



HAL
open science

Mesophilic and thermophilic biohydrogen and bioelectricity production from real and synthetic wastewaters

Paolo Dessi

► **To cite this version:**

Paolo Dessi. Mesophilic and thermophilic biohydrogen and bioelectricity production from real and synthetic wastewaters. Environmental Engineering. Université Paris-Est; Tampereen yliopisto, 2018. English. NNT: 2018PESC2056 . tel-02373735

HAL Id: tel-02373735

<https://theses.hal.science/tel-02373735>

Submitted on 21 Nov 2019

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Joint PhD degree in Environmental Technology



Docteur de l'Université Paris-Est
Spécialité : Science et Technique de l'Environnement



Dottore di Ricerca in Tecnologie Ambientali



Degree of Doctor in Environmental Technology



TAMPERE UNIVERSITY OF TECHNOLOGY

Thesis for the degree of Doctor of Philosophy in Environmental Technology

Tesi di Dottorato – Thèse – PhD thesis – Väitöskirja

Paolo Dessì

Mesophilic and thermophilic biohydrogen and bioelectricity production from real and synthetic wastewaters

23/05/2018, Tampere

In front of the PhD evaluation committee

Prof. Alan Guwy	Reviewer
Dr. Serge Hiligsmann	Reviewer
Prof. Jóhann Örlygsson	Reviewer
Prof. Piet N.L. Lens	Promotor
Prof. Giovanni Esposito	Co-Promotor
Prof. Hab. Eric D. van Hullebusch	Co-Promotor
Asst. Prof. Aino-Maija Lakaniemi	Co-Promotor
Prof. Jukka Rintala	Chair



Evaluation committee

Chair

Prof. Jukka Rintala
Laboratory of Chemistry and Bioengineering
Faculty of Natural Sciences
Tampere University of Technology
Finland

Reviewers/Examiners

Prof. Alan Guwy
Sustainable Environment Research Centre
Faculty of Computing, Engineering and Science University of South Wales
United Kingdom

Dr. Serge Hilgsmann
3BIO-BioTech
Brussels School of Engineering
Université Libre de Bruxelles
Belgium

Prof. Jóhann Örlygsson
Faculty of Natural Resource Sciences
University of Akureyri
Iceland

Thesis Promotor

Prof. P.N.L. Lens
Department of Environmental Engineering and Water Technology
IHE Delft
Delft, The Netherlands

Thesis Co-Promotors

Prof. Giovanni Esposito
Department of Civil and Mechanical Engineering
University of Cassino and Southern Lazio
Italy

Prof. Hab. Eric D. van Hullebusch
University of Paris-Est Marne-la-Vallée
France

Asst. Prof. Aino-Maija Lakaniemi
Laboratory of Chemistry and Bioengineering
Faculty of Natural Sciences
Tampere University of Technology
Finland

Supervisory team

Thesis Supervisor

Prof. P.N.L. Lens
Department of Environmental Engineering and Water Technology
IHE Delft
Delft, The Netherlands

Thesis Co-Supervisors

Prof. Giovanni Esposito
Department of Civil and Mechanical Engineering
University of Cassino and Southern Lazio
Italy

Asst. Prof. Aino-Maija Lakaniemi
Laboratory of Chemistry and Bioengineering
Faculty of Natural Sciences
Tampere University of Technology
Finland

Dr. Gavin Collins
Microbial Communities Laboratory
School of Natural Sciences
National University of Ireland Galway
Ireland

Thesis Instructors

Asst. Prof. Luigi Frunzo
Department of Mathematics and Applications
University of Naples Federico II
Italy

Dr. Estefania Porca
Microbial Communities Laboratory
School of Natural Sciences
National University of Ireland Galway
Ireland

This research was conducted in the framework of the Marie Skłodowska-Curie European Joint Doctorate (EJD) in Advanced Biological Waste-to-Energy Technologies (ABWET) and supported by from Horizon 2020 under grant agreement no. 643071.

Abstract

In the last century, fossil fuels have been intensively used for energy production causing a dramatic increase of CO₂ level in the atmosphere and the related environmental issues, such as global warming and ozone layer depletion. In 2015, the increased awareness about climate change led to the signature of the Paris agreement, in which 195 countries committed to cut off their greenhouse gases emission by 40% (compared to emissions in 1990) by 2030. The achievement of such an ambitious target is strictly linked to a gradual switch from fossil fuels to sustainable and renewable sources for energy production. This is driving many industries producing organic and inorganic waste towards a biorefinery concept, in which side streams, wastes and wastewaters are seen as a potential feedstocks for biofuel and/or biochemical production.

Dark fermentation and microbial fuel cells (MFCs) are two emerging technologies for biological conversion of the chemical energy of organic compounds into hydrogen (H₂) and electricity, respectively. Although these technologies can potentially replace fossil fuels for energy production, their establishment is hindered by their low energy output. Due to kinetic and thermodynamic advantages, high temperature can be the key for increasing both dark fermentative H₂ production and electricity production in MFCs. Therefore, this thesis focuses on delineating how temperature influences biological production of H₂ and electricity from organic carbon-containing wastewaters.

Start-up and selection of a suitable microbial community is a crucial phase in dark fermentation. Two heat-treated inocula (fresh and digested activated sludge) were compared, in four consecutive batch cycles, for H₂ production from xylose at 37, 55 and 70 °C. At both 37 and 55 °C, a higher H₂ yield was achieved by the fresh than the digested activated sludge, whereas a very low H₂ yield was obtained by both inocula at 70 °C. Then, four different inoculum pretreatments (acidic, alkaline, heat and freezing shocks) were evaluated, in a single-stage batch experiment, for creating an efficient mesophilic (37 °C) and thermophilic (55 °C) H₂ producing community. Acidic and alkaline shocks selected known H₂ producing microorganisms belonging to *Clostridiaceae* at the expenses of lactate producing bacteria, resulting in the highest H₂ yield at 37 and 55 °C, respectively. Although a heat shock resulted in a low H₂ yield in a single batch, H₂ production by the heat-treated fresh activated sludge was shown to increase in the experiment with four consecutive batch cycles. This suggests that H₂ producing microbial communities may develop in the long-term as long as culture conditions are optimized for growth of H₂ producers.

Heat-treated fresh activated sludge was selected as inoculum for continuous H₂ production from a xylose-containing synthetic wastewater in a mesophilic (37 °C) and a thermophilic (55-70 °C, increased stepwise) fluidized bed reactor (FBR). A higher H₂ yield was obtained in the thermophilic than in the mesophilic FBR. Furthermore, H₂ production at 70 °C, which failed in the earlier batch study, was successful in the FBR, with a stable yield of 1.2 mol H₂ mol⁻¹ xylose_{added}, by adapting the microbial community from 55 °C to 70 °C stepwise at 5 °C intervals. Operation temperature of 70 °C was also found optimal for H₂ production from thermomechanical pulping (TMP) wastewater in a temperature gradient incubator assay, as batch cultivation at 70 °C enriched the H₂ producing *Thermoanaerobacterium* sp. and repressed homoacetogenic microorganisms.

A detailed knowledge of microbial communities, and particularly the active subpopulation, is crucial in order to adjust the conditions to favor the growth of exoelectrogenic microorganisms in MFCs. A RNA approach was used to study the structure and role of the anode-attached, membrane-attached and planktonic microbial communities in a mesophilic (37 °C) and a thermophilic (55 °C) two-chamber, xylose-fed MFC. An anode-attached community dominated by *Geobacteraceae* sustained electricity production at 37 °C, whereas the establishment of methanogenic and H₂ oxidizing microorganisms resulted in a low electricity production at 55 °C. However, the development of a thermophilic exoelectrogenic community can be promoted by applying a start-up strategy which includes imposing a negative potential to the anode and chemical inhibition of methanogens. At both 37 and 55 °C, aerobic membrane-attached microorganisms were likely involved in consuming the oxygen diffusing from the cathodic to the anodic chamber, thus favoring the exoelectrogenic microorganisms, which are strictly anaerobic, but competing with them for the substrate. A mesophilic exoelectrogenic community was also shown to produce electricity from TMP wastewater in an upflow MFC operated at 37 °C.

In conclusion, a higher and more stable H₂ yield can be achieved in thermophilic rather than mesophilic dark fermentation. Dark fermentation at 70 °C is particularly suitable for treatment of TMP wastewater as it is released at high temperature (50-80 °C) and could be treated on site with minimal energy requirement for heating the bioreactor. TMP wastewater can be also used as substrate for electricity production in mesophilic MFCs. Electricity production in thermophilic MFCs is feasible, but enrichment of thermophilic exoelectrogenic microorganisms may require a long start-up period with optimized conditions. The detailed RNA-level microbial community analysis performed in this study may help in selecting a start-up and operation strategy to optimize electricity production.

Tiivistelmä

Fossilisia polttoaineita on hyödynnetty energian tuotannossa laajasti yli sadan vuoden ajan. Tämä on lisännyt merkittävästi hiilidioksidin määrää ilmakehässä edistään ilmastomuutosta sekä otsonikerroksen ohenemista. Satayhdeksänkymmentäviisi valtiota allekirjoitti Pariisin ilmastosopimuksen vuonna 2015 ja sitoutui vähentämään kasvihuonepäästöjään 40 % vuoteen 2030 mennessä vuoden 1990 tasosta. Kunnianhimoisen tavoitteen saavuttaminen vaatii energian tuotannon siirtymistä fossilisista polttoaineista kestäviin ja uusiutuviin energialähteisiin. Yhtenä ratkaisuna tämän tavoitteen saavuttamiseksi monet orgaanisia ja epäorgaanisia jätteitä tuottavat teollisuuden alat pyrkivät muuntumaan biojalostamoiksi, joissa sivuvirrat, jätteet ja jätevedet ovat mahdollisia raaka-aineina biopolttoaineiden ja/tai bioperäisten kemikaalien tuotannossa.

Pimeäfermentaatio ja mikrobipolttokennot ovat kehittyviä prosesseja, joissa orgaanisten yhdisteiden kemiallinen energia muunnetaan mikrobien avulla vedyksi tai sähköksi. Vaikka nämä bioprosessit voivat korvata fossilisia polttoaineita, niiden teollista hyödyntämistä hidastavat toistaiseksi suhteellisen matalat energiasaannot ja tuotantoprosessien epävakaus. Korkeassa lämpötilassa reaktiokinetiikka ja termodynamiikka voivat edistää vedyntuottoa pimeäfermentaatiolla sekä sähköntuottoa mikrobipolttokennoissa. Tässä väitöstyössä tutkittiin lämpötilan vaikutusta vedyn ja sähkön biologiseen tuotantoon biohajoavia orgaanisia yhdisteitä sisältävistä jätevesistä.

Yksi tärkeimmistä vaiheista pimeäfermentaation prosessikehityksessä on fermentaation käynnistys ja soveltuvien mikrobien valinta. Tässä työssä vertailtiin kahden lämpökäsittelyn mikrobiviljelmän (tuore ja mädätetty aktiiviliete) vedyntuottoa ksyloosista neljän perättäisen panosrikastusvaiheen jälkeen eri lämpötiloissa (37, 55 ja 70 °C). Vetyasaannot olivat suurempia tuoreella aktiivilietteellä 37 ja 55 °C:ssa. Sen sijaan 70 °C:ssa vedyntuotto oli hyvin vähäistä molemmilla rikastusviljelmillä. Lisäksi neljää eri esikäsittelymenetelmää (happo-, emäs-, lämpö- ja jäädytyskäsittely) vertailtiin yksivaiheisessa panoskokeessa tehokkaan mesofiilisen tai termofiilisen vetyä tuottavan mikrobisyhteisön aikaansaamiseksi. Happo- ja emäskäsittelyt valikoivat tunnettuja vetyä tuottavia mikroorganismeja (*Clostridiaceae*) maitohappoa tuottavien mikrobien kustannuksella. Happokäsittely mahdollisti korkeimman vedyntuoton 37 °C:ssa, kun taas emäskäsittely johti korkeimpaan vedyntuottoon 55 °C:ssa. Vaikka lämpökäsittely aktiiviliete tuotti varsin vähän vetyä yksittäisessä panoskasvatuksessa, lämpökäsittelyn aktiivilietteen vedyntuotanto kasvoi merkittävästi neljän perättäisen rikastusvaiheen aikana. Tämä osoittaa, että viljelmän esikäsittelyn lisäksi optimoidut kasvatusolosuhteet rikastavat tehokkaasti vetyä tuottavia mikrobisyhteisöjä.

Lämpökäsiteltyä aktiivilietettä käytettiin mikrobien lähteenä myös jatkuvatoimisissa leijupetireaktoreissa. Niissä tutkittiin vedyntuottoa ksyloosia sisältävästä synteettisestä jätevedestä 37 °C:ssa sekä 55-70 °C:ssa. Termofiilisessa leijupetireaktorissa vedyntuotto oli suurempaa kuin mesofiilisessa ja suurimmat vetysaannot saavutettiin 55 ja 70 °C:ssa, joissa molemmissa saanto oli 1.2 mol H₂ mol⁻¹ ksyloosi. Leijupetireaktorissa vetyä onnistuttiin tuottamaan 70 °C:ssa kasvatettaessa mikrobeja vaiheittain (5 °C) 55 °C:sta 70 °C:een nousevassa lämpötilassa, vaikka panoskokeessa vetyä ei saatukaan tuotettua 70 °C:ssa tuoreella aktiivilietteellä. Erillisessä panoskokeessa havaittiin, että 70 °C oli optimaalinen lämpötila vedyntuotolle myös termomekaanisen sellunvalmistuksen jätevedestä käytettäessä leijupetireaktorissa rikastettua mikrobiviljelmää.

Mikrobiyhteisöjen ja erityisesti aktiivisten mikrobiyhteisöjen tuntemus edistää eksoelektrogeenisten mikro-organismien kasvun optimoimista mikrobipolttokennoissa. Kaksi-kammioisissa mikrobipolttokennoissa tutkittiin anodille ja membraanille kiinnittyneiden sekä planktonisten mikrobiyhteisöjen rakennetta ja eri mikrobien rooleja sekä mesofiilissä että termofiilissä olosuhteissa. Anodille kiinnittyneet mikrobit (*Geobacteraceae*) tuottivat sähköä 37 °C:ssa, mutta metanogeenien ja vetyä kuluttavien mikrobien läsnäolo heikensi sähköntuottoa merkittävästi 55 °C:ssa. Sekä 37 °C:ssa että 55 °C:ssa aerobiset membraanille kiinnittyneet mikrobit kuluttivat katodikammioista anodikammioon virtaavaa happea. Samalla ne kuitenkin kilpailivat substraatista anaerobisten eksoelektrogeenisten mikrobien kanssa. Erillisessä ylöspäinvirtausmikrobipolttokennolla tehdyssä kokeessa sähköä tuotettiin onnistuneesti myös termomekaanisen sellunvalmistuksen jätevedestä 37 °C:ssa.

Tämä tutkimus osoitti, että termofiiliset mikrobit tuottavat enemmän ja vakaammin vetyä kuin mesofiiliset mikrobit. Pimeäfermentaatio 70 °C:ssa sopii erityisesti mm. termomekaanisen sellunvalmistuksen jätevesien käsittelyyn, koska termomekaanisessa prosessissa syntyvät jätevedet ovat lämpimiä (50-80 °C). Tällöin bioreaktorin lämmittämiseen ei tarvittaisi paljon energiaa. Tämän tutkimuksen perusteella termomekaanisen sellunvalmistuksen jätevesi soveltuu käsiteltäväksi sähköä tuottavissa mesofiilissä mikrobipolttokennoissa. Sähköntuotto termofiilissä mikrobipolttokennoissa oli myös mahdollista, vaikka termofiilisten eksoelektrogeenisten mikrobien rikastus vaatii pitkän aloitusvaiheen optimoiduissa olosuhteissa. Lisäksi yksityiskohtainen, RNA-tason mikrobianalyysi auttaa sähköntuotannon optimoimisessa.

