are involved in the mineralization of organic matter (Nielsen et al., 2001). In a two-staged production process, the first optimized for dark fermentative H$_2$ production and the second for methane generation via anaerobic digestion, it’s possible to produce biohythane (mixture of H$_2$ and CH$_4$), which can be used as energy carrier. From an environmental point of view, hythane has potential in the reduction of the GHG emissions into atmosphere due to the presence of H$_2$ which reduces the carbon content of this gaseous blend (Bolzonella et al., 2018; Liu et al., 2018; Pasupuleti and Venkata Mohan, 2015; Si et al., 2016).

Compared to conventional H$_2$ production from natural gas by steam reforming, gasification and water electrolysis which use non-renewable energy sources to produce H$_2$, biological hydrogen production is less energy intensive and a variety of waste-derived feedstocks can be utilized as carbon sources for dark fermentative H$_2$ production to facilitate waste recycling. Compared to dark fermentation under mesophilic conditions (25 to 40 °C), processes operated under thermophilic (55 to 80 °C) conditions have garnered interest over the recent years due to several advantages. Thermophilic processes can often be operated at a slightly lower retention times than corresponding mesophilic processes, since microbiological activity and kinetics of chemical reactions increase with increased temperature (O-Thong et al., 2011). Thermophilic processes for H$_2$ production have usually reduced presence of H$_2$ consumers and other unwanted microorganisms and seem to enable more stable H$_2$ production in long-term operation (Dong et al., 2011; Ferrer et al., 2010). Another important advantage of dark fermentation under thermophilic conditions is the natural sanitation it provides by getting rid of undesired pathogens in the system (Abreu et al., 2012). High temperatures may also increase availability of certain organic compounds because their solubility increases with increasing temperature (Meegoda et al., 2018).

The major challenges that have prevented the large-scale utilization of dark fermentative H$_2$ production include: selection of suitable and efficient microorganisms or microbial communities,
low substrate conversion efficiency, low hydrogen yield, as well as mixture of hydrogen and carbon dioxide as products requiring separation (Stetson, 2013). The performance of microbial communities is often affected by factors such as pH, temperature, substrate and inoculum pretreatment (Cisneros-Pérez et al., 2015; Penteado et al., 2013). Changes in environmental conditions during dark fermentative H₂ production cause change in the population dynamics, which in turn can lead to instability of H₂ producing systems (Bakonyi et al., 2014; Koskinen et al., 2007). Previous studies investigating the effects of temperature on fermentative H₂ production have focused on comparing batch and reactor performances at different fixed operating temperatures (Dessì et al., 2018; Zhang and Shen, 2006). However, very little is known about the impact of short-term temperature fluctuations on H₂ production and microbial community dynamics. Therefore, assessing the impact of such factors on the structure and composition of mixed microbial communities is important, as the detrimental effects of operational variations can only be delineated by understanding their effect on well-performing microbial community. This knowledge is also required for developing strategies to enable fast and efficient recovery of H₂ production. This is the first study to demonstrate the influence transient downward temperature fluctuations on the stability of H₂ production and the use of bioaugmentation strategy to ensure fast recovery of biological systems exposed to stress periods. Understanding microbial community composition, changes that occur during process disturbances and extreme conditions (such as high substrate concentration or temperature fluctuations) is required for enhancing the process stability. In-depth understanding of the microbial community dynamics requires fast and efficient microbial monitoring methods.

1.2. Research objectives

The main objective of this study was to enhance thermophilic hydrogen production by using microbial strategies (bioaugmentation and synthetic co-cultures) and by increasing the understanding on the microbial community dynamics especially during stress conditions. The specific objectives were:
• To study the impact of sudden short-term temperature fluctuations on thermophilic dark fermentative H₂ production and microbial community composition with and without augmenting a mixed culture with known H₂ producers before and after the fluctuations (Chapters 3 and 4).
• To enhance H₂ production by using a synthetic co-culture of two ecologically distant species, *Thermatoga neapolitana* and *Caldicellulosiruptor saccharolyticus* (Chapter 5).
• To develop a quantitative polymerase chain reaction (qPCR) based method for monitoring the growth of *T. neapolitana* in synthetic co-cultures (Chapter 5) and after bioaugmenting mixed cultures with *T. neapolitana* (Chapter 6).
• To examine the effects of different feed glucose concentrations on H₂ production and microbial community composition of a thermophilic mixed culture with and without bioaugmenting the culture with *T. neapolitana* (Chapter 6).

1.3. Thesis structure
This thesis is composed of seven chapters (Figure 1.2). In Chapter 1, the rationale for this study is explained and an overview of the thesis is provided. The Chapter starts by presenting the importance of waste-to-energy conversion processes and description of current state-of-the-art of dark fermentative hydrogen production. This is followed by the problem statement and proceeds with explaining objectives of the study.

Chapter 2 provides a theoretical background on the existing knowledge on H₂ production methods, and biological H₂ production focusing on the microbiology and factors affecting dark fermentative H₂ production and microbial strategies for enhancing H₂ production.

Chapter 3 reports on the effects of short-term upward and downward temperature fluctuations on thermophilic dark fermentative H₂ production and the dynamics of microbial communities in response to these changes. In Chapter 4, bioaugmentation was applied to cultures undergoing short-term temperature fluctuations as a strategy to enhance H₂ production process during and after the temperature fluctuations.

Chapter 5 of this study focuses on the co-cultivation of two ecologically distant organisms (*Caldicellulosiruptor saccharolyticus* and *Thermotoga neapolitana*) to improve H₂ production and the development of a quantitative PCR method for genus and species level monitoring of *T. neapolitana*. A 16S rRNA gene method was designed to target eight members of the *Thermotoga* genus. Given the high degree of similarity and absence of correlation that usually occur between
16S rRNA gene, a more specific method was further developed targeting the hydA gene for a more comprehensive evaluation of *T. neapolitana* in synthetic co-cultures and mixed cultures.

In **Chapter 6** the effect of different feed glucose concentrations on H₂ production in a thermophilic mixed culture with and without augmenting the culture with *T. neapolitana* is reported. A pre-adaptation strategy (incubation of *T. neapolitana* in a mixed culture for several batch cycles) was employed prior to the experiment to make *T. neapolitana* a stable member of the microbial community.

**Chapter 7** summarizes the knowledge obtained in this thesis and further discusses the practical implications of the research work. Chapter 7 also provides recommendations for further studies and includes main conclusions drawn based on the work.

**Figure 1.2** Overview of the structure of this PhD thesis.
References


CHAPTER 2

Theoretical background on H₂ production
2.1. H₂ utilization and production

Currently, H₂ is used in the chemical industry as a fundamental building block for the production of ammonia-fertilizers and methanol used for polymer manufacturing (Andrews and Shabani, 2012). It is also used for obtaining high grade petrol in a process called reforming and also to remove sulfur compounds, which otherwise would contaminate the catalytic converters in cars (Cheremisinoff and Rosenfeld, 2009). H₂ is currently being tested as a potential energy source in the transportation sector. For example, in aviation, H₂-powered fuel cells are considered as a potential energy source for aircrafts where the fuel cell modules can supply electricity to the electrical system as emergency generator or as an auxiliary power unit (Alazemi and Andrews, 2015; Ball and Weeda, 2015; Hua et al., 2014). Similarly, the uses of H₂-powered fuel cells are currently being tested as potential energy source for ships (Bicer and Dincer, 2018; Sharma and Ghoshal, 2015).

Hydrogen can be generated through several methods including thermochemical and biological processes. Approximately 95% of the hydrogen produced is derived primarily from non-renewable, fossil raw materials and thus generates GHG emissions (Demirbas, 2004). Thermal production process, in which steam reforming is used to produce H₂ from natural gas or other light hydrocarbons, is most common with worldwide annual H₂ production of approximately 50 million tons (Armaroli and Balzani 2011; US Department of Energy 2013). Steam reforming of natural gas is currently the least expensive method of producing H₂. Hydrogen can be produced also by electrolysis of water, a process which uses electricity to split water into H₂ and oxygen (Armaroli and Balzani, 2011; Holladay et al., 2009; Kapdan and Kargi, 2006). However, about 80% of the operation cost goes to electricity consumption. About to 50% of the global H₂ demand is generated from steam reforming of natural gas, approximately 30% from oil and naphtha reforming from refinery or chemical industrial off-gases, 18% via coal gasification, about 3.9% from water electrolysis, and 0.1% from other sources (Kalamaras and Efstathiou, 2013; Muradov and Veziroğlu, 2005). Hydrogen can be produced biologically from renewable feedstocks such as biomass and organic wastes, but the biological processes (described in detail in the next section) are still in research and development phase. However, compared to thermochemical and electrochemical H₂ production, biological H₂ production is preferable due to its low energy requirement. Most biological H₂ production processes also involve the production of CO₂ but it is worth noting that this CO₂ released from biomass is consumed during photosynthesis unlike fossil fuels where the CO₂ released has been built up over time (Vijayaraghavan and Mohd Soom, 2006).
2.2. Biological H₂ production mechanisms

Biological H₂ production can occur via various microbial-driven processes such as direct or indirect biophotolysis, photofermentation, bioelectrochemical hydrogen production in microbial electrolysis cells, dark fermentation or a combination of some of these methods (Gómez et al., 2011; Hallenbeck et al., 2012; Schütz et al., 2004; Zurrer and Bachofen, 1979). Biological H₂ production methods have been widely studied and are considered preferable to the traditional H₂ production methods mentioned in section 2.1 because of the possibility to use renewable feedstocks in the production process (Benemann and Benemann, 2000; Chen, 2006; Guwy et al., 2011; Hallenbeck, 2013). For decades, biological H₂ production from organic wastes has been considered as a sustainable means for energy production (Benemann, 1996; Harper and Pohland, 1986). Such wastes include for example the organic fraction of municipal solid waste, agricultural wastes and pulp and paper manufacturing waste streams (Benemann, 1997; Claassen et al., 1999; Sen et al., 2008).

2.2.1. Biophotolysis of water by green algae and cyanobacteria

Biophotolysis can either be direct or indirect and is carried out by green microalgae or cyanobacteria utilizing sunlight to split water into oxygen (O₂) and H⁺ ions. The H⁺ ions can then be combined through direct (Equation 1) or indirect routes (Equations 2 and 3) to produce H₂ gas (Azwar et al., 2014; Dasgupta et al., 2010; Yu and Takahashi, 2007). H₂ production by direct photolysis utilizes energy from sunlight and microalgal photosynthetic systems to convert water into chemical energy (Equation 1).

\[
2H_2O + \text{solar energy} \rightarrow 2H_2 + O_2 \quad (1)
\]

Apart from having the ability to fix CO₂ via photosynthesis, many types of green algae and cyanobacteria also have the ability to fix nitrogen from the atmosphere and produce enzymes that can catalyze the H₂ generating step via indirect biophotolysis (Rahman et al., 2016). Indirect biophotolysis consists of two stages that occur in series. The first step is the biomass production (carbohydrate) through photosynthetic system (Equation 2). During this process, O₂-evolving photosynthesis is used to fix and store carbon, thus producing reduced carbon compounds that can later be used in the second stage, which utilises the biomass rich-carbohydrate for H₂-producing fermentation (Equation 3) (Yu and Takahashi, 2007).

\[
6H_2O + 6CO_2 + \text{light} \rightarrow C_6H_{12}O_6 + 6O_2 \quad (2)
\]
\[ C_6H_{12}O_6 + 6H_2O \rightarrow 12H_2 + 6CO_2 \] (3)

Direct biophotolysis is an advantageous process because \( H_2 \) is produced directly from water and sunlight. However, high light intensity is required and the rate of \( H_2 \) production is typically low. Additionally, oxygen is inhibitory to the process, which makes the process difficult to optimize (Benemann and Benemann, 2000; Chandrasekhar et al., 2015; Yu and Takahashi, 2007). Similarly, \( H_2 \) production by indirect biophotolysis is sensitive to oxygen as the oxygen produced photosynthetically tends to inactivate the key enzymes of biophotolysis (nitrogenase and hydrogenase), thereby causing instability in \( H_2 \) production. Some cyanobacteria exhibit temporal separation mechanism by carrying out oxygenic photosynthesis in the day and nitrogen fixation at night time (Compaoré and Stal, 2010).

2.2.2. Bioelectrochemical \( H_2 \) production in microbial electrolysis cells

\( H_2 \) can be produced in a microbial electrolysis cell (MEC) in which exoelectrogenic microorganisms degrade organic materials (e.g. acetate) producing protons, electrons and CO\(_2\) (Figure 2.2) Protons migrate through a proton exchange membrane to the cathode and electrons are transported through an external circuit to the cathode to produce \( H_2 \) with the help of small voltage applied between the electrodes (Liu et al., 2012; Wang et al., 2012). MECs should not be confused with microbial fuel cells as the latter is a fuel cell that generates electricity via the microbial activity on the anode and/or cathode (Logan et al., 2008). Equation 4 and 5 shows an example of the evolution of \( H_2 \) in a MEC from acetate as the carbon source.

Anode: \( CH_3COOH + 2H_2O \rightarrow 2CO_2 + 8e^- + 8H^+ \) (4)

Cathode: \( 8H^+ + 8e^- \rightarrow 4H_2 \) (5)

Some exoelectrogens capable of transferring electrons to the anode in a MEC include bacterial species belonging the genera *Geobacter*, *Shewanella* and *Pseudomonas* (Bond and Lovley, 2003; Call et al., 2009; Geelhoed and Stams, 2011; Liu et al., 2005; Moreno et al., 2015). The required applied voltage (typically 0.5 to 0.9 V) (Singh et al., 2015) in MECs for the formation of \( H_2 \) is low when compared to the theoretical minimum voltage of 1.23 V that is required for water electrolysis (Rozendal et al., 2006). While MEC is a potentially attractive green technology to tackle the global warming and energy crisis, there are existing challenges such low \( H_2 \) production rate and expensive materials which hinders commercialization (Kadier et al., 2016; Zhang and
Chapter 2: Theoretical background on hydrogen production

Angelidaki, 2014). Therefore, new approaches are needed in biofilm engineering, materials for electrodes and reactor configuration to enable successful large scale applications.

Figure 2.1 Schematic representation of bioelectrochemical hydrogen production in a two-chamber bioelectrochemical system (adapted from Singh et al. 2015). Examples of reactions occurring at the anode and cathode are presented in Equation 4 and 5, respectively.

### 2.2.3. Photofermentation

Photofermentation is carried out by purple non-sulfur (PNS) bacteria through a light-driven biochemical process, in which organic acids such as acetate, butyrate, lactate and/or propionate are converted to H₂ and CO₂ under anaerobic conditions as shown in Equations 6–9 (Adessi et al., 2012; Chen et al., 2008a).

\[
\text{Acetate (C}_2\text{H}_4\text{O}_2) + 2\text{H}_2\text{O} + \text{Light energy} \rightarrow 4\text{H}_2 + 2\text{CO}_2 \quad (6)
\]

\[
\text{Butyrate (C}_4\text{H}_8\text{O}_2) + 6\text{H}_2\text{O} + \text{Light energy} \rightarrow 10\text{H}_2 + 4\text{CO}_2 \quad (7)
\]

\[
\text{Lactate (C}_3\text{H}_6\text{O}_3) + 3\text{H}_2\text{O} + \text{Light energy} \rightarrow 6\text{H}_2 + 3\text{CO}_2 \quad (8)
\]

\[
\text{Propionate (C}_3\text{H}_6\text{O}_2) + 4\text{H}_2\text{O} + \text{Light energy} \rightarrow 7\text{H}_2 + 3\text{CO}_2 \quad (9)
\]
As organic acids are present in many waste streams, photofermentative microorganisms could be utilized for simultaneous waste treatment and H₂ production (Ghosh et al., 2017; Keskin et al., 2011; Lee et al., 2017). The most widely known PNS bacteria that are able to produce H₂ via photofermentation belong to the genera of *Rhodospirillum*, *Rhodopseudomonas* and *Rhodobacter* (Boran et al., 2012; Carlozzi and Lambardi, 2009; Zurrer and Bachofen, 1979). The advantage of this process over biophotolysis is that the process does not result in generation of inhibitory O₂ and therefore the H₂ production rates are typically higher than those obtained with direct or indirect biophotolysis (Holladay et al., 2009; Oh et al., 2004; Singh et al., 2015). However a major limitation is the low photochemical efficiencies since the process is light dependent (Hay et al., 2013; Levin et al., 2004; Özgür and Peksel, 2013). Furthermore, the H₂ yield and rate of H₂ production are too low for practical applications (Afsar et al., 2011; Androga et al., 2011; Özgür et al., 2010; Sabourin-Provost and Hallenbeck, 2009). Further research is therefore necessary to improve the H₂ yield or production rate.

### 2.2.4. Dark fermentation

During dark fermentation, microorganisms break down complex organic polymers such as cellulose to monomers which are then further utilized to produce H₂ and other by-products. H₂ is produced by fermentative microorganisms as a means of disposing electrons derived from the oxidation of organic compounds (Yang, 2007). H₂ can be produced by facultative anaerobic or obligate anaerobic bacteria using different pathways. The most common pathway utilized by obligate anaerobic H₂ producing microorganisms is the glycolytic or Embden-Meyerhoff pathway (Verhaart et al., 2010; Zhang et al., 2012). In this pathway, glucose is oxidized to pyruvate, leading to the generation of reduced nicotinamide adenine dinucleotide (NADH) and adenosine triphosphate (ATP). The pyruvate is further oxidized to acetyl-coA via the reduction of ferredoxin (Fd). If acetyl-CoA is converted to acetate, both the reduced Fd and NADH are then used to convert H⁺ to H₂. Alternatively, pyruvate from glucose may be converted by facultative microorganisms to formate by the catalysis of pyruvate formate lyase, and the produced formate can be further degraded into H₂ and CO₂ with formate hydrogen lyase (Chandrasekhar et al., 2015; Vardar-Schara et al., 2008). The pathway utilized for H₂ production depends on the microorganisms involved as well as the environmental conditions (Chandrasekhar et al., 2015; Ramirez-Morales et al., 2015). H₂ production rate in dark fermentation is typically higher than those observed for photofermentation and biophotolysis (Chandrasekhar et al., 2015).
Although H\textsubscript{2} can be produced from various organic materials (for a review, see e.g. Khanna and Das, 2013; Lin et al., 2012; Show et al., 2010), glucose is one of the most abundant sugars on earth and a building block of many organic compounds such starch, glycogen and cellulose. Therefore, the biochemical pathways of dark fermentation will be presented for glucose in this section (Figure 2.2). Due to thermodynamic constraints, the theoretical yield of H\textsubscript{2} in dark fermentation is only 4 moles because a large part of the substrate is converted into various soluble metabolic products such as acetate, butyrate, propionate or ethanol (Tapia-Venegas et al., 2015). The maximum theoretical yield, also known as the Thauer limit (4 mol H\textsubscript{2} mol\textsuperscript{-1} glucose), can only be achieved through the acetate pathway (Equation 10), whereas H\textsubscript{2} production from glucose via the butyrate pathway produces 2 moles of H\textsubscript{2} (Equation 11). The production of other soluble metabolic products such as propionate, ethanol and lactate (Equation 12-14) (Wicher et al., 2012) reduces the maximum H\textsubscript{2} yield obtainable via dark fermentation, because these pathways consume glucose and do not result in H\textsubscript{2} generation or even consume H\textsubscript{2} (Frascari et al., 2013; Masset et al., 2012). Thus, the energy that is stored chemically in the organic material is not fully recoverable in the form of gaseous H\textsubscript{2} (Kumar et al., 2016).

\[
\text{C}_6\text{H}_{12}\text{O}_6 + 2\text{H}_2\text{O} \rightarrow 2\text{CH}_3\text{COO}^- + 2\text{CO}_2 + 4\text{H}_2 \text{ (Acetate pathway)} \tag{10}
\]

\[
\text{C}_6\text{H}_{12}\text{O}_6 \rightarrow \text{CH}_3\text{CH}_2\text{CH}_2\text{COO}^- + 2\text{CO}_2 + 2\text{H}_2 \text{ (Butyrate pathway)} \tag{11}
\]

\[
\text{C}_6\text{H}_{12}\text{O}_6 + 2\text{H}_2 \rightarrow 2\text{CH}_3\text{CH}_2\text{COO}^- + 2\text{H}_2\text{O} \text{ (Propionate pathway)} \tag{12}
\]

\[
\text{C}_6\text{H}_{12}\text{O}_6 \rightarrow 2\text{CH}_3\text{CH}_2\text{OH} + 2\text{CO}_2 \text{ (Ethanol pathway)} \tag{13}
\]

\[
\text{C}_6\text{H}_{12}\text{O}_6 \rightarrow 2\text{CH}_3\text{CHOHCOOH} \text{ (Lactate pathway)} \tag{14}
\]

In some cases, the metabolic pathways lead to ethanol and acetate production, lowering the stoichiometric H\textsubscript{2} yield to 2 mol of H\textsubscript{2} per mole of glucose (Equation 15). This is often dependent on the temperature condition and the microorganism involved (Li and Fang, 2007).

\[
\text{C}_6\text{H}_{12}\text{O}_6 + 2\text{H}_2\text{O} \rightarrow \text{CH}_3\text{CH}_2\text{OH} + \text{CH}_3\text{COOH} + 2\text{CO}_2 + 2\text{H}_2 \tag{15}
\]
2.3. Microbiology of dark fermentative H$_2$ production

Dark fermentative H$_2$ production can be carried out at mesophilic (25–40 °C), thermophilic (40–65 °C) or hyperthermophilic (>80 °C) conditions depending on the microbial inoculum used (Sinha and Pandey, 2011). H$_2$-producers are quite diverse and widespread in nature. They have been found in environments such as animal manure (Yokoyama et al., 2007), sewage sludge (Kotay and Das, 2006) and hot springs (Koskinen et al., 2008). Fermentative microorganisms are either
facultative anaerobes or obligate anaerobes (Cabrol et al., 2017; Ghimire et al., 2015; Rittmann and Herwig, 2012).

Facultative anaerobic H\textsubscript{2} producers are attractive for their lower oxygen sensitivity and their presence in dark fermentative process is useful for rapidly depleting O\textsubscript{2} possibly present in the culture (Khanna et al., 2011). However, their presence is often limited by harsh inoculum pretreatment methods (described in more detail in section 2.4) such as heat treatment and acid treatment used to inactivate H\textsubscript{2} consuming organisms, which are typically found from the same environments as H\textsubscript{2} producing microorganisms (Cabrol et al., 2017). Expect for Bacillales (phylum Firmicutes, class Bacilli) all other known facultative H\textsubscript{2}-producers belong to the class gammaproteobacteria (phylum proteobacteria) and are able to thrive at mesophilic conditions. These include for example, \textit{Citrobacter} spp., \textit{Klebsiella} spp., \textit{Enterobacter} spp. (Patel et al., 2014), \textit{Shewanella oneidensis} (Meshulam-Simon et al., 2007) and \textit{Pseudomonas stutzeri} (Goud et al., 2014). An interesting characteristic of the H\textsubscript{2} producing Bacillales is that some of the members of this group possess the ability to resist shock conditions (such as heat-shock) via sporulation (Kumar et al., 2013).

Obligate anaerobes constitute a diverse array of microorganisms which are either spore formers or non-spor formers (For review, see e.g. Cabrol et al., 2017). One of the most studied obligately anaerobic microorganisms able to produce H\textsubscript{2} at mesophilic conditions is \textit{Clostridium butyricum}, which belongs to the class Clostridia. Clostridia have been found to dominate mesophilic fermentative microbial communities irrespective of the source of inoculum or pre-treatment method (Cabrol et al., 2015; Das and Veziroglu, 2008; Ueno et al., 2001).