Sommario

Nell'ultimo secolo, i combustibili fossili sono stati intensivamente usati per produzione di energia, causando un drammatico aumento della concentrazione di CO₂ nell'atmosfera e le relative problematiche ambientali, come il riscaldamento globale e il consumo dello strato di ozono. Nel 2015, l'aumento della consapevolezza sui cambiamenti climatici ha portata all'firma dell'accordo di Parigi, nel quale 195 nazioni hanno deciso di impegnarsi a tagliare le loro emissioni di gas-serra del 40% (rispetto ai valori del 1990) entro il 2030. Il raggiungimento di questo ambizioso obiettivo è strettamente legato a un graduale passaggio da combustibili fossili a fonti sostenibili e rinnovabili per la produzione di energia. Questo sta portando varie industrie produttrici di rifiuti organici verso l'implementazione di un concetto di bioraffineria, nel quale sottoprodotti industriali, rifiuti e acque di scarico sono visti come una potenziale materia prima per la produzione biologica di sostanze chimiche e combustibili.

Dark fermentation e microbial fuel cells (MFCs) sono due tecnologie emergenti per la conversione biologica dell'energia chimica dei composti organici in idrogeno (H₂) ed energia elettrica, rispettivamente. Nonostante tali tecnologie abbiano il potenziale per rimpiazzare i combustibili fossili per la produzione di energia, la loro affermazione è ostacolata dal loro scarso rendimento energetico. In base ai suoi vantaggi cinetici e termodinamici, l'alta temperatura potrebbe essere la chiave per migliorare sia la produzione di H₂ via dark fermentation, sia la produzione di energia elettrica in MFCs. Perciò, questa tesi focalizza sul determinare come la temperatura influenza la produzione biologica di H₂ ed energia elettrica da acque di scarico contenenti contaminanti organici.

L'avvio del processo e la selezione di un'adeguata popolazione microbiologica è una fase cruciale nella dark fermentation. Due inoculi sottoposti a shock termico caldo (fango attivo fresco e digestato) sono stati confrontati, in quattro esperimenti consecutivi in batch, per la loro produzione di H₂ a partire da xilosio a 37, 55 e 70 °C. A 37 e 55 °C, una più alta produzione di H₂ è stata ottenuta dal fango attivo fresco rispetto al digestato, mentre una produzione di H₂ molto bassa è stata ottenuta da entrambi gli inoculi a 70 °C. Quindi, quattro diversi pretrattamenti dell'inoculo (shock acido, shock basico, shock termico caldo e congelamento) sono stati valutati, in un singolo esperimento in batch, per ottenere un'efficiente popolazione microbica produttrice di H₂ in condizioni mesofile (37 °C) e termofile (55 °C). Gli shock acido e basico hanno selezionato con successo microrganismi noti produttori di H₂, appartenenti alle *Clostridiaceae*, a discapito dei batteri produttori di acido lattico, risultando nella più alta produzione di

idrogeno a 37 e 55 °C, rispettivamente. Sebbene lo shock termico caldo abbia causato una bassa produzione di H₂ in un singolo batch, la produzione di H₂ dal fango attivo fresco sottoposto a trattamento termico caldo è aumentata nei quattro batch consecutivi. Questo indica che una popolazione microbiologica produttrice di H₂ può svilupparsi nel tempo, se le condizioni sono ottimizzate per la sua crescita.

Fango attivo fresco sottoposto a trattamento termico caldo è stato selezionato come inoculo per la produzione di H₂ in continuo a partire da un'acqua di scarico sintetica contenente xilosio in un bioreattore a letto fluidizzato (FBR) operato in condizioni mesofile (37 °C) e termofile (55-70 °C, aumentata gradualmente). Una produzione di H₂ più alta è stata ottenuta nel FBR termofilo rispetto al mesofilo. Inoltre, la produzione di H₂ a 70 °C, fallimentare nell'esperimento in batch, è stata ottenuta nel FBR, con una produzione stabile di 1.2 mol H₂ mol⁻¹ xilosio, adattando gradualmente la popolazione microbica da 55 a 70 °C a salti di 5 °C. Il processo a 70 °C si è anche dimostrato ottimale per la produzione di H₂ a partire da acque di scarico proveniente da pulping termomeccanico (TMP) in un esperimento con incubatore a gradiente termico, in quanto la coltivazione in batch a 70 °C ha supportato la crescita del microorganismo produttore di H₂ *Thermoanaerobacterium* sp. e represso i microorganismi omoacetogenici.

Una conoscenza dettagliata delle popolazioni microbiche, e in particolare della frazione attiva, è cruciale per impostare condizioni favorevoli alla crescita di microorganismi elettrogeni nelle MFC. Un approccio basato sul RNA è stato usato per studiare la struttura e il ruolo delle popolazioni microbiche sviluppatesi attaccate all'anodo, attaccate alla membrana o in forma planctonica in MFC a due camere operate in condizioni mesofile (37 °C) e termofile (55 °C). Una popolazione anodica dominata da *Geobacteraceae* ha supportato la produzione di energia elettrica a 37 °C, mentre la crescita di microorganismi metanogeni e H₂-ossidanti ha causato una produzione bassa a 55 °C. Tuttavia, lo sviluppo di una popolazione elettrogena termofila può essere promossa applicando una strategia iniziale che includa l'imposizione di un potenziale anodico negativo e un'inibizione chimica dei metanogeni. A 37 e 55 °C, i microorganismi aerobici attaccati alla membrana hanno probabilmente consumato l'ossigeno che stava diffondendo dalla camera catodica a quella anodica, favorendo i microorganismi elettrogeni strettamente anaerobici, ma competendo con loro per il substrato. Una popolazione elettrogena mesofila ha prodotto energia elettrica anche da acqua di scarico da TMP in una MFC a flusso verticale operata a 37 °C.

In conclusione, una produzione di idrogeno più alta e stabile può essere ottenuta tramite dark fermentation in condizioni termofile piuttosto che mesofile. Dark fermentation a 70 °C è particolarmente indicata per il trattamento di acqua di scarico da TMP, in

quanto tale acqua viene rilasciata ad alta temperatura (50-80 °C) e può essere trattata in situ, con una richiesta di energia minima per riscaldare il bioreattore. L'acqua di scarico da TMP può anche essere usata come substrato per produzione di energia elettrica in MFCs mesofiliche. La produzione di energia elettrica in MFCs termofiliche è fattibile, ma lo sviluppo di microorganismi elettrogeni termofili può richiedere un lungo periodo in condizioni ottimizzate. L'analisi dettagliata sul RNA eseguita in questo studio potrebbe aiutare nella selezione di una strategia all'avvio e durante l'operazione per ottimizzare la produzione di energia elettrica.

Résumé

Au siècle dernier, les combustibles fossiles ont été intensivement utilisés pour la production d'énergie, entraînant une augmentation dramatique du niveau de CO₂ dans l'atmosphère et les problèmes environnementaux connexes, tels que le réchauffement de la planète et l'appauvrissement de la couche d'ozone. En 2015, la sensibilisation accrue au changement climatique a conduit à la signature de l'accord de Paris, dans lequel 195 pays s'engageaient à réduire leurs émissions de gaz à effet de serre de 40% (par rapport aux émissions de 1990) d'ici 2030. La réalisation d'un objectif aussi ambitieux est strictement liée à un passage progressif des énergies fossiles à des sources durables et renouvelables de production d'énergie. Cela conduit de nombreuses industries produisant des déchets organiques et inorganiques vers un concept de bioraffinerie dans lequel les effluents secondaires, les déchets et les eaux usées sont considérés comme des matières premières potentielles pour la production de biocarburant et / ou de biochimie.

La fermentation sombre et les piles à combustible microbiennes (MFC) sont deux technologies émergentes respectivement pour la conversion biologique de l'énergie chimique des composés organiques en hydrogène (H₂) et en électricité. Bien que ces technologies puissent potentiellement remplacer les combustibles fossiles pour la production d'énergie, leur établissement est entravé par leur faible production d'énergie. En raison des avantages cinétiques et thermodynamiques, la température élevée peut être la clé pour augmenter à la fois la production d'H₂ via fermentation sombre et la production d'électricité dans les MFC. Par conséquent, cette thèse se concentre sur la manière dont la température influence la production biologique de H₂ et d'électricité à partir d'eaux usées contenant du carbone organique.

Le démarrage et la sélection d'une communauté microbienne appropriée est une phase cruciale dans la fermentation sombre. Deux inocula traités thermiquement (à boues activées fraîches et digérées) ont été comparés, sur quatre cycles consécutifs, pour la production de H₂ à partir de xylose à 37, 55 et 70 °C. A la fois à 37 et 55 °C, on obtient un meilleur rendement en H₂ par les boues activées fraîches comparé aux boues digérées tandis qu'un très faible rendement en H₂ est obtenu par les deux inocula à 70 °C. Ensuite, quatre prétraitements d'inoculum différents (chocs acides, alcalins, thermiques et de congélation) ont été évalués, dans une expérience batch en une étape, pour créer une efficace communauté productrice de H₂ mésophile (37 °C) ou thermophile (55 °C). Les chocs acides et alcalins ont sélectionné des micro-organismes producteurs de H₂, appartenant aux *Clostridiaceae*, au détriment des bactéries produisant du lactate, ce qui a donné respectivement le rendement en H₂ le plus élevé à 37 et 55 °C. Bien que le choc

thermique ait abouti à un faible rendement en H₂ dans un seul lot, il a été montré que la production de H₂ par les boues activées fraîches traitées thermiquement augmentait dans l'expérience avec quatre cycles consécutifs. Ceci suggère que les communautés microbiennes productrices de H₂ peuvent se développer à long terme tant que les conditions de culture sont optimisées pour la croissance des producteurs de H₂.

Des boues activées fraîches et traitées thermiquement ont été sélectionnées comme inoculum pour la production continue de H₂ à partir d'une eau usée synthétique contenant du xylose dans un réacteur à lit fluidisé (FBR) mésophile (37 °C) et thermophile (55-70 °C, augmenté par étapes). Un rendement en H₂ plus élevé a été obtenu dans le FBR thermophile que dans le FBR mésophile. En outre, la production de H₂ à 70 °C, qui a échoué dans l'étude précédente, a été couronnée de succès dans le FBR, avec un rendement stable de 1.2 mol H₂ mol⁻¹ xylose, en adaptant la communauté microbienne de 55 °C à 70 °C à intervalles de 5 °C. La température de fonctionnement de 70 °C s'est également révélée optimale pour la production de H₂ à partir d'eaux usées thermomécaniques (TMP) dans un incubateur à gradient de température, car la culture en batch à 70 °C enrichissait le *Thermoanaerobacterium* sp. produisant du H₂ et réprimait les micro-organismes homoacétogènes.

Une connaissance détaillée des communautés microbiennes, et en particulier de la sous-population active, est cruciale pour ajuster les conditions favorables à la croissance de micro-organismes exoélectrogènes dans les MFC. Une approche de l'ARN a été utilisée pour étudier la structure et le rôle des communautés microbiennes attachées à l'anode, attachées à la membrane et planctoniques dans un MFC mésophile (37 °C) et thermophile (55 °C) alimenté au xylose. Une communauté anodine dominée par *Geobacteraceae* a soutenu la production d'électricité à 37 °C, alors que l'établissement de micro-organismes méthanogènes et H₂ oxydants a entraîné une faible production d'électricité à 55 °C. Cependant, le développement d'une communauté exoélectrogène thermophile peut être favorisé en appliquant une stratégie de démarrage qui comprend l'imposition d'un potentiel négatif à l'anode et l'inhibition chimique des méthanogènes. A 37 et 55 °C, les micro-organismes aérobies attachés à la membrane étaient probablement impliqués dans la consommation de l'oxygène diffusant de la chambre cathodique à la chambre anodique, favorisant les micro-organismes exoélectrogéniques strictement anaérobies mais étaient en compétition avec eux pour le substrat. Une communauté exoélectrogénique mésophile a également été mise en évidence pour produire de l'électricité à partir d'eaux usées de TMP dans un MFC à flux ascendant exploité à 37 °C.

En conclusion, une production de H₂ plus élevée et plus stable peut être obtenue dans une fermentation sombre thermophile plutôt que mésophile. La fermentation sombre à 70 °C est particulièrement appropriée pour le traitement des eaux usées de TMP car elle est libérée à haute température (50-80 °C) et pourrait être traitée sur site, avec des besoins énergétiques minimum pour chauffer le bioréacteur. Les eaux usées de TMP peuvent également être utilisées comme substrat pour la production d'électricité dans les MFC mésophiles. La production d'électricité dans les MFC thermophiles est faisable, mais l'enrichissement des micro-organismes exoélectrogènes thermophiles peut nécessiter une longue période de démarrage avec des conditions optimisées. L'analyse détaillée de la communauté microbienne au niveau de l'ARN réalisée dans cette étude peut aider à choisir une stratégie de démarrage et d'exploitation pour optimiser la production d'électricité.

Samenvatting

In de vorige eeuw werden fossiele brandstoffen intensief gebruikt voor de productie van energie, waardoor het CO₂-niveau in de atmosfeer en de daarmee samenhangende milieuproblemen, zoals het broeikaseffect en de uitputting van de ozonlaag, dramatisch toenamen. In 2015 leidde de toegenomen bewustwording van de klimaatverandering tot de ondertekening van de overeenkomst van Parijs, waarin 195 landen zich ertoe verbonden om hun emissie van broeikasgassen tegen 2030 met 40% te verminderen (vergeleken met emissies in 1990). De verwerkelijking van zo'n ambitieuze doelstelling is strikt gekoppeld aan een geleidelijke overgang van fossiele brandstoffen naar duurzame en hernieuwbare bronnen voor energieproductie. Dit stimuleert veel industrieën die organisch en anorganisch afval produceren in de richting van een bioraffinageconcept, waarbij zijstromen, afval en afvalwater worden gezien als potentiële grondstoffen voor biobrandstoffen en/of bioproductie van chemicaliën.

Donkere gisting en microbiële brandstofcellen (MFC's) zijn twee opkomende technologieën voor de biologische omzetting van chemische energie van organische verbindingen in respectievelijk waterstof (H₂) en elektriciteit. Hoewel deze technologieën potentieel fossiele brandstoffen voor energieproductie kunnen vervangen, wordt hun toepassing gehinderd door hun lage energieoutput. Vanwege kinetische en thermodynamische voordelen kan hoge temperatuur de sleutel zijn voor het verhogen van zowel de productie van donkere fermentatieve H₂ productie als elektriciteitsproductie in MFC's. Daarom richt dit proefschrift zich op het bepalen van de invloed van temperatuur op de biologische productie van waterstof en elektriciteit uit organisch koolstofhoudend afvalwater.

Start-up en selectie van geschikte microbiële gemeenschap is een cruciale fase in donkere fermentatie. Twee hittebehandelde inocula (vers en vergist actief slib) werden vergeleken in vier opeenvolgende batch cycli, voor H₂-productie uit xylose bij 37, 55 en 70 °C. Zowel bij 37 als bij 55 °C werd een hogere H₂-opbrengst bereikt door het verse dan vergiste actief slib, terwijl een zeer lage H₂-opbrengst werd verkregen door beide inocula bij 70 °C. Vervolgens werden vier verschillende inoculumvoorbehandelingen (zure, basische, hitte- en vriesschokken) geëvalueerd in een eenstaps batch-experiment, voor het creëren van een efficiënte mesofiele (37 °C) of thermofiele (55 °C) H₂ producerende gemeenschap. Zure en basische schokken selecteerden bekende waterstofproducerende micro-organismen die tot *Clostridiaceae* behoren ten koste van lactaatproducerende bacteriën, resulterend in de hoogste H₂-opbrengst bij respectievelijk 37 en 55 °C. Hoewel de hitteshock in een lage H₂-opbrengst resulteerde

in batch, bleek de H₂-productie van het hitte behandelde verse actief slib in het experiment met vier opeenvolgende batch cycli toe te nemen. Dit suggereert dat H₂-producerende microbiële gemeenschappen zich op lange termijn kunnen ontwikkelen zolang de kweekomstandigheden geoptimaliseerd zijn voor de groei van H₂-producenten.

Met hitte behandeld actief slib werd als inoculum gekozen voor continue H₂-productie uit een xylose-bevattend synthetisch afvalwater in een mesofiel (37 °C) en een thermofiele (55-70 °C, verhoogde stapsgewijze) wervelbedreactor (FBR). Een hogere H₂-opbrengst werd verkregen in de thermofiele dan in de mesofiele FBR. Bovendien was de H₂-productie bij 70 °C, die mislukte in het eerdere batchonderzoek, succesvol in de FBR, met een stabiele opbrengst van 1.2 mol H₂ mol⁻¹ xylose, door de microbiële gemeenschap stapsgewijs aan te passen van 55 °C tot 70 °C met intervallen van 5 °C. Eentemperatuur van 70 °C werd ook optimaal bevonden voor H₂-productie uit thermomechanisch pulp (TMP) afvalwater in een temperatuur gradiënt incubator test, omdat de batch-incubatie bij 70 °C de H₂ producerende *Thermoanaerobacterium* verrijkte en homoacetogene micro-organismen onderdrukte.

Een gedetailleerde kennis van microbiële gemeenschappen, en met name de actieve subpopulatie, is cruciaal om de omstandigheden aan te passen om de groei van exo-electrogene micro-organismen in MFCs te bevorderen. Een RNA-benadering werd gebruikt om de structuur en de rol van de aan anode gehechte, membraan-gebonden en planktonische microbiële gemeenschappen te bestuderen in een mesofiele (37 °C) en een thermofiele (55 °C) tweekamer xylose-gevoed MFC. Een anode-gehechte gemeenschap gedomineerd door *Geobacteraceae* gaf een continue stroomproductie bij 37 °C, terwijl de colonisatie van methanogene en H₂ oxiderende micro-organismen resulteerde in een lage stroomproductie bij 55 °C. De ontwikkeling van een thermofiele exo-electrogene gemeenschap kan echter worden bevorderd door een opstart strategie, bestaande uit het opleggen van een negatieve potentieel aan de anode en chemische remming van methanogenen. Zowel bij 37 als bij 55 °C waren aërobe membraangehechte micro-organismen waarschijnlijk betrokken bij het verbruiken van zuurstofverstrooiing van de kathode naar de anode kamer, waarbij de strikt anaërobe exo-electrogene micro-organismen de voorkeur kregen, maar met hen concurreerden voor substraat. Een mesofiele exo-electrogenische gemeenschap bleek ook elektriciteit te produceren uit TMP-afvalwater in een MFC met opwaartse vloeistofstroming bij 37 °C.

Concluderend kan een hogere en stabielere H₂-opbrengst worden bereikt in thermofiele dan in mesofiele donkere fermentatie. Donkere fermentatie bij 70 °C is met name geschikt voor de behandeling van TMP-afvalwater, omdat het wordt geloosd bij hoge temperatuur (50-80 °C) en ter plaatse kan worden behandeld met een minimale energiebehoefte voor het verwarmen van de bioreactor. TMP-afvalwater kan ook worden

gebruikt als substraat voor elektriciteitsproductie in mesofiele MFC's. Elektriciteitsproductie in thermofiele MFC's is haalbaar, maar verrijking van thermofiele exo-elektrogene micro-organismen kan een lange opstartperiode met geoptimaliseerde omstandigheden vereisen. De gedetailleerde microbiële gemeenschapsanalyse op RNA-niveau die in dit onderzoek is uitgevoerd, kan helpen bij het selecteren van een opstart- en bedrijfsstrategie om de energieproductie te optimaliseren.

Preface

This thesis summarizes three years of experimental work mainly performed at Tampere University of Technology (TUT), and during the research exchange periods at National University of Ireland Galway (NUIG) and Università di Cassino e del Lazio Meridionale (UNICAS). The research project was part of the Marie Skłodowska-Curie European Joint Doctorate in Advanced Biological Waste-To-Energy Technologies (ABWET). It received economical support from the European Union Horizon 2020 framework under grant agreement no. 643071.

I would like to thank my supervisor, Prof. Piet Lens, for his guidance through my research and for his valuable comments on my manuscripts. I gratefully thank my instructor, Asst. Prof. Aino-Maija Lakaniemi, which always supported me and gave useful comments on my research results and manuscripts. I am thankful to my co-supervisors Dr. Gavin Collins and prof. Giovanni Esposito, and to Dr. Estefania Porca and Dr. Luigi Frunzo, which supervised my research during my research exchange periods. I also thank prof. Jóhann Örlygsson, from University of Akureyri, Dr. Serge Hiligsmann, from Université Libre de Bruxelles, and prof. Alan Guwy, from University of South Wales for pre-reviewing and commenting my thesis.

I wish to thank all the past and present co-workers, particularly the bioelectrochemical system group in TUT, my colleagues from ABWET and ETeCoS³ European joint degree programmes and the “microbiology” research group in Galway. I thank them for the amazing time spent together both during work and free time. I would thank also the technicians, which successfully accomplished the difficult task of making things work in the lab. Particularly, I would thank Antti Nuottajärvi and Tarja Ylijoki-Kaiste from TUT, Maurice Martyn from NUIG and Gelsomino Monteverde from UNICAS. I thank also Prof. Jukka Rintala, Suvi-Päivikki Ikonen, Kirsi Viitanen from TUT and all the other people from partner universities for dealing with all the administrative and economical tasks related to the joint doctorate.

Finally, I would thank my family, for always support my decisions and for coming to Tampere in the day of my PhD defense and to all my friends from all over the world for making my days wonderful.

Tampere, March 2018

Paolo Dessì

Contents

ABSTRACT.....	I
TIIVISTELMÄ.....	III
SOMMARIO.....	V
RESUME	VIII
SAMENVATTING	XI
PREFACE.....	XIV
CONTENTS.....	XV
LIST OF SYMBOLS AND ABBREVIATIONS	XVIII
LIST OF PUBLICATIONS	XIX
AUTHOR'S CONTRIBUTION	XX
1 INTRODUCTION	1
2 TOWARDS A BIOREFINERY CONCEPT FOR A SUSTAINABLE ECONOMY....	5
2.1 Sources of waste and wastewaters	5
2.2 Opportunities for energy recovery from waste streams.....	7
3 HYDROGEN PRODUCTION VIA DARK FERMENTATION.....	9
3.1 Fundamentals of dark fermentation	9
3.1.1 Dark fermentation pathways.....	9
3.1.2 Hydrogen producing bacteria	11
3.1.3 Hydrogen consuming bacteria and other competitors in dark fermentative mixed cultures.....	12
3.1.4 Selective enrichment of hydrogen producing bacteria	13

3.2	Bioreactors for dark fermentative hydrogen production	17
3.3	Biological hydrogen production at high temperature	21
4	MICROBIAL FUEL CELLS FOR BIOLOGICAL ELECTRICITY PRODUCTION .	23
4.1	Principle of microbial fuel cells	23
4.1.1	Electron transfer mechanisms	23
4.1.2	Exoelectrogenic microorganisms.....	25
4.1.3	Microorganisms competing with exoelectrogens	26
4.1.4	Selective enrichment of exoelectrogens	27
4.2	Microbial fuel cell design: state of the art.....	28
4.3	Thermophilic microbial fuel cells.....	31
5	HYPOTHESIS AND AIMS OF THE PRESENT WORK	33
6	MATERIALS AND METHODS	35
6.1	Hydrogen production via dark fermentation	35
6.1.1	Overview of the hydrogen production experiments.....	35
6.1.2	Inocula and their pretreatment.....	36
6.1.3	Experimental set-up of batch experiments.....	37
6.1.4	Experimental set-up of continuous hydrogen production experiment....	38
6.2	Electricity production in microbial fuel cells.....	40
6.2.1	Overview of the experiments	40
6.2.2	Inoculum preparation	41
6.2.3	Mesophilic and thermophilic bioelectricity production from xylose	42
6.2.4	Power and polarization curves	42
6.3	Chemical and bioelectrochemical analyses	42

6.4	Microbial community analyses.....	43
6.4.1	Polymerase chain reaction-denaturing gradient gel electrophoresis.....	43
6.4.2	Illumina Miseq sequencing.....	44
6.5	Calculations	45
7	RESULTS AND DISCUSSION.....	47
7.1	Dark fermentative hydrogen production.....	47
7.1.1	Comparison of hydrogen production potential of different inocula	47
7.1.2	Inoculum pretreatments.....	51
7.1.3	Continuous hydrogen production.....	51
7.1.4	Hydrogen production from thermomechanical pulping wastewater	53
7.1.5	Microbial community composition.....	54
7.1.6	Metabolic pathways.....	55
7.2	Microbial fuel cells for biological electricity production.....	57
7.2.1	Bioelectricity production in mesophilic MFCs.....	57
7.2.2	Bioelectricity production in thermophilic MFCs	59
7.2.3	Metabolic pathways.....	60
7.2.4	Structure and role of the attached and planktonic microbial communities	61
8	CONCLUSIONS	63
9	RECOMMENDATIONS FOR FUTURE RESEARCH	65
	REFERENCES	67

List of Symbols and Abbreviations

AEM	Anion exchange membrane
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
BES	Bioelectrochemical system
BESA	2-bromoethanesulfonic acid
CE	Coulombic efficiency
CEM	Cation exchange membrane
COD	Chemical oxygen demand
CSTR	Continuous stirred tank reactor
FBR	Fluidized bed reactor
HPR	Hydrogen production rate
HRT	Hydraulic retention time
LAB	Lactic acid bacteria
MBR	Membrane bioreactor
MEC	Microbial electrolysis cell
MFC	Microbial fuel cell
NADH	Nicotinamide adenine dinucleotide
OLR	Organic loading rate
PBR	Packed bed reactor
PCR-DGGE	Polymerase chain reaction-denaturing gradient cell electrophoresis
PEM	Proton exchange membrane
SHE	Standard hydrogen electrode
TBR	Trickling bed reactor
TMP	Thermomechanical pulping
TS	Total solids
UASB	Upflow anaerobic sludge blanket
VFA	Volatile fatty acid
VS	Volatile solids

List of Publications

- I. Dessì, P., Lakaniemi, A.-M., Lens, P.N.L. (2017) Biohydrogen production from xylose by fresh and digested activated sludge at 37, 55 and 70 °C. *Water Research* 115:120–129.
- II. Dessì, P., Porca, E., Frunzo, L., Lakaniemi, A.-M., Collins, G., Esposito, G., Lens, P.N.L. (2018) Inoculum pretreatment differentially affects the active microbial community performing mesophilic and thermophilic dark fermentation of xylose. *International Journal of Hydrogen Energy* 43:9233–9245.
- III. Dessì, P., Porca, E., Waters, N.R., Lakaniemi, A.-M., Collins, G., Lens, P.N.L. (2018) Thermophilic versus mesophilic dark fermentation in xylose-fed fluidised bed reactors: Biohydrogen production and active microbial community. *International Journal of Hydrogen Energy* 43:5473–5485.
- IV. Dessì, P., Porca, E., Lakaniemi, A.-M., Collins, G., Lens, P.N.L. (2018) Temperature control as key factor for optimal biohydrogen production from thermomechanical pulping wastewater. *Submitted for publication*.
- V. Dessì, P., Porca, E., Haavisto, J., Lakaniemi, A.-M., Collins, G., Lens, P.N.L. (2018) Composition and role of the attached and planktonic active microbial community in mesophilic and thermophilic xylose-fed microbial fuel cells. *RSC Advances* 8: 3069–3080.

Author's Contribution

Paper I: Paolo Dessì performed the experiments and all the related analysis, wrote the manuscript and is the corresponding author. Aino-Maija Lakaniemi and Piet Lens participated in planning the experiments, helped in data interpretation and thoroughly revised the manuscript.

Paper II: Paolo Dessì performed the experiments and all the related analysis (with the exception of gas composition analysis), wrote the manuscript and is the corresponding author. Estefania Porca contributed to the planning and performing the experiments and microbial community analysis and commented on the manuscript. Gavin Collins helped in data interpretation. Luigi Frunzo and Giovanni Esposito commented the manuscript. Aino-Maija Lakaniemi and Piet Lens participated in planning the experiments, helped in data interpretation and thoroughly revised the manuscript.

Paper III: Paolo Dessì performed the experiments and all the related analysis, wrote the manuscript and is the corresponding author. Estefania Porca and Nicholas Waters helped in performing the microbial community analysis and commented the manuscript. Gavin Collins helped in data interpretation. Aino-Maija Lakaniemi and Piet Lens participated in planning the experiments, helped in data interpretation and thoroughly revised the manuscript.

Paper IV: Paolo Dessì performed the experiments and all the related analysis, wrote the manuscript and is the corresponding author. Estefania Porca helped in performing the microbial community analysis and commented the manuscript. Gavin Collins helped in data interpretation. Aino-Maija Lakaniemi and Piet Lens participated in planning the experiments, helped in data interpretation and thoroughly revised the manuscript.

Paper V: Paolo Dessì performed the experiments and all the related analysis, wrote the manuscript and is the corresponding author. Estefania Porca helped in performing the microbial community analysis and commented the manuscript. Johanna Haavisto helped performing the experiments and commented the manuscript. Gavin Collins helped in data interpretation. Aino-Maija Lakaniemi and Piet Lens participated in planning the experiments, helped in data interpretation and thoroughly revised the manuscript.

1 Introduction

The intensive use of fossil fuels for energy production is leading to a rapid depletion of the global energy reserves and to the emission of greenhouse gases. Despite the economic and environmental concerns, energy production is strictly dependent on fossil fuels, as evidenced by their 80% share of the total energy supply in 2016 (International Energy Agency 2017). Although the concentration of carbon dioxide (CO₂) in the atmosphere, which recently exceeded 400 ppm, already caused irreversible climate changes (Solomon et al. 2009), a societal commitment to increasing awareness is needed to mitigate such detrimental environmental issues.

An important starting point towards a more sustainable world was issued in December 2015, when the Paris agreement on climate change was signed. In the agreement, 195 countries committed to reach the ambitious target of reducing their greenhouse gas emission by 40% (compared to emission level in 1990) by 2030 (Liobikienė and Butkus 2017). The completion of this target is linked to the decrease of the overall energy consumption, increasing energy efficiency and a gradual substitution of fossil fuels, which account for about 65% of the total greenhouse gas emissions (IPCC 2014), with renewable and clean energy sources. Accordingly, the European Union set the target of contributing 20% of the annual energy consumption from renewable sources by 2020 and 27% by 2030 (European Commission 2014).

It is globally recognized that biomasses and biodegradable waste streams (organic matter convertible to fuels) have the potential of replacing fossil fuels for energy production (Cherubini 2010; Venkata Mohan et al. 2016; Özdenkçi et al. 2017). This is driving many industries producing organic side- and waste streams towards the implementation of integrated systems called biorefineries (Cherubini 2010). In a biorefinery concept, wastes are considered as an opportunity to generate value-added

products such as biofuels or biochemicals, and at the same time reduce waste disposal costs and fulfil the environmental regulations on waste emissions (Machani et al. 2014; Kinnunen et al. 2015).