H\textsubscript{2} producing microorganisms that can produce H\textsubscript{2} at thermophilic conditions include both bacteria and archaea (Pawar and van Niel, 2013). Some thermophilic H\textsubscript{2} producers, such as \textit{Caldicellulosiruptor saccharolyticus} have the ability to effectively hydrolyze complex carbohydrates for their metabolism (Carver et al., 2011; Nissilä et al., 2011). Their ability to hydrolyze complex carbohydrates makes them valuable for H\textsubscript{2} production as well as other industrial processes for enhancing the rate of hydrolysis (De Vrije et al., 2009; Islam et al., 2009). Among the thermophilic H\textsubscript{2} producers that have been reported in literature, \textit{Thermoanaerobacterium} spp. have been the most often found from mixed cultures (Karadag and Puhakka, 2010; Koskinen et al., 2008; O-Thong et al., 2011). Other genera of thermophilic H\textsubscript{2} producing microorganisms include for example \textit{Caldicellulosiruptor} (Willquist et al., 2010), \textit{Thermotoga} (Nguyen et al., 2008), and \textit{Thermoanaerobacter} (Koskinen et al., 2008).
Thermophilic microorganisms typically produce higher H\(_2\) yields than H\(_2\) producers thriving at mesophilic conditions, because H\(_2\) yielding reactions are thermodynamically more favorable at higher temperatures (Foglia et al., 2011; Qiu et al., 2016; Verhaart et al., 2010). For example, H\(_2\) yield of approximately 96% of the theoretical value (4 mol H\(_2\) mol\(^{-1}\) glucose) has been reported with *Thermotoga neapolitana* at 80 °C (Ippolito et al., 2010), while about 90% of the theoretical yield has been obtained with *Caldicellulosiruptor saccharolyticus* at 72.5 °C (De Vrije et al., 2007).

Dark fermentative H\(_2\) production at both mesophilic and thermophilic conditions can be carried out using either mixed or pure cultures (Hung et al., 2011; Li and Fang, 2007; Zhang et al., 2007). Pure microbial cultures have mainly been utilized at laboratory-scale for studying H\(_2\) production mechanisms, the effect of environmental conditions on dark fermentation and the ability of different species to utilize different carbon sources (Table 2.1). However, Van Groenestijn et al. (2009) demonstrated pilot-scale H\(_2\) production (400 L cylindrical stainless steel trickle bed reactor of 1.2 m height filled with 190 L polyurethane foam) using sucrose for studying the performance of pure cultures of *C. saccharolyticus* at a temperature of 73 °C under non-aseptic conditions (Van Groenestijn et al., 2009). They obtained a yield of 2.8 mol H\(_2\) mol\(^{-1}\) hexose and a volumetric productivity of 22 mmol H\(_2\) L\(^{-1}\) filter bed h\(^{-1}\). Even though pure cultures often generate H\(_2\) with very high efficiency, they are typically quite sensitive to contamination and can require aseptic conditions throughout the fermentation, which is not feasible for large scale production (Taherdanak et al., 2015). Conversely, mixed cultures do not require an aseptic environment for H\(_2\) production (for a review, see e.g. Hallenbeck, 2009). Additionally, single microorganisms have less hydrolytic capacity when compared to a consortium of microorganism which offers the possibility to work on a wide spectrum of low-cost, easily available substrates. Therefore, mixed microbial cultures are preferred for H\(_2\) production especially when complex organic materials such as lignocellulosic biomass or wastewaters are used as substrates (Anh et al., 2011; Cappelletti et al., 2012; Kim et al., 2008; Kumar et al., 2015; Hiroshi Yokoyama et al., 2007).

The microbial diversity often observed in mixed cultures can sometimes be disadvantageous for dark fermentative H\(_2\) production due to the activity of H\(_2\) consuming microorganisms and/or microorganisms that compete with H\(_2\) producers for substrates without producing H\(_2\) (Cabrol et al., 2017). Microorganisms that contribute negatively towards H\(_2\) production in mixed cultures are phylogenetically diverse. Hydrogenotrophic methanogens and homoacetogens are the most common H\(_2\) consuming microorganisms (Cabrol et al., 2017). Examples of hydrogenotrophic methanogens, which use H\(_2\) as the major electron donor to reduce CO\(_2\) and produce methane, include methanoarchaea in the order *Methanobacteriales* and *Methanomicrobiales* (Chaganti et
al., 2012). Homoacetogens are strict anaerobes, which can consume H$_2$ and CO$_2$ to form acetate and include e.g. certain *Clostridium* spp. such as *C. aceticum*, *C. thermoautotrophicum*, *C. thermoaceticum* and *C. stercorarium* (Guo et al., 2010; Ueno et al., 2006). Their growth is typically favored at acidic pH and long hydraulic retention time (Guo et al., 2010; Ren et al., 2007). Microorganisms that compete with the organic substrate and are not able to produce H$_2$ include for example lactic acid bacteria and nitrate-reducing bacteria (Bundhoo and Mohee, 2016; Cabrol et al., 2017; Ghimire et al., 2015; Mizuno et al., 1998; Wang and Wan, 2009). The presence of such microorganisms in the mixed cultures can lead to low H$_2$ yields and increase in the formation of soluble metabolites.
### Table 2.1 Dark fermentative \( \text{H}_2 \) yields obtained with selected pure cultures under different growth conditions.

<table>
<thead>
<tr>
<th>Microbial species</th>
<th>Mode</th>
<th>Substrate</th>
<th>Temperature (°C)</th>
<th>pH</th>
<th>Substrate concentration (g L(^{-1}))</th>
<th>( \text{H}_2 ) yield (mol ( \text{H}_2 ) mol(^{-1}) glucose)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mesophiles</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Enterobacter aerogenes</em></td>
<td>Batch</td>
<td>Glucose</td>
<td>38</td>
<td>6.5</td>
<td>10</td>
<td>1.0</td>
<td>Yokoi et al., 1995</td>
</tr>
<tr>
<td><em>Enterobacter cloacae</em></td>
<td>Batch</td>
<td>Glucose</td>
<td>36</td>
<td>6</td>
<td>10</td>
<td>2.2</td>
<td>Kumar and Das, 2000</td>
</tr>
<tr>
<td><em>Clostridium beijerinckii</em></td>
<td>Batch</td>
<td>Glucose</td>
<td>35</td>
<td>7.2</td>
<td>3</td>
<td>2.8</td>
<td>Lin et al., 2007</td>
</tr>
<tr>
<td><em>Clostridium butyricum</em></td>
<td>Batch</td>
<td>Glucose</td>
<td>36</td>
<td>7.2</td>
<td>3</td>
<td>2.3</td>
<td>Lin et al., 2007</td>
</tr>
<tr>
<td><em>Clostridium butyricum</em></td>
<td>Batch</td>
<td>Palm oil mill effluent</td>
<td>37</td>
<td>5.5</td>
<td>15–100</td>
<td>0.22</td>
<td>Chong et al., 2009a</td>
</tr>
<tr>
<td><strong>Thermophiles</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Caldicellulosiruptor saccharolyticus</em></td>
<td>Batch</td>
<td>Glucose</td>
<td>70</td>
<td>7</td>
<td>10</td>
<td>3.4</td>
<td>Budde et al., 2010</td>
</tr>
<tr>
<td></td>
<td>Batch</td>
<td>Miscanthus hydrolysate</td>
<td>72</td>
<td>7</td>
<td>28</td>
<td>2.4</td>
<td>De Vrije et al., 2009</td>
</tr>
<tr>
<td><em>Clostridium thermocellum</em></td>
<td>Batch</td>
<td>Cellulose</td>
<td>60</td>
<td>6.8</td>
<td>1</td>
<td>1.9</td>
<td>Islam et al., 2009</td>
</tr>
<tr>
<td><em>Thermoanaerobacterium thermosaccharolyticum</em></td>
<td>Batch</td>
<td>Glucose</td>
<td>60</td>
<td>6.5</td>
<td>10</td>
<td>2.4</td>
<td>O-Thong et al., 2008</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Xylose</td>
<td>60</td>
<td>6.5</td>
<td>10</td>
<td>2.6</td>
<td>Ren et al., 2008</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Xylose</td>
<td>75</td>
<td>7</td>
<td>5</td>
<td>3.4</td>
<td>Ngo et al., 2012</td>
</tr>
<tr>
<td><em>Thermotoga neapolitana</em></td>
<td>Batch</td>
<td>Glucose</td>
<td>75</td>
<td>7</td>
<td>10</td>
<td>3.5</td>
<td>De Vrije et al., 2010</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Carrot pulp hydrolysate</td>
<td>75</td>
<td>7</td>
<td>10</td>
<td>2.7</td>
<td>De Vrije et al., 2010</td>
</tr>
</tbody>
</table>
2.4. Factors affecting dark fermentative H₂ production

2.4.1. Temperature

Changes in operating temperature can influence the substrate degradation rate and the activity of the H₂ producing microorganisms (Nazlina et al., 2009). Temperature changes can also result in shifts in the metabolic pathways in a mixed culture as different organisms might become predominant at different temperatures (Dessì et al., 2018c). Increasing the temperature in dark fermentative H₂ production within a range in which the H₂-producing organisms are still active might help to suppress the activity of certain H₂ consuming microorganisms such as methanogens thereby enhancing H₂ production (Ghimire et al., 2015; Luo et al., 2011). Even though dark fermentation can occur at a wide temperature range, it has been shown that small or sudden changes in the operating temperature can cause significant changes in microbial community composition and H₂ yield (Dessì et al., 2018a, 2018b; Qiu et al., 2017b). Even temporal temperature fluctuations caused by e.g. technical failures or environmental changes can lead to changes in the enzymatic activities, growth rates and/or loss of microbial diversity (El-Mashad et al., 2004; Wu et al., 2006).

Dark fermentative H₂ production at high temperatures accelerates the metabolic rates and growth rates of microorganisms compared to lower temperatures (Westerholm et al., 2018). Dark fermentative H₂ production at relatively high temperatures is advantageous because of low viscosity and high gas stripping as a result of low solubility of H₂ gas (Chandrasekhar et al., 2015). Furthermore, high operational temperature can help to inactivate H₂ consuming microorganisms naturally present in e.g. wastewaters (Karakashev and Angelidaki, 2011). One drawback of thermophilic processes is that the energy required for heating the bioreactors may be higher than the energy gained from the produced H₂ (Ketheesan and Nirmalakhandan, 2011; Lin et al., 2012; Perera et al., 2012, 2010). Thus, it is not considered economically feasible due to the energy required to maintain the high temperatures. To make the operation at high temperatures feasible, either waste heat from some other process or a very hot substrate would be required. For example, some industrial wastewaters such as thermomechanical pulp and paper wastewater which are produced at elevated temperatures as high as 50 to 70 °C can be treated on site with minimum energy requirement (Hubbe et al., 2016).

2.4.2. pH

The pH of the cultivation medium has direct influence on the metabolic activities of microorganisms and thereby on H₂ production (Fang and Liu, 2002; Liu et al., 2011). The optimum
pH range for dark fermentative H\textsubscript{2} production varies from pH 4.5 to 9 (La Licata et al., 2011; Lee et al., 2009; Wongthanate and Chinnacotpong, 2015). However, Mota et al. (2018) recently reported H\textsubscript{2} production from acidogenic reactors fed with sucrose at pH below 3.0. For dark fermentative H\textsubscript{2} production at mesophilic conditions, pH below 6 has been widely used as it inhibits the activity of methanogens which consume H\textsubscript{2} (La Licata et al., 2011; Lee et al., 2009; Qiu et al., 2017a). Depending on the inoculum source, thermophilic H\textsubscript{2} production processes have been carried out at pH below 6 (Dessi et al., 2018c) or at neutral pH (Table 2.1).

In several studies, acetate and butyrate are the major end products of favorable H\textsubscript{2} production. A neutral operational pH has been observed to mostly favor the acetate pathway, while acidic pH conditions mostly favor the butyrate pathway (Abreu et al., 2013; Ciranna et al., 2012; Karlsson et al., 2008; Prakasham et al., 2009; Zhang et al., 2015, 2014). During dark fermentative H\textsubscript{2} production test at 37 °C, Luo et al. (2011) showed acetate as the major metabolic product at operational pH of 7, while butyrate dominated at an operational pH 5.5. A study on dark fermentation of cheese whey at different pH ranges (5.5 to 7.7) and a temperature of 39 °C showed pH 6 as the optimal pH and acetate levels were higher in all the tests except at pH 6.5 where butyrate and propionate levels exceeded those of acetate (De Gioannis et al., 2014). Nonetheless, Khanal et al. (2004) studied the effects of pH and intermediate products and submitted that acetate and butyrate levels were independent of pH in the range studied (4.5 to 6.5). Furthermore, Luo et al. (2010) reported butyrate as the major VFA in the dark fermentation of cassava stillage in both H\textsubscript{2} production test carried out at the initial pH 5 and 7. Example of optimal pH ranges for some H\textsubscript{2} producing organisms include pH of 7 applied for H\textsubscript{2} production by \textit{Thermotoga neapolitana} (Maru et al., 2012) and pH of 6.5 for H\textsubscript{2} production by \textit{Thermoanaerobacterium thermosaccharolyticum} (Saripan and Reungsang, 2013).

### 2.4.3. H\textsubscript{2} partial pressure

Partial pressure of H\textsubscript{2} is an important factor in dark fermentation, as it has been shown to affect the H\textsubscript{2} yield. High H\textsubscript{2} partial pressure of the headspace in a bioreactor leads to increased H\textsubscript{2} concentrations in the liquid phase (Levin et al., 2004). If the concentration of H\textsubscript{2} produced in the dark fermentation system is too high, it makes the thermodynamics of H\textsubscript{2} production even less advantageous and as such, can be inhibitory to H\textsubscript{2} production process (Chong et al., 2009b; Logan et al., 2002; Nath and Das, 2004). Continuous or intermittent removal of the produced biogas lowers the H\textsubscript{2} partial pressure and the H\textsubscript{2} concentration in the liquid phase which typically increases the obtainable H\textsubscript{2} yield (Bundhoo and Mohee, 2016; Lee et al., 2012; Mandal et al., 2012; Mota et al., 2018).
Dreschke et al. (2019) reported that high dissolved H$_2$ concentration can significantly reduce H$_2$ production although H$_2$ partial pressure would not be seemingly high and that H$_2$-rich gas can be used for stripping dissolved H$_2$ from the culture without causing any dilution of the produced gas. Thus, the recirculation of produced H$_2$-rich gas back to the bioreactor can be used to enhance H$_2$ yields although the gas recirculation does not necessarily reduce the H$_2$ partial pressure, but reduces the H$_2$ concentration in the liquid phase. Other methods used for lowering H$_2$ partial pressure include increase in headspace to culture volume ratio and sparging with external gas such as CO$_2$ (Dreschke et al., 2019). Kim et al. (2006) showed that sparging a continuously stirred tank reactor with either N$_2$ or CO$_2$ improved H$_2$ production. They further compared the effectiveness of using either N$_2$ or CO$_2$ and reported that using CO$_2$ improved H$_2$ yield better than N$_2$ (Kim et al., 2006). The use of N$_2$ or CO$_2$ would however lead to dilution of the produced gas and likely to increased costs of H$_2$ recovery. Additionally, the effectiveness of gas sparging also depends on a number of factors such as the gas flow rate and the reactor configuration.

### 2.4.4. Substrate concentration

For dark fermentative H$_2$ production to be economically viable, high substrate loads should be converted efficiently to H$_2$ (Ljunggren and Zacchi, 2010). The increase in substrate concentration to a certain extent can be beneficial for enhancing H$_2$ production. However, the limitations of using high substrate concentrations for H$_2$ production are imminent as increased accumulation of organic acids and alcohols have inhibitory effects on H$_2$ production (Elbeshbishy et al., 2017) as illustrated in Figure 2.6. Inhibition resulting from high substrate concentrations may lead to growth inhibition, incomplete substrate utilization and/or unwanted changes in the metabolic products (Nicolaou et al., 2010). Even though several types of substrates have been studied for dark fermentative H$_2$ production, a vast majority of the reports are based on hexose and pentose sugars, which are often used at initial concentrations ranging from 1 to 50 g COD L$^{-1}$ (Elbeshbishy et al., 2017; Guo et al., 2010; Kargi et al., 2012; Kim et al., 2006; Wang and Wan, 2008a). While, most of these studies have suggested substrate concentrations above 20 g COD L$^{-1}$ to be inhibitory to H$_2$ production, the threshold is not consistent as it may vary as a result of other factors such as inoculum characteristics and reactor configuration. Optimizing substrate concentration or the food to microorganism ratio (F/M) and the organic loading rate is necessary for preventing substrate inhibition (Ghimire et al., 2015; Mohammadi et al., 2017, 2012; Wongthanate and Chinnacotpong, 2015).
Figure 2.3 Inhibition mechanisms induced by high substrate concentration, low pH and undissociated acids during dark fermentative H₂ production (modified from Elbeshbishy et al. 2017).

2.5. Microbial strategies for optimizing dark fermentation

It has been stated that in order to maximize net energy gain via dark fermentation, appropriate H₂-producing cultures capable of high H₂ yield have to be employed (Chandrasekhar et al., 2015; Kumar et al., 2016). The relatively low yield and production rate obtained and the often observed H₂ production instability of mixed culture fermentation especially at mesophilic conditions are common denominators that prevent biological H₂ production from becoming a practical means for industrial-scale H₂ production (Cabrol et al., 2017; Chandrasekhar et al., 2015). The following sections focus on the strategies that have been employed for optimizing H₂ production via dark fermentation.

2.5.1. Enrichment of H₂-producers in mixed cultures

In order to obtain higher H₂ yields by inactivating H₂ consuming and other competing microorganisms, different pretreatment methods such as heat-shock, acid treatment, base addition, aeration, freezing and thawing, and addition of specific chemicals such as chloroform, sodium 2-bromoethanesulfonate or 2-bromoethanesulfonic acid and iodoproppane have been used (for a review, see e.g. Wang and Wan, 2008b). Some of these pretreatments, including the widely-used heat treatment, select for spore-forming H₂-producers (Baghchehsaraee et al., 2008; Dessi et al., 2018a; Rafieenia et al., 2018). These pretreatments have been used as a strategy to enhance H₂ production. However, the most efficient/optimal pretreatment seems to depend of
the inoculum source and the H₂ producing conditions (e.g. substrate and temperature). Cheong and Hansen (2006) evaluated the different pretreatment methods (acid, sodium 2-bromoethanesulfonate, dry heat-shock and freezing and thawing) for enriching H₂-producing bacteria from cattle manure sludge. They showed that H₂ production from the pre-treated inocula were 1.9–9.8 times greater when compared to the control sludge. Furthermore, they concluded that the acid pretreatment method was the best among the five methods studied (Cheong and Hansen, 2006). On the other hand, Zhu and Béland (2006) studied six different pretreatment methods (acid, base, heat-shock, aeration, 2-bromoethanesulfonic acid and iodopropane) for enriching H₂ producers and concluded that the base pretreatment resulted in highest H₂ production. Dessì et al. (2018a) reported that the most promising pretreatment method is different for mesophilic and thermophilic cultures. They reported that at mesophilic condition, acid shock was the most optimal pretreatment while alkaline shock was optimal for enrichment of H₂ producers under thermophilic conditions (Dessì et al., 2018a). Therefore, more research is needed to understand the effects of pretreatments on different microbial communities. In addition, inoculum pretreatments are not typically efficient in removing all H₂ consuming homoacetogens, as spore-forming Clostridium spp. include both H₂-producing and homoacetogenic species (Diekert and Wohlfarth, 1994; Hugenholtz and Ljungdahl, 1990). Thus, it is very difficult to select conditions that would promote one and inactivate the other (Cabrol et al., 2017)

H₂ production can also be enhanced by controlling the operational parameters at an optimum level for H₂ producing organisms, but unsuitable for most typical H₂ consumers. For example, since the growth of methanogens is inhibited at pH below 6, many studies have controlled pH <6 to optimize H₂ production by inhibiting the growth of methanogenic microorganisms (Cabrol et al., 2017; Luo et al., 2011; Mohan, 2008). The use of low hydraulic retention time is another strategy that has been used to optimize H₂ production due to washout of the slow-growing methanogens (Liu et al., 2008). Furthermore, H₂ consumption by homoacetogens has been shown to become more favorable at high H₂ partial pressure (Kotsyurbenko et al., 2001; Peters et al., 1998). Homoacetogens have also been shown to be inhibited at pH of 5.5 at thermophilic conditions (Ghimire et al., 2015; Luo et al., 2011).

2.5.2. Synthetic co-cultures
Cultivation of two or more syntrophic H₂ producing microorganisms together as a synthetic co-culture is also a potential strategy to enhance substrate degradation and conversion to H₂ compared to pure cultures without introducing H₂ consuming organisms to the culture (for a
review, see Laxman Pachapur et al., 2015). Synthetic co-cultures appear to be advantageous over single microorganisms because of the potential synergy between the different metabolic pathways of the species involved, leading to increased H\textsubscript{2} yield. For example, co-cultures have the ability to improve hydrolysis of complex substrates (Elsharnouby et al., 2013). Many studies have investigated the use of co-cultures of two bacterial species, one with the capability of hydrolyzing cellulose, and the other with the ability to produce H\textsubscript{2} efficiently from simple sugars (Abreu et al., 2016; Chen et al., 2015; Kotay and Das, 2006; Vatsala et al., 2008). For example, co-cultures of \textit{Clostridium thermocellum} and \textit{Thermoanaerobacterium thermosaccharolyticum} were successfully used to convert cellulose directly to H\textsubscript{2} at 60 °C and pH of 4.4. Alone, the H\textsubscript{2} yield obtained by \textit{C. thermocellum} from cellulose was about 0.8 mol H\textsubscript{2} mol\textsuperscript{-1} glucose, with lactate as the main product. However, when used as a co-culture with \textit{T. thermosaccharolyticum}, the H\textsubscript{2} production increased about 2-fold to 1.8 mol H\textsubscript{2} mol\textsuperscript{-1} glucose (Liu et al., 2008). In addition, a co-culture of \textit{Citrobacter freundii} and \textit{Clostridium butyricum} was shown to enhance H\textsubscript{2} production by dark fermentation compared to the respective pure cultures (Beckers et al., 2010). Given the characteristics of \textit{C. freundii} as a facultative anaerobe, such a culture would not require the addition of any reducing agents as \textit{C. freundii} consumes oxygen and provides the anaerobic conditions required for \textit{C. butyricum} growth (Beckers et al., 2010).

### 2.5.3. Bioaugmentation

Apart from the natural adaptation and selection of H\textsubscript{2} producing microbial communities to specific operational conditions, bioaugmentation of mixed cultures with pure cultures with desired metabolic characteristics has been proposed as a strategy to increase microbial biomass and diversity of indigenous microbial populations (Chandrasekhar et al., 2015; Kumar et al., 2016). Over the years, bioaugmentation has been successfully used to reduce the start-up time of dark fermentation (Pandit et al., 2015) and to help regain stable operation of biological processes after process disturbances (Goud et al., 2014; Guo et al., 2010), to enable operation at shorter hydraulic retention time (Baek et al., 2016) and to decrease the recovery time of anaerobic digesters after organic overloading (Acharya et al., 2015; Goud et al., 2014).

Bioaugmentation with known H\textsubscript{2} producers has been studied for the improvement of H\textsubscript{2} production performance from complex organic materials such as vegetable waste, cellulosic waste and digested sludge (Guo et al., 2010; Marone et al., 2012; Martín-Hernández et al., 2012; Sompong O-Thong, 2017; Wang et al., 2008). For example, \textit{Escherichia coli}, \textit{Enterobacter aerogenes} or \textit{Bacillus subtilis} were added to municipal digester sludge to study the effect of bioaugmentation
on \( \text{H}_2 \) production from organic fraction of municipal solid waste (Sharma and Melkania, 2018). The authors reported improvement of \( \text{H}_2 \) production with bioaugmentation of each three different organisms, whilst bioaugmentation with \( B. \text{subtilis} \) resulted in higher \( \text{H}_2 \) production than bioaugmentation with \( E. \text{coli} \) and \( E. \text{aerogenes} \) (Sharma and Melkania, 2018). Recently, \( \text{Thermoanaerobacterium thermosaccharolyticum} \) was added to three types of seed sludge: rotten corn stover, cow dung compost, and anaerobic digester sludge, to investigate the effect of bioaugmentation on thermophilic \( \text{H}_2 \) production (Zhang et al., 2019). The bioaugmentation with 5% (volume) of \( T. \text{thermosaccharolyticum} \) increased the \( \text{H}_2 \) yield by 12.8%, 2.9% and 7.1% in rotten corn stover, cow dung compost and sludge from anaerobic digestion, respectively (Zhang et al., 2019). Bioaugmentation has also been shown to protect the existing microbial community from organic loading shocks or reduce the susceptibility of the \( \text{H}_2 \) producing bioreactors to process fluctuations (Ács et al., 2015; Goud et al., 2014; Kuo et al., 2012; Venkata Mohan et al., 2009). Goud et al. (2014) used acidogenic bacteria for bioaugmentation to enhance \( \text{H}_2 \) production at an elevated organic loading with food waste. Fermentative bacteria capable of producing \( \text{H}_2 \) were added to a native mixed microflora by Marone et al. (2012) to improve its \( \text{H}_2 \) yield from vegetable waste.