Hydrogen (H_2) is promising biofuel candidate to replace fossil fuels in the near future, as it is widely available in nature, has the highest heating value per gram among fuels and is a carbon neutral fuel (Crabtree et al. 2004; Dincer 2012). However, hydrogen does not occur in nature as molecular H_2 , requiring to be chemically or biologically transformed from carrier molecules (1H containing molecules) such as water or hydrocarbons. At present, 90% of the global H_2 production relies on fossil fuels, being chemically produced by processes such as steam reforming, coal gasification or thermal cracking of fossil-based materials (Rafieenia et al. 2018). However, biological approaches are less energy intensive, utilize renewable, inexhaustible feedstocks and can be integrated into the waste recycling process (Nikolaidis and Poullikkas 2017). Studies on biological H_2 production have focused on bio-photolysis of water, the water-gas shift reaction and photo or dark fermentation of organic compounds (Holladay et al. 2009). Among them, dark fermentation is the most potential approach as it is applicable to a variety of organic substrates which are converted to H_2 at relatively high conversion rates (Arimi et al. 2015). However, the H_2 yield obtained by dark fermentation is still not enough to make this technology competitive at commercial scale (Kumar et al. 2017b).

Bioelectrochemical systems (BES) are another rapidly emerging technology for sustainable production of energy and/or chemicals from inorganic and organic compounds, including biomasses and high-strength wastewaters (Pant et al. 2012). Microbial fuel cells (MFCs), which enable the conversion of chemical energy of organic compounds into electrical energy, are so far the most studied among BES applications. Abiotic fuel cells require high temperature and/or costly catalysts, and sometimes corrosive electrolytes, whereas MFCs can be operated at mild temperatures and pH conditions, using inexpensive catalysts and cheap anodic substrates such as organic waste (Santoro et al. 2017).

Mesophilic dark fermentation and electricity production in MFCs have been intensively studied, whereas thermophilic processes has received less attention. High temperature can be advantageous for both H_2 and bioelectricity production, because it can enhance H_2 yields (Verhaart et al. 2010) and electron production rates of exoelectrogenic microorganisms (Du et al. 2007). High temperature also enhances microbial growth rates (Ratkowsky et al. 1982) and biological hydrolysis of complex substrates into simpler substances available for microbial H_2 or electricity production (Gadow et al. 2012), and reduce pathogen contamination (Suryawanshi et al. 2010). In dark fermentation, thermophilic conditions have been demonstrated to favor H_2 producing bacteria at the

expenses of competing microorganisms such as homoacetogens (Luo et al. 2011) and lactate producing bacteria (Noike et al. 2002). Furthermore, some industrial processes involving organic compounds, such as thermomechanical pulping (TMP) used in pulp and paper industry, produce wastewater at high temperature (Rintala and Lepistö 1992), which can be treated on site without requiring extra heating.

This thesis focuses on comparing mesophilic and thermophilic dark fermentation and MFCs for production of H₂ and electricity from organic carbon-containing synthetic and real wastewaters in batch systems and bioreactors operated in continuous mode. The composition of the microbial communities involved in both processes was also studied in detail, at both DNA and RNA level, enabling information on the presence and activity of microorganisms. This is the first study reporting the effect of inoculum pretreatment and operation temperature on the active dark fermentative microbial communities utilizing RNA-level community analysis. In addition, no previous studies have reported such of details about the composition of microbial populations (and the active subpopulation) from different sampling points in MFCs. This is also the first study attempting to understand the role of membrane-attached microbial communities on MFC performance. Furthermore, TMP wastewater was studied for the first time as a possible substrate for dark fermentative H₂ production and electricity production in MFCs.

2 Towards a biorefinery concept for a sustainable economy

The increase of population, especially in the developing countries, and the new wave of urbanization are increasing the societal need of energy and materials (Venkata Mohan et al. 2016; Nizami et al. 2017). At present, energy conversion and production of chemicals and materials depend highly on fossil fuels, raising concerns about the availability of fossil fuel reserves and the emission of greenhouse gases to the atmosphere (Parajuli et al. 2015). A biorefinery concept, in which the society realizes the potential of renewable sources for a sustainable economy, was proposed as an alternative to the fossil fuel-based economy (Cherubini 2010). In the same way as petroleum refineries, in which multiple fuels and chemical are produced from crude oil, the aim of biorefineries is to exploit organic substrates from industrial or municipal waste and side streams to generate value-added products such as biofuels and biochemicals.

2.1 Sources of waste and wastewaters

In the past, biological treatment approaches have been proposed to recover energy or value-added products from various organic wastes and wastewaters. The increasing world population has raised the attention on municipal solid waste, as its organic fraction has a huge potential that can be exploited for sustainable energy production (Venkata Mohan et al. 2016). Lignocellulosic biomass is also of high importance, being the most abundant renewable source in nature for energy and chemical production (Özdenkçi et al. 2017). Possible lignocellulosic substrates for biorefineries include crops, grass, wood and industrial side streams, such as agricultural residues, bark, sawdust and black liquors (Özdenkçi et al. 2017). Animals are also an important source of organic

substrates suitable for bioenergy or biochemical production, such as manure and waste generated by food processing industries (Nizami et al. 2017). Due to its increasing production and low biodegradability, plastic waste has become a problem at global scale and a serious hazard for marine environments (Willis et al. 2017). Although hardly suitable for biological processes, plastic can be recovered as raw material, or can also be an abundant substrate for liquid fuel production by pyrolysis (Das and Tiwari 2018).

Due to their abundance and environmental concerns, wastewaters containing organic substrates fit well in the biorefinery strategy. Most of such wastewaters are produced in food or manufacturing industries, in which water is used to process the raw materials. It is estimated that about 90% of the wastewater generated worldwide is released in rivers, lakes or oceans without treatment (Nizami et al. 2017). Among industrial effluents, wastewater from processing of agricultural products, as well as distillery and brewery wastewaters have demonstrated high potential for bioenergy or biochemical production due to the high content of easily biodegradable carbohydrates (Garcia-Nunez et al. 2016; Lu et al. 2017; Laurinavichene et al. 2018). Another valuable substrate for biorefineries is dairy wastewater, which is characterized by a high organic load, in particular carbohydrates, proteins and lipids (Demirel et al. 2005). Although poor in organic compounds, wastewater from textile industries is rich in nutrients and was recently proposed for growing microalgae, which can be used as substrate for biodiesel production (Fazal et al. 2018).

The pulp and paper industry is one of the most water-intensive industrial sectors (Toczyłowska-Mamińska 2017). It is estimated that 10-100 m³ of water is consumed per ton of produced paper (Meyer and Edwards 2014), generating enormous amounts of wastewater to be treated. The composition of this wastewater depends on the type of wood raw material used and the overall manufacturing process, which can be roughly divided into wood debarking, pulp making and processing and paper making (Kamali and Khodaparast 2015). Despite their heterogeneous composition, wastewaters from the different manufacturing processes are generally characterized by a chemical oxygen demand (COD) concentration of 1-14 g L⁻¹ (Meyer and Edwards 2014). Effluents from thermomechanical pulping (TMP) mills, in which the pulp is obtained by steaming the wood under high temperature and pressure (Pokhrel and Viraraghavan 2004), are rich in carbohydrates (25-40 % of the total COD). Furthermore, the lack of possible inhibitors such as sulphite, sulphate, hydrogen peroxide, fatty acids and resin acid makes it particularly suitable for biological treatment processes (Rintala and Puhakka 1994; Ekstrand et al. 2013).

2.2 Opportunities for energy recovery from waste streams

Many biotechnological processes have been applied to recover energy and other valuable products from waste, wastewaters and gaseous effluents. Depending on the municipal or industrial waste stream to valorize, such processes can be integrated and applied in different ways. The combinations of bioprocesses can be referred as biorefinery models (Venkata Mohan et al. 2016).

A model in which organic substrates are converted to H_2 and carboxylate compounds such as short chain fatty acids or alcohols by fermentation has been proposed by several research groups (Motte et al. 2015; Venkata Mohan et al. 2016; Nizami et al. 2017). Such carboxylate compounds can be further exploited to produce more H_2 , methane (which can be combined with H_2 to produce hythane), bioalcohols, bioplastics, bioelectricity or long chain fatty acids (Figure 2.1) (Guwy et al. 2011; Bundhoo 2017). This approach can be applied to both solid waste and wastewaters, and can be further improved by including a chemical, physical or biological nutrient recovery step. Among biological processes, constructed wetlands with plants is a promising way to remove nitrogen and phosphorous from wastewaters (Masi et al. 2018). Microalgae can also be used to recover nutrients from wastewaters (Cai et al. 2013) and digestates (Tao et al. 2017).

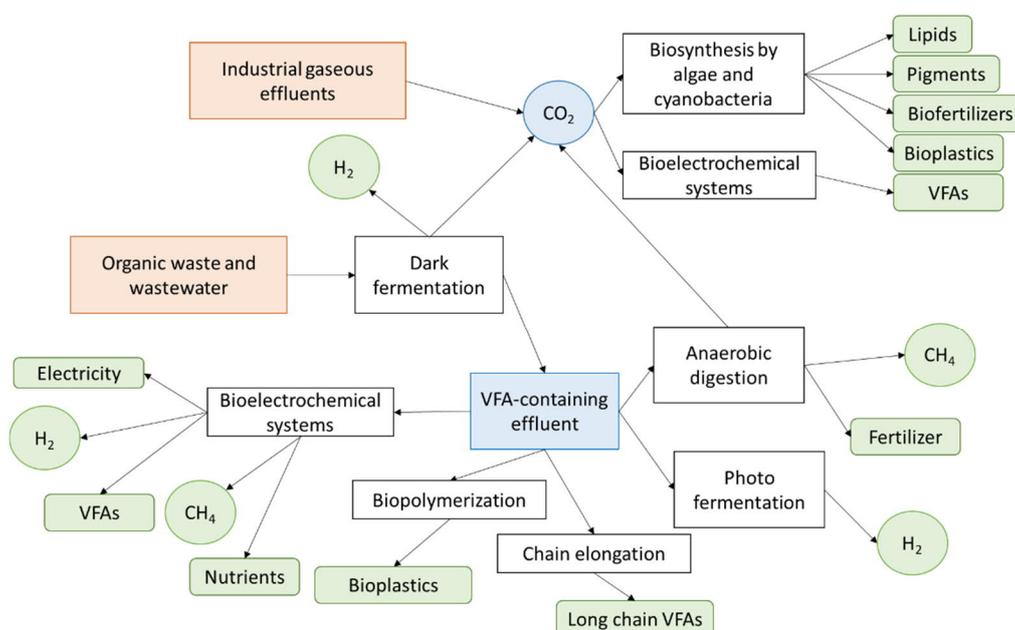


Figure 2.1: Schematic representation of different possibilities for biofuel, biochemical and bioenergy recovery from wastes, wastewaters and gaseous effluents by combining dark fermentation and bioelectrochemical systems to various biological processes (modified from Venkata Mohan et al. 2016).

The potential of organic carbon containing wastewaters can be exploited by using microalgae, which combine solar energy uptake and heterotrophic or autotrophic carbon sequestration to produce high-value bioproducts such as lipids (precursors of biodiesel) or pigments (Venkata Mohan et al. 2015; Duppeti et al. 2017). The algal biomass can be further treated biologically to produce H₂ and methane, or thermo-chemically to produce biochar and biofuel by pyrolysis, or syngas by gasification (Singh et al. 2016). However, post-treatment of algal biomass is limited by the need of cost intensive processes such as harvesting and dewatering (Venkata Mohan et al. 2016).

Bioelectrochemical systems (BES) can be applied to wastewaters with various types of organic or inorganic contaminants. BES rely on the capacity of microorganisms to catalyze the electron transfer from organic compounds to a solid anode electrode, and/or the electron uptake from a cathode electrode to an electron acceptor (Butti et al. 2016). The electron flow between the anode and cathode electrodes can be exploited for power production in microbial fuel cells (MFCs) (Hernández-Fernández et al. 2015), or for enabling production or recovery of valuable products at the cathode. BES have in fact many possible applications other than power production, including H₂ production in microbial electrolysis cells (MECs) (Lu and Ren 2016), bioelectrochemical methane production (Geppert et al. 2016), long chain fatty acids electrosynthesis (Batlle-Vilanova et al. 2017), wastewater remediation (Zhang et al. 2017b), metal recovery (Sulonen et al. 2018), desalination (Al-Mamun et al. 2018) and nutrient recovery (Kelly and He 2014).

Carbon dioxide (CO₂) is by far the most abundant pollutant present in gaseous industrial effluents, and its emission is limited and regulated in most industrialized countries according to the Paris agreement (Liobikienė and Butkus 2017). However, CO₂ can be seen as a resource for biological conversion to value-added products (Figure 2.1). Among the possible applications, CO₂ can be used by eukaryotic algae and cyanobacteria to produce valuable compounds such as lipids (precursors of biodiesel), pigments, biofertilizers and bioplastics (Venkata Mohan et al. 2015; Duppeti et al. 2017; Kumar et al. 2018). Another emerging technology is chain elongation of CO₂ to fatty acids by using BES (Arends et al. 2017; Batlle-Vilanova et al. 2017).

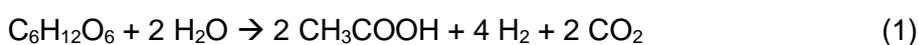
Selection of the biorefinery concept strictly depends on the waste or wastewater to be treated, as well as from the local conditions (temperature, availability of light) and local legislation (incentives, emission limits). In general, the concept must be selected with the aim to maximize the production of value-added compounds and reduce disposal cost. For example, the composition of pulp and paper wastewaters, rich in carbohydrates and/or volatile fatty acids (VFAs), makes them particularly suitable for acidogenic and bioelectrogenic processes.

3 Hydrogen production via dark fermentation

3.1 Fundamentals of dark fermentation

3.1.1 Dark fermentation pathways

In dark fermentation, H₂ is produced by fermentative microorganisms as a way to dispose the electrons resulting from oxidation of organic compounds during their catabolism (Li and Fang 2007). H₂ can be produced through many different pathways (Figure 3.1), depending on the microbial species involved, operating parameters and substrates used. Glycolysis or the Embden-Meyerhoff pathway is the most common sugar degradation route for mesophilic and thermophilic H₂ producing microorganisms, such as *Clostridium* sp. (Lee et al. 2011) and *Thermoanaerobacter* sp. (Vipotnik et al. 2016). In glycolysis, sugars are oxidized to pyruvate, resulting in the generation of reduced nicotinamide adenine dinucleotide (NADH) and energy in the form of adenosine triphosphate (ATP) (Figure 3.1). Pyruvate can be further oxidized to acetyl-CoA through ferredoxin reduction. If acetyl-CoA is then converted to acetate, both NADH and reduced ferredoxin are used to convert H⁺ to H₂ through a metalloenzyme called hydrogenase (Figure 3.1), yielding the theoretical maximum (also called Thauer limit) of 4 mol H₂ mol⁻¹ glucose (Eq. 1) (Thauer et al. 1977). This is also valid for pentose sugars such as xylose, which yields 3.3 mol H₂ mol⁻¹ xylose through the acetate pathway (Eq. 2).