Establishing synthetic interactions between species is, thus, becoming quite common in laboratory studies. However, in some cases, bioaugmentation can be unsuccessful due to the inability of the added species to compete with the indigenous microbial population under the operational conditions of the process (Chandrasekhar et al., 2015; Ferguson et al., 2014; Herrero and Stuckey, 2015; Venkata Mohan, 2009). Different species possess different growth properties (e.g. growth rates and substrate preferences) as well as different capacities to cope with stress, which arise from variations in growth conditions such as temperature and pH. Therefore, the microorganisms chosen for bioaugmentation purposes should have the desirable properties needed to perform the required function under the planned operational conditions.

### 2.5.4. Genetically engineered microorganisms

Metabolic engineering is another optimization strategy that has been considered for dark fermentation to improve \( \text{H}_2 \) production (Hallenbeck, 2013; Hallenbeck et al., 2012; Kothari, 2017). It has been used to increase \( \text{H}_2 \) production in bacteria by the causing the overexpression of certain enzymes that are responsible for \( \text{H}_2 \) production and/or to block pathways that might result in \( \text{H}_2 \) consumption (Abo-Hashesh et al., 2011; Hallenbeck and Ghosh, 2012; Maeda et al., 2008; Vardar-Schara et al., 2008). Metabolic engineering has received increasing attention for
improving H₂ production and application of this technique can significantly improve H₂ production rate and yields (Akhtar and Jones, 2009, 2008; Ma et al., 2009; Maeda et al., 2012). But the challenge might be that tools to engineer some of the species shown to produce high H₂ yields do not exist yet. An example of metabolic engineering include the study by Maeda et al. (2007) where multiple, stable mutations were introduced into *Escherichia coli* which allowed for increase of H₂ production from 0.65 to 1.3 mol H₂ mol⁻¹ glucose. Further studies showed that genetically modified *Escherichia coli* produced 68.5% more H₂ from glucose fermentation compared to the wild-type strain (Maeda et al., 2012). At the moment, continuous large-scale production of H₂ from organic waste materials with genetically engineered strain(s) is not feasible due to restraints such as possible contamination, competition with endogenous microorganisms and loss of the genetically engineered strain(s) (Cabrol et al., 2017).

### 2.5.5. Integration of different biological H₂ production processes

The combinations some of the biological H₂ production processes described in section 2.2 have been studied in order to improve the overall H₂ production yield and to utilize the chemical energy present in the dark fermentation effluents (Chen et al., 2008b; Hallenbeck et al., 2012; Nath et al., 2008). For example, Li et al. (2014) integrated dark fermentation with microbial electrolysis cells and obtained a H₂ yield of 130 mL H₂ per gram of corn stalk from dark fermentation and a yield of 257 mL H₂ per gram of corn stalk by employing the fermentation effluent as feedstock in microbial electrolysis cell, thereby obtaining an overall H₂ yield of 387 mL H₂ per gram of corn stalk. Nath and Das (2004) reported coupling of dark fermentation with photofermentation in an overall process in which glucose was used as the substrate for dark fermentation and the fermentation effluent as a substrate for *Rhodobacter sphaeroides* to achieve higher overall H₂ yield.

Another technology under consideration includes the co-production of a mixture of H₂ and CH₄ known as biohythane which is carried out through a two-stage anaerobic digestion reactor configuration (Monlau et al., 2015). This technology is gaining more attention as a renewable energy carrier in the transport sector due to its energetic and environmental advantages. The presence of H₂ contributes to improving the engine combustion yield as well as reduce the CO₂ emissions from the combustion (Bolzonella et al., 2018; Liu et al., 2018, 2013; Roy and Das, 2016; Si et al., 2016).
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CHAPTER 3

Impacts of short-term temperature fluctuations on biohydrogen production and resilience of thermophilic microbial communities

ABSTRACT

Anaerobic microflora enriched for dark fermentative $\text{H}_2$ production from a mixture of glucose and xylose was used in batch cultivations to determine the effects of sudden short-term temperature fluctuations on $\text{H}_2$ yield and microbial community composition. Batch cultures initially cultivated at 55°C (control) were subjected to downward (from 55°C to 35°C or 45°C) or upward (from 55°C to 65°C or 75°C) temperature shifts for 48 h after which, each culture was transferred to a fresh medium and cultivated again at 55°C for two consecutive batch cycles. The average $\text{H}_2$ yield obtained during the first cultivation at 55°C was 2.1 ± 0.14 mol $\text{H}_2$ mol$^{-1}$ hexose equivalent. During the temperature shifts, the obtained $\text{H}_2$ yields were 1.8 ± 0.15, 1.6 ± 0.27 and 1.9 ± 0.00 mol $\text{H}_2$ mol$^{-1}$ hexose equivalent at 35°C, 45°C and 65°C, respectively, while no metabolic activity was observed at 75°C. The sugars were completely utilized during the 48 h temperature shift to 35°C but not at 65°C and 45°C. At the end of the second cycle after the different temperature shifts, the $\text{H}_2$ yield obtained was 96.5, 91.6, 79.9 and 54.1% (second cycle after temperature shift to 35°C, 45°C, 65°C and 75°C, respectively) when compared to the average $\text{H}_2$ yield produced in the control at 55°C. Characterization of the microbial communities present in the control culture at 55°C showed the predominance of *Thermoanaerobacteriales*, *Clostridiales* and *Bacilliales*. The microbial community composition differed based on the fluctuation temperature with *Thermoanaerobacteriales* being most dominant during the upward temperature fluctuations and *Clostridiales* being the most dominant during the downward temperature fluctuations.
3.1. INTRODUCTION

The increasing global demand for energy and fuels, and the environmental hazards that fossil fuels contribute, strongly demand for alternative energy resources. \( \text{H}_2 \) has been considered as a possible sustainable alternative (Hosseini and Wahid, 2016; Johnston et al., 2005). Although \( \text{H}_2 \) is a very abundant element on earth, it does not typically exist as \( \text{H}_2 \) in nature. \( \text{H}_2 \) is usually found combined with other elements, whilst it can be produced locally from numerous sources. \( \text{H}_2 \) is produced industrially via electrolysis, coal gasification, and fossil fuel reforming (Dincer, 2012; Holladay et al., 2009). Known biological \( \text{H}_2 \) production (BHP) methods include direct and indirect biophotolysis by green algae and cyanobacteria (Yu and Takahashi, 2007), photofermentation by phototrophic bacteria (Adessi et al., 2017) and dark fermentation by fermentative bacteria (Hu et al., 2013). BHP via dark and photofermentation has drawn increasing interest because of the ability to generate \( \text{H}_2 \) from various organic resources, such as industrial waste streams and lignocellulosic materials (Li and Fang, 2007; Zhang et al., 2003). Photosynthetic \( \text{H}_2 \) production on the other hand is of interest because it needs only light and water and does not produce any \( \text{CO}_2 \) (Ghirardi, 2006; Melis, 2007).

However, \( \text{H}_2 \) production by dark fermentation is advantageous over the other BHP processes because of higher \( \text{H}_2 \) production rates and the possibility to use a wide variety of organic materials as substrates (Chong et al., 2009b). Dark fermentation can be carried out under different temperatures, i.e. mesophilic (35-40 °C), thermophilic (52-60 °C) and extremely thermophilic (>65 °C) conditions by different groups of fermentative bacteria (Dessì et al., 2018c; Verhaart et al., 2010). Given the faster rates and higher yields of \( \text{H}_2 \), the use of thermophilic dark fermentation is often preferred over mesophilic processes (Pawar and van Niel, 2013). However, thermophilic operations can require higher energy input for heating and are prone to inhibition (Angelidaki and Ahring, 1994) and sudden environmental changes (Biey et al., 2003) which can eventually result in reduced process stability or productivity (Pawar and van Niel, 2013; M. Wu et al., 2006).

Studies on anaerobic biological processes have shown that even small changes in the operating temperature can cause significant changes in microbial community composition and \( \text{H}_2 \) yields (Dessì et al., 2018c, 2018b; Qiu et al., 2017b). Temporal temperature fluctuations can also lead to changes in the enzymatic activities, growth rates and/or loss of microbial diversity, which directly affect \( \text{H}_2 \) production (Gadow et al., 2013b). Temperature is therefore a key parameter to be controlled in dark fermentative processes. Previous studies investigating the effects of temperature on fermentative \( \text{H}_2 \) production have focused on comparing batch incubations and
reactor performances at different fixed operating temperatures (Dessì et al., 2018b; Zhang and Shen, 2006). However, short-term temperature fluctuations can occur e.g., due to technical failures and therefore it is important to study the effects of short-term temperature fluctuations because they might lead to unwanted changes of the microbial growth and activities. Several studies focusing on anaerobic digestion have been carried out to establish the relationship between sudden temperature fluctuations and biogas production (Chae et al., 2008; Gao et al., 2012; Kundu et al., 2014). However, to our knowledge only one study has been conducted to evaluate the influence of transient downward temperature fluctuations on the stability of H$_2$ production (Gadow et al., 2013b). To date, no study has been done to correlate the outcome of temperature fluctuations in dark fermentation to the resilience and stability of the microbial populations involved.

The present study therefore aims to evaluate the effects of sudden short-term downward and upward temperature fluctuations during thermophilic dark fermentative H$_2$ production and reveal connections between effect of temperature changes and the microbial community structure. Chemical and molecular methods were used to monitor and characterize the microbial responses to transient temperature fluctuations (two different upward and two different downward temperature fluctuations). Understanding the resilience and stability as well as the dynamics of microbial communities to transient temperature fluctuations will help in developing strategies for optimization of dark fermentation processes, especially after possible process disturbances.

### 3.2. MATERIALS AND METHODS

#### 3.2.1. Enrichment culture: medium composition and inoculum source
The enrichment of H$_2$ producing microbial community for this study was carried out in a continuous stirred tank reactor (CSTR) after which the enriched culture was used in batch bottle experiments to study the effects of sudden short-term temperature fluctuations on H$_2$ production and microbial community composition. An anaerobically digested sludge was used as inoculum for the CSTR after heat shock pretreatment at 90°C for 20 min. Two hundred milliliters of the pretreated sludge (10% v/v, final concentration, 40 mg L$^{-1}$ of volatile solids) was inoculated to 1800 mL of the following culture medium (mg/L): K$_2$HPO$_4$, 500; NH$_4$Cl, 100; MgCl$_2$ $\cdot$ 6H$_2$O, 120; H$_6$FeN$_2$O$_8$S$_2$ $\cdot$ 6H$_2$O, 55.3; ZnCl$_2$, 1.0; MnCl$_2$ $\cdot$ 4H$_2$O, 2.0; CuSO$_4$, 000.4; (NH$_4$)$_6$Mo$_7$O$_24$, 1.2; CoSO$_4$, 1.3; H$_3$BO$_3$, 0.1; NiCl$_2$ $\cdot$ 6H$_2$O, 0.1; Na$_2$O$_3$Se, 0.01; CaCl$_2$ $\cdot$ 2H$_2$O, 80; yeast extract, 500 and 0.055 mL HCl (37%). The culture was fed with glucose (800 mg L$^{-1}$) and xylose (1200 mg L$^{-1}$ xylose). Xylose was used as substrate because it is a major component of hemicellulose and therefore commonly
present in lignocellulosic biomass, lignocellulose hydrolysates, and pulp and paper wastewaters. Utilizing both glucose and xylose is a practical way to move towards efficient bioconversion of lignocellulosic wastes to H₂. The total working volume of the CSTR was two liters. The reactor was flushed with nitrogen for 5 min and then operated in continuous mode at hydraulic retention time of 6 h and at 55 °C for 21 days. The pH was maintained at 6.5.

3.2.2. H₂ production batch experiments
Prior to exposing the cultures to temporal temperature fluctuations, the H₂-producing enrichment culture (step 1 in Figure 1) was acclimatized to batch growth conditions at 55°C using the same cultivation medium as in the CSTR (step 2 in Figure 1). The acclimatized culture was then divided into ten anaerobic cultivation bottles containing fresh medium and subjected to a one-time temperature shock. This was done by placing duplicate bottles to 35 and 45 °C (downward temperature shocks), to 55 °C (control) as well as to 65 and 75 °C (upward temperature shocks) for 48 h (Figure 1, step 3). At the end of the 48 h incubation period, the cultures were centrifuged for 5 minutes, transferred to fresh medium and incubated at the original temperature of 55 °C for 48 h (step 4 in Figure 1). This step was repeated one more time (step 5 in Figure 1).
Figure 1 Experimental setup to study the effects of different temperature fluctuations during dark fermentation. First, $H_2$ producers were enriched in a continuous stirred tank reactor (CSTR) at 55 °C for 21 days (step 1). This was followed by the acclimatization of the enriched mixed culture to batch conditions (step 2) and then, specific temperature shock described as the downward temperature fluctuation (35 °C or 45 °C) and the upward temperature fluctuation (65 °C or 75 °C) were imposed (step 3). Cultures incubated at 55 °C (C) were used as control. After the temperature shocks, the $H_2$ production was followed for two more batch cycles at 55 °C (step 4 and step 5) to delineate how the culture can recover from the different temperature fluctuations.

3.2.3. Analysis of $H_2$ production

The biogas volume and composition measurement was carried out at the respective incubation temperatures mentioned in section 2.2 while keeping the culture bottles in water baths to maintain a constant temperature. Gas production was monitored was periodically measured with a digital manometer to determine the volume of gas produced. $H_2$ partial pressure can have a significant effect on fermentation as some thermophilic microorganisms may change their metabolism to the production of more reduced substrates such as lactate when the $H_2$ partial pressure increases. The biogas volume was therefore measured before and after releasing the pressure in each culture bottle. The total volume of produced $H_2$ was calculated at standard temperature using Equation 1 (Logan et al., 2002).
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\[ V_{H_2,t} = V_{H_2,t-1} + C_{H_2,t} (V_{G,t} - V_{G,t-1}) + V_H (C_{H_2,t} - C_{H_2,t-1}) \]  

(1)

Where \( V_{H_2,t} \) is the cumulative \( H_2 \) gas produced at time \( t \), \( V_{H_2,t-1} \) is the cumulative \( H_2 \) gas produced at \( t-1 \), \( V_{G,t} \) 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 \( H_2 \) gas fraction in the headspace at time \( t \), \( C_{H_2,t-1} \) is the \( H_2 \) gas fraction in the headspace at time \( t-1 \) and \( V_H \) is the total headspace volume in the culture bottle. \( H_2 \) production in moles was calculated on the basis that one mole of an ideal gas will occupy 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 gas composition was analyzed using a gas chromatograph (Clarus 580, Perkin Elmer) with a thermal conductivity detector. The columns used were a RtQbond column for \( H_2 \), \( O_2 \), \( N_2 \) and \( CH_4 \) quantification and a RtMolsieve column for \( CO_2 \) quantification. The carrier gas was argon under a pressure of 3.5 bars. A gas tight Hamilton syringe was used for gas sampling.

3.2.4. Analysis of the liquid metabolites

Culture suspension samples were collected before and after each experimental step for chemical analysis of the metabolic products. The samples were centrifuged at 13000 rpm for 15 min and the supernatant was filtered with 0.2 μm filter before the analyses. Glucose, xylose, organic acid and alcohol concentrations were measured by high performance liquid chromatography (HPLC) using a refractive index detector (Waters R410) as described previously by (Monlau et al., 2013).

3.2.5. Microbial community analysis

Genomic DNA was extracted using the PowerSoil™ DNA Isolation Sample Kit (MoBio Laboratories, Inc., Carlsbad, CA, USA) according to the manufacturer’s instructions. The following primers; 515_532U and 909_928U (Wang and Qian, 2009) including their respective linkers, were used to amplify the V4_V5 region of the 16S rRNA gene over 30 amplification cycles at an annealing temperature of 65 °C. The resulting products were purified and loaded onto the Illumina MiSeq cartridge prior to sequencing. Sequencing and library preparation were performed at the Genotoul Lifescience Network Genome and Transcriptome Core Facility in Toulouse, France (get.genotoul.fr). The sequences analysis was done as described by Venkiteshwaran et al. (2016).
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3.2.6. Calculations

$H_2$ yield was calculated by dividing the mol $H_2$ per mole of hexose equivalent using the conversion factor of 5/6 for converting xylose to its hexose equivalent. The theoretical $H_2$ produced was calculated based on the measured acetate and butyrate concentrations (mM) using the Equation 2 (Luo et al., 2010) in order to determine the ratio of experimental to theoretical $H_2$ yield (Akinbomi and Taherzadeh, 2015). If the calculated ratio is above 100%, the $H_2$ is produced also via other pathways than acetate and butyrate while a ratio below 100% indicates homoacetogenic activity.

Theoretical $H_2$ produced = $2 \times \sum (\text{acetate concentration} + \text{butyrate concentration})$ (2)

The relative $H_2$ yield compared to the control was calculated for each culture by comparing the $H_2$ yields obtained during or after the temperature fluctuation period to the average yield (steps 3 - 5) obtained from the control cultures kept at 55 °C (Equation 3).

Relative $H_2$ yield compared to the control ($\%$) = \frac{H_2 \text{ yield obtained during/after temperature shift}}{\text{average } H_2 \text{ yield obtained from the control}} \times 100$ (3)

Total COD of soluble compounds was calculated based on the sum of acids, ethanol and residual sugars by using the following conversion factors: 1 mM glucose = 192 mg COD L$^{-1}$, 1 mM xylose = 160 mg COD L$^{-1}$, 1 mM acetate = 64 mg COD L$^{-1}$, 1 mM propionate = 112 mg COD L$^{-1}$, 1 mM lactate = 96 mg COD L$^{-1}$, 1 mM butyrate = 160 mg COD L$^{-1}$ and 1 mM ethanol = 96 mg COD L$^{-1}$ (Gonzales and Kim, 2017; Sivagurunathan and Lin, 2016). The COD mass balance was calculated using the endpoint concentrations of the residual sugars and soluble metabolites.

3.3. RESULTS AND DISCUSSION

3.3.1. Thermophilic $H_2$ production at constant temperature in CSTR and first batch test (55 °C)

Methane was not detected in any of the incubations performed in this study, which indicates that the initial heat-shock pretreatment was sufficient to totally suppress the activity of methanogens (Cai et al., 2004; Venkata Mohan et al., 2008). The maximum $H_2$ yield obtained during the enrichment in the CSTR was 1.9 mol $H_2$ mol$^{-1}$ hexose equivalent. The $H_2$ yield obtained in the batch cultivation at 55°C (control) was $2.2 \pm 0.07$, $2.1 \pm 0.06$ and $1.9 \pm 0.14$ mol $H_2$ mol$^{-1}$ hexose equivalent (in steps 3, 4 and 5 respectively). The $H_2$ yield was seen to decrease in steps 4 and 5.
During the cultivation in the different steps (3, 4 and 5) at 55 °C, the concentration of ethanol and acetate increased in step 4 compared to steps 3 and 5 (Table 1). The increase in these metabolites coincided with a decrease in butyrate concentration. The butyrate to acetate ratio (HBu/HAc, mM:mM) has been used in previous studies as an indicator of relative contribution of these two pathways to H\textsubscript{2} production (Lin et al., 2006; Sangyoka et al., 2016). In the cultures incubated at 55 °C, it was 1.2, 0.5 and 1.5 in steps 3, 4 and 5, respectively. The values from step 3 and 5 are in line with previous studies which have reported HBu/HAc ratios ranging between 1.5 and 4.0 (Lin et al., 2006; Sangyoka et al., 2016). The ratio of experimental to theoretical H\textsubscript{2} yield was 99, 91 and 83% in steps 3, 4 and 5, respectively. Based on the H\textsubscript{2} yields, HBu/HAc ratios and the ratios of experimental to theoretical H\textsubscript{2} yield, there might have been a slight shift in the metabolic pathway towards acetate production during the thermophilic dark fermentative H\textsubscript{2} production in step 4 and an increasing homoacetogenic activity from step 3 to 5.

In a dark fermentation, the maximum theoretical H\textsubscript{2} that can be obtained from glucose under standard temperature and pressure is 4 mol H\textsubscript{2} per mol glucose with acetate as the only metabolite while 2 mol H\textsubscript{2} per mol of glucose is produced when butyrate is the only metabolite (Vardar-Schara et al., 2008). However, during dark fermentation, H\textsubscript{2} is produced along with other metabolites such as alcohols, lactate and propionate, the production of which are either consume substrate without production of any H\textsubscript{2} or even consume H\textsubscript{2} and therefore lead to low H\textsubscript{2} yields (Buyukkamaci and Filibeli, 2004; Hawkes et al., 2007). The metabolites produced by a bacterium or mixed cultures depend on the environmental conditions.

**Table 1** Concentration of soluble metabolites and their contribution to end-point COD in the control cultures incubated at 55 °C including results from the incubations steps 3, 4 and 5.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Concentration (mmol L\textsuperscript{-1})</th>
<th>Percentage of end-point COD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Step 3</td>
<td>Step 4</td>
</tr>
<tr>
<td>Lactate</td>
<td>n.d</td>
<td>n.d</td>
</tr>
<tr>
<td>Acetate</td>
<td>5.01 ± 0.4</td>
<td>7.63 ± 0.0</td>
</tr>
<tr>
<td>Ethanol</td>
<td>1.22 ± 0.3</td>
<td>3.85 ± 0.1</td>
</tr>
<tr>
<td>Butyrate</td>
<td>5.96 ± 0.4</td>
<td>4.00 ± 0.1</td>
</tr>
<tr>
<td>Propionate</td>
<td>0.73 ± 0.0</td>
<td>0.63 ± 0.0</td>
</tr>
</tbody>
</table>

n.d: Not detected
3.3.2. Sudden transient downward temperature shift and its effects on H₂ metabolism

During the downward temperature shifts, the H₂ yield slightly decreased compared to the control (55 °C), being 1.8 ± 0.15 and 1.6 ± 0.27 mol H₂ mol⁻¹ hexose equivalent at 35 and 45 °C, respectively. However, H₂ production recovered rapidly when the cultures were transferred to a fresh medium and incubated at the original temperature of 55 °C. H₂ yield of 2.2 ± 0.07 and 2.0 ± 0.01 mol H₂ mol⁻¹ hexose equivalent (steps 4 and 5, respectively) were obtained for cultures exposed to 35°C shift, and 2.0 ± 0.08 and 1.9 ± 0.00 mol H₂ mol⁻¹ hexose equivalent (steps 4 and 5, respectively) for cultures exposed to 45°C shift. The substrates were fully consumed during and after the temperature shift to 35°C. Meanwhile during the temperature shift to 45°C, 6.4 ± 0.5% of the substrate was not consumed (Table 2) but all substrates given were completely depleted after the cultures were returned to 55 °C. During the downward temperature fluctuation, similar metabolic patterns were observed at 35 and 45 °C shifts except that the concentration of lactate and butyrate was higher at the 35 °C than 45 °C shift (Figure 2, Table 2). Meanwhile, the concentration of acetate was higher at 45 °C than 35 °C. When cultures after both fluctuating temperatures where returned to 55 °C, they both showed similar patterns in their metabolite distribution with only slight variations in their frequencies (Figure 2, Table 2).

The proportion of experimental to theoretical H₂ yield (calculated based on acetate and butyrate concentrations) during and after the temperature fluctuation to 35 °C was 79, 99 and 90% in steps 3, 4 and 5, respectively. For cultures exposed to 45 °C, the ratio was 71, 88 and 89% in steps 3, 4 and 5, respectively. Lactate and propionate, have been reported to be involved in H₂ consuming pathways, which leads to H₂ yields which are significantly lower than the theoretical values calculated using only the concentrations of acetate and butyrate (Buyukkamaci and Filibeli, 2004; Hawkes et al., 2007). However, it is also possible that the decrease in H₂ production during the downward temperature fluctuation was influenced by homoacetogenic activity and to minor extent lactate production. This lead to a reduction in the H₂ yield compared to the controls incubated at 55 °C.
Chapter 3: Impacts of short-term temperature fluctuations on biohydrogen production and resilience of thermophilic microbial communities

Figure 2. Metabolites and H$_2$ produced during the downward temperature fluctuations. H$_2$ and soluble metabolite production during temperature fluctuation at A) 35 °C (step 3) and B) 45 °C and after returning the cultures back to 55 °C (steps 4 and 5).

Table 2 Contribution of each metabolite and the residual sugars to end-point COD during and after the downward temperature fluctuations.

<table>
<thead>
<tr>
<th>Parameters (%) COD</th>
<th>Fluctuation to 35 °C</th>
<th></th>
<th>Fluctuation to 45 °C</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Step 3</td>
<td>Step 4</td>
<td>Step 5</td>
<td>Step 3</td>
</tr>
<tr>
<td>Residual sugars</td>
<td>n.d</td>
<td>0.2 ± 0.2</td>
<td>n.d</td>
<td>6.4 ± 0.5</td>
</tr>
<tr>
<td>Lactate</td>
<td>3.2 ± 1.7</td>
<td>0.1 ± 0.1</td>
<td>0.4 ± 0.3</td>
<td>2.3 ± 0.7</td>
</tr>
<tr>
<td>Acetate</td>
<td>14.0 ± 0.2</td>
<td>22.1 ± 0.0</td>
<td>19.2 ± 2.4</td>
<td>16.1 ± 0.2</td>
</tr>
<tr>
<td>Ethanol</td>
<td>n.d</td>
<td>18.5 ± 0.4</td>
<td>13.3 ± 4.6</td>
<td>n.d</td>
</tr>
<tr>
<td>Butyrate</td>
<td>48.8 ± 1.0</td>
<td>30.6 ± 0.5</td>
<td>36.3 ± 6.7</td>
<td>44.1 ± 0.3</td>
</tr>
<tr>
<td>Propionate</td>
<td>n.d</td>
<td>2.9 ± 0.0</td>
<td>3.6 ± 0.1</td>
<td>n.d</td>
</tr>
<tr>
<td>Sum</td>
<td>66.0</td>
<td>74.4</td>
<td>72.8</td>
<td>68.9</td>
</tr>
</tbody>
</table>

n.d: Not detected

3.3.3. Sudden transient upward temperature shift and its effects on H$_2$ metabolism

During the upward temperature shift at 65 °C, the H$_2$ yield was 1.9 ± 0.0 mol H$_2$ mol$^{-1}$ hexose equivalent which corresponded 10.5% decrease compared to the average H$_2$ yield in the controls. The H$_2$ yield in the subsequent cultivation steps at 55°C was 12.1% (1.9 ± 0.05 mol H$_2$ mol$^{-1}$ hexose equivalent) and 21.3% (1.7 ± 0.27 mol H$_2$ mol$^{-1}$ hexose equivalent) lower in steps 4 and 5, respectively, compared to the average H$_2$ yield in the control. Temperature shift to 75 °C resulted in a complete stop of the dark fermentative microbial activity. Hence, no substrate
consumption was observed. \( \text{H}_2 \) production recovered as soon as the cultures were transferred to a fresh medium and incubated again at 55°C. However, the \( \text{H}_2 \) yield was only 0.7 ± 0.26 mol \( \text{H}_2 \) mol\(^{-1}\) hexose equivalent (67.3% decrease) and 1.13 ± 0.17 mol \( \text{H}_2 \) mol\(^{-1}\) hexose equivalent (44.9% \( \text{H}_2 \) decrease) in steps 4 and 5, respectively.

During the temporal temperature shift to 65°C, 25.5 ± 2.4% of the substrates was not consumed at the end of the 48 h period (Table 3). However, all the substrates added were consumed when the cultures were returned to 55°C. Upon the shift to 65°C, the share of acetate and ethanol increased while that of butyrate and lactate reduced (Figure 3). This was different compared to the metabolite formation obtained in the control at 55 °C where butyrate was the most abundant metabolite, followed by acetate. The ratio of experimental to theoretical \( \text{H}_2 \) yield calculated from the sum of acetate and butyrate was higher than expected (145%) for the acetate–butyrate pathway. When the temperature was returned to 55 °C, butyrate and acetate once again became the major liquid metabolites (Table 3). The ratio of experimental to theoretical \( \text{H}_2 \) yield calculated from the sum of acetate and butyrate were 82 and 74% in steps 4 and 5, respectively. Due to the higher percentage of acetate and ethanol than butyrate, it is possible that the temperature fluctuation to 65 °C induced a metabolic shift towards ethanol–acetate pathway. The ethanol–acetate pathway have a theoretical maximum of 2 mol of \( \text{H}_2 \) mol\(^{-1}\) glucose (Hwang et al., 2004; Ren et al., 2007)

\[
\text{C}_6\text{H}_{12}\text{O}_6 + \text{H}_2\text{O} \rightarrow \text{C}_2\text{H}_5\text{OH} + \text{CH}_3\text{COOH} + 2\text{CO}_2 + 2\text{H}_2
\] (4)

Similar findings where butyrate and acetate were the major liquid metabolites at 35–60°C while at 65°C the main by-product was ethanol have been reported by Qiu et al. (Qiu et al., 2017b). Some members of the order Clostridiales such as Ruminococcus albus are capable of \( \text{H}_2 \) production via the ethanol-acetate pathway. Additionally, Thermoanaerobacterium spp. has been reported to produce ethanol and acetate as the major by-products at 65 °C from xylose (Qiu et al., 2017b).

As mentioned earlier, \( \text{H}_2 \) production came to a complete stop during the temperature shift to 75°C with no substrate consumption. However, only 95% of the total substrate given was detected in the end of the incubation, indicating that the existing microbial population used 5% of the substrates for their survival mechanisms or other metabolic pathways. When the cultures were returned to 55°C after the high rise in temperature to 75°C, the substrate removal was again complete at the end of the incubation period in steps 4 and 5. After the fluctuation period at 75 °C,
H$_2$ producing activity commenced with varying concentrations of the metabolites (Figure 3, Table 3). The ratios of experimental to theoretical H$_2$ yield calculated from the sum of acetate and butyrate were 40 and 51% in steps 4 and 5, respectively.

![Figure 3 Metabolites and H$_2$ produced during the upward temperature fluctuations at A) 65 °C (step 3) and B) 75 °C and after returning the cultures back to 55 °C (steps 4 and 5).](image)

**Table 3** Contribution of each metabolite and the residual sugars to the end-point COD during and after upward temperature fluctuations.

<table>
<thead>
<tr>
<th>Parameters (% COD)</th>
<th>Fluctuation to 65 °C</th>
<th>Fluctuation to 75 °C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Step 3</td>
<td>Step 4</td>
</tr>
<tr>
<td>Residual sugars</td>
<td>22.5 ± 2.4</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>Lactate</td>
<td>3.6 ± 0.4</td>
<td>1.0 ± 0.0</td>
</tr>
<tr>
<td>Acetate</td>
<td>16.4 ± 0.7</td>
<td>21.4 ± 0.0</td>
</tr>
<tr>
<td>Ethanol</td>
<td>21.9 ± 1.2</td>
<td>19.1 ± 0.1</td>
</tr>
<tr>
<td>Butyrate</td>
<td>8.0 ± 0.4</td>
<td>31.2 ± 0.1</td>
</tr>
<tr>
<td>Propionate</td>
<td>2.8 ± 0.0</td>
<td>2.9 ± 0.2</td>
</tr>
<tr>
<td>Sum</td>
<td>75.2</td>
<td>75.6</td>
</tr>
</tbody>
</table>

n.d.: Not detected

**3.3.4. Microbial community composition during the altered temperature conditions**

The microbial community in the thermophilic control cultures incubated at 55°C was dominated by three major orders: *Thermoanaerobacterales* (94, 98 and 77% in steps 3, 4 and 5, respectively) > *Clostridiales* (3, 1 and <1%) > *Bacillales* (2, <1 and 22%). The sudden increase in *Bacillales* in
step 5 was concomitant with the decreased H\textsubscript{2} yield. The dominant member of this order was *Tumebacillus* spp., which corresponded up to 20% of relative abundance of all microorganisms detected in step 5. *Tumebacillus* spp. are gram positive, aerobic, rod shaped, and spore forming bacteria, which are able to degrade carbohydrates and have been detected from anaerobic processes (Gagliano et al., 2015). However, their role in the consortium is not known and it is not certain whether its presence was the reason of the lower yield observed in step 5, during incubation at 55°C.

**Downward temperature shifts**

Decreasing the temperature to 35°C or to 45°C for 48 h considerably influenced the microbial community composition. *Clostridiales* became the dominant order in the community (relative abundance of 84% and 74% at 35 °C and 45 °C, respectively) as seen in Figure 4. During both downward shifts, *Thermoanaerobacterales* was present in lower abundance (10% and 25% at 35 °C and 45 °C, respectively) compared to the control cultures. *Bacillales* (6% and <1% at 35 °C and 45 °C, respectively) was also present at low relative abundance at this point. The members of the order of Clostridiales identified belonged to *Clostridium* spp. The increase in temperature back to 55 °C lowered the relative abundance of *Clostridiales*, and *Thermoanaerobacterales* became again the dominant order (Figure 4). The share of other members of the consortium, some of which were known homoacetogens, was below 1%. Though in very low abundance, the metabolic capacities of this group of bacteria might have had noticeable influence on the dark fermentative metabolism (Rafrafi et al., 2013). When compared to the control cultures (incubated constantly at 55 °C), *Thermoanaerobacterales* quickly became the most dominant species in steps 4 and 5 after downward temperature fluctuations which means that short-term downward temperature fluctuation leads to the suppression of thermophilic microorganisms but due to their resilient nature, are able quickly re-adapt when the temperature becomes again favorable for their growth.
Figure 4. Microbial community composition as relative abundance of different microbial orders (>1%) during (step 3) and after (steps 4 and 5) the downward temperature shifts.

**Upward temperature shifts**

In contrast to the downward temperature shifts, temporal upward temperature shifts did not cause a significant alteration in the microbial community. *Thermoanarobacterales* remained the dominant order during and after the temperature shifts. *Thermoanaerobacterales* (97%) and *Clostridiales* (3%) were the main orders during the temperature shift at 65°C. Interestingly, during this step, it was observed that H₂ might have also been produced via the acetate–ethanol pathway due to the high amount of ethanol produced and on the fact that the ratio of experimental to theoretical H₂ produced was greater than 100%. Members of the group *Thermoanaerobacterium*, which were the most dominant group of the order *Thermoanaerobacterales*, are known to produce H₂ via the acetate and butyrate pathway. However, the ratio of experimental to theoretical H₂ yield (calculated based on acetate and butyrate) reported in the previous section, indicated
that \( \text{H}_2 \) production could not have been achieved only via the acetate and butyrate pathways. A fraction of the \( \text{H}_2 \) produced might have come from some members of the order \textit{Clostridiales} via the acetate-ethanol pathway (Ren et al., 2007), despite their low abundance. It has been shown that sub-dominant bacteria can influence the global microbial metabolic network in mixed cultures (Cabrol et al., 2017; Rafrafi et al., 2013). It would be important to identify bacteria, which are able to utilize this pathway for \( \text{H}_2 \) production in order to optimize of \( \text{H}_2 \) production efficiency even under unstable conditions.

After the cultures were taken back from 65°C to 55°C, the relative abundance of \textit{Thermoanaerobacterales} was 97%, \textit{Clostridiales} was 1% and \textit{Bacillales} was 2% (step 4). In step 5, proportion of \textit{Thermoanaerobacterales} decreased to 79%, \textit{Clostridiales} seemed to disappear from the microbial consortium, and \textit{Bacillales} increased to 21% (Figure 5).

No activity was observed during the temperature shift to 75°C suggesting that the bacteria present, did not have enough time to initiate \( \text{H}_2 \) production activity and perhaps, needed more time to adapt to such a high temperature. However, \( \text{H}_2 \) production started as soon as the culture was returned to 55 °C. In the first cycle after the fluctuation (step 4), the microbial community consisted of \textit{Thermoanaerobacterales} (91%) and \textit{Bacillales} (9%). In the second cycle after the temperature shift to 75°C (step 5), \textit{Thermoanaerobacterales} dominated the microbial community with 99.9% abundance. While \textit{Clostridiales} are able to withstand temperatures up to 55° C (Chen et al., 2012; Y. Liu et al., 2008), a further increase in the temperature to 65° C led to the decrease in their relative abundance and even to complete disappearance after the 75°C fluctuation. The decrease in the \( \text{H}_2 \) yield after the 65°C and 75°C shift can be linked to the disappearance of \textit{Clostridiales} from the consortium. Although most of the studies on dark fermentative \( \text{H}_2 \) production have focused on key-stone species as having the most significant impact on biological processes, it has been shown that sub-dominant bacteria can also have a significant effect despite their low abundance (Rafrafi et al., 2013). Therefore, it is suggested that the presence of \textit{Clostridiales} in the consortium had a significant role in \( \text{H}_2 \) productivity, hence the low yield obtained following its disappearance from the microbial consortium. When compared to the control cultures (incubated constantly at 55 °C), Thermoanaerobacterales remained the most abundant species in steps 4 and 5 after upward temperature fluctuations. However, the relative abundance was higher, compared to the control cultures (especially after the 75 °C fluctuation) while Clostridiales disappeared from the consortium.
Figure 5 Microbial community composition as relative abundance of different microbial orders (>1%) during (step 3) and after (steps 4 and 5) the upward temperature shifts.

3.3.5. Comparison between cultures exposed to temporal downward and upward temperatures

H₂ production recovered faster after the downward temperature fluctuations than after the upward temperature fluctuations. This is in line with results of Huang et al. (2004) (Huang et al., 2004) who suggested that for thermophilic systems, a longer adaptation time is often required for new temperature conditions (see Table 4).
Table 4. Percentage maximum relative H$_2$ yield (compared to H$_2$ yield obtained in the controls) obtained during the temperature fluctuations and after the fluctuation periods.

<table>
<thead>
<tr>
<th>Culture conditions</th>
<th>Temperature Fluctuation</th>
<th>Step 4 (55 °C)</th>
<th>Step 5 (55 °C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shift to 35 °C</td>
<td>84.3</td>
<td>107.6</td>
<td>96.5</td>
</tr>
<tr>
<td>Shift to 45 °C</td>
<td>75.9</td>
<td>96.8</td>
<td>91.6</td>
</tr>
<tr>
<td>Shift to 65 °C</td>
<td>90.9</td>
<td>89.3</td>
<td>79.9</td>
</tr>
<tr>
<td>Shift to 75 °C</td>
<td>0</td>
<td>30.9</td>
<td>54.1</td>
</tr>
</tbody>
</table>

The resilience of a microbial consortium is the capacity of the microorganisms in the consortium to recover quickly from process disturbances. Resilience and functional redundancy are the basic mechanisms via which microbial communities are able to maintain community function when disturbance is introduced into a biological system (Konopka et al., 2015; Werner et al., 2011). The current result suggests that the performance during and after the temperature fluctuations was a consequence of a microbial community with higher resilience to the downward temperature shifts than to the upward temperature shifts. Change in the relative abundance of Clostridiales and Thermoanaerobacteriales due to the transient changes in the incubation temperature illustrates the robustness and adaptability of the mixed microbial community to the new incubation conditions. The presence of members of these orders helped to maintain continuous H$_2$ production process during the fluctuating conditions, despite the lower H$_2$ yield observed. The species belonging to Thermoanaerobacteriales are known for their abilities to survive in environments of extremely high temperature (O-Thong et al., 2011a; Qiu et al., 2017a). Thermoanaerobacterium spp. belonging to the Thermoanaerobacteriales was the most abundant genus in the consortium and demonstrated the ability to function at all the studied temperature. Clostridiales was the most abundant order at 35 °C. Its level of relative abundance decreased with increasing temperatures up to 65 °C and disappearance at 75 °C. Clostridiales differ in their optimal growth conditions compared to Thermoanaerobacteriales (Bader and Simon, 1980; Mtimet et al., 2016). However, both groups are metabolically similar which allows for flexibility in H$_2$ production performance when process disturbances occur. Other bacteria present in the consortium enabled higher microbial diversity and increase of the system robustness. Nonetheless, the presence of homoacetogens, though in very low abundance, created a negative impact on the H$_2$ production. The results obtained in this study showed that H$_2$ production due to temperature disturbances is a result of changes occurring in the metabolic networks and the
microbial community composition. Temperature therefore plays an important role in microbial community stability and resilience.

Additionally, as observed in this study, the effects of short-term temperature fluctuations on the microbial communities depend on the extent of the temperature fluctuation. For example, after the downward temperature fluctuations, the thermophilic microorganisms were able to re-adapt to their original conditions without any identifiable loss in microbial diversity in the community. On the contrary, after the upward temperature fluctuations, the microbial community was different from that observed in the control incubated constantly at 55 °C. In the control, Clostridiales were present, although in low abundance and were considered to play a role in H\textsubscript{2} production, as their disappearance after the temperature fluctuations led to reduced H\textsubscript{2} production in steps 4 and 5. This might lead to reduced H\textsubscript{2} production also in the long term due to loss of microbial diversity. Therefore, recovery strategies such as bioaugmentation to optimize the H\textsubscript{2} production could be required after unexpected upward temperature fluctuations. However, this subject requires further studies.

3.4. CONCLUSIONS

Sudden, even temporal upward and downward temperature fluctuations had a direct impact on microbial community structure, the soluble metabolites produced and the H\textsubscript{2} production. A mixed microbial culture enriched for H\textsubscript{2} production at 55 °C recovered more rapidly enabling similar H\textsubscript{2} yield (92-108%) after returning to original temperature of 55 °C as the control culture kept at constant temperature (55 °C). Upward temperature shifts from 55 to 65 or 75 °C had more significant negative effect on dark fermentative H\textsubscript{2} production than downward temperature shifts and H\textsubscript{2} yield remained lower than the control (55 °C) after the temperature was returned to 55 °C (31-89%). The likely reason for this was that upward temperature shifts resulted in more significant loss of microbial diversity. Based on the observations made in this study, attention should be paid towards optimizing operational parameters during bioreactor operations, especially with regards to factors that may lead to unexpected increase in temperature such as high organic loading rate. Thus, cooling systems are recommended. Alternatively, adding known H\textsubscript{2} producers (i.e. bioaugmentation) characterized by wide temperature ranges might help to improve the robustness of the system by making up for the loss in microbial diversity enhancing the stability and resilience of the microbial consortium to adverse environmental changes and consequently improve the performance of the H\textsubscript{2} production process.
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REFERENCES


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CHAPTER 4

Bioaugmentation enhances dark fermentative hydrogen production in cultures exposed to short-term temperature fluctuations

ABSTRACT

Hydrogen-producing mixed cultures were subjected to a 48-hours (h) downward or upward temperature fluctuation from 55 to 35 or 75 °C. Hydrogen production was monitored during the fluctuations and for three consecutive batch cultivations at 55 °C to evaluate the impact of temperature fluctuations and bioaugmentation with synthetic mixed culture of known H₂-producers either during or after the fluctuation. Without augmentation, H₂ production was significantly reduced during the downward temperature fluctuation and no H₂ was produced during the upward fluctuation. H₂ production improved significantly during temperature fluctuation when bioaugmentation was applied to cultures exposed to downward or upward temperatures. However, when bioaugmentation was applied after the fluctuation, i.e., when the cultures were returned to 55 °C, the H₂ yields obtained was between 1.6 to 5% higher than when bioaugmentation was applied during the fluctuation. Thus, the results indicate the usefulness of bioaugmentation in process recovery, especially if bioaugmentation time is optimized.
4.1. INTRODUCTION

Biological methods for H₂ production, including biophotolysis, photo fermentation, dark fermentation and biocatalyzed electrolysis, have received increasing attention due to their ability to utilize renewable feedstocks such as organic wastes, plant biomass residues or sunlight for H₂ generation (Dincer, 2012; Hallenbeck and Benemann, 2002). Among the above stated biological H₂ production methods, dark fermentation offers several advantages, such as no requirement for light, higher H₂ production rates, simple bioreactor setup and wide versatility in the choice of the substrate (Chong et al., 2009; Kargi et al., 2012).

Dark fermentative H₂ production can be carried out at mesophilic, thermophilic and hyperthermophilic conditions (Kargi et al., 2012; Shin et al., 2004) but is thermodynamically more favourable at higher temperatures (Zhang et al., 2014; Zheng et al., 2015). In addition, thermophilic bioprocesses typically result in higher H₂ production rates as well as inhibition of pathogens (Sahlström, 2003) and H₂ consumers such as methanogens and homoacetogens (Dessì et al., 2018b, 2018a). However, decreased bacterial diversity at higher temperatures can lead to instability of the bioprocess (Westerholm et al., 2018). Thermophilic processes are typically more sensitive to temperature fluctuations and require more consistent organic loading rate than mesophilic dark fermentation processes (Angelidaki and Ahring, 1994). At high loading rates, temperature of anaerobic processes can also increase due to high microbial activity (Daverio et al., 2003; Lindorfer et al., 2006). Possible upsets might also occur much faster at high temperatures due to faster microbial metabolism. However, the extent of the impact of varying bioreactor temperatures depend on the microbial communities present and the magnitude and duration of the temperature change (Okonkwo et al., 2019). A sudden, even transient temperature changes can produce varying responses in microbial populations resulting in an imbalanced metabolism and low process performance (Jiang and Morin, 2007; Okonkwo et al., 2019).

In the event of unwanted temperature fluctuation, restoring the activity of the microorganisms catalysing the biological processes can be time-consuming and cost intensive. One way to stabilise performance of a bioreactor during process disturbances, is to augment the bioreactor with H₂ producing microorganisms (Ren et al., 2010; Wang et al., 2008). Over the years, bioaugmentation has been successfully applied for example to reduce the start-up time of dark fermentation (Pandit et al., 2015). Bioaugmentation was shown to protect the existing microbial community from organic loading shocks and reduce the susceptibility of the H₂-producing bioreactors to process disturbances (Ács et al., 2015; Goud et al., 2014; Venkata Mohan et al., 2015).
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2009). In a study by Guo et al. (2010), *Escherichia coli, Enterobacter aerogenes*, and *Bacillus subtilis* were separately used under mesophilic conditions to improve H$_2$ production from organic fraction of municipal solid waste (Guo et al., 2010). In another study, Goud et al. (2014) used acidogenic bacteria to enhance the H$_2$ production from food waste at an elevated organic load of 50 g L$^{-1}$ of the waste. Bioaugmentation strategy has also been applied as a means to decrease the recovery time of anaerobic digesters stressed by organic overloading (Acharya et al., 2015; Goud et al., 2014).

Different bacterial species possess varied optimal growth requirements and capacities to cope with stress related to changes in cultivation conditions such as changes in temperature or high H$_2$ partial pressures (Pawar and van Niel, 2013). Therefore, the microorganisms chosen for bioaugmentation purposes should have the desirable properties needed to perform the required function under the specific operational conditions. In our previous study, we showed that effects of temporal temperature fluctuations on dark fermentative H$_2$ production were more severe during and after upward temperature fluctuations (from 55 °C to 65 °C or 75 °C) than during and after downward temperature fluctuations (from 55 °C to 35 °C and from 55 °C to 45 °C) (Okonkwo et al., 2019). The aim of this study was to investigate the effects of augmenting thermophilic mixed cultures during and after periods of temperature shock, with a synthetic mixture of well-known H$_2$ producers and the importance of bioaugmentation time on dark fermentative H$_2$ production. To our knowledge, bioaugmentation with known H$_2$ producing bacteria has not been previously studied as a means to resolve the adverse effects caused by sudden temperature fluctuations.