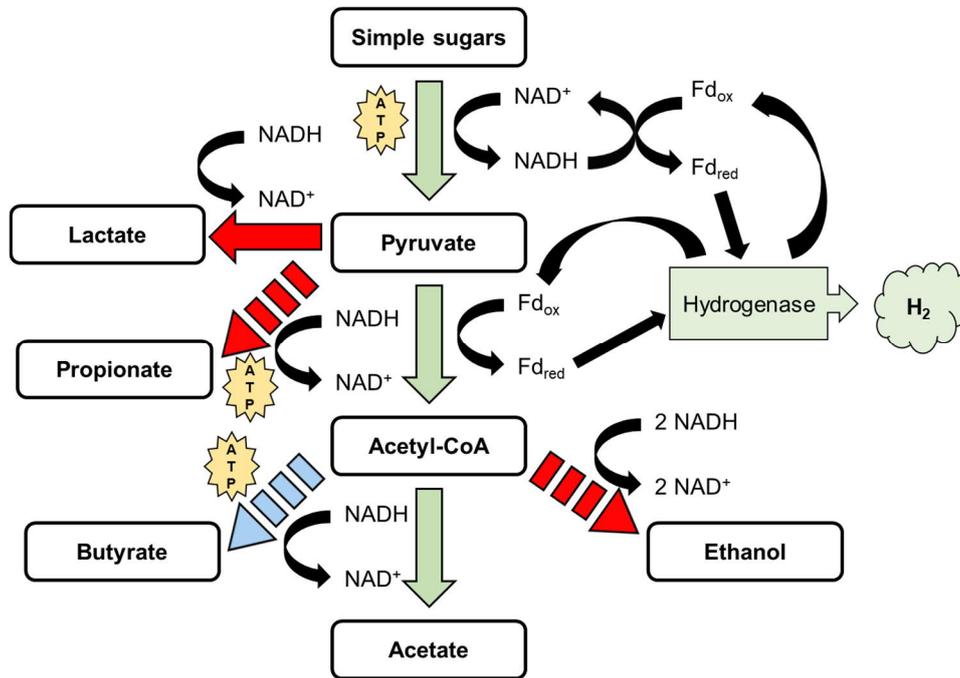
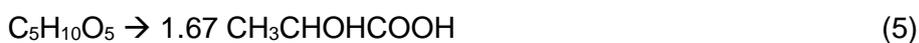
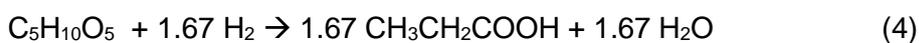


Figure 3.1: Metabolic pathways for fermentation of sugars, modified from Saady (2013) and Shaw et al. (2008). The green arrows represent the pathway producing the highest H_2 yield. The blue and red arrows represent the pathways in which NADH is partially or totally, respectively, consumed through non-hydrogenic pathways. The discontinuous arrows represent intermediate steps, which are not shown in the figure for simplicity.

However, the acetate production pathway is exergonic only at high temperatures and low partial pressure of H_2 (Verhaart et al. 2010). The dark fermentative process can be inhibited by the accumulation of produced H_2 in the medium, making an efficient gas stripping necessary to enable high H_2 yield (Beckers et al. 2015). Due to those limitations, several different fermentation pathways usually occur simultaneously in H_2 producing bioreactors. In practical systems, NADH is partially or totally consumed for the production of VFAs more reduced than acetate, such as butyrate (Eq. 3), propionate (Eq. 4) and lactate (Eq. 5), or alcohols such as ethanol (Eq. 6), and only the remaining NADH and ferredoxin is used for H_2 production (Figure 3.1). This results in H_2 yields ranging between 14 and 70% of the Thauer limit (Saady 2013).



3.1.2 Hydrogen producing bacteria

Dark fermentative H₂ production is carried out by obligate or facultative anaerobic microorganisms active at ambient (15-30 °C), mesophilic (30-39 °C), thermophilic (50-64) or hyperthermophilic (> 65 °C) conditions (Lee et al. 2011). Such microorganisms are spread around in both natural and anthropogenic environments. Biological samples of e.g. sewage sludge (Baghchehsaraee et al. 2008; Hasyim et al. 2011; Reilly et al. 2014), digested sludge (Elbeshbishy et al. 2010; Bakonyi et al. 2014), landfill leachate (Wong et al. 2014), compost (Song et al. 2012) and hot spring cultures (Koskinen et al. 2008) can be used as source of microorganisms for dark fermentative bioreactors.

H₂ producing microorganisms thriving at ambient and mesophilic conditions belong in general to the strictly anaerobic family of *Clostridiaceae* or to the facultative anaerobic family of *Enterobacteraceae*. *Clostridium* sp. are the most studied H₂ producing microorganisms, as they have been often reported to dominate the mesophilic fermentative microbial communities regardless of the inoculum source, inoculum pretreatment and reactor type (Baghchehsaraee et al. 2008; Mäkinen et al. 2012; Jeong et al. 2013; Si et al. 2015; Chatellard et al. 2016). Most H₂ producing *Clostridium* sp., such as *C. butyricum* and *C. acetobutylicum*, produce H₂ through both the acetate and butyrate pathway under mildly acidic conditions, with optimum H₂ yield at pH close to 5 (Grupe and Gottschalk 1992; Masset et al. 2010). Various *Clostridium* sp., and even different strains of the same species, have shown different H₂ yielding capabilities (Hilgsmann et al. 2011).

Microorganisms of the genus *Thermoanaerobacterium*, and specifically *T. thermosaccharolyticum*, are among the most widely studied thermophilic H₂ producers (O-Thong et al. 2009; Cao et al. 2014; Santos et al. 2014b; Ottaviano et al. 2017). Karadag and Puhakka (2010) showed that *Thermoanaerobacterium* sp. become dominant in a dark fermentative bioreactor, at the expenses of *Clostridium* sp., after increasing the temperature from 37-45 °C to 50-60 °C. However, the genus *Clostridium* includes some thermophilic species as well, such as *C. thermopalmarium* (Lawson Anani Soh et al. 1991) and *C. thermosaccharolyticum* (Islam et al. 2016). Due to their high H₂ production capabilities, many hyperthermophilic microorganisms, including *Thermoanaerobacter* sp. (Xue et al. 2001; Soboh et al. 2004; Sigurbjornsdottir and Orlygsson 2012; Vipotnik et al. 2016), *Thermotoga* sp. (Van Niel et al. 2002; D'Ippolito et al. 2010; Nguyen et al. 2010), *Caldicellulosiruptor* sp. (Van Niel et al. 2002; Ivanova et al. 2009; Zeidan and Van Niel 2010), *Caloramator* sp. (Ciranna et al. 2014) and *Thermococcus* sp. (Kanai et al. 2005) have been studied for H₂ production as pure cultures. However, mixed cultures do not require sterilization, are easy to operate and control and offer more versatility, and are therefore preferable for full-scale applications

(Li and Fang 2007). Among studies with mixed cultures, *Thermoanaerobacter* sp. and *Thermoanaerobacterium* sp. were the dominant microorganism detected after hyperthermophilic (70 °C) dark fermentation of glucose and cellulose, respectively, using sewage sludge as inoculum (Hasyim et al. 2011; Gadow et al. 2013). Qiu et al. (2011) reported that a microbial community constituted by *Caldicellulosiruptor* sp., *Coprothermobacter* sp., *Caldanaerobacter* sp., *Thermobrachium* sp., and *Thermotoga* sp. was enriched from cow manure digestate while performing both batch and continuous dark fermentative H₂ production from bioethanol distillery wastewater in the temperature range 65-80 °C.

3.1.3 Hydrogen consuming bacteria and other competitors in dark fermentative mixed cultures

Methanogenic archaea and homoacetogenic bacteria are the most common H₂ consuming microorganisms affecting dark fermentative processes. *Methanosarcina* sp., *Methanosaeta* sp. and *Methanobacteria* sp. are the most common methanogenic archaea in anaerobic bioreactors (Abbassi-Guendouz et al. 2013; De Vrieze et al. 2017). Homoacetogenesis (autotrophic conversion of H₂ and CO₂ to acetate) is carried out by a group of phylogenetically diverse bacteria, including many mesophilic and thermophilic *Clostridium* sp. (Ryan et al. 2008). Other mesophilic homoacetogenic microorganisms belong to the genera *Acetobacterium*, *Butyribacterium*, *Peptostreptococcus* and *Sporomusa* (Saady 2013), whereas *Moorella thermoacetica* is one of the most commonly found thermophilic homoacetogen. Also propionate producers consume H₂ for their metabolism, and have been reported to become dominant in reactors operated at low hydraulic retention time (HRT) and high organic loading rate (OLR) (Santos et al. 2014b; Sivagurunathan et al. 2016a). Other H₂ consuming microorganisms are nitrate or sulfate reducers (Bundhoo and Mohee 2016), but such microorganisms are not commonly found in dark fermentative bioreactors because the nitrate or sulfate concentration in the feeds is typically too low to support their growth.

Lactic acid bacteria (LAB), widely found in dark fermentative bioreactors (Etchebehere et al. 2016), compete with H₂ producers by converting sugars to lactate, or to a combination of lactate, acetate and/or ethanol (Makarova et al. 2006). Furthermore, LAB have been reported to outcompete other microorganisms, including *Clostridium* sp., by synthesizing and excreting antibodies (Noike et al. 2002). The most common LAB found in dark fermentative reactors are bacilli such as *Lactobacillus* sp. (Etchebehere et al. 2016) and the spore-forming *Sporolactobacillus* sp. (Fujita et al. 2010).

3.1.4 Selective enrichment of hydrogen producing bacteria

Inoculum pretreatment processes have been widely applied on mixed microbial cultures to select H₂ producing microorganisms by repressing H₂ consumers. Many of the pretreatment processes rely on the fact that some H₂ producers, including *Clostridium* sp., *Bacillus* sp. and *Thermoanaerobacterium* sp., produce spores when exposed to harsh conditions, and germinate when environmental conditions become favorable again (Collins et al. 1994; Galperin 2013). However, most H₂ consuming microorganisms are non-spore forming, and thus not able to resist the harsh conditions.

Temperature and pH shocks are the most widely applied pretreatments to select spore forming microorganisms (for a review, see Wang and Yin 2017). High temperature causes cell lysis and protein denaturation (Appels et al. 2008), whereas low temperature causes membrane lipid stiffening, formation of ice crystals in the cell and protein gelling (Sawicka et al. 2010). Low or high pH may inactivate key enzymes, change the electrical charge of the membrane and modify the internal pH of the cell (Rafieenia et al. 2018). Other pretreatment processes that have been studied include chemical treatment with 2-bromoethansulphonate acid (BESA) or chloroform, aeration, ionizing irradiation, ultrasonication, microwaves and electric shock (for a review, see Wang and Yin, 2017).

Many studies have compared the effect of various inoculum pretreatments on mesophilic and thermophilic dark fermentation, but the different inoculum, substrate and operating conditions used in the various studies makes it difficult to draw conclusions about their feasibility (Table 3.1). In most cases, the comparisons were done in terms of H₂ yields, giving little attention to the effect of the various pretreatments on the microbial communities, which were analyzed only in a few cases at DNA-level (Table 3.1).

Despite inoculum pretreatments have been extensively used for dark fermentation, they have some drawbacks, which may discourage their application in full-scale bioreactors. Inoculum pretreatments may eliminate non-sporulating H₂ producers, while sporulating H₂ consumers and competitors may survive the treatment (Bundhoo et al. 2015). Furthermore, the effect of the pretreatment is hardly maintained in the long term, especially when actual waste or wastewater is used as feedstock (Saady 2013). In fact, wastes and wastewaters likely contain microorganisms which can grow and restore H₂ consumption in the long term, making it necessary to repeat periodically the pretreatment, which is not a sustainable approach.

The growth of a H₂ producing microbial community can be also promoted by controlling the operating parameters. Methanogenesis is usually inhibited at pH < 6. Methanogenic archaea may be flushed out by setting a short (0.5-12 hours) HRT in bioreactors with

suspended biomass (Sivagurunathan et al. 2016a). Due to their phylogenic diversity, getting rid of homoacetogenic microorganisms in fermentative bioreactors is challenging. However, homoacetogenesis is not favorable at thermophilic conditions. Luo et al. (2011) reported that homoacetogenesis was inhibited by the combination of high temperature (55 °C) and low pH (5.5). The same applies to most LAB, which are mainly mesophiles and will be eliminated at temperatures > 50 °C (Noike et al. 2002). However, *Sporolactobacillus* sp. was found even at 70 °C in expanded granular sludge bed reactors performing dark fermentation of glucose and arabinose (Abreu et al. 2012). H₂ consuming bioconversions become more favorable at high H₂ partial pressure, but an intermittent gas-sparging with e.g. N₂ may mitigate this issue, although diluting the H₂ concentration in the gas (Kongjan et al. 2009).

Table 3.1: Summary of studies comparing the effect of inoculum pretreatments on H₂ yield from various substrates, showing pH, temperature of incubation and microbial community analysis applied. The pretreatment resulting in the highest H₂ yield in the specific study is underlined and written in bold.

Inoculum	Pretreatment applied ^a	Substrate (g COD L ⁻¹)	T ^b	pH ^c	H ₂ yield (mmol H ₂ g ⁻¹ COD) ^d	Microbiological analysis	Reference
Sludge from soybean treatment plant	AS, BS, <u>DHT</u> , FT, HT	Glycerol (30.7)	35	7.0	n.a.	Plate count and 16S rRNA gene sequencing	Misturini Rossi et al. (2011)
Anaerobic microflora from lab-scale UASB	AS, AS+CT, AS+HT, AS+CT+HT, <u>CT</u> , CT+HT, HT	Dairy wastewater (10.4)	29	6.3	0.03	None	Venkata Mohan et al. (2008)
None (substrate was assumed to contain also the necessary microorganisms)	ENZ, <u>HT</u> , MW	Waste sludge (19.5)	35	7.9	0.29	PCR-DGGE and 16s rRNA gene sequencing	Guo et al. (2015)
Granular sludge from brewery	AS, BS, CT, FT, <u>HT</u>	Palm oil mill effluent (49.0)	35	5.5	0.44	None	Mohammadi et al. (2012)
Digested sludge from plant treating the organic fraction of municipal waste	AE, AS, FT <u>HT</u> , US	Rice and lettuce powder (24.8)	37	5.5	0.80	None	Dong et al. (2010)
Anaerobic digested sludge	AS, BS, CT, HT, <u>LS</u>	Sucrose (22.5)	60	5.5	2.14	PCR-DGGE, 16s rDNA sequencing, phylogenetic tree	O-Thong et al. (2009)
None (substrate was assumed to contain also the necessary microorganisms)	AS, BS, HT, US, <u>US+AS</u> , <u>US+BS</u> <u>US+HT</u>	Food waste (18.4)	37	5.5	2.65	None	Elbeshbishy et al. (2011)
Anaerobic sludge from cassava ethanol plant	AS, BS, CT, HT, LS ^e	Cassava stillage (60.1)	60	6.0	2.91	None	Luo et al. (2010)
Sludge from cattle manure treatment plant	<u>AS</u> , CT, DHT, FT, HT	Glucose (25.0)	35.5	7.0	4.40	None	Hansen (2006)
Marine intertidal sludge	<u>AS</u> , BS, FT HT,	Glucose (21.3)	37	7.2	4.48	PCR-DGGE and 16s rRNA gene sequencing	Liu et al. (2009)
Sewage sludge	US, <u>UV</u>	Apple pomace (n.a. ^f)	37	5.0	4.77	None	Wang et al. (2010)
Suspended and granular anaerobic sludge	CT, <u>HT</u> , HT+CT	Wheat straw powder (20.0)	37	7.0	5.20	None	Argun and Kargi (2009)
Anaerobic sewage sludge and granular sludge	AS, <u>CT</u> , HT	Glucose (20.0)	35	7.0	6.08	None	Hu and Chen (2007)

Table 3.1: continued.