4.2. MATERIALS AND METHODS

4.2.1. Enrichment culture and medium composition

The inoculum used in this study was obtained from a H$_2$ producing thermophilic (55 °C) continuous stirred tank reactor (CSTR) and the medium composition used is as described by Okonkwo et al. (2019). For the enrichment, anaerobically digested sludge heat treated at 90°C for 20 min was used for inoculation of the CSTR by adding 10% of the sludge to a final working volume of two litres. The reactor was flushed with nitrogen for 5 min and then operated in continuous mode at hydraulic retention time of 6 h and at 55 °C for a period of 21 days maintaining the pH at 6.5. The enriched microbial community consisted of members belonging to the genus *Thermoanaerobacterium, Clostridium* and *Bacillus* (Okonkwo et al., 2019). The medium contained the following compounds in mg L$^{-1}$: K$_2$HPO$_4$, 500; NH$_4$Cl, 100; MgCl$_2$ · 6H$_2$O, 120; H$_8$FeN$_2$O$_6$S$_2$ · 6H$_2$O, 55.3; ZnCl$_2$, 1.0; MnCl$_2$ · 4H$_2$O, 2.0; CuSO$_4$, 000.4; (NH$_4$)$_6$Mo$_7$O$_24$, 1.2;
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\[ \text{C}_6\text{H}_2\text{O}_3, 1.3; \text{H}_2\text{BO}_3, 0.1; \text{NiCl}_2 \cdot 6\text{H}_2\text{O}, 0.1; \text{Na}_2\text{O}_3\text{Se}, 0.01; \text{CaCl}_2 \cdot 2\text{H}_2\text{O}, 80; \text{yeast extract}, 500 \]
and 0.055 mL 37% HCl. The culture was fed with glucose (800 mg L\(^{-1}\)) and xylose (1200 mg L\(^{-1}\)). Utilizing both glucose and xylose is a practical way to move towards efficient bioconversion of lignocellulosic wastes to H\(_2\).

4.2.2. Synthetic mixed culture used for bioaugmentation

The following bacterial strains from DSMZ, Germany were selected for bioaugmentation: *Thermoanaerobacter thermohydrosulfuricus* (DSM-567), *Caldicellulosiruptor saccharolyticus* (DSM-8903), *Clostridium thermocellum* (DSM-1237), *Thermoanaerobacterium thermosaccharolyticum* (DSM-571) and *Thermotoga neapolitana* (DSM-4359). All the species are strictly anaerobic thermophiles except for *T. neapolitana* which can tolerate low oxygen concentration (Van Ooteghem et al., 2004). Together these bacteria form a synthetic consortium that has the following properties: Thermophilic with broad range of temperatures, not auxotrophic to any amino acids, and able to degrade wide range of organic substrates (Pawar and van Niel, 2013). Table 1 further shows the properties of the bacteria inoculated to the synthetic mixed culture used for bioaugmentation. The bacterial strains were cultivated individually at 65 °C using the medium previously described above and were then mixed together in a 1:1 ratio based on optical density to obtain the synthetic mixed culture. As individual cultures, the selected microorganisms are efficient H\(_2\) producers and are able to proliferate in the medium provided for this study. The synthetic culture was cultivated in batch (for 4 days) at 65 °C for three consecutive transfers to a final OD\(_{600}\) of 1.1 (on day 4 of the third batch cultivation cycle). The final synthetic culture was then used to study the effect of bioaugmentation during and after the temperature fluctuation periods as described in the next section. Since the bacteria in the consortium have growth temperatures ranging between 55 and 80 °C, 65 °C was considered as suitable temperature for the pre-cultivation. In addition, as our previous study showed that upward temperature fluctuations had more severe impacts on H\(_2\) production than downward temperature fluctuations (Okonkwo et al., 2019), incubation of the synthetic mixed culture at a temperature higher than 55 °C was hypothesized to provide the needed enhancement of H\(_2\) production especially in the cultures exposed to upward temperature fluctuations.
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Table 1 Properties (growth temperature, pH range and oxygen sensitivity) of bacteria inoculated to the synthetic mixed culture used for bioaugmentation.

<table>
<thead>
<tr>
<th>Bacterial species</th>
<th>Temperature (°C)</th>
<th>pH ranges</th>
<th>Oxygen sensitivity</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thermoanaerobacter thermohydrosulfuricus</td>
<td>55-75</td>
<td>5.8-8.5</td>
<td>Obligate anaerobe</td>
<td>Vos et al. 2011</td>
</tr>
<tr>
<td>Caldicellulosiruptor saccharolyticus</td>
<td>65-75</td>
<td>5.2-9</td>
<td>Obligate anaerobe</td>
<td>Vos et al. 2011</td>
</tr>
<tr>
<td>Clostridium thermocellum</td>
<td>60-64</td>
<td>5.6-5.8</td>
<td>Obligate anaerobe</td>
<td>Vos et al. 2011</td>
</tr>
<tr>
<td>Thermoanaerobacter thermosaccharolyticum</td>
<td>50-80</td>
<td>5.5-9</td>
<td>Obligate anaerobe</td>
<td>Vos et al. 2011</td>
</tr>
<tr>
<td>Thermotoga neapolitana</td>
<td>55-95</td>
<td>5.5-9</td>
<td>Obligate anaerobe but tolerates low oxygen</td>
<td>Jannasch et al. 1988; Van Ooteghem et al. 2004</td>
</tr>
</tbody>
</table>

4.2.3. Experimental procedure

The batch experiments were carried out in gas-tight 500 mL polypropylene centrifuge tubes (69 x 160 mm, Beckman Coulter) designed for gas and liquid sampling as well as centrifugation and cultivation. The microbial inoculum used in each batch corresponded to 10% of the total working volume (200 mL). Each cultivation tube was flushed with nitrogen for 5 min before starting the incubation.
Figure 1 Experimental setup to study the effects of bioaugmentation with a synthetic mixed culture on H₂ production during and after temperature stress periods. All cultures were first incubated in batch at 55 °C for 48 h. Then some of the cultures were subjected to a 48-h temperature fluctuation at 35 or 75 °C (step 1) and subsequently incubated at 55 °C for three consecutive 48-h batch cultivation steps (steps 2, 3 and 4) (a). The experiment included also control cultures, which were incubated after the acclimatization period at 55 °C for four consecutive batch cultivation steps with and without bioaugmentation in step1 (b).
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Prior to exposing the cultures to temporal temperature fluctuations, the mixed culture inoculum was first acclimatized to batch growth conditions by incubating at 55°C for 48 h as shown in Figure 1. At 55 °C, the substrates were completely consumed by the end of the 48 h period. After the acclimatization, the cultures were subjected to either downward (from 55 to 35 °C) or upward temperature fluctuation (from 55 to 75 °C) of 48 h (referred to as step 1 in Figure 1) to evaluate the impact of temperature fluctuation on H₂ production. After the stress period, each culture was centrifuged for 5 minutes; the spent medium was decanted and replaced with fresh medium, and the culture was then incubated again at 55 °C for 48 h. This was carried out for altogether three consecutive batch cultivations (steps 2, 3 and 4) to estimate the H₂ production recovery after the temperature fluctuations. To determine the impact of bioaugmentation, cultures exposed to similar conditions (downward or upward temperature fluctuation) were augmented with the synthetic mixed cultures described in the previous section, either during or after the temperature fluctuation period (in the beginning of step 1 or in the beginning of step 2). The reason for the different bioaugmentation times was to monitor the effect of bioaugmentation time on H₂ production. As a control, batch cultivations were also carried out at 55 °C for four consecutive cycles (step 1 to 4). The cultures were either unaugmented or augmented with the synthetic mixed culture in the beginning of step 1, to study the effect of bioaugmentation under stable incubation temperature. The synthetic mixed culture / inoculum (volume of synthetic mixed culture to volume of mixed culture inoculum) ratio that was used for enhancing H₂ production during or after temperature fluctuation was 0.2. This bioaugmentation ratio was chosen, because according to Sharma and Melkania (2018), the highest cumulative H₂ production and volumetric H₂ production was obtained with the bacteria/sludge ratio of 0.2 and 0.25, when they studied the effect of bioaugmentation on H₂ production from organic fraction of municipal solid waste.

The pH during the experiments was adjusted to 6.5 and the incubation temperatures were attained using temperature-controlled water baths. The experimental design described is similar to that employed by Okonkwo et al. (2019) to study the impact of temperature fluctuations on dark fermentative H₂ production.

4.2.4. Analytical techniques and calculations conducted

Glucose, xylose, organic acid and alcohol concentrations were measured by high performance liquid chromatography (HPLC) using a refractive index detector (Waters R410) as described previously by Monlau et al. (2013). The gas composition was analysed by a gas chromatograph (Clarus580, Perkin Elmer, Waltham, USA) equipped with a thermal conductivity detector (399152,
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Linde, Munich, Germany) and two columns: RtQBond to split H₂, O₂, N₂, CH₄ and RtMolsieve (5Å) to quantify CO₂. The carrier gas was argon at a pressure of 3.5 bar. The temperatures of the oven, injector and detector were 60 °C, 250 °C and 150 °C, respectively. The gas volume and composition measurements were conducted at the respective incubation temperatures mentioned in section 2.3 by retaining the cultivation bottles in the water baths during the sampling. The total volume of produced H₂ was calculated at standard temperature using Equation 1 (Logan et al., 2002).

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

(1) 

Where \( V_{H_2,t} \) is the cumulative H₂ gas produced at time t, \( V_{H_2,t-1} \) is the cumulative H₂ gas produced at 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 H₂ gas fraction in the headspace at time t, \( C_{H_2,t-1} \) is the H₂ gas fraction in the headspace at time t-1 and \( V_H \) is the total headspace volume in the culture bottle. H₂ production in moles was calculated on the basis that one mole of an ideal gas will occupy a volume of 22.4 L at standard temperature and pressure according to the ideal gas law. H₂ yield was calculated by dividing the mol H₂ per mole of hexose equivalent using the conversion factor of 5/6 for converting xylose to its hexose equivalent. Total chemical oxygen demand (COD) of soluble compounds was calculated based on the sum of acids, ethanol and residual sugars by using the following conversion factors: 1 mM glucose = 192 mg COD L⁻¹, 1 mM xylose = 160 mg COD L⁻¹, 1 mM acetate = 64 mg COD L⁻¹, 1 mM propionate = 112 mg COD L⁻¹, 1 mM lactate = 96 mg COD L⁻¹, 1 mM butyrate = 160 mg COD L⁻¹ and 1 mM ethanol = 96 mg COD L⁻¹ (Gonzales and Kim, 2017; Sivagurunathan and Lin, 2016).

The relative H₂ yield obtained during and after the temperature fluctuations was calculated using the equations obtained in the unaugmented control using Equation 2:

\[ \text{Relative } H_2 \text{ yield (\%) } = \frac{\text{H}_2 \text{ yield obtained during/after temperature shift}}{\text{average } H_2 \text{ yield obtained from the unaugmented control}} \times 100 \]  

(2) 

4.2.5. Microbial analysis for unaugmented and augmented samples during temperature fluctuation

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 (Wang and Qian, 2009) including their respective linkers were used to amplify the V4_V5 region of the 16S rRNA gene. The resulting products were purified and sequenced using Illumina MiSeq. Sequencing and library preparation were performed at the Genotoul Lifescience
Network Genome and Transcriptome Core Facility in Toulouse, France. Sequence analysis was performed as previously described by Venkiteshwaran et al. (2016). The 16S rRNA sequences used to support the findings of this study have been deposited in the NCBI Sequence Read Archive under project file SUB6057113: MN203768 - MN203978.

4.3. RESULTS

4.3.1. Characterization of bacteria in the synthetic mixed culture

Bacteria belonging to the genera *Thermoanaerobacter*, *Caldicellulosiruptor*, *Clostridium*, *Thermoanaerobacterium* and *Thermotoga* were added to the synthetic mixed culture that was used for bioaugmentation in this study. After incubation of the synthetic mixed culture for three consecutive 4-day batch cultivations at 65 °C, the microbial characterization revealed that the community included all the added bacterial genera with the exception of *Clostridium* (Figure 2). Compared to all the other bacteria in the culture, *Thermoanaerobacter* was seen to have the highest relative abundance (60%), followed by *Thermoanaerobactium* (25%). *Thermotoga* and *Caldicellulosiruptor* had an abundance of 8 and 7%, respectively.

![Figure 2](image)

*Figure 2* Relative abundance (%) of each genera in the synthetic mixed culture used for bioaugmentation of the native mixed culture during and after the temperature fluctuations.
4.3.2. **Comparison between augmented and unaugmented cultures at constant temperature of 55 °C**

All the substrates (800 mg L\(^{-1}\) glucose and 1200 mg L\(^{-1}\) xylose) added to the unaugmented control cultures incubated at constant temperature of 55 °C were consumed within the 48 h period. The H\(_2\) yield obtained from the unaugmented cultures was 1.85 ± 0.01, 1.80 ± 0.03, 1.86 ± 0.06 and 1.89 ± 0.10 mol H\(_2\) mol\(^{-1}\) hexose equivalent in steps 1, 2, 3 and 4, respectively, resulting in an average H\(_2\) yield of 1.85 ± 0.04 mol H\(_2\) mol\(^{-1}\) hexose equivalent.

To determine the influence of bioaugmentation without any temperature stress, cultures incubated at constant temperature of 55 °C were augmented with the synthetic mixed culture in the beginning of step 1. The H\(_2\) yield obtained in the bioaugmented control cultures was 2.19 ± 0.08, 2.07 ± 0.06, 2.07 ± 0.16 and 1.94 ± 0.01 mol H\(_2\) mol\(^{-1}\) hexose equivalent in step 1, 2, 3 and 4, respectively. Thus, the average H\(_2\) yield was 2.07 ± 0.09 mol H\(_2\) mol\(^{-1}\) hexose equivalent. The average H\(_2\) yield obtained with bioaugmentation was significantly higher than the average yield obtained from the unaugmented control cultures. A one-way ANOVA between the unaugmented and the augmented control cultures showed that the difference in H\(_2\) yield was statistically significant (p < 0.05).

Bioaugmentation had also a clear impact on the distribution of the soluble metabolites. The metabolites observed in all conditions included acetate, butyrate, ethanol and traces of lactate and propionate. The unaugmented cultures had a higher percentage of butyrate in all steps (Figure 3a), while bioaugmentation altered the metabolic profile by increasing the share of acetate production (Figure 3b). Propionate was also detected in steps 2 and 4 in the bioaugmented cultures.
Figure 3 Hydrogen yield and the contribution of the residual sugars and soluble metabolites to the endpoint COD at 55 °C in (a) the unaugmented control cultures and (b) in the augmented control cultures. Data represents mean values and standard deviation from duplicate cultivations. The red rectangle indicate the point at which bioaugmentation was applied.

4.3.3. Process recovery after the downward temperature shift and the impact of bioaugmentation

A downward temperature fluctuation during dark fermentation was shown to have a negative impact on H₂ production in the unaugmented cultures. The temperature shift from 55 to 35 °C in step 1 resulted in a H₂ yield of 1.35 ± 0.13 mol H₂ mol⁻¹ hexose equivalent (Figure 4a), which is 27% lower than the average H₂ yield obtained under stable conditions with the unaugmented control (Figure 3a). After the temperature fluctuation period, the H₂ yield increased gradually from step 2 to step 3 (Figure 4a). The H₂ yields obtained in steps 2, 3 and 4 (after temperature fluctuation) were 1.52 ± 0.13, 1.75 ± 0.04, 1.66 ± 0.09 mol H₂ mol⁻¹ hexose equivalent, respectively.

Cultures to which the bioaugmentation was applied at the beginning of temperature fluctuation (step 1) gave a H₂ yield of 1.84 ± 0.04 mol H₂ mol⁻¹ hexose equivalent during the fluctuation. The obtained H₂ yield was similar to that observed with unaugmented control cultures incubated at 55 °C (1.85 ± 0.04 mol H₂ mol⁻¹ hexose equivalent), which indicated that the negative impact
caused by the downward temperature fluctuation could be compensated by bioaugmentation with the synthetic mixed culture. The $\text{H}_2$ yield further increased to a maximum of $1.87 \pm 0.01 \text{ mol H}_2 \text{ mol}^{-1}$ hexose equivalent in step 3 when the temperature was returned to $55 \degree \text{C}$ (Figure 4b).

In cultures to which the bioaugmentation was applied after the temperature fluctuation (step 2), $\text{H}_2$ yield was higher than in the cultures to which the bioaugmentation was applied at the beginning of the temperature fluctuation, being $1.91 \pm 0.05$, $1.92 \pm 0.02$ and $1.92 \pm 0.01 \text{ mol H}_2 \text{ mol}^{-1}$ hexose equivalent in steps 2, 3 and 4, respectively (Figure 4c).

**Figure 4** Hydrogen yield and the contribution of the residual sugars and soluble metabolites to the endpoint COD during and after the downward temperature fluctuation from 55 to 35 °C (a) without bioaugmentation, (b) with bioaugmentation applied in the beginning the temperature fluctuation (step 1) and (c) with bioaugmentation applied after the temperature fluctuation in the beginning of step 2. Data represents mean values and standard deviation from duplicate cultivations. The red rectangles indicate the point at which bioaugmentation was applied.

The total soluble metabolic end-products calculated based on their COD was between 74 and 90% of the initial COD added as xylose and glucose (Figure 4). In the unaugmented cultures, the metabolites produced during the temperature fluctuation (step 1) were acetate, butyrate, ethanol, lactate and propionate (Figure 4a). The shift in temperature back to $55 \degree \text{C}$ caused a shift in the
distribution of the soluble metabolic products as acetate and ethanol showed a slight increase while the concentration of lactate reduced significantly and was not at all detected in steps 3 or 4 (Figure 4a). In cultures to which the bioaugmentation was applied during the temperature fluctuation, the share of acetate and ethanol increased significantly compared to the unaugmented cultures. Lactate slightly increased from step 1 to step 2 but was not detected in steps 3 and 4. Meanwhile, very low concentrations of propionate were also detected in steps 3 and 4 (Figure 4b). When the bioaugmentation was applied after the temperature fluctuation, acetate and ethanol share also increased compared to the unaugmented culture. Lactate was detected in step 2 but was not detected in steps 3 and 4 (Figure 4c), similarly to the cultures to which bioaugmentation was conducted during the fluctuation (Figure 4b).

During the downward temperature shift, *Thermoanaerobacterium* spp. was less dominant in the unaugmented cultures compared to incubation at 55 °C with a share of 27%, while *Clostridium* and *Bacillus* had a relative abundance of 22% and 31%, respectively. Other genera accounted for 18% of the community. Bioaugmentation in the beginning of the temperature fluctuation caused an increase in the relative abundance of *Thermoanaerobacterium* spp. from 27% in the unaugmented culture to 72%, which suggests that the *Thermoanaerobacterium* added was involved in H₂ production during the downward temperature fluctuation despite the low temperature.

### 4.3.4. Process recovery after the upward temperature shift and impact of bioaugmentation

Upward temperature fluctuation from 55 to 75°C was shown to have a severe impact on microbial metabolism as no H₂ production was observed within the 48 h incubation period in the unaugmented culture or in the cultures to which bioaugmentation was done in the beginning of the temperature fluctuation (step 1). In the unaugmented cultures, when the temperature was taken back to 55 °C (step 2), H₂ production occurred (1.27 ± 0.06 mol H₂ mol⁻¹ hexose equivalent) and gradually increased to 1.43 ± 0.08 and 1.45 ± 0.01 mol H₂ mol⁻¹ hexose equivalent in steps 3 and 4, respectively (Figure 5a). However, the highest H₂ yield which was obtained in step 4 was still significantly lower (approximately 21%) than the average H₂ yield obtained under stable temperature conditions (unaugmented control at 55 °C).

In the cultures augmented in the beginning of the temperature fluctuation period, H₂ yield was 1.45 ± 0.05, 1.74 ± 0.13, 1.89 ± 0.08 mol H₂ mol⁻¹ hexose equivalent in steps 2, 3 and 4,
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respectively (Figure 5b). The H₂ production was higher than in the unaugmented culture exposed to upward temperature fluctuation and the highest H₂ yield obtained (in step 4) was comparable to the H₂ yield in the unaugmented control kept at 55 °C. In the cultures augmented after the temperature fluctuation period (in step 2), H₂ yield was 1.53 ± 0.06, 1.76 ± 0.08, and 1.93 ± 0.04 mol H₂ mol⁻¹ hexose equivalent in steps 2, 3 and 4, respectively (Figure 5c).

**Figure 5** H₂ yield and the contribution of the residual sugars and soluble metabolites to the endpoint COD during and after the downward temperature fluctuation from 55 to 75 °C (a) without bioaugmentation, (b) with bioaugmentation applied during temperature fluctuation (step 1) and (c) with bioaugmentation applied after temperature fluctuation in the beginning of step 2. Data represents mean values and standard deviation from duplicate cultivations. The red rectangles indicate the point at which the bioaugmentation was applied.

During the upward temperature fluctuation, no VFAs and alcohols were formed in the unaugmented or in the cultures augmented in the beginning of the fluctuation, which further verified the absence of microbial activity. In the unaugmented cultures, the metabolites produced were acetate, butyrate, ethanol and lactate, when the temperature was shifted back to 55 °C (Figure 5a). Butyrate had the highest share of produced soluble metabolites, followed by ethanol and acetate. Lactate, which contributed 12% in step 2, reduced to 4% in step 4, while the share
of acetate and ethanol increased slightly. However, the contribution of butyrate remained relatively constant (Figure 5a). In the cultures to which the bioaugmentation was applied in the beginning of the temperature fluctuation, butyrate remained the most abundant soluble metabolite (Figure 5b). In the cultures to which the bioaugmentation was applied after the temperature fluctuation, there was also increased acetate and ethanol production observed and the share of lactate reduced gradually with each incubation step (Figure 5c), similar to the cultures augmented in the beginning of the fluctuation (Figure 5b).

4.4. DISCUSSION

In this study, it was observed that bioaugmenting native microbial communities with synthetic mixed cultures during and after upward or downward temperature fluctuation enhanced H₂ production and thus limited the negative impact observed in the control cultures. Prior to the augmentation, microbial data of the synthetic mixed culture used showed differences in the microbial distribution with *Thermoanaerobacter* having a higher relative abundance (60%) than the other species added, followed by *Thermoanaerobacterium*, *Thermotoga* and *Caldicellulosiruptor* (Figure 2). The difference in the relative distribution observed in the synthetic mixed culture was likely a result of the different growth rates of the different bacteria at the selected growth conditions (Akinosho et al., 2014; Vanfossen et al., 2009; Yu and Drapcho, 2011). Of all the species added to the synthetic culture, only *C. thermocellum* was not detected in the final synthetic mixed culture used for bioaugmenting. This was likely because the ability of the other bacteria to utilize xylose gave them a competitive advantage over *C. thermocellum*, since it does not metabolize xylose (Wilson et al., 2013) and has been shown to grow poorly on glucose (Ng and Zeikus, 1982). The preferred soluble sugars of *C. thermocellulum* are cellulose, cellobiose or cellodextrins (Stevenson and Weimer, 2005; Zhang and Lynd, 2005). Therefore, *C. thermocellulum* was already lost before the bioaugmentation of the synthetic cultures (Figure 2).

Compared to the unaugmented control culture incubated at constant temperature of 55 °C, the bioaugmented control cultures demonstrated an increase in H₂ production compared to the unaugmented cultures. Additionally, the relative abundance of *Thermoanaerobacterium* spp., increased in the augmented cultures compared to the unaugmented cultures. Relating this observation to H₂ production, suggests that *Thermoanaerobacterium* spp. had the most significant impact on the H₂ production and might have influenced the increase in acetate concentration since it is capable of producing large amounts of acetate (Cao et al., 2010; O-Thong et al., 2008). The relative abundance of the other genera representing the added species was quite low.
(Caldicellulosiruptor had a relative abundance of 0.02%, Thermoanaerobacter, 0.3% and Thermotoga, 0.04%).

For the cultures exposed to the downward temperature fluctuation, the relative H₂ yield (H₂ yield compared to the unaugmented control) after the temperature fluctuation period was 5 to 10% lower than the H₂ yield obtained the unaugmented control at 55 °C. This implied that the downward temperature fluctuation caused a reduction in H₂ yield even after three consecutive batch incubations at 55 °C. Gadow et al. (2013) reported similar observations when a H₂ producing stirred tank reactor operated in a continuous mode with hydraulic retention time of 10 days was exposed to 24-h temperature fluctuation from 52 to 32 °C. They demonstrated a decrease of 27% in the H₂ content during the temperature shock. Furthermore, the maximum H₂ content they achieved after 10 days of recovery (at 55 °C) was 9% lower than the value before the temperature fluctuation. As shown in Figure 4c, H₂ production was enhanced when bioaugmentation was applied in the beginning of the temperature fluctuation (in step 1). However, based on the differences in H₂ production observed with the different bioaugmentation times, the short-term temperature fluctuation also caused stress on the microorganisms used in the bioaugmentation even though the differences were not statistically significant (p > 0.05). Thus, in order to maximize the recovery of H₂ production after downward temperature fluctuations, it seems advisable to conduct the bioaugmentation after the fluctuation period. Alternatively, repeated bioaugmentation applied as soon as an unwanted temperature fluctuation is observed and after the temperature has been restored to a desired level, might also enable maximal process recovery after a temperature fluctuation period. For example, Yang et al. (2016) showed improved performance of anaerobic digestion with the application of repeated bioaugmentation.

The addition of synthetic mixed cultures during or after downward temperature fluctuation showed a positive impact in the enhancement of H₂ production and improved the recovery time of bacterial activity when compared to the unaugmented cultures. Comparison between the unaugmented and the augmented cultures exposed to the downward temperature fluctuation showed that Thermoanaerobacterium spp. added into the microbial consortium played a significant role in the H₂ production observed during the downward temperature fluctuation. Other species present in the synthetic mixed culture used for bioaugmentation had a much lower relative abundance during the downward temperature fluctuation, as Thermoanaerobacter had a relative abundance of 0.8%, Caldicellulosiruptor 0.02% and Thermotoga 0.2%. Thus, they might have had little or no influence on the enhancement of H₂ production, although Rafrafi et al. (2013) have reported that,
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despite low levels of abundance, subdominant species are able to influence the global microbial metabolic network in mixed cultures.

Cultures exposed to the upward temperature fluctuation had a completely different response to H₂ production when compared to the cultures exposed to the downward temperature fluctuation. During the upward temperature fluctuation (75 °C), no metabolic activity was observed for the 48 h period. However, this did not imply complete deterioration of the culture, as microbial activity was observed when the temperature was brought back to the original incubation temperature of 55 °C. Nonetheless, H₂ production observed was significantly lower compared to the control cultures maintained at 55 °C. It was unexpected that no H₂ production was observed in the cultures augmented in the beginning of the upward temperature fluctuation as the synthetic mixed culture used for the bioaugmentation contained *Caldicellulosiruptor* and *Thermotoga* capable of producing H₂ at extremely high temperatures of 70 and 80 °C, respectively (Abreu et al., 2016). Additionally, based on the wide temperature and pH range of *T. neapolitana*, it was expected that it would have been an excellent member of the microbial community during the temperature fluctuation at 75 °C. Nonetheless, it is possible that the 48 h fluctuation period was too short for the bacteria to get adapted to the high temperature, which is why there was no sign of microbial activity observed. When comparing the cultures which were augmented at different times, higher H₂ yields were obtained when the bioaugmentation was applied after the upward temperature fluctuation than when the augmentation was applied in the beginning of the temperature fluctuation. Although the differences in H₂ yield obtained from the different bioaugmentation times were not statistically significant (P > 0.05), it is likely that also the bacteria used for bioaugmentation were negatively affected by the upward temperature stress. Nonetheless, bioaugmentation proved to be an effective strategy for enhancing H₂ production after the temperature stress. Furthermore, the bioaugmentation is also considered important for boosting the microbial diversity especially after upward temperature fluctuations, as even short-term upward temperature fluctuations have been demonstrated to result in loss of microbial diversity (Gadow et al., 2013; Okonkwo et al., 2019).

It was expected that *Thermoanaerobacter thermohydrosulfuricus* would be an active participant in the consortium during the downward or upward temperature fluctuation due to its relatively high abundance (60%) observed in the synthetic mixed culture (Figure 2). Furthermore, *Thermoanaerobacter* has been shown to grow in conditions, which are similar to the cultivation conditions used in this study (Table 1). Even though *Thermoanaerobacter* was the dominant
genus in the synthetic mixed cultures prior to augmentation, while the results obtained from the augmented cultures showed that *Thermoanaerobacterium* became the most dominant species during or after bioaugmentation under all studied conditions except in unaugmented cultures undergoing downward temperature fluctuation. It is possible that the pre-existence and dominance of *Thermoanaerobacterium* spp. prior to augmentation ensured optimal growth/survival of the specie and better metabolic adaptation compared to the other species added in the consortium. It has been reported that *Thermoanaerobacterium thermosaccharolyticum* (which was among of the bacteria in the synthetic mixed culture) is able to grow at 35-37 °C if spores are first germinated at a higher temperature (Ashton, 1981). Hence, the dominance of *Thermoanaerobacterium* spp. might have been as a result of its ability to better cope with temperature stress as opposed to the other species. As seen in Figures 4 and 5 between unaugmented and augmented cultures, stress factors such as temperature slows adaptation time and might have prevented the activity of the other species or their proliferation. Chen et al. (2015) demonstrated that the successful application of bioaugmentation relied upon the adaptation or coexistence of the bioaugmented bacteria to indigenous microorganisms. The increase in the abundance of *Thermoanaerobacterium* in the augmented cultures caused a relative decrease in abundance of the other microbial genera in the consortium.

The addition of bacteria into a native consortium has been shown in previous studies to affect the metabolic distribution and depending on the metabolic pathways utilized by the bacteria added, an additional pathway might be observed (Yang et al., 2016a). It is therefore important to choose bacteria, which are directly involved with H₂ production, for bioaugmentation. Furthermore, it is likely that during the heat shock, some of the microorganisms formed spores as a mechanism to overcome the heat shock, which would explain the gradual increase in H₂ yield from step 2 to 4 after the upward temperature fluctuation. For example, *Thermoanaerobacterium* has been reported to form spores, which are heat resistant (Lee et al., 1993; Mtimet et al., 2016). Thus, the strengthening of a microbial consortium by bioaugmentation improves recovered activity during or after stress periods. This study demonstrated that bioaugmenting a H₂ producing mixed culture with a synthetic mixed culture consisting of known H₂-producing bacteria can be used as an effective approach for enhancing H₂ production performance during temperature fluctuations. However, the positive effects of bioaugmentation were even higher, when it was applied after the temperature fluctuation. Thus, bioaugmentation both during and after the temperature fluctuation could also be a valid option.
REFERENCES


Chapter 4: Bioaugmentation enhances dark fermentative hydrogen production in cultures exposed to short-term temperature fluctuations


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CHAPTER 5

Quantitative real-time PCR monitoring dynamics of \textit{Thermotoga neapolitana} in synthetic co-culture for biohydrogen production

ABSTRACT

This study demonstrates the potential for biohydrogen production in a co-culture of two ecologically distant species, \textit{Thermatoga neapolitana} and \textit{Caldicellulosiruptor saccharolyticus}, and the development of a quantitative real-time PCR (qPCR) method for quantifying the hyperthermophilic bacterium of the genus \textit{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$^{-1}$ glucose for \textit{C. saccharolyticus}, \textit{T. neapolitana}, and their co-culture respectively. Statistical analysis comparing the H$_2$ production rate of the co-culture to either \textit{C. saccharolyticus} or \textit{T. neapolitana} pure cultures indicated a significant difference in the H$_2$ production rate (p<0.05: t-test), with the highest 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 \textit{T. neapolitana} in the bioprocess, we developed a qPCR method using 16S rRNA gene and hydrogenase (hydA) gene targets. The qPCR data using hydA primers specific to \textit{T. neapolitana} showed an increase in hydA gene copies from $3.32 \times 10^7$ to $4.4 \times 10^8$ hydA gene copies per mL confirming the influence of \textit{T. neapolitana} in the synthetic consortium.
Chapter 5: Quantitative real-time PCR monitoring dynamics of Thermotoga neapolitana in synthetic co-culture for biohydrogen production

5.1. INTRODUCTION

The global trends of fossil fuel depletion and impact on climate change due to over-exploitation of natural resources have led to a search for alternative measures to produce renewable energy (Fino, 2014; International Energy Agency, 2006). Today, $\text{H}_2$ 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 (Andrews and Shabani, 2012). $\text{H}_2$ is presently produced from natural gas, heavy oils, naphtha and coal (Nath and Das, 2003; Suzuki, 1982) which are not sustainable feedstocks. Hence, there is a need for alternative $\text{H}_2$ production routes. One of the means that have been highly considered for sustainable energy is biological $\text{H}_2$ production (Hallenbeck, 2012, 2009; Hallenbeck et al., 2012; Hallenbeck and Benemann, 2002).

Research on biological $\text{H}_2$ 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 (Hemschemeier et al., 2009; Melis et al., 2000). Dark fermentation has garnered interest due to the ability to utilize a wide variety of waste streams and energy crops as substrate for $\text{H}_2$ production, and high $\text{H}_2$ production rates ($10-15 \times 10^3 \text{ ml H}_2 \text{ L}^{-1} \text{ h}^{-1}$) (Hallenbeck et al., 2012; Zeidan and van Niel, 2010). Dark fermentative $\text{H}_2$ 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 $\text{H}_2$ production (De Vrije et al., 2007; Kádár et al., 2004), 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 (Stams, 1994; Verhaart et al., 2010).

Several bacterial species have been identified for their ability to produce high volumes of $\text{H}_2$ at hyperthermophilic conditions. An example is the bacterium *Thermotoga neapolitana*. Substantial efforts in research have been carried out by studying the interactivity of co-culture systems to increase $\text{H}_2$ production. The use of co-cultures in $\text{H}_2$ production has been studied extensively and has been shown to offer various advantages such as the reduction in lag phase, resistance to environmental fluctuations as well improvement in $\text{H}_2$ production (Chang et al., 2008; Cheng and Zhu, 2013; Laxman Pachapur et al., 2015; Li and Liu, 2012; Y. Liu et al., 2008; Pachapur et al., 2015). Recently, there has been a renewed interest in using designed co-culture microorganisms for improving yields of various products and improving the range of substrate utilization (Chang et al., 2008; Cheng and Zhu, 2013; Li and Liu, 2012; Vatsala et al., 2008). Using defined
mesophilic microorganisms, the elaboration of a co-culture system was studied in a pilot scale, treating distillery effluent. The results showed the potential of H\textsubscript{2} production with viable application towards industrialization (Vatsala et al., 2008). However, only few studies, have reported the use of designed thermophilic co-cultures for H\textsubscript{2} production (Li and Liu, 2012; Pawar et al., 2015; Zeidan et al., 2010; Zeidan and Van Niel, 2009).

A variety of interesting interactions can be expected by combining microorganisms from different habitats, some of which might be potentially useful for H\textsubscript{2} production such as, tolerance to higher H\textsubscript{2} partial pressure, high substrate concentration. Being potentially more robust to environmental changes, microbial co-cultures are also better able to withstand periods of nutrient limitation (Li and Liu, 2012; Y. Liu et al., 2008; Zeidan et al., 2010; Zeidan and Van Niel, 2009). Until now, the creation of a stable microbial community consisting of two or more members has been quite challenging due to the diverse growth rates of different members and the subsequent imbalance between the consumption and production metabolites.

In recent years, there has been a significant increase in the studies on \textit{T. neapolitana}, as it has enabled one of the best H\textsubscript{2} yields (3.8 mol H\textsubscript{2} mol\textsuperscript{-1} glucose) that have been reported in literature (Laxman Pachapur et al., 2015) (Li and Liu, 2012). Combined with its ability to produce high H\textsubscript{2} yields at elevated temperatures (55 - 85 °C), \textit{T. neapolitana}, is capable of metabolizing a wide range of simple and complex carbohydrates such as hexoses, pentoses, disaccharides, glucan and amorphous cellulose (D’Ippolito et al., 2010; Frascari et al., 2013; Maru et al., 2012; Van Ooteghem et al., 2004). Previous studies on \textit{T. neapolitana} have focused on optimizing growth and H\textsubscript{2} production conditions for pure cultures of members of \textit{Thermotoga} sp. (Han et al., 2014; Van Ooteghem et al., 2004). However, molecular methods are still needed to better understand and answer questions relating to \textit{T. neapolitana}’s physiological, ecological and metabolic features. A deeper understanding of the bacterium will allow for its metabolic engineering and use in biotechnological applications. Developing molecular methods for monitoring the activity of \textit{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 (OD) (biased in the case of floc formers like \textit{T. neapolitana}) or microscopy (Eriksen et al., 2008; Han et al., 2014; Ngo et al., 2012, 2011; Van Ooteghem et al., 2004, 2002). Some of these methods are only suitable for monocultures and fail to differentiate between different species. Furthermore, members of the genus \textit{Thermotoga} undergo floc
formation which often causes ambiguities in cell enumeration (Lakhal et al., 2011). With the continued increase in the number of sequenced microbial genomes, unveiling the different mechanisms of interspecies relationships can be facilitated by use of molecular and metabolic modeling based approaches (Bouchez et al., 2000; Herrero and Stuckey, 2015; Vanwonterghem et al., 2014).

In the present work, co-cultivation of two ecologically distant organisms for improved H₂ evolution and the development of a quantitative PCR assay for genus and species-level monitoring of Thermotoga was carried out. The 16S rRNA gene 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 rRNA gene and H₂ producing activity in T. neapolitana, we further developed a qPCR approach targeting the 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.

5.2. MATERIALS AND METHODS

5.2.1. 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): NH₄Cl, 1.0; K₂HPO₄, 0.3; KH₂PO₄, 0.3; MgCl₂ x 6 H₂O, 0.2; CaCl₂ x 2H₂O, 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 milliliters 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. For the inocula, 3 mL of pre-cultivated T. neapolitana and C. saccharolyticus having OD₆00nm of 0.2 and 0.1 respectively (10% of the total culture volume) was used in the pure culture incubations. In case of the co-culture, 1.5 mL of the pre-cultivated T. neapolitana and C. saccharolyticus were used (total inoculum accounted for 10% of
the culture volume). The cultures were incubated at 75°C and 150 rpm. To validate the specificity of the primers developed in this study, microbial community from a H₂ producing fluidized bed bioreactor without *Thermotoga* was used (Dessì et al., 2017) as well as pure cultures of *T. neapolitana* and *C. saccharolyticus*.

### 5.2.2. Analytical techniques

The cell concentration of the culture suspension containing bacterial cells was determined by measuring the absorbance spectrophotometrically with an Ultraspec 200 Pro spectrophotometer (Amersham Biosciences, Munich, Germany) at 600 nm. H₂ 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 bottle using a syringe method (Owen et al., 1979). 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 (Dessì et al., 2017; Logan et al., 2002):

\[
V_{H₂,t} = V_{H₂,t-1} + C_{H₂,t}(V_{G,t} - V_{G,t-1}) + V_{H}(C_{H₂,t} - C_{H₂,t-1})
\]

Where \(V_{H₂,t}\) is the cumulative H₂ gas produced at time \(t\), \(V_{H₂,t-1}\) is the cumulative H₂ 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 \(t-1\), \(C_{H₂,t}\) is the H₂ gas fraction in the headspace at time \(t\), \(C_{H₂,t-1}\) is the H₂ gas fraction in the headspace at time \(t-1\) and \(V_{H}\) is the total headspace volume in the culture bottle. H₂ production was calculated and 75 °C this temperature was used for the gas law calculations. 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 H₂SO₄ at a flow rate of 0.6 mL min⁻¹. At the end of 100 h period of incubation, samples from the co-cultures were harvested, centrifuged and stored at -20 °C for genomic isolation and molecular analyses.

### 5.2.3. 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.

5.2.4. 16S rRNA gene and hydA primer design

In this study 16S rRNA and hydA genes were targeted for genus and species level monitoring of Thermotoga sp. The 16S rRNA 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 the primer designing using the Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). Primer specificities towards 16S rRNA gene 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 rRNA non-redundant reference dataset, SSURef 108 NR (Klindworth et al., 2013).

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 rRNA gene of Thermotoga sp. (Fwd, 5’-TACCCCATACGCTCCATCAA-3’; Rev, 5’-CCGTTACCCCAACCACTAC-3’) and T. neapolitana hydA (hydA_F, AGTACACGGCATGAAGGAGA; hydA_R, CGCAGAACACAACTATCCAC-3’). The applicability and specificity of the developed primers was tested using the H₂-producing cultures of T. neapolitana, T. maritima, C. saccharolyticus and mixed cultures from fluidized bed bioreactor, known to contain members of the genus Thermoanaerobacterium (Dessì et al., 2017). 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).

5.2.5. Real-time quantitative PCR

Optimization of primer annealing temperatures for 16S rRNA and hydA genes were performed by testing six annealing temperatures for 16S rRNA 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 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 (Tolvanen et al., 2008). Gene copies per ng of DNA extracted and the gene copies per sample were evaluated according to equation 2 and 3, respectively (Ritalahti et al., 2006). The amplification efficiencies were calculated using equation 4.

\[
\text{Gene copies} = \text{DNA}_{\frac{\text{ng}}{\mu L}} \times \frac{1\ g}{1000\ g} \times \frac{1\ \text{mol bp DNA}}{660\ g\ DNA} \times \frac{6.023 \times 10^{23}\ \text{bp mol}}{\text{bp}} \times \frac{1\ \text{copy}}{\text{genome size bp}} \times \text{vol. DNA used}_{\mu L} \tag{2}
\]

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

\[
\text{Efficiency} = [-1 + 10^{-\left(\frac{1}{\text{slope}}\right)}] \tag{4}
\]

### 5.3. RESULTS AND DISCUSSION

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

The H₂ 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 H₂ production rate obtained was 24.65 mL L⁻¹ h⁻¹. The growth obtained from OD measurements reached a maximum OD₆₀₀nm 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 H₂ yield obtained was 2.7 ± 0.05 mol H₂ mol⁻¹ 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 (Chandrasekhar et al., 2015; Nualsri et al., 2017; Sharma and Arya, 2017).

![Figure 1](image)

**Figure 1** H$_2$ production, glucose consumption, OD600nm of the culture suspensions and soluble metabolites produced by *C. saccharolyticus* (●) H$_2$ production, (■) Glucose consumption, (▲) 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 of 19 h 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$^{-1}$ glucose and compared to *C. saccharolyticus*, the consumption of glucose was slower (Figure 2). Similar to *C. saccharolyticus*, 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 (Lakhal et al., 2011). 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*.

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$^{-1}$ 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. saccharolyticus* and 0.014 (p<0.05: t-test) with *T. neapolitana* showing a significant difference in the H$_2$ production rate.
Overall, the highest $\text{H}_2$ 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 the 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** $\text{H}_2$ production by co-culture of *T. neapolitana* and *C. saccharolyticus* (*●*) $\text{H}_2$ production, (*■*) Glucose consumption, (*△*) 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 assessed for biohydrogen production under hyperthermophilic conditions and have been shown to be a promising strategy for improved $\text{H}_2$ production (Cheng and Zhu, 2013; Laxman Pachapur et al., 2015; Li and Liu, 2012). 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 $\text{H}_2$ production (Abreu et al., 2016; De Vrije et al., 2009). Additionally, *C.*
saccharolyticus has been recognized as being relatively insensitive to H₂ partial pressure (Willquist et al., 2010). Due to the faster initialization of growth of C. saccharolyticus (Pradhan et al., 2016; Vanfossen et al., 2009), a higher OD of T. neapolitana was used in the co-culture as mentioned in section 3.1. Pradhan et al. (2016) reported a doubling time of 4 h for T. neapolitana while Vanfossen et al. (2009) reported a doubling time of 95 minutes for C. saccharolyticus. Results in Figure 3 indicate that the synthetic co-culture demonstrated a positive effect to both H₂ production rate and H₂ yield compared to the pure cultivations grown under similar conditions. This therefore, suggests a synergistic effect between T. neapolitana and C. saccharolyticus. Compared to the pure strains, the co-cultures demonstrated a rapid glucose utilization and higher H₂ production rate. The results obtained from this study is in line with the literature reporting enhanced H₂ production using synthetic co-cultures (Abreu et al., 2016; Ács et al., 2015; Kovács et al., 2013; Mohan et al., 2005; Seppälä et al., 2011; Vatsala et al., 2008; Wang, 2008; Yang et al., 2016a).

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

<table>
<thead>
<tr>
<th>Strain/co-culture</th>
<th>Substrate</th>
<th>Temperature (°C)</th>
<th>Maximum H₂ yield (mol H₂/mol of glucose)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. saccharolyticus and T. maritima</td>
<td>Xylose, Cellubiose</td>
<td>70</td>
<td>2.7 ± 0.1</td>
<td>(Abreu et al., 2016)</td>
</tr>
<tr>
<td>C. Thermocellum JN4 and T. thermosaccharolyticum GD17</td>
<td>Microcrystalline cellulose, cellobiose or filter paper</td>
<td>60</td>
<td>1.8 ± 0.09</td>
<td>(Y. Liu et al., 2008)</td>
</tr>
<tr>
<td>C. saccharolyticus and T. neapolitana</td>
<td>Glucose</td>
<td>75</td>
<td>2.81 ± 0.09</td>
<td>This study</td>
</tr>
</tbody>
</table>

5.3.2. 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 rRNA and hydA genes. The hydA gene has been used in the quantitative monitoring of Clostridium butyricum in mesophilic H₂ producing bioprocess systems (Tolvanen et al., 2010, 2008). The specificities of the newly designed primers were evaluated by in silico PCR of 16S rRNA gene primer and Primer-BLAST for both 16S rRNA 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 rRNA 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>
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<th>Specificity (%)</th>
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<td>CP010967</td>
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<td>190530</td>
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</table>

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 rRNA amplicons had different lengths, the melt curve analysis for T. neapolitana and T. maritima showed a slightly different melting temperature (Tm). However, the 16S rRNA 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.
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Figure 4 qPCR profile of Thermotoga sp. 16S rRNA 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 rRNA 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.

5.3.3. Real-time quantitative PCR

The number of gene copies calculated from the constructed T. neapolitana 16S rRNA 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 (Nicolet and Fontecilla-Camps, 2012; Pei et al., 2010). The regression coefficients showed strong linear correlations for all targets during qPCR analysis (Figure 5).
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Figure 5 Standard curve for Thermotoga 16S rRNA and hydA amplicons shown are: a) Thermotoga sp. specific 16S rRNA 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 the 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 rRNA 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 3.32×10⁷ to 4.4×10⁸ 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 rRNA 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 rRNA amplification showed that increasing the annealing temperature from 60 °C to 72 °C had a significant effect on the amplification efficiency.
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Figure 5 Comparison of quantification results obtained from 16S rRNA 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 H$_2$ production suggesting the growth of T. neapolitana in the co-culture culture. In a similar study, co-cultivation of C. saccharolyticus and T. maritima (Abreu et al., 2016) improved the H$_2$ 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 H$_2$ 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% (Ritalahti et al., 2006; Tolvanen et al., 2010, 2008). 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 rRNA and hydA amplicons at the qPCR annealing temperature (60 °C) used in the experiment was calculated. There was a significant variation in the 16S rRNA 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 rRNA 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 rRNA amplicon sequence was increased to 72 °C in silico (using the Mfold web server DNA folding form), a $\Delta G$ value of -8.80 kcal mol$^{-1}$ 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 rRNA primers. The high R-squared values obtained in the 16S rRNA amplifications confirm that the reactions were consistent with an absence of any non-specific product or primer dimer formation. Overall, 16S rRNA and hydA based quantitative methods established were specific for the genus Thermotoga and T. neapolitana respectively.

5.4. CONCLUSIONS

This study demonstrates that ecologically distant H$_2$-producing organisms with different cultivation conditions such as T. neapolitana and C. saccharolyticus can be used for the 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 H$_2$ production rate compared to the respective monocultures. Additionally, qPCR methods were successfully developed for genus and specie-specific quantitative monitoring of Thermotoga sp. in H$_2$ producing systems. The hydA provided a promising target to complement with the existing 16S rRNA 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 H₂ production by the co-culture. Furthermore, the work presents an alternative quantification method for genus-level monitoring of *Thermotoga* spp. and specie-specific monitoring of *T. neapolitana* which can be further explored for its ability to form biofilm and retain biomass for improved H₂ production under different cultivation conditions.
REFERENCES


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CHAPTER 6

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

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 \(\text{H}_2\) 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 \(\text{H}_2\) production rate was improved when the initial glucose concentration was increased. In contrast, the highest \(\text{H}_2\) yield (1.68 mol \(\text{H}_2\) 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, \(\text{H}_2\) 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.
6.1. INTRODUCTION

Fermentative hydrogen ($H_2$) production is possible with both pure and mixed microbial cultures originated from natural or engineered environments (Barca et al., 2015; Bundhoo et al., 2015; Hallenbeck, 2009). 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 (Call et al., 2009; Li and Fang, 2007; Valdez-Vazquez and Poggi-Varaldo, 2009; Wang and Wan, 2008; Yu et al., 2002; Zhang et al., 2007). 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 (Boboescu et al., 2014; Dreschke et al., 2019b; Hallenbeck, 2009; Kothari, 2017; Yue et al., 2011).