Inoculum	Pretreatment applied ^a	Substrate (g _{COD} L ⁻¹)	T ^b	pH ^c	H ₂ yield (mmol H ₂ g ⁻¹ COD) ^d	Microbiological analysis	Reference
Sludge from secondary settler of municipal wastewater treatment plant	<u>AE</u> , AS, BS, HT,	Glucose (10.7)	35	6.8	6.26	PCR-DGGE and 16s rRNA gene sequencing	Ren et al. (2008)
Digested sewage sludge	<u>EF</u> , HT	Glucose (20.0)	37	8.0	7.44	RNA concentration and 16S rRNA gene sequencing	Jeong et al. (2013)
Digester anaerobic sludge	AS, BS, HT, <u>US</u>	Glucose (8.5)	37	6.5	8.07	None	Elbeshbishy et al. (2010)
Anaerobic sludge from plant treating soybean wastewater	AS, BS, <u>HT</u>	Sucrose (28.1)	35	5.5	8.83	None	Mu et al. (2007)
Anaerobic sludge from ethanol manufacturing	AS, BS, HT, <u>LA</u>	Glucose (n.a.)	37	5.5	8.84	Plate count	Chaganti et al. (2012)
Activated sludge	AE, <u>AS</u> , BS, CT, HT	Glucose (10.7)	35	7.0	8.89	None	Chang et al. (2011)
Digested sewage sludge	AE, AS, BS, CT, <u>HT</u> ,	Glucose (7.1)	35	7.0	9.26	Optical density	Wang and Wan (2008)
Granular and flocculated anaerobic sludge from municipal wastewater treatment	AS, BS, <u>CT</u> , HT, LA, LS	Glucose (5.3)	37	6.0	9.38	Genomic DNA extraction, separation in polyacrylamide and sequencing	Pendyala et al. (2012)
Digested anaerobic sludge from municipal wastewater treatment plant	AS, BS, HT, <u>II</u>	Glucose (10.7)	36	7.0	11.20	None	Yin et al. (2014)
Anaerobic sludge from pig manure	AS, BS, <u>HT</u>	Glucose (21.3)	35	6.2	11.25	None	Wang et al. (2011)
Cow dung compost	AE, AE+LS, HT, <u>IR</u>	Sucrose (11.2)	36	7.0	11.50	Plate count	Song et al. (2012)
Fresh cattle dung	AS, CT, <u>HT</u>	Sago wastewater (11.6)	30	7.0	12.33	None	Sen and Suttar (2012)
Digested activated sludge	AE, AS, BS, <u>CT</u> , HT	Sucrose (11.2)	35	6.3-8.0	14.67	None	Zhu and Béland (2006)

^a AE, Aeration; AS, acidic shock; BS, base (alkaline) shock; CT, chemical treatment; DHT, dry heat treatment; EF, electric field; ENZ, enzymatic; FT, freeze and thawing; HT, heat treatment; II, ionizing irradiation; IR, infrared; LA, linoleic acid; LS, loading shock; MW, microwave; US, ultra sonication; UV, ultra violet. ^b Incubation temperature. ^c Initial pH of incubation. ^d Highest H₂ yield obtained per g COD_{added} in the first batch culture of pretreated or untreated inoculum. ^e The highest H₂ yield was obtained using the untreated inoculum. ^f Not available.

3.2 Bioreactors for dark fermentative hydrogen production

Bioreactors relying on activity of suspended and biofilm-attached biomass have both been widely studied for dark fermentative H₂ production (Wang and Wan 2009) (Figure 3.2). Suspended biomass bioreactors such as continuous stirred tank reactors (CSTRs) ensure a good mixing, but can result in microorganism flush out (Li and Fang 2007). Self-granulation could reduce the loss of active microorganisms (Show et al. 2011), but according to Sivagurunathan et al. (2016a) self-granulation is not easily controllable in dark fermentative bioreactors. Biofilm-based attached biomass systems such as packed bed reactors (PBRs), membrane bioreactors (MBRs), fluidized bed reactors (FBRs) and upflow anaerobic sludge blanket reactors (UASBs) operated with a carrier material are characterized by adhesion of microorganisms as biofilm on inert supporting surfaces, and thus a higher biomass retention time (Show et al. 2011) (Figure 3.2). However, in the case of MBRs, the adhesion of microorganisms causes biofouling of the membrane and a consequent increase of the operating costs (Show et al. 2011) (Figure 3.2). Trickling bed reactors (TBRs) have also been proposed for dark fermentative H₂ production, as they ensure low gas retention, high cell density and an easy control of pH and temperature (Oh et al. 2004b).

Among the attached biomass systems, FBRs are particularly efficient for H₂ production as the turbulent regime improves the mass transfer between biomass and substrate (Barca et al. 2015). Furthermore, FBRs favor the stripping of the produced H₂, avoiding its accumulation and the consequent inhibition by high H₂ partial pressure (Barca et al. 2015). Several carrier materials have been used as support for biofilm growth, including activated carbon (Zhang et al. 2007; Muñoz-Páez et al. 2013), expanded clay (Cavalcante de Amorim et al. 2009; Barros et al. 2010), celite (Koskinen et al. 2006), polystyrene (Barros et al. 2010; Barros and Silva 2012), as well as grounded tyre and plastic material (Barros and Silva 2012). FBRs have also been operated without a carrier material by using self-granulating biomass (Zhang et al. 2008) or biomass immobilized within silicone gel (Lin et al. 2006). FBRs have been operated with a HRT ranging from 0.5 h (Chang et al. 2002) to 24 h (Muñoz-Páez et al. 2013). A low HRT is usually related with higher H₂ yields as it enhances the washout of H₂ consuming microorganisms such as methanogens and homoacetogens due to their slower growth rates compared to H₂ producing bacteria. However, a too low HRT may result in an incomplete substrate utilization. For example, the effect of HRT has been studied in the range of 1-8 h in a FBR reactor with glucose as the substrate (Dos Reis and Silva 2011), obtaining a maximum yield of 2.55 mol H₂ mol⁻¹ glucose at an HRT of 2 h.

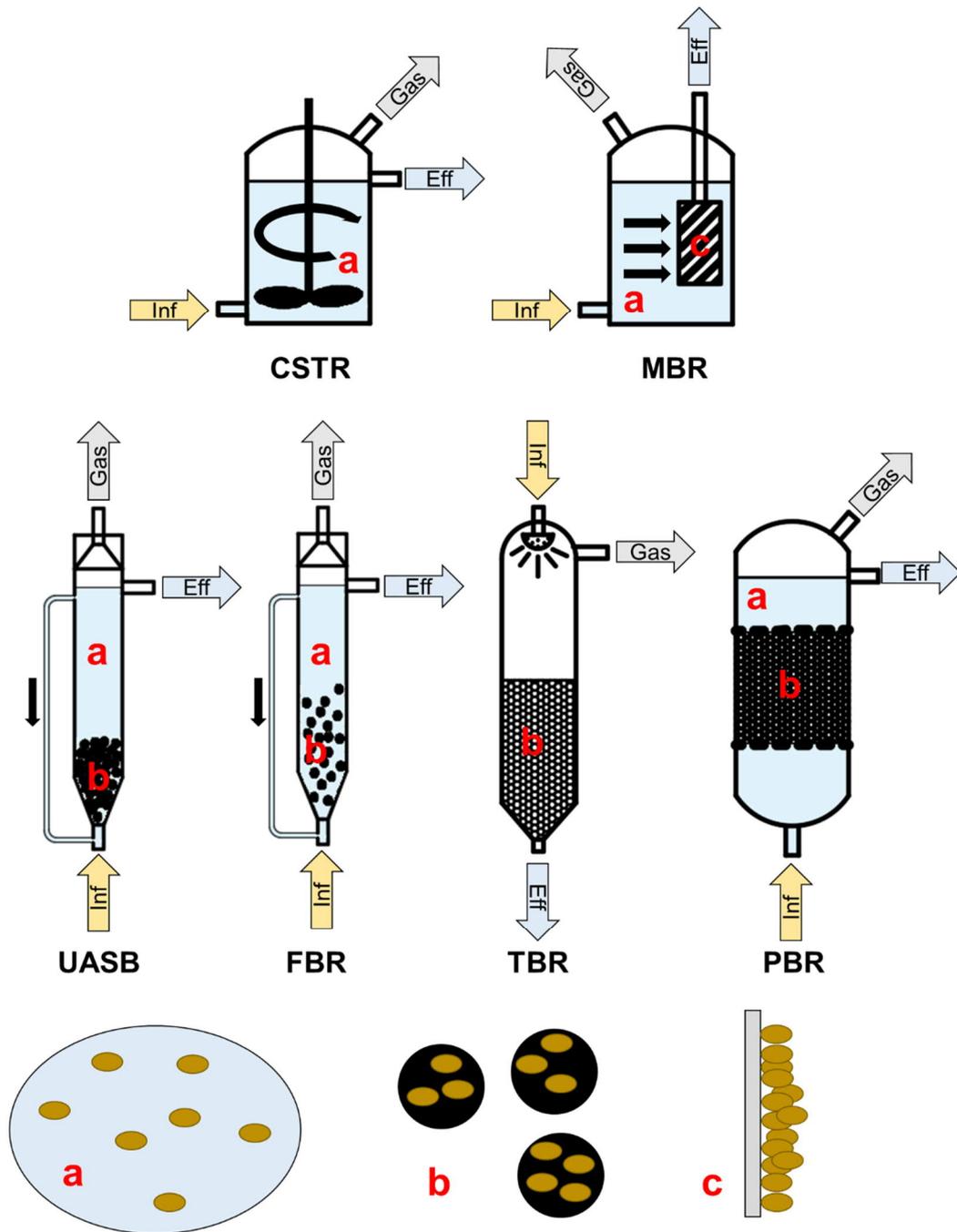


Figure 3.2: Bioreactor configurations for dark fermentative H_2 production and biomass retention systems involved. The colored arrows indicate the influent inlet (inf) and effluent (eff) or gas outlet. The figure includes schematic diagrams of continuous stirred tank reactor (CSTR), membrane bioreactor (MBR), upflow anaerobic sludge blanket (UASB), fluidized bed reactor (FBR), trickling bed reactor (TBR) and packed bed reactor (PBR). The microorganisms can be retained in the bioreactors as planktonic microorganisms (a), attached on carrier materials (b) and/or growing as biofilm on membrane (c).

Besides the conventional bioreactors, alternative dark fermentative bioreactor designs have been proposed in order to optimize biomass retention, mixing and gas stripping from the fermentation broth. Hiligsmann et al. (2014) studied a sequenced-batch horizontal fixed bed and a biodisc-like bioreactor for dark fermentation of glucose by a pure culture of *Clostridium butyricum* immobilized in polyurethane cubes or in a polypropylene rotating disc, respectively. The biodisc-like bioreactor resulted in the highest H₂ production rate (HPR) of 703 mL H₂ L⁻¹ h⁻¹ with a remarkably high yield of 2.4 mol H₂ mol⁻¹ glucose (Hiligsmann et al. 2014). Hassan Khan and Gueguim Kana (2016) proposed a reactor configuration with 3D-printed porous cartridges filled with pellets of heat treated anaerobic sludge immobilized in sodium alginate. A 30% higher H₂ yield, as well as a 60% shorter lag phase, was obtained using this novel configuration compared to the control bioreactor without cartridges. Gas sparging with N₂ or CO₂ can help decreasing the H₂ partial pressure inside dark fermentative bioreactors (Kim et al. 2006), but it also results in a dilution of the H₂ produced. To overcome this problem, two gas separation membranes were connected to a dark fermentative CSTR in order to separate the H₂ from the CO₂ (Bakonyi et al. 2017). The CO₂-rich gas stream was then recirculated from the bottom of the CSTR, stripping H₂ from the liquid and resulting in a 30% higher HPR compared to a control CSTR operated without membrane (Bakonyi et al. 2017). Alternatively, a low H₂ partial pressure can be achieved by operating the dark fermentation bioreactor at low pressure by vacuuming (Lee et al. 2012).

Despite the improvements achieved in the last two decades, the establishment of dark fermentation at industrial scale is still hindered by the low H₂ yields, as well as by the costs of handling and processing of organic substrates (Kumar et al. 2017b). Therefore, research on dark fermentation is still mainly carried out at laboratory scale. However, scale-up of dark fermentative bioreactors have been attempted in a few studies (Table 3.2). A remarkably high HPR of 312.5 mL H₂ h⁻¹ L⁻¹ was obtained by dark fermentation of hydrolyzed corn stover in a 3000 L baffle reactor (Table 3.2). Furthermore, in the same study, the effluent of dark fermentation was used as substrate for photofermentation, resulting in an additional HPR of 196 mL H₂ h⁻¹ L⁻¹.

Table 3.2: H₂ production rates (HPR) reported in pilot-scale studies on dark fermentation of different substrates conducted using different inocula, reactor types, temperature, pH and organic loading rates (OLR).

Inoculum	Reactor type ^a	Volume (L)	Substrate	T (°C)	pH	HRT (h)	OLR (g COD L ⁻¹ h ⁻¹)	HPR (mL H ₂ h ⁻¹ L ⁻¹)	Reference
Food waste and tap water	CSTR	200	Food waste	55	5-6	79.2	21.6	39.6	Cavinato et al. (2012)
Heat treated seacost sludge	CSTR	400	Sucrose	37	5.6-5.8	8	2.5	558	Lin et al. (2011)
Heat treated digested sludge	Tank reactor	500	Liquid from food waste pressing	33	5.3	21	12.3-71.3	0.16	Lee and Chung (2010)
Sludge from fructose processing	FBR	1000	Gluten processing wastewater	35	6.0	36	0.56	9.3	Cheng et al. (2011)
Sludge from municipal wastewater treatment plant	CSTR	1480	Molasses	35	7.0	4.2	2.8	232	Ren et al. (2006)
Heat treated sewage sludge	Baffle reactor	3000	Hydrolized corn stover	35	4.5	16	0.63	313	Zhang et al. (2018)

^a Continuous stirred tank reactor (CSTR); fluidized bed reactor (FBR).

3.3 Biological hydrogen production at high temperature

Temperature is a key parameter in dark fermentation as it can widely affect the composition and productivity of the fermentative microbial communities (Karadag and Puhakka 2010). Dark fermentation at high temperature can be advantageous because the acetate production pathway, which leads to the highest H₂ yield, becomes more favorable as the temperature increases (Verhaart et al. 2010). Thermophilic conditions are also beneficial for the kinetics of the reactions and the growth rate of microorganisms surviving at high temperature (Ratkowsky et al. 1982). For those reasons, dark fermentation at high temperature typically results in higher H₂ yields than mesophilic processes with a similar substrate and inoculum (Yokoyama et al. 2007; Hasyim et al. 2011; Kargi et al. 2012; Zheng et al. 2014). Technical advantages of high temperature include low viscosity and high gas stripping due to the low solubility of gas (and thus low H₂ partial pressure). Furthermore, thermophilic conditions are not favourable for most H₂ consuming (Hasyim et al. 2011) and pathogenic microorganisms. The main drawback of thermophilic processes is the energy required for heating the bioreactors, which may overcome the energy gained by the increased H₂ yield (Perera et al. 2010). However, some industrial wastewaters, such as TMP wastewater (Rintala and Lepistö 1992; Suvilampi et al. 2001), are produced at elevated temperatures, and can therefore be treated on site with a minimum or even without any energy requirement for heating.

Continuous H₂ production at high temperature (55-70 °C) from simple sugars by mixed cultures has been studied using various reactor types and at HRT varying from a few hours to 3 days (Table 3.3). More complex substrates have also been studied for thermophilic dark fermentative H₂ production. For example, H₂ was successfully produced from diluted sugarcane vinasse in a FBR at 55 °C, with a maximum HPR of 780 mL H₂ h⁻¹ L⁻¹, but the process was inhibited by the high concentration of volatile fatty acids with undiluted sugarcane vinasse as the substrate (Santos et al. 2014a). H₂ production from cellulose has been evaluated by using a hyperthermophilic (70°C) CSTR reactor, operated in continuous mode, inoculated with digested sewage sludge (Gadow et al. 2013). The CSTR produced H₂ steadily for 150 days with an average HPR of 20 mL H₂ h⁻¹ L⁻¹. Continuous H₂ production from bioethanol distillery wastewater has been studied at 70 °C in a CSTR inoculated with cow manure digestate, obtaining a steady HPR of 52 mL H₂ h⁻¹ L⁻¹ (Qiu et al. 2011).

Table 3.3: Highest stable H₂ production rate (HPR) obtained in continuous studies on thermophilic (T ≥ 55°C) dark fermentation of simple sugars using different inoculum, reactor type, pH and hydraulic retention time (HRT).