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 $H_2$-producing mixed culture (Goud et al., 2014) and a hyperthermophilic *Thermotoga neapolitana* pure culture (Dreschke et al., 2019c). Increasing the substrate concentration and, thus, the organic load of the system allows to save the energy required for heating of $H_2$-producing bioreactors, as high substrate concentrations lead to increased microbial activity and heat generation by microbial metabolism (Kyazze et al., 2006). 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 (Argun and Kargi, 2011; Gadhamshetty et al., 2010). 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 (Cai et al., 2011).

Bioaugmentation has been proposed in several studies as a potential strategy for enhancing dark fermentation under stress conditions (Goud et al., 2014; Kumar et al., 2016; Okonkwo et al., 2019a). 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 system (da Silva and Alvarez, 2009; Han et al., 2010; Herrero and Stuckey, 2015). Bioaugmentation is an emerging strategy for industrial wastewater treatment (Nzila et al., 2016) and has been used to shorten the lag phase and improve the chemical oxygen demand (COD) removal during dark fermentation from the organic fraction of municipal solid waste (Sharma and
Melkania, 2018). It has also been used to enhance thermophilic H₂ production from corn stover hydrolysate (Zhang et al., 2019) and beverage wastewater (Sivagurunathan et al., 2014). Okonkwo et al., (2019b) applied bioaugmentation with a synthetic co-culture to enhance the H₂ 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 H₂ production also at high substrate concentrations (Cabrol et al., 2017). 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 (Chandrasekhar et al., 2015). Some studies have used strategies such as repeated bioaugmentation to promote the persistence of the added bacterium in the system (Yang et al., 2016a, 2016b). 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 H₂ 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 (Eriksen et al., 2008; Ngo et al., 2011a). To the best of our knowledge, this is the first study to use pre-adaptation as a strategy for making T. neapolitana a stable member of a native H₂-producing microbial community and for enhancing H₂ production.

6.2. MATERIALS AND METHODS

6.2.1. Experimental Setup

The medium used for the cultivation consisted of the following components (g/L): NH₄Cl, 1.0; K₂HPO₄, 0.3; KH₂PO₄, 0.3; MgCl₂ x 6 H₂O, 0.2; CaCl₂ x 2H₂O, 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 with a thermophilic mixed culture augmented with *Thermotoga neapolitana* (b).

### 6.2.2. Experimental procedure

The inoculum used in this study was obtained from a laboratory scale continuously stirred tank bioreactor producing H₂ at 55 °C (Okonkwo et al., 2019). The cultivation 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). 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) 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, 27.8 and 55.5 to 111.0 mmol L$^{-1}$ in order to determine the impact of increasing substrate concentration on H$_2$ production, biomass concentration and metabolic patterns.

### 6.2.3. 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 (Logan et al., 2002):

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

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\textsubscript{2} gas produced was divided by 22.4 L in order to obtain H\textsubscript{2} produced in moles. The H\textsubscript{2} yield and productivity were calculated using Equations 2 and 3, respectively.

\[
H_2 \text{ yield} = \frac{\text{mol } H_2}{\text{mol glucose consumed}} \tag{2}
\]

\[
H_2 \text{ productivity} = \frac{\text{mmol } H_2}{\text{reaction volume } \times \text{fermentation time (hour)}} \tag{3}
\]

6.2.4. 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 (Wang and Qian, 2009) 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 Venkiteswaran et al. (2016). Real-time quantitative polymerase chain reaction (qPCR) monitoring of T. neapolitana was carried out using HydA primers following the method described by Okonkwo et al. (2018). 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.

6.3. RESULTS AND DISCUSSION

6.3.1. H\textsubscript{2} production rates and yields at increasing glucose concentrations

In the unaugmented cultures, the highest H\textsubscript{2} yield was 1.42 mol H\textsubscript{2} mol\textsuperscript{-1} of glucose consumed at an initial concentration of 27.8 mmol L\textsuperscript{-1} of glucose. The H\textsubscript{2} yield dropped to 1.17 mol H\textsubscript{2} mol\textsuperscript{-1} of glucose consumed at 111 mmol L\textsuperscript{-1} of feed glucose concentration (Figure 2a). The H\textsubscript{2} yield obtained in the augmented cultures was higher than that obtained in the unaugmented cultures. Nonetheless, similar to the unaugmented cultures, the H\textsubscript{2} yield decreased by increasing the substrate concentration. With bioaugmentation, H\textsubscript{2} yield increased by 37, 16 and 12% at 5.6, 55.5 and 111 mmol L\textsuperscript{-1} of feed glucose, respectively, compared to the unaugmented cultures. The highest H\textsubscript{2} yield (1.68 mol H\textsubscript{2} per mol of consumed glucose) was obtained at the feed glucose concentration of 5.6 mmol L\textsuperscript{-1}. Qiu et al., (2017) studied the effect of xylose concentrations (ranging from 16.7 to 100.0 mmol L\textsuperscript{-1}) on dark fermentative H\textsubscript{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 (Lee et al., 2008; Zheng et al., 2008).

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 (Azbar et al., 2009; Chong et al., 2009). 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.

![Figure 2](image)

**Figure 2** H$_2$ yield and H$_2$ production rate obtained with the unaugmented (a) and the augmented (b) culture at different initial glucose concentrations.
6.3.2. Effect of glucose concentration on the composition of soluble metabolites

The main soluble microbial products associated with glucose degradation were ethanol, acetate and lactate (Figure 3). In the unaugmented cultures, ethanol was the main soluble metabolite produced and corresponded to 67–77% COD of the total soluble metabolites produced. The share of acetate decreased with increasing glucose concentration (from 18 to 12%), while that of lactate increased from 4.5 to 18% (Figure 3a). In the augmented cultures, the share of ethanol decreased with increasing glucose concentration (from 58% at 5.6 mmol L\(^{-1}\) glucose to 27% at 111 mmol L\(^{-1}\) glucose). Thus, the share of ethanol was lower in the augmented culture compared to the unaugmented culture at all studied glucose concentrations. The percentage of acetate decreased in the augmented culture from 29% at 5.6 mmol L\(^{-1}\) of feed glucose to 16% at 111 mmol L\(^{-1}\) of feed glucose, while the share of lactate significantly increased from 13% at initial concentration of 5.6 mmol L\(^{-1}\) glucose to 57% at 111 mmol L\(^{-1}\) of feed glucose (Figure 3b).

![Bar chart showing the distribution of soluble metabolites as COD equivalents at the endpoint of fermentation for different initial glucose concentrations in unaugmented and augmented cultures.](image)

**Figure 3** The distribution of soluble metabolites as chemical oxygen demand (COD) equivalents at the endpoint of fermentation the different initial glucose concentrations in the unaugmented (a) and augmented (b).

In many previous studies, dark fermentation of glucose has resulted in the production of mainly butyrate and acetate as soluble metabolites under mesophilic, thermophilic and hyperthermophilic...
conditions (Abreu et al., 2016; Gadow et al., 2013; Ngo et al., 2011b; Reilly et al., 2014; Zhao et al., 2010). However, it seems that ethanol-based fermentation was the major pathway leading to H₂ production in this study due to the high ethanol yields obtained especially with the unaugmented cultures. The ethanol-type fermentation (Equation 4) has a theoretical maximum of 2 mol of H₂ per mol of glucose and has been reported to occur under mesophilic conditions (Hwang et al., 2004; Ren et al., 2007) but not for mixed cultures at temperatures as high as 70 °C.

$$C_6H_{12}O_6 + H_2O \rightarrow C_2H_5OH + CH_3COOH + 2CO_2 + 2H_2$$

(4)

Previous studies have reported yields of 1.8 mol ethanol mol⁻¹ glucose with pure culture of T. ethanolicus (Wiegel and Ljungdahl, 1981) and 1.5 mol ethanol mol⁻¹ glucose from T. hydrosulfuricus (Lacis and Lawford, 1991) at 72 and 69 °C respectively. The highest ethanol yield obtained with the unaugmented culture in this study was 1.4 mol H₂ 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 H₂ 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 (Dreschke et al., 2019a; Ippolito et al., 2010; Okonkwo et al., 2018). The direction of the metabolic pathway towards acetate production allows producing more H₂ 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 (Dreschke et al., 2019c), 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 H₂ production thereby reducing the H₂ yield (Kim et al., 2014; Mishra et al., 2019).

6.3.3. Quantity of T. neapolitana in 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 (Okonkwo et al., 2018). 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 H₂ 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](image_url)

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

### 6.3.4. 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 (Zhang et al., 2016). 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%.
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Figure 5 Microbial community composition and relative abundance of genera identified at different feed glucose concentrations in the unaugmented (a) and the 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* spp., respectively. However, at the higher initial glucose concentrations, *Bacillus* spp. were not detected anymore from the microbial community and the shares of *Thermoanaerobacter* and *Thermotoga* spp. were 62-80% and 20-37%, respectively (Figure 5). The abundance of *Thermotoga* in the community was higher at the two highest initial glucose concentrations, which is accordance with the qPCR results (Figure 4).

The bioaugmentation of a *Thermoanaerobacter*-dominated mixed culture with *T. neapolitana* improved both the H\(_2\) production yield and rate. *Thermoanaerobacter* species are well known thermophilic bacteria capable of producing H\(_2\), ethanol and acetate (Claassen et al., 1999; Koskinen et al., 2008; Lin and Tanaka, 2006). Thus, their presence explains also the high ethanol production observed. Bacteria within this genus have also been reported to use the Embden–Meyerhof–Parnas pathway for sugar degradation and produce ethanol, acetate and lactate as major volatile end products (Lee et al., 1993), which is in accordance with the metabolite profiles observed in this study. In the bioaugmented culture, the presence of *T. neapolitana* resulted in lower ethanol production, while the shares of acetate and lactate increased compared to the
unaugmented cultures. Thus, the differences observed in the abundance of different soluble metabolites in the unaugmented and augmented cultures can be explained with the observed differences in the microbial community composition.

The pre-adaptation as a strategy to make *T. neapolitana* a stable member of the native microbial community was successful based on the molecular monitoring methods used this study, as both the *T. neapolitana* hydA gene copy numbers and relative abundance of *Thermotoga* were shown to increase towards the end of the study. The pre-adaptation of a bacteria to a mixed culture prior to its application to a 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 (Okonkwo et al., 2019b). However, pre-adaptation may not be feasible in the case of sudden transient disturbances due to the fact that it is time consuming.

Except for the switch in the metabolic pathways and an enhanced H₂ 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 (Bastida et al., 2009; Lacerda and Reardon, 2009; Wang et al., 2016), as this could enable further process optimization.

### 6.4. CONCLUSIONS

The bioaugmentation of a *Thermoanaerobacter*-dominated mixed culture with *Thermotoga neapolitana* improved both the H₂ production yield and rate. Thus, the results of this study indicate that the use of a single strain with required characteristics can be enough for improving the performance of a biological process. The H₂ production rate of the augmented cultures increased when initial glucose concentration was increased from 5.6 to 55.5 mmol L⁻¹, while the highest H₂ production yield, 1.68 mol H₂ 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.
Chapter 6: Enhancing thermophilic dark fermentative hydrogen production at high glucose concentrations via bioaugmentation with *Thermotoga neapolitana*

REFERENCES


Han, W., Li, Y.F., Yue, L.R., 2010. Biohydrogen production: microbiological aspects and bioaugmentation, in: 2010 international conference on mechanic automation and control engineering, MACE2010. https://doi.org/10.1109/MACE.2010.5535490


Chapter 6: Enhancing thermophilic dark fermentative hydrogen production at high glucose concentrations via bioaugmentation with *Thermotoga neapolitana*


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CHAPTER 7

General discussion and conclusions
Chapter 7: General discussion and conclusion

7.1. General discussion

In this work, thermophilic dark fermentative H₂ production was studied using different biological cultivation systems and under varying conditions. As can be seen from Table 7.1, the H₂ yield obtained with the synthetic co-culture of *T. neapolitana* and *C. saccharolyticus* (chapter 5) was higher than those obtained in any of the mixed cultures used in this study with or without bioaugmentation (chapters 3, 4 and 6). Analysis of mass balance in COD equivalents of substrates added to the incubations and the soluble metabolites present in the end of the incubations as described by Gonzales and Kim (2017) and Sivagurunathan and Lin (2016), enabled comparison of substrate conversion efficiencies of the different experiments (Table 7.2). The results showed that the COD removal efficiency was higher in the pure cultures and in the synthetic co-culture compared to mixed cultures, with pure culture of *T. neapolitana* having the highest COD removal efficiency. However, it should be noted that the cultivation conditions were different in the different experiments of this study as shown in Table 7.1.

In chapters 3 and 4, where cultures were exposed to either upward or downward temperature fluctuation, it was observed that a larger share of the substrate was converted to butyrate compared to either acetate or lactate. In the experiments conducted at 75 °C with initial pH and glucose concentration of 7.5 and 20 mM (chapter 5), acetate was the major metabolite produced in both pure cultures and in the synthetic co-culture. Meanwhile in chapter 6 where cultures were exposed to increased substrate concentration and bioaugmentation applied, a higher share of ethanol was observed compared to ethanol or lactate. However, at increasing substrate concentrations, the share of lactate increased especially in the bioaugmented cultures. The differences observed in the distribution of the metabolites across the different studies was a result of the difference in metabolic pathways due to the differences in incubation conditions and the existing microbial community. For example, in chapters 3 and 4 where cultures were exposed to temperature fluctuation and the impact of bioaugmentation on the full recovery of H₂ produced, the main player in the microbial community was *Thermoanaerobacterium* spp. while *Thermoanaerobacter* spp. was the major player in chapter 6 where cultures were exposed to increased substrate concentration and bioaugmentation applied.
Table 7.1 Comparison of $H_2$ production obtained under thermophilic conditions in different experiments conducted at constant temperature. It should be noted that these results have been obtained using different pH, substrates and inoculum source.

<table>
<thead>
<tr>
<th>Inoculum</th>
<th>Mode</th>
<th>pH</th>
<th>Temperature (°C)</th>
<th>Substrate concentration (mM)</th>
<th>Substrate degradation rate (mM h$^{-1}$)</th>
<th>$H_2$ production (mmol L$^{-1}$)</th>
<th>Maximum $H_2$ production rate (mmol L$^{-1}$ h$^{-1}$)</th>
<th>Maximum $H_2$ yield (mol $H_2$ mol$^{-1}$ glucose consumed)</th>
<th>Major metabolites</th>
<th>Reference</th>
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</thead>
<tbody>
<tr>
<td>Unaugmented mixed culture</td>
<td>Batch</td>
<td>6.0</td>
<td>55</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>1.85</td>
<td>Butyrate, acetate, lactate, ethanol</td>
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<td>Augmented mixed culture</td>
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<td></td>
<td></td>
<td>4.4 glu$^a$ + 8.0 xyl$^b$</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>2.07</td>
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</tr>
<tr>
<td>T. neapolitana</td>
<td>Batch</td>
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<td>75</td>
<td>20.0 glu$^a$</td>
<td>0.45</td>
<td>42.06</td>
<td>0.69</td>
<td>2.53</td>
<td>Acetate, lactate</td>
<td>Chapter 5</td>
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<td>C. saccharolyticus</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.59</td>
<td>50.78</td>
<td>1.09</td>
<td>2.69</td>
<td>Acetate</td>
<td></td>
</tr>
<tr>
<td>Co-culture of T. neapolitana +</td>
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<td></td>
<td></td>
<td></td>
<td>1.27</td>
<td>53.73</td>
<td>1.61</td>
<td>2.77</td>
<td>Acetate</td>
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<tr>
<td>C. saccharolyticus</td>
<td>Co-culture of T. neapolitana + C. saccharolyticus</td>
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<td>5.6 glu$^a$</td>
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<td>0.31</td>
<td>1.22</td>
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<td>27.8 glu$^a$</td>
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<td>27.14</td>
<td>0.62</td>
<td>1.42</td>
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<td></td>
<td></td>
<td>55.5 glu$^a$</td>
<td>1.0</td>
<td>23.14</td>
<td>0.75</td>
<td>1.24</td>
<td>Anthropose, butyrate, lactate</td>
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<td>Enriched hydrogenogenic culture</td>
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<td></td>
<td></td>
<td>111.0 glu$^a$</td>
<td>1.1</td>
<td>29.45</td>
<td>0.92</td>
<td>1.17</td>
<td>Anthropose, butyrate, lactate</td>
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<td></td>
<td>5.6 glu$^a$</td>
<td>0.3</td>
<td>9.54</td>
<td>0.41</td>
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<td>0.53</td>
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<td>1.0</td>
<td>29.92</td>
<td>1.20</td>
<td>1.44</td>
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<tr>
<td>Enriched hydrogenogenic culture</td>
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<td></td>
<td></td>
<td></td>
<td>111.0 glu$^a$</td>
<td>1.2</td>
<td>35.99</td>
<td>1.06</td>
<td>1.31</td>
<td></td>
</tr>
</tbody>
</table>

glu$^a$ = glucose, xyl$^b$ = xylose, NA = data not available
### Table 7.1 Continued

<table>
<thead>
<tr>
<th>Inoculum</th>
<th>Mode</th>
<th>pH</th>
<th>Temperature (°C)</th>
<th>Substrate (mM)</th>
<th>H₂ production (mmol L⁻¹)</th>
<th>Maximum H₂ production rate (mmol L⁻¹ h⁻¹)</th>
<th>Maximum H₂ yield (mol H₂ mol⁻¹ glucose equivalent consumed)</th>
<th>Major metabolites</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. thermocellum + C. thermopalmarium</td>
<td>Batch</td>
<td>7.0</td>
<td>55</td>
<td>Cellulose</td>
<td>61.89</td>
<td>NA</td>
<td>1.36</td>
<td>Acetate, butyrate, ethanol</td>
<td>Geng et al., 2010</td>
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<tr>
<td>C. saccharolyticus + C. kristjanssonii</td>
<td>Continuous</td>
<td>6.7</td>
<td>70</td>
<td>27.8 gluᵃ + 33.3 xylᵇ</td>
<td>NA</td>
<td>4.8</td>
<td>3.70</td>
<td>Acetate</td>
<td>Zeidan et al., 2010</td>
</tr>
<tr>
<td>Activated sludge</td>
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<td>55, 70</td>
<td>50 - xylose</td>
<td>NA</td>
<td>NA</td>
<td>1.2</td>
<td>Acetate, butyrate, ethanol</td>
<td>(Dessì et al., 2018b)</td>
</tr>
<tr>
<td>Activated sludge</td>
<td>Continuous</td>
<td>NA</td>
<td>35</td>
<td>Glucose</td>
<td>NA</td>
<td>3.7</td>
<td>1.9</td>
<td>Acetate, butyrate, ethanol, propionate</td>
<td>(Guo et al., 2013)</td>
</tr>
<tr>
<td>Anaerobic granular sludge</td>
<td>Continuous</td>
<td>4.5</td>
<td>35</td>
<td>Glucose</td>
<td>NA</td>
<td>3.19</td>
<td>2.6</td>
<td>Acetate, butyrate</td>
<td>(Hernández-Mendoza and Buitrón, 2014)</td>
</tr>
<tr>
<td>Palm oil mill wastewater treatment plant</td>
<td>Batch</td>
<td>5.5</td>
<td>60</td>
<td>Palm oil mill effluent</td>
<td>NA</td>
<td>2.60</td>
<td>1.08</td>
<td>Acetate, butyrate</td>
<td>O-Thong et al., 2008</td>
</tr>
<tr>
<td>Anaerobic digested sludge</td>
<td>Batch</td>
<td>5.5</td>
<td>60</td>
<td>Sucrose</td>
<td>53.99</td>
<td>4.4</td>
<td>1.96</td>
<td>Acetate, butyrate</td>
<td>O-Thong et al., 2009</td>
</tr>
</tbody>
</table>

gluᵃ = glucose, xylᵇ = xylose, NA = data not available
Table 7.2 A fraction of COD (%) from the substrate presented by each soluble metabolite in the end of batch incubation calculated for each experiment conducted in this study.

<table>
<thead>
<tr>
<th>Culture/Temperature</th>
<th>pH</th>
<th>Substrate (mM)</th>
<th>Residual sugars (%)</th>
<th>Lactate (%)</th>
<th>Acetate (%)</th>
<th>Ethanol (%)</th>
<th>Butyrate (%)</th>
<th>Propionate (%)</th>
<th>COD removal (%)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>55 °C (control-average)</td>
<td>6.0</td>
<td>ND</td>
<td>4.4 glu&lt;sup&gt;a&lt;/sup&gt; + 8.0 xyl&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.4</td>
<td>2.3</td>
<td>16.2</td>
<td>ND</td>
<td>44.0</td>
<td>ND</td>
<td>28.6</td>
</tr>
<tr>
<td>Fluctuation to 35 °C (step 3)</td>
<td>6.0</td>
<td>ND</td>
<td>4.4 glu&lt;sup&gt;a&lt;/sup&gt; + 8.0 xyl&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.4</td>
<td>2.3</td>
<td>16.2</td>
<td>ND</td>
<td>44.0</td>
<td>ND</td>
<td>28.6</td>
</tr>
<tr>
<td>55 °C after fluctuation (step 5)</td>
<td>6.0</td>
<td>ND</td>
<td>4.4 glu&lt;sup&gt;a&lt;/sup&gt; + 8.0 xyl&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.4</td>
<td>2.3</td>
<td>16.2</td>
<td>ND</td>
<td>44.0</td>
<td>ND</td>
<td>28.6</td>
</tr>
<tr>
<td>Fluctuation to 45 °C (step 3)</td>
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<td>ND</td>
<td>4.4 glu&lt;sup&gt;a&lt;/sup&gt; + 8.0 xyl&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.4</td>
<td>2.3</td>
<td>16.2</td>
<td>ND</td>
<td>44.0</td>
<td>ND</td>
<td>28.6</td>
</tr>
<tr>
<td>55 °C after fluctuation (step 5)</td>
<td>6.0</td>
<td>ND</td>
<td>4.4 glu&lt;sup&gt;a&lt;/sup&gt; + 8.0 xyl&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.4</td>
<td>2.3</td>
<td>16.2</td>
<td>ND</td>
<td>44.0</td>
<td>ND</td>
<td>28.6</td>
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<td>Fluctuation to 65 °C (step 3)</td>
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<td>4.4</td>
<td>2.3</td>
<td>16.2</td>
<td>ND</td>
<td>44.0</td>
<td>ND</td>
<td>28.6</td>
</tr>
<tr>
<td>55 °C after fluctuation (step 5)</td>
<td>6.0</td>
<td>ND</td>
<td>4.4 glu&lt;sup&gt;a&lt;/sup&gt; + 8.0 xyl&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.4</td>
<td>2.3</td>
<td>16.2</td>
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<td>4.4</td>
<td>2.3</td>
<td>16.2</td>
<td>ND</td>
<td>44.0</td>
<td>ND</td>
<td>28.6</td>
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<tr>
<td>55 °C after fluctuation (step 5)</td>
<td>6.0</td>
<td>ND</td>
<td>4.4 glu&lt;sup&gt;a&lt;/sup&gt; + 8.0 xyl&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.4</td>
<td>2.3</td>
<td>16.2</td>
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glu<sup>a</sup> = glucose, xyl<sup>b</sup> = xylose, ND = not detected, NA = data not available
### Table 7.2 Continued

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<th>Culture/Temperature</th>
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<th>Substrate (mM)</th>
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<th>Lactate (%)</th>
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<td>Enriched hydrogenogenic culture augmented with T. neapolitana. (70 °C)</td>
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<td>24.7</td>
<td>32.1</td>
<td>4.1</td>
<td>27.3</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>11.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>55.5 glu&lt;sup&gt;a&lt;/sup&gt;</td>
<td>56.9</td>
<td>15.3</td>
<td>3.9</td>
<td>10.5</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>8.9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>111.0 glu&lt;sup&gt;a&lt;/sup&gt;</td>
<td>75.5</td>
<td>10.6</td>
<td>2.0</td>
<td>5.0</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>4.2</td>
<td></td>
</tr>
</tbody>
</table>

glu<sup>a</sup> = glucose, xyl<sup>b</sup> = xylose, ND = not detected, NA = data not available
H₂-producing inoculum enriched at 55 °C was used to evaluate the impact of short-term temperature fluctuations on H₂ production and the impact of adding known H₂ producers belonging to five different genera into the existing microbial community during or after the temperature fluctuation periods (chapters 3 and 4). From the results obtained, it is evident that H₂ production was affected by sudden short-term temperature changes. The effects of the fluctuations included decrease in H₂ yield, shifts in the metabolic product quantity and distribution, and changes in the microbial community composition. The major metabolic products observed in these studies were butyrate, acetate, ethanol and lactate and traces of propionate (chapters 3 and 4). Short-term temperature fluctuations did not trigger the formation of new metabolites although bacteria often function during changes in environmental conditions by expressing a range of metabolic capabilities leading to diversion in the metabolic pathway (Ergal et al., 2018; Meyer et al., 2004; Palomo-Briones et al., 2017). Different species possess different growth properties as well as different abilities to cope with stress that occurs as a result of variations in growth conditions (Braga et al., 2016; Melbinger and Vergassola, 2015). The shifts in the share of the different metabolites can therefore be largely attributed to the dominance of different microorganisms under the different temperature conditions.

Bioaugmentation played a significant role in enhancing H₂ production during and after the temperature fluctuations (chapter 4). Choosing bacteria from different genera with different growth rates and wide temperature ranges was considered as an effective strategy to overcome the negative effects of temperature fluctuations. However, there were limitations observed from adding five different species in the consortium. Amongst the species added, only the abundance of T. thermosaccharolyticum seemed to correlate with enhanced H₂ production. This could have been a result of the used cultivation pH since optimum H₂ production by Thermoanaerobacterium thermosaccharolyticum has been reported at pH of 6.5 (Saripan and Reungsang, 2013). Nonetheless, it was demonstrated that bioaugmentation helps to improve the diversity of the microbial community by making up for the loss in microbial diversity occurring during especially upward temperature fluctuations and this seemed to be crucial for optimum performance. Previous studies on the effects of temperature fluctuations on mesophilic and thermophilic conditions have shown that temperature fluctuations tend to result in unstable bioreactor performance, and in some cases in complete failure of the system (El-Mashad et al., 2004; Gadow et al., 2013; Wu et al., 2006). It is important that operators of bioreactors have strategies to cope with unexpected interruptions or technical failures that can arise during bioreactor operation and based on the results of this study, bioaugmentation with carefully selected microorganisms could...
be a viable option. The stability of a bacterial community depends on its structure, which can change due to environmental disturbances such as changes in operational conditions or ecological interactions (Shade et al., 2012). This study demonstrated that having specialized microorganisms such as *Thermoanaerobacterium* in the consortium can combat the negative effects of sudden disturbances.

In chapter 5, H₂ production of pure cultures of *Thermotoga neapolitana* and *Caldicellulosiruptor saccharolyticus* and their synthetic co-culture was compared. Both microorganisms were selected for their ability to efficiently utilize hexose and pentose sugars to obtain high H₂ yields. *T. neapolitana* can produce up to 3.8 mol H₂ mol⁻¹ glucose (Munro et al., 2009) and *Caldicellulosiruptor saccharolyticus* has been reported to produce up to 3.6 mol H₂ mol⁻¹ glucose (De Vrije et al., 2007). The H₂ production by co-culture of *T. neapolitana* and *C. saccharolyticus*, showed a 29 and 13% increase in H₂ production compared to pure cultures of *T. neapolitana* and *C. saccharolyticus*, respectively. The synthetic co-culture of these two ecologically distant species could also be used for treating complex carbohydrates as *C. saccharolyticus* has the ability to hydrolyze complex carbohydrates such as cellulose to glucose (van de Werken et al., 2008; Zurawski et al., 2015) which can also be utilized by both microorganisms (*C. saccharolyticus* and *T. neapolitana*) to produce H₂.

The effect of different feed glucose concentrations (from 5.6 to 111.0 mmol L⁻¹) on H₂ production with and without augmenting the culture with *T. neapolitana* further illustrated that bioaugmentation enhances H₂ production. The unaugmented mixed culture was dominated by *Thermoanaerobacter spp.*, which are well known thermophilic bacteria capable to produce H₂, ethanol and acetate (Claassen et al., 1999; Koskinen et al., 2008; Lin and Tanaka, 2006). Thus, their presence explains also the high ethanol production that was observed during the incubations. In the batch studies, bioaugmentation increased the H₂ production at the different substrate concentrations. H₂ production in the bioaugmented cultures increased by 38% at 5.6 mmol⁻¹ glucose compared to the unaugmented culture with the same initial concentration of glucose. Even though there was an overall decreased in H₂ yield with the increase in glucose concentration in both unaugmented and augmented cultures, bioaugmented cultures showed higher yields compared to the unaugmented cultures.

Bioaugmentation also caused a shift in the metabolic networks towards when compared to the unaugmented culture, resulting in a lower share of ethanol and a higher share of acetate and lactate in the fermentation broth. Lactate as an electron sink was responsible for the lower H₂
yield observed at higher glucose concentrations, as lactate production takes a large amount of reducing power away from H\textsubscript{2} production (Kim et al., 2014; Mishra et al., 2019). Previous studies have shown high concentrations of lactate by *T. neapolitana* at increased substrate concentrations (Dreschke et al., 2019b), which explains the increased share of lactate observed in the augmented culture compared to the unaugmented culture. This study demonstrated that the augmented cells were able to adapt to their new environment and co-existed with the native consortium. The persistence of *T. neapolitana* in the native microbial consortium is also attributed to the pre-adaptation method that was used in the study.

Even though bioaugmentation has been shown to enhance H\textsubscript{2} yield in dark fermentative H\textsubscript{2} production (Goud et al., 2014; Kumar et al., 2015; Ma et al., 2009; Wang et al., 2008; Zhang et al., 2019), only very few studies have used molecular methods to validate the effect of bioaugmentation. QPCR technique developed in chapter 5 was used to validate the growth of *T. neapolitana* in the synthetic co-culture and to quantify *T. neapolitana* from the mixed cultures bioaugmented with *T. neapolitana* (chapter 6) at different substrate concentrations. The qPCR data showed that the gene copies of *T. neapolitana* increased in the bioaugmented mixed culture with increase in glucose concentration. High-throughput sequencing was used to characterize the bacteria and investigate whether the added cells could be linked to H\textsubscript{2} production performance. The data obtained from high-throughput sequencing showed that *Thermoanaerobacter* was the major player in dark fermentation in both unaugmented and augmented cultures. However, with the augmented cultures, it was observed that *Thermotoga* was also an active participant and its relative abundance increased with increase in substrate concentration. The application of qPCR technique indicated the significant increase in the population of *T. neapolitana* added with respect to increase in substrate concentration. Combined with qPCR, high-throughput sequencing served as a useful tool for monitoring the survival and activity of bioaugmented strains (chapter 6).

### 7.1.1. Microbial community stability during process disturbances and its implication for H\textsubscript{2} production

Understanding of the resistance (insensitivity to disturbance) and resilience (the rate of recovery after disturbance) is important for predicting community response to a disturbance. In chapters 3 and 4, *Thermoanaerobacterium* was dominant genus in the mixed cultures at 55 °C with an average of 90% relative abundance. Other genera observed from the culture included *Clostridium* and *Bacillus* and their relative abundance changed with respect to temporal fluctuations. All of these bacterial genera have been widely observed during thermophilic dark fermentative H\textsubscript{2}
production (Cabrol et al., 2017; Pawar and van Niel, 2013). However, different H\textsubscript{2} producing bacteria become dominant at different operating conditions (Dessì et al., 2018; Gómez et al., 2009; Kothari, 2017). A look into the microbial communities showed that *Thermoanaerobacterium* was more resistant to upward or downward temperature fluctuations. Nonetheless, *Clostridium* spp. became dominant during the downward temperature fluctuations (45 and 35 °C) especially at 35 °C. The distribution of *Clostridium* during the downward temperature fluctuations was significantly higher as opposed to their share at 55 °C. This shows that in the event of a sudden downward temperature fluctuation, *Clostridium* had a competitive advantage over the other microorganisms and thus preventing complete loss in H\textsubscript{2} metabolism or minimizing the negative effect of temperature fluctuation on H\textsubscript{2} metabolism. *Thermoanaerobacterium* remained dominant during and after the upward temperature fluctuations (65 and 75 °C). However, a significant amount of the microbial diversity, e.g. *Clostridium* spp., was lost as a result of the high degree of temperature fluctuation. Despite the high temperature fluctuation at 75 °C, *Thermoanaerobacterium* spp. were able to restore the H\textsubscript{2} metabolism after the fluctuation period. Therefore, the greater the range of species that are capable of responding differently to diverse environmental fluctuations (either by resisting the fluctuation or being able to recover from it), the more likely it is that the microbiome will stabilize in response to the applied fluctuation (Allison and Martiny, 2008; Elmqvist et al., 2003; Holling, 2013). Our results indicate that microbial communities were more resilient to downward temperature fluctuations compared to upward temperature fluctuations due to the thermostolerant *Clostridium* spp. present in the culture. Table 7.3 provides a summary of the effects of sudden temperature fluctuations on the hydrogen yield during and after temperature fluctuation and the effect of bioaugmentation on the obtained hydrogen yield in batch conditions.
Table 7.3 Summary of the effects of sudden temperature fluctuations on the hydrogen yield during and after temperature fluctuation and the effect of bioaugmentation on the obtained hydrogen yield in batch conditions.

<table>
<thead>
<tr>
<th>Inoculum</th>
<th>pH</th>
<th>Substrate concentration (mM)</th>
<th>Temperature (°C)</th>
<th>Maximum H₂ yield (mol H₂ mol⁻¹ glucose equivalent)</th>
<th>Percentage increase in H₂ yield from augmentation</th>
<th>Major metabolites</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enriched hydrogenogenic culture</td>
<td>6.0</td>
<td>4.4 glu⁴ + 8.0 xyl⁵</td>
<td>55</td>
<td>2.1</td>
<td>NA</td>
<td>butyrate, acetate, lactate, ethanol</td>
<td>Chapter 3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>35 → 55</td>
<td>1.8 → 2.2</td>
<td>NA</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>45 → 55</td>
<td>1.6 → 2.0</td>
<td>NA</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>65 → 55</td>
<td>1.9 → 1.9</td>
<td>NA</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>75 → 55</td>
<td>0.0 → 1.1</td>
<td>NA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unaugmented (Control)</td>
<td>6.0</td>
<td>4.4 glu⁴ + 8.0 xyl⁵</td>
<td>55</td>
<td>1.9</td>
<td>NA</td>
<td>butyrate, acetate, lactate, ethanol</td>
<td>Chapter 3</td>
</tr>
<tr>
<td>Augmented</td>
<td>55</td>
<td>1.9</td>
<td>55</td>
<td>2.1</td>
<td>NA</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Upward temperature fluctuation</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unaugmented</td>
<td>35 → 55</td>
<td>1.4 → 1.8</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bioaugmentation during temperature fluctuation</td>
<td>35 → 55</td>
<td>1.8 → 1.9</td>
<td></td>
<td></td>
<td>36.3% → 6.8%</td>
<td>Butyrate, acetate, lactate, ethanol</td>
<td>Chapter 4</td>
</tr>
<tr>
<td>Bioaugmentation after temperature fluctuation</td>
<td>6.0</td>
<td>4.4 glu⁴ + 8.0 xyl⁵</td>
<td>35 → 55</td>
<td>1.4 → 1.9</td>
<td>0 → 9.7%</td>
<td></td>
<td></td>
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<tr>
<td><strong>Upward temperature fluctuation</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unaugmented</td>
<td>75 → 55</td>
<td>0.0 → 1.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bioaugmentation during temperature fluctuation</td>
<td>75 → 55</td>
<td>0.0 → 1.9</td>
<td></td>
<td></td>
<td>0.00% → 30%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bioaugmentation after temperature fluctuation</td>
<td>75 → 55</td>
<td>0.0 → 1.9</td>
<td></td>
<td></td>
<td>0 → 33%</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

glu⁴ = glucose, xyl⁵ = xylose, NA = data not available
In chapter 6, the gradual increase of glucose did not cause any significant shift in the microbial distribution in unaugmented cultures. *Thermoanaerobacter* remained the most dominant member of the consortium even though lactate concentration increased with increase in glucose concentration. Zhao et al. (2018) showed that increase in acetate concentration caused a shift in the microbial structure as well as a redirection in the degradation pathway during methane production. In this study, it is possible that the microbial community structure remained the same due to the low diversity observed under thermophilic conditions. However, the impact of different substrate concentration during dark fermentative H$_2$ production under mesophilic conditions could have more significant effects on the microbial community structure due to higher microbial diversity. With cultures augmented with *T. neapolitana*, *Thermoanaerobacter* remained the dominant member of the consortium with 75% relative abundance while *Thermotoga* was 23% at 5.6 mmol L$^{-1}$ initial glucose concentration. However, the increase in substrate concentration led to increase in the abundance of *Thermotoga* to 37%, causing a reduction in the relative abundance of *Thermoanaerobacter* (62%).

Based on the results of this study, it is therefore suggested that microbial diversity plays an important role during stress conditions such as temperature fluctuations or increase in substrate concentration. However, when comparing the effects of temperature fluctuations on the microbial community structure and the effect of increasing substrate concentration, it is obvious that temperature changes during dark fermentative H$_2$ production have a more significant effect on the microbial community composition.

### 7.1.2. Practical implications and limitations of bioaugmentation

Having bioreactors equipped to efficiently degrade organic materials require the presence of microbial consortia that have the ability to degrade the available waste materials. The concept of bioaugmentation has been studied extensively in bioremediation processes for removal of recalcitrant compounds such as chlorinated compounds by *Dehalococcoides* from groundwater (Schaefer et al., 2009). This study showed that bioaugmentation can be successfully used as a strategy for enhancing H$_2$ production in batch systems and was validated by integrating molecular methods, i.e., qPCR and/or high-throughput sequencing (Chapter 4 and 6). However, previous studies have reported the loss of the added specie over time due reasons such as, inability to compete with members of the native microbial community, phage infection and weak biofilm formation or washout (Bouchez et al., 2000; Fu et al., 2009; Nzila et al., 2016; Zhang et al., 2017). Some studies have used repeated bioaugmentation as a strategy to ensure the enhancement of
biological processes (Yang et al., 2016a, 2016b). Though the use of this method has been shown to be effective, to save time and cost, based on this study, the pre-adaptation of bacterial species to a native microbial community prior to its application in a biological process is considered practical. With this method, it would also be easier to pre-determine whether or not, the added species is compatible with the native microbial community. The pre-adaptation method used in this study (chapter 6) was successful as T. neapolitana was shown to be an active member of the microbial consortium based on the microbial methods applied. Figure 7.1 gives an illustration of how bioaugmentation can be applied to an industrial process and how microbiological applications such as qPCR can be integrated with engineering principles to enhance the efficiency of biological processes. Based on previous studies, the persistence and survival of a bacteria added into a microbial community depends on the augmentation pattern (Yang et al., 2016a, 2016b). For example, Yang et al (2016a) compared one time bioaugmentation and repeated bioaugmentation with Hydrogenispora ethanolica LX-B for enhancing hydrogen production. They observed that repeated bioaugmentation ensured the persistence of the added bacterium in the system while with one-time bioaugmentation, the H. ethanolica became undetectable after three batch incubation cycles. Similarly, Yang et al (2016b) also showed higher methane production with repeated bioaugmentation compared to one time bioaugmentation.

For a robust and commercially viable application of dark fermentative hydrogen production, chemical and physical factors such as pH, temperature, and the partial pressure of H₂ influencing the hydrogen yield and production rate need to be addressed. Furthermore, inoculum selection is crucial to ensure operational stability and stress tolerance. Coupled with the low H₂ yield obtained during dark fermentative H₂ production, the incomplete degradation of organic materials is one of the bottlenecks in the commercialization of dark fermentative H₂ production. Nonetheless, the results of chapter 5 show that H₂ yields can be enhanced using synthetic co-cultures. Furthermore, results of chapter 4 also illustrated that H₂ yields can be maintained during unstable conditions by bioaugmentation with highly specializes H₂-producers. The microbial community structure and its variation during the operation of a fermenter is of prime significance to a biohydrogen production process. As observed in this study, any notable change in the microbial community structure could lead to a significant variation in system performance. It is therefore, suggested that microbial monitoring should be conducted while scaling-up biohydrogen production processes to understand the potential implications of stressed experienced in pilot- and large-scale systems (e.g. inhomogeneities in pH, temperature and substrate concentration due to non-optimal mixing) on the microbial community. This study demonstrated that the use of
selective primers for monitoring a specific microbial community dominated in dark fermentation can be designed and applied successfully to monitor the fluctuation or dynamics of the specific microorganisms. Similarly, in order to understand the long-term effects of bioaugmentation, it is important to determine the fate or survival of exogenous microorganisms added into a biological system. On this note, the use of microbial monitoring methods is therefore essential and should be integrated with engineering principles in order to enhance biological processes.
Chapter 7: General discussion and conclusion

Figure 7.1 Illustration of ways to integrate bioaugmentation and molecular monitoring methods to dark fermentation processes.
Chapter 7: General discussion and conclusion

7.2. Future research perspectives

In this study bioaugmentation was found beneficial especially during shock conditions, but bioaugmentation should also be studied in continuously fed systems to determine the effects of bioaugmentation and fate of added microorganism(s) in more realistic conditions for commercial H₂ production. Additionally, given the enhancement of H₂ production from the use of synthetic co-cultures in batch conditions, further studies should also be conducted to evaluate the H₂ production efficiency of the co-culture of _T. neapolitana_ and _C. saccharolyticus_ over a long period and preferably in a system operated in continuous mode. Furthermore, the use of more complex substrates from wastewater sources or lignocellulosic wastes would enable the evaluation of the effectiveness of both the synthetic co-culture and bioaugmented cultures in utilizing organic waste materials for H₂ production. For example, it is expected that adding _T. neapolitana_ to an existing microbial community will improve the catabolism of complex organic compounds due to its ability to degrade a wide variety of carbohydrates (De Vrije et al., 2009; Kazanov et al., 2013; Rodionov et al., 2013).

As bioaugmentation of a thermophilic mixed culture with _T. neapolitana_ was demonstrated to enhance H₂ production, it would be useful to understand better the underlying mechanisms. As the genomic data of _T. neapolitana_ is available, it should be possible to identify specific peptides for _T. neapolitana_ (most preferably, peptides of hydrogenase which would closely link with H₂ production) as a substitute for monitoring the activity of _T. neapolitana_ in a mixed culture. Proteomic quantification provides a better resolution than genomic or transcriptomic quantification for monitoring the activity of the bacteria and it enables the quantification of the actual enzyme responsible for the process (Sidoli et al., 2017). Furthermore, the use of molecular methods in dark fermentative H₂ production to monitor cause and effect of the process should be extended to time series of shotgun metagenomics or meta-transcriptomics data. This would allow researchers to quantify the number of functions shared across taxa from the same community and identify the taxa that are expressing genes for specific functions at a given time point (Aguiar-Pulido et al., 2016; Mallick et al., 2017). Obtaining such information through time and in response to disturbances would provide quantitative insight as to how often and under what scenarios the microbial community structure and function are linked, and whether the linkages observed are relevant for H₂ production. Applying these molecular tools to carefully designed disturbance experiments will additionally help to unravel mechanisms of community stability. Thus, such information-rich datasets can be used in predicting stability of microbial communities in the face of new disturbances.
Finally, the diversification of the end products of metabolism (organic acids and alcohols) obtained from thermophilic dark fermentation may help to overcome the low H$_2$ yield obtained from the process. Volatile fatty acids such as acetate and butyrate which are key intermediate products observed in this study can also be considered as intermediates for currently emerging bioprocesses such as medium chain fatty acids generation (Atasoy et al., 2018; Baumann and Westermann, 2016; Bhatia and Yang, 2017). However, it is very difficult to separate the volatile fatty acids from one another to provide pure products and this might make their commercial utilization challenging (Kim et al., 2013). Another possibility would be the use of the effluents of dark fermentation as substrate for anaerobic digestion to produce methane (Mamimin et al., 2015; Martinez-Jimenez et al., 2017; Mishra et al., 2017; Si et al., 2016), for microbial fuel cells to produce electricity (Wang et al., 2011) or to photofermentation (Chen et al., 2008). It should be noted, however, that for now from these options, biogas production is the only commercialized conversion process.

### 7.3. Conclusions

Results of this study showed that the ability of a microbial consortium to withstand temperature fluctuations depends on the degree and direction of the temperature shift. On one hand, sudden decrease in temperature led to a reduction in H$_2$ production despite due to re-adaptation of thermotolerant species to the lower incubation temperature. On the other hand, higher temperatures lead to loss in microbial diversity, decrease in H$_2$ production and possible temporal termination of H$_2$ production.

Bioaugmentation was shown to be an effective strategy for enhancing H$_2$ production. When the mixture of five different microorganisms (*Caldicellulosiruptor saccharolyticus*, *Clostridium thermocellum*, *Thermoanaerobacterium thermosaccharolyticum* and *Thermotoga neapolitana*) were used for bioaugmentation, only the relative abundance of *T. thermosaccharolyticum* correlated with the improvement in H$_2$ yield based on results obtained from molecular analyses. Bioaugmentation with a single strain (*T. neapolitana*) showed that the use of a single strain with the required properties needed in a biological system is sufficient for improving dark fermentative H$_2$ production. However, bioaugmentation did not only enhance the H$_2$ yield, it also improved the production rate as well as the substrate degradation efficiency. The addition of a specialized bacterium or a group of specialized microorganisms into an existing consortium helps the microbiome to become more adaptable towards deviations in environmental conditions.
In this study, the highest H\textsubscript{2} yield was obtained with a synthetic co-culture containing \textit{C. saccharolyticus} and \textit{T. neapolitana} which resulted to about 3.3 or 12\% increase in H\textsubscript{2} yield when compared to pure cultures of \textit{C. saccharolyticus} and \textit{T. neapolitana}, respectively. The H\textsubscript{2} yield obtained with the synthetic co-culture was also higher than the yields obtained with any of the mixed cultures used in this study. However, it should be taken into account that the incubation conditions varied between the experiments conducted using different cultures and therefore the results are not fully comparable.

In summary, this study showed that thermophilic dark fermentative H\textsubscript{2} production can be enhanced by using synthetic co-cultures or bioaugmentation. The qPCR method used to target the HydA gene was an important tool developed for monitoring the survival of \textit{T. neapolitana} in mixed culture systems. The monitoring method targeting the 16S rRNA was also beneficial not only for \textit{T. neapolitana} but also other members of the genus \textit{Thermotoga}.

The use of molecular methods improved the understanding on the role of certain species in the consortium and improved the understanding of the microbial community dynamics under varying conditions. The use of molecular methods is therefore important in biological applications as it helps to create a link between the microbial community structure and observed H\textsubscript{2} production.
REFERENCES


Chapter 7: General discussion and conclusion


Appendixes: supporting information for Chapters

Table S6.1 Production yields of acetate lactate and ethanol per mol of glucose consumed in the unaugmented and the T. neapolitana-augmented culture at different feed glucose concentrations.

<table>
<thead>
<tr>
<th>Culture</th>
<th>Feed glucose concentration (mmol L⁻¹)</th>
<th>Soluble metabolites (mol mol⁻¹ glucose consumed)</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Acetate</td>
<td>Ethanol</td>
<td>Lactate</td>
<td>Acetate</td>
</tr>
<tr>
<td>Cultures without bioaugmentation</td>
<td>5.6</td>
<td>0.21</td>
<td>1.17</td>
<td>0.09</td>
<td>0.25</td>
</tr>
<tr>
<td></td>
<td>27.8</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>55.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>111.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cultures bioaugmented with T. neapolitana</td>
<td>5.6</td>
<td>0.43</td>
<td>0.87</td>
<td>0.20</td>
<td>0.16</td>
</tr>
<tr>
<td></td>
<td>27.8</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td></td>
<td>55.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>111.0</td>
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</tbody>
</table>