Inoculum	Pretreatment	Reactor type ^a	Substrate (g COD L ⁻¹)	T (°C)	pH ^b	HRT (h)	HPR (mL H ₂ h ⁻¹ L ⁻¹)	Reference
Biomass from H ₂ -producing CSTR	None	UASB	Sucrose (11.2)	55	4.5-5.0 (nc)	3	112.5	Keskin et al. (2012)
Anaerobic digester sludge	Heat treatment (105°C, 5 min)	UASB	Sucrose (11.2)	55	5.0-5.5 (nc)	1.5	124.2	Keskin et al. (2011)
Anaerobic sludge	Heat treatment (80°C, 60min)	FBR	Sucrose (5.0)	60	5.5 (i)	12	60.5	Lutpi et al. (2016)
Biomass from H ₂ -producing CSTR	None	TBR	Glucose (7.3)	60	5.5 (c)	2	980.6	Oh et al. (2004b)
Biomass from H ₂ -producing CSTR	None	CSTR	Xylose (1.1)	70	6.7 (nc)	72	2.6	Kongjan et al. (2009)
Biomass from H ₂ -producing CSTR	None	UASB	Glucose (2.1)	70	7.0 (i)	24	12.7	Zheng et al. (2008)
Biomass from CSTR producing methane	Chemical treatment (BESA)	UASB	Glucose (4.8)	70	7.2 (i)	27	47.3	Kotsopoulos et al. (2006)
Anaerobic sludge from thermophilic CSTR	None	CSTR	Glucose (4.6)	70	5.5 (c)	21.6	91.7	Zhang et al. (2014)

^a Continuous stirred tank reactor (CSTR), fluidized bed reactor (FBR), trickling bed reactor (TRB), upflow anaerobic sludge blanket (UASB). ^b The reported pH refers either to the initial pH (i) or the operation pH, which can be either controlled to a stable value by automatic titration (c) or not controlled (nc).

4 Microbial fuel cells for biological electricity production

4.1 Principle of microbial fuel cells

Microbial fuel cells (MFCs) are devices which convert the chemical energy of some organic and inorganic compounds to electric energy through biological-mediated reactions (Logan et al. 2006). MFCs generally consist of an anode electrode, which acts as electron acceptor for the biological oxidation of organic compounds, and a cathode electrode, which acts as electron donor for biotic or abiotic reactions (Logan et al. 2006). The reactions at the two electrodes create a potential difference, which results in migration of electrons from the anodic to the cathodic chamber of the MFC through an external circuit, producing current (for a review, see Butti et al. 2016). At the same time, protons migrate through the anodic solution to the cathode and combine with a terminal acceptor and the electrons. If oxygen is the terminal electron acceptor, water is formed as the end-product (Figure 4.1).

4.1.1 Electron transfer mechanisms

Biological electricity production in MFCs is carried out by a certain group of anaerobic or facultative anaerobic bacteria, called exoelectrogens, able to oxidize the substrates and transfer electrons outside the cell to the solid anode electrode (Logan 2009). Electron transfer essentially occurs as direct (short-range or long-range) or mediated transport of electrons (Schröder 2007; Kumar et al. 2015) (Figure 4.1). Short-range electron transfer occurs through redox-active proteins on the surface of the cell membrane, such as *c*-type cytochromes, whereas long-range electron transfer occurs via conductive pili called

nanowires (Snider et al. 2012; Kumar et al. 2016). In the mediated electron transfer, a mediator compound is reduced by receiving the electrons from the membrane-bound proteins and then oxidized by transferring the electrons to the anode surface, becoming reusable for further electron transfer (Kumar et al. 2016). Some exoelectrogenic microorganisms can produce and excrete mediators such as flavin, riboflavin, pyocyanin and quinone (Kumar et al. 2016). Once mediators are released outside the cell, also microorganisms that do not themselves produce mediators can utilize them for electron transfer (Pham et al. 2008). Artificial mediators such as ferricyanide, neutral red, methyl viologen, phenazines, phenoxazines, phenothiazines, quinine and benzoquinone can be added to facilitate the electron transfer, but this approach is not economically sustainable in continuous operation (Butti et al. 2016).

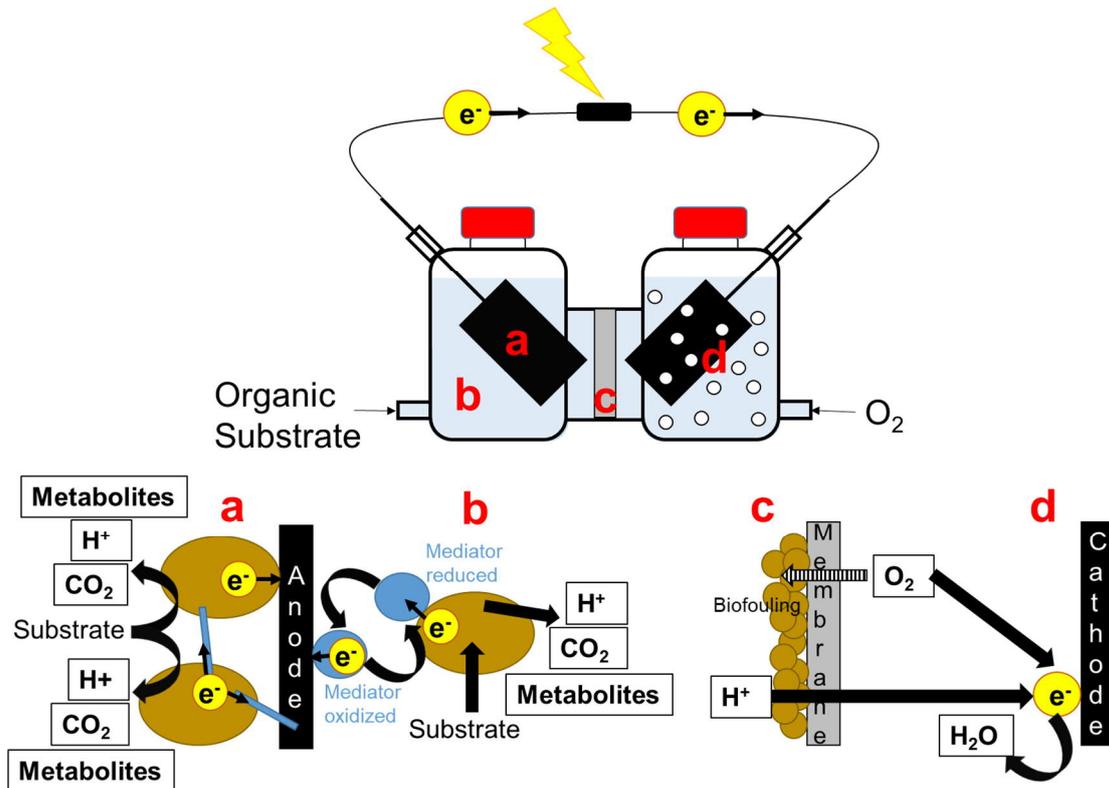


Figure 4.1: Schematic representation of a two-chamber microbial fuel cell with a biotic anode fed with organic compounds and with dissolved oxygen used as electron acceptor at the cathode (top part of the figure). Direct (a) and mediated (b) electron transfer mechanisms, proton flow through the membrane, which can be limited in case of biofouling (c), and cathodic reaction (d) are depicted in more detail on the bottom part of the figure.

4.1.2 Exoelectrogenic microorganisms

Exoelectrogens are a wide group of strictly and facultative anaerobic microorganisms. They can be found in both natural (lakes, rivers, oceans, groundwater, sediments, and soil) and anthropogenic (wastes, wastewaters, contaminated soils, compost and industrial systems) environments, even at extreme pH, temperature and pressure conditions (Miceli et al. 2012; Chabert et al. 2015). The most typical inocula for laboratory-scale experiments include aerobic sludge (Mohd Yusoff et al. 2013; Zhang et al. 2016), anaerobic sludge or digestates (Borole et al. 2009; Torres et al. 2009; Chae et al. 2010a; Coronado et al. 2013; Modestra and Mohan 2017) and microorganisms from previously operated BES (Rabaey et al. 2005; Haddadi et al. 2014; Vicari et al. 2017). Anaerobic sludge has been demonstrate to be richer in exoelectrogenic microorganisms than aerobic sludge, but it also contains competing methanogenic microorganisms (Lobato et al. 2012).

Exoelectrogenic bacteria mainly belong to the *Proteobacteria*, *Firmicutes* and *Acidobacteria* phyla (Kumar et al. 2015). The most studied exoelectrogenic microorganisms are dissimilatory iron reducers such as *Geobacter* sp. and *Shewanella* sp., which can perform short-range electron transfer via membrane-bound proteins (Kumar et al. 2017a). *Geobacter* sp. can also perform long-range electron transfer by excreting conductive pili, whereas *Shewanella* sp. can increase the electron transfer distance by extending its outer membrane and periplasm, or performs mediated electron transfer through self-produced shuttles (Kumar et al. 2017a). *Geobacter sulfurreducens* can form multilayer biofilms in which the different cells are connected through nanowires (Bonanni et al. 2012), resulting in a conductive net which allows to transport the electrons to the anode from long distances. This usually results in a higher electricity generation compared to bacteria forming a monolayer biofilm, as multilayer biofilms enable long-range electron transfer to the anode via interspecies electron transfer (Logan 2009). However, thick biofilms may result in a high diffusion resistance, limiting the electrochemical activity of microorganisms (Sun et al. 2016). *Geobacter* sp. has been widely reported as the dominant microorganism in mesophilic MFCs, regardless of the MFC set-up, substrate and inoculum source (Gao et al. 2014; Lesnik and Liu 2014; Jiang et al. 2016; Haavisto et al. 2017). Other known mesophilic exoelectrogenic microorganisms include *Pseudomonas aeruginosa*, which excretes pyocyanin to mediate electron transfer (Shen et al. 2014), and *Acidithiobacillus ferrooxidans*, which was shown to produce conductive nanowires (Li and Li 2014). However, *Acidithiobacillus* sp. grows only on inorganic substrates, such as ferrous iron, and is active only at pH lower than 3 (Meruane and Vargas 2003).

Most of the known exoelectrogenic bacteria are active at ambient or mesophilic conditions. However, few *Firmicutes* such as *Thermincola* sp. (Wrighton et al. 2008; Marshall and May 2009; Parameswaran et al. 2013), *Caloramator* sp. (Fu et al. 2013a), and *Thermoanaerobacter* sp. (Lusk et al. 2015), and *Deferribacteres* such as *Calditerrivibrio* sp. (Fu et al. 2013b) have been reported to produce electricity at thermophilic conditions (55-60 °C). Although most *Firmicutes* perform only mediated electron transfer upon addition of external mediators, evidence of direct electron transfer was given for *Thermincola potens* (Wrighton et al. 2011) and *Thermincola ferriacetica* (Parameswaran et al. 2013). In particular, *T. ferriacetica* was shown to develop a thick, multilayer conductive biofilm, in which the cells are connected through a network of extracellular pili, similarly to *Geobacter* sp. biofilms (Parameswaran et al. 2013). Interestingly, *Thermoanaerobacter pseudethanolicus* was shown to generate current from acetate produced by sugar fermentation, but electricity production was negligible in a medium containing acetate as the only organic substrate (Lusk et al. 2015). Although it has not been studied for electricity production as a pure culture, *Coprothermobacter* sp. was reported to dominate the electroactive mixed microbial community in an acetate-fed MFC (Jong et al. 2006) and it is therefore a possible thermophilic anode-respiring microorganism. *Caldanaerobacter* sp. and *Thermodesulfobacterium* sp. were detected at temperatures up to 98 °C in a glucose-fed MFC inoculated with water from a petroleum reservoir, with a sampling temperature of about 98 °C (Fu et al. 2015). However, their exoelectrogenic activity needs to be proven in pure culture experiments.

4.1.3 Microorganisms competing with exoelectrogens

Non-exoelectrogenic microorganisms can compete with exoelectrogens by directing a share of electrons available in the substrate away from the anode through other pathways than electricity generation. Acetoclastic and hydrogenotrophic methanogenic archaea, which consume electrons to form methane from acetate or from H₂ and CO₂, respectively, have been widely reported to compete with exoelectrogens in MFCs operated at ambient (Chung and Okabe 2009), mesophilic (Rismani-Yazdi et al. 2013) and even thermophilic (Hussain et al. 2012) conditions. Among the archaeal microorganisms colonizing MFCs, *Methanosarcina* sp. can perform both acetoclastic and hydrogenotrophic methanogenesis, whereas *Methanobacteriaceae* do not have cytochromes and methanophenazine, and thus can perform only hydrogenotrophic methanogenesis (Thauer et al. 2008).

Although methanogens are the prevalent competitor of exoelectrogenic microorganisms, other microorganisms can consume electrons through pathways other than electricity production in MFCs. Fermentative bacteria may prevail in case of MFCs fed with fermentable fuel, such as carbohydrates, as fermentation is thermodynamically more

favorable than the respiratory mechanism of exoelectrogens (Borole et al. 2011). However, exoelectrogens can create a synergy with fermentative bacteria by producing electricity from volatile fatty acids and alcohols resulting from sugar fermentation (Kiely et al. 2011). Other potential competitors include H₂ oxidizing microorganisms (Hayashi et al. 1999) and nitrate or sulfate reducing microorganisms (Borole et al. 2011). A share of electrons can also be consumed by aerobic metabolism in case on oxygen intrusion to the anodic chamber (Kim et al. 2007).

4.1.4 Selective enrichment of exoelectrogens

A fast development of a stable electroactive biofilm is the key to decrease start-up time and minimize competition by non-exoelectrogenic pathways in MFCs (Boghani et al. 2013). Selection of an inoculum rich in exoelectrogenic species which can form a biofilm in the operational conditions of the specific MFC (e.g. temperature, pH and substrate), and poor in competitors, is crucial for optimal MFC performance (Lobato et al. 2012). In the same way as in the case of dark fermentation (see section 3.1.4), the inoculum can be pretreated to eliminate methanogenic archaea, but in this case also non-spore forming exoelectrogenic microorganisms can be eliminated. Methanogens can also be detached from the anode by operating the MFC in open circuit mode, favoring their elimination by flushing out (Kaur et al. 2014). Methanogens can also be inhibited by prolonged starvation, without affecting the exoelectrogenic microorganisms (Kaur et al. 2014).

The anode potential influences the metabolic pathways of the microbial communities in MFCs (Busalmen et al. 2008; Carmona-Martínez et al. 2013). Therefore, a certain anode potential can be applied and kept constant, e.g. with a potentiostat, to select the desired metabolic routes. Imposing a positive potential (e.g. from 0.2 to 0.4 V vs. standard hydrogen electrode, SHE) typically increases the energy gain of the anode respiring microorganisms, resulting in the establishment of a highly diverse microbial community. On the other hand, a negative potential (e.g. from -0.05 to -0.3 V vs. SHE) will generally favor the exoelectrogenic microorganisms able to respire at low potential (Torres et al. 2009; Commault et al. 2013). For example, Torres et al. (2009) reported a 97% relative abundance of *Geobacter sulfurreducens* in a MFC started up imposing a potential of -0.15 V (vs. SHE), whereas a diverse microbial community was obtained in an identical MFC started up imposing a potential of 0.37 V (vs. SHE). However, Zhu et al. (2014) reported only minor changes in the microbial communities applying potentials from -0.25 to 0.81 V, showing that certain bacteria can self-regulate the electron transfer pathways to adapt to the anode potential.

Continuously imposing a potential, and thus operating the system as a microbial electrolysis cell (MEC) is not feasible in full-scale applications, as a current must be continuously added to the system to keep stable the potential. However, the anode potential can be indirectly controlled by selecting an appropriate external load. A low external load increases the availability of the electron acceptor, and thus the energy available for the growth of exoelectrogenic microorganisms (Zhang et al. 2017a). However, the maximum power is obtained when the external load is similar to the internal resistance of the MFC (Logan et al. 2006). Therefore, it can be convenient to apply different external loads at the different stages of MFC operation. In fact, an external load varying in the range 50-800 Ω , controlled by an algorithm, was installed to a MFC resulting in a 5-times higher power production than a similar MFC with a static external load of 200 Ω (Premier et al. 2011).

The composition of the anolyte solution can also be modified in order to favor the growth of exoelectrogenic microorganisms. It was shown that MFCs fed with fermentable substrates can result in a wide microbial community but low Coulombic efficiency (CE), due to electron consumption via non-electrogenic metabolism, whereas exoelectrogenic pathways prevail in acetate-fed MFCs (Chae et al. 2009). Therefore, it can be useful to enrich a microbial community on acetate prior to utilization for producing electricity from more complex substrates.

4.2 Microbial fuel cell design: state of the art

MFCs can be constructed with a single chamber or two-chamber configuration. In the first case, only the anode electrode is submerged in a liquid medium, whereas the cathode is exposed to surrounding air. In the second case, both anode and cathode electrodes are submerged in a liquid medium (Butti et al. 2016). Generally, higher power production is associated to dual chamber MFCs, but the higher fabrication and operating costs make their scale up challenging.

Traditional MFC set-ups includes cubic single chamber MFC, with the cathode exposed to the air, and two chamber cubic or h-type MFCs, which consists of two chambers or two bottles, respectively, connected through a membrane (Logan et al. 2006). Upflow MFC, in which the anolyte is supplied from the bottom of the reactor, is an easily scalable alternative, which can be operated at low HRT (Haavisto et al. 2017). Tubular MFCs, in which the electrodes are located concentrically in a cylindrical frame, optimize the surface area of the anode, cathode and membrane (if present), which can result in a high power output (Kim et al. 2010). A tubular, upflow MFC, consisting of five modules

connected in series, with copper mesh and stainless steel coil composite electrodes, successfully treated (78% COD_{soluble} removal) concentrate of domestic wastewater at an HRT of 0.75 days producing a maximum power of 916 mW m⁻³ (Koroglu et al. 2016).

Anode and cathode chambers in dual chamber MFCs are often connected by a proton exchange membrane (PEM), a cation exchange membrane (CEM) or an anion exchange membrane (AEM) and less commonly by bipolar membranes, salt bridge or size-selective separators (Kim et al. 2007; Li et al. 2011; Butti et al. 2016). The main drawback of CEM is the competitive transport of cations other than H⁺, leading to a pH split between the two chambers. AEM promote the transfer of cations, but are more permeable to the substrates used in MFCs, such as acetate, and more liable to deformation (Kim et al. 2007; Li et al. 2011). MFCs can also be operated without a membrane: in this case, power may increase due to the lower internal resistance of the MFC, but the CE often decreases due to the increased diffusion of electron acceptor (e.g. oxygen) from the cathodic to the anodic chamber (Liu and Logan 2004).

In MFCs, optimal electrodes are highly conductive, inexpensive, as well as mechanically and chemically resistant (Butti et al. 2016). Generally, metals have higher conductivity than carbon, but their smooth surface is not favorable for the adhesion of microorganisms. Furthermore, many metals are not suitable for long-term MFC operation due to corrosion, and can be even toxic for exoelectrogenic microorganisms (Zhu and Logan 2014; Sonawane et al. 2017). Carbonaceous materials, in particular graphite, are commonly used because they have relative high conductivity, chemical stability, good biocompatibility and because they are relatively cost-efficient (Hernández-Fernández et al. 2015). The anode surface is crucial for energy production. Many different anode configurations are commercially available, including carbon-based electrodes such as carbon cloth, brush, rods, felt, mesh, granules and paper, and metal-based electrodes such as stainless steel or Ti plates, stainless steel mesh and scrubber, and Ag, Ni, Cu and Au sheets (for a review, see Santoro et al. 2017). Metal foams have also been proposed to increase the anodic surface (Mapelli et al. 2013). Anode pretreatment, such as ammonia gas treatment (Cheng and Logan 2007), electrochemical treatment (Tang et al. 2011) and polymer coatings (Mehdinia et al. 2013) have shown to significantly increase power generation in MFCs by improving the surface charge of the electrode, generating new functional groups and increasing the surface for microorganisms attachment, respectively. Composite, multi-material anodes have also been proposed, such as graphite-polymer, carbon nanotube, graphene-based and carbon-metal composites (Yong et al. 2012; Chen et al. 2013; Sonawane et al. 2017). A low-cost alternative is to utilize natural anode materials, such as carbonized plants, which are porous and conductive (Karthikeyan et al. 2015).

Cathodic reactions may be the limiting factor of the whole electricity production process if the kinetics of the cathodic reactions are slow (Rismani-Yazdi et al. 2008). A good electron acceptor must have a high redox potential and be cheap and easily available (Venkata Mohan et al. 2014). Oxygen is a promising electron acceptor for MFC scale up as it has a high redox potential, it is abundant in the atmosphere and has low cost (Lu et al. 2012). Despite its high redox potential, however, the performance of oxygen for the cathodic reaction is limited by its slow reduction kinetics, as well as by its low solubility (Rismani-Yazdi et al. 2008). Other catholytes used in MFCs include ferricyanide, permanganate, perchlorate and persulfate (Lu et al. 2012). Wei et al. (2012) reported a significantly higher power generation in a two-chamber MFC with a ferricyanide cathode compared to an air-sparged cathode, with an optimum concentration of 0.1 M ferricyanide. This can be attributed to the strong oxidizing nature of ferricyanide, and to the higher mass transfer and lower activation energy for the cathodic reaction compared to oxygen (Venkata Mohan et al. 2014). However, the use of ferricyanide is not sustainable for full scale applications, as it is toxic and becomes consumed during MFC operation, requiring periodic regeneration (Ucar et al. 2017). Another interesting approach is to use metal ions (e.g. Ag^+ , Au^{3+} , Cd^{2+} , Co^{3+} , Co^{2+} , Cr^{6+} , Cu^{2+} , Fe^{3+} , Ni^{2+} and U^{6+}) as terminal electron acceptor, enabling simultaneous reduction and recovery of metals in their elemental form from polluted wastewaters, such as effluents from metallurgical industries or mining (Nancharaiyah et al. 2015; Sulonen et al. 2015; Sulonen et al. 2018). Bioelectrochemical recovery of Cu has even been attempted at pilot scale, but the set-up used requires improvement, as the high internal resistance limited both electricity production and copper recovery (Rodenas Motos et al. 2017).

Different strategies can be applied to improve the cathode performance, such as increasing the electrode surface area and using cathode-membrane assembly, in which the electrodes and membrane are bound together for a better proton transfer (Rismani-Yazdi et al. 2008; Kim et al. 2009). Catalysts can be introduced to accelerate reduction kinetics, especially if oxygen is used as electron acceptor (Liu et al. 2014). Pt and Pd are commonly used as catalysts in bioelectrochemical systems due to their excellent catalytic activity and high resistance to corrosion. However, their high cost and scarcity is driving the search on cheaper and more abundant catalysts. The addition of non-precious metals, such as Ni, not only decreases the cost of the catalyst, but was even shown to increase the power production 3-4 times compared to a Pt catalyst when added in a 1:1 proportion with Pt (Cetinkaya et al. 2015). Recently, the potential of living microorganisms for catalyzing cathodic reactions has also been considered as a promising low cost alternative. It has been reported that a wide variety of microorganisms such as *Shewanella* sp., *Pseudomonas* sp., *Acinetobacter* sp., and *Acidithiobacillus* sp.,

and even some green algae are able to use the cathode as electron donor for their metabolism (for a review, see Liu et al. 2014).

4.3 Thermophilic microbial fuel cells

In the same way as biohydrogen production, thermophilic electricity production could be advantageous because of the high electron production rates of thermophilic bacteria (Du et al. 2007) and the elimination of pathogens. Microorganisms such as *Thermincola* sp., *Calditerrivibrio* sp., and *Thermoanaerobacter* sp. have been reported to produce electricity under thermophilic conditions (Table 4.1). Bioelectricity production under thermophilic conditions by mixed cultures was reported for the first time by Jong et al. (2006), who obtained a maximum power output of 1030 mW m⁻² using acetate as the substrate. Most studies on thermophilic bioelectricity production have been performed at 55 or 60 °C using either MFC or MEC configurations, in which a potential is constantly applied to the device to enrich certain microorganisms (Table 4.1). A hyperthermophilic MFC has been successfully operated by Fu et al. (2015), who reported electricity production from glucose at 75-98 °C, with a maximum power density at 95 °C using water from a petroleum reservoir as inoculum (Table 4.1).

Thermophilic MFCs are still in an early stage of development, and it should be noted that most studies have been performed using basic reactor configurations, as they aimed to study the principles of thermophilic electricity production rather than to improve power production. This may lead to underestimating the potential of thermophilic MFCs when compared with the more technologically advanced MFCs operated at lower temperatures (see section 4.2). However, a remarkably high power production of 1 W m⁻², with a maximum CE of 89%, was obtained from distillery wastewater using a thermophilic two-chamber MFC, which is in line with experiments on mesophilic electricity production from wastewaters (Ha et al. 2012). A thermophilic MFC has been designed to specifically prevent evaporation (Carver et al. 2011), which is one of the main practical issues of thermophilic MFCs. This MFC consisted of a sealed anodic chamber made of glass and a plastic cathodic chamber floating on the anolyte, with a composite electrode in contact with the anolyte at one side and with air at the other side (Carver et al. 2011). However, the high internal resistance of the device resulted in a low power production (Table 4.1).

Table 4.1: Summary of studies reporting current or power generation at thermophilic conditions. Microbial electrolysis cells (MECs) used with electricity generation purposes have also been included in the table.

Inoculum	Substrate	Reactor type	T (°C)	Power or current density	CE (%)	Reference
Thermophilic digester sludge	Acetate (32.9 mM)	Two-chamber MFC	55	823 mW m ⁻²	n.a.	Fu et al. (2013b)
<i>Calditerrivibrio nitroreducens</i>	Acetate (32.9 mM)	Two-chamber MFC	55	272 mW m ⁻²	n.a.	Fu et al. (2013b)
Thermophilic digester sludge	Acetate (32.9 mM)	Two-chamber MFC	55	512 mW m ⁻²	n.a.	Fu et al. (2013a)
<i>Caloramator australicus</i>	Yeast extract (0.2% w/v)	Single chamber MEC	55	3.2 mW m ⁻²	n.a.	Fu et al. (2013a)
Thermophilic digester sludge	Acetate (3.4 mM)	Two-chamber MFC	55	1030 mW m ⁻²	80	Jong et al. (2006)
Thermophilic digester sludge	Distillery wastewater (300 mg COD L ⁻¹)	Two-chamber MFC	55	1000 mW m ⁻²	89	Ha et al. (2012)
Thermophilic digester sludge	Acetate (10 mM)	Two-chamber MFC	55	37 mW m ⁻²	89	Wrighton et al. (2008)
Thermophilic compost	Glucose (25 mM)	Two-chamber MFC	57	4.0 mW m ⁻²	n.a.	Carver et al. (2011)
<i>Thermincola ferriacetica</i> DSMZ 14005	Acetate (35 mM)	Two-chamber MEC	60	7-8 A m ⁻²	93	Parameswaran et al. (2013)
<i>Thermoanaerobacter pseudethanolicus</i>	Xylose (20 mM)	Two-chamber MEC	60	5.8 A m ⁻²	35	Lusk et al. (2015)
<i>Thermoanaerobacter pseudethanolicus</i>	Glucose (10 mM)	Two-chamber MEC	60	4.3 A m ⁻²	65	Lusk et al. (2015)
<i>Thermoanaerobacter pseudethanolicus</i>	Cellobiose (7.5 mM)	Two-chamber MEC	60	5.2 A m ⁻²	28	Lusk et al. (2015)
Marine sediment	Acetate (25 mM)	Single chamber MFC	60	207 mW m ⁻²	35.5	Mathis et al. (2008)
<i>Thermincola ferriacetica</i> strain Z-0001	Acetate (10 mM)	Single chamber MFC	60	160 mW m ⁻²	97	Marshall and May (2009)
Water from a petroleum reservoir	Glucose (11.1 mM)	Two-chamber MFC	95	165 mW m ⁻²	n.a.	Fu et al. (2015)

5 Hypothesis and aims of the present work

Due to the thermodynamic advantage of operation of dark fermentation at high temperatures, it was hypothesized that thermophilic biohydrogen production may result in a higher hydrogen yield than a mesophilic process. The same concept applies to thermophilic MFCs, which have not been studied as intensively as mesophilic MFCs, and thus deserve more attention.

Both mesophilic and thermophilic dark fermentation are widely affected by the selection of the initial inoculum. Many aerobic and anaerobic inocula have been tested for dark fermentation, but a direct comparison between an aerobic and an anaerobic inoculum originating from a same source may help in defining the start-up strategy for dark fermentative bioreactors.

Inoculum pretreatments have been widely applied in laboratory-scale dark fermentation studies, and their effect on H₂ production has been compared in many studies. However, only few studies took into account the effect of the various pretreatments on the microbial community. A RNA-level approach was proposed with the aim of better understanding the impact of pretreatments on H₂ producing microorganisms and competitors.

It was hypothesized that a thermophilic (55 °C) dark fermentative bioreactor can be adapted for biohydrogen production at temperatures up to 70 °C, and that a mesophilic (37 °C) microbial community can be converted to thermophilic (55 °C) without need for reinoculation.

According to recent literature, it was hypothesized that DNA-level microbial community analysis may drive to erroneous conclusions on the role of microorganisms in bioreactors. Therefore, a RNA-based analysis was proposed to study the microbial communities in

MFCs. Also, it was hypothesized that not only anode-attached microorganisms, but also planktonic and membrane-attached species may directly or indirectly contribute to power generation in MFCs.

Despite the thermodynamic advantages, thermophilic MFCs often produce less power than mesophilic MFCs due to the low number of thermophilic exoelectrogenic species and their difficult enrichment. Based on the results obtained in the RNA-level microbial community analysis on the thermophilic MFC, a start-up strategy was developed to favor the growth of thermophilic exoelectrogens in MFCs.

Thermomechanical pulping (TMP) wastewater, being rich in carbohydrates, low in possible inhibitory chemicals and produced at high temperature, would be an excellent substrate for thermophilic dark fermentation, but its hydrogen producing potential has not yet been investigated. The same applies for its potential for bioelectricity production from TMP in MFCs.

Therefore, the specific objectives of this thesis were as follows:

- Compare fresh and digested activated sludge, collected from the same municipal wastewater treatment plant for biohydrogen production under mesophilic (37°C), thermophilic (55°C) and hyperthermophilic (70°C) conditions (Paper I)
- Evaluate the effect of temperature (heat, freeze) and pH (acidic and alkaline) pretreatments on mesophilic (37 °C), thermophilic (55 °C) and hyperthermophilic (70°C) biohydrogen production considering both H₂ yield and composition of the active microbial community (Paper II)
- Evaluate the continuous biohydrogen production from xylose in the temperature range 55-70 °C (Paper III)
- Delineate whether TMP wastewater is a suitable substrate for dark fermentation, and what is the optimal temperature for dark fermentation of TMP wastewater (Paper IV)
- Study the H₂-producing and H₂-consuming microbial communities at both DNA and RNA level, and their metabolic pathways (Paper I, II, III, IV)
- Evaluate composition and role of the anode-attached, planktonic and membrane-attached microbial communities in mesophilic (37 °C) and thermophilic (55°C) two-chamber MFCs (Paper V)
- Develop a strategy to improve power production in thermophilic (55°C) MFCs (unpublished results)
- Evaluate whether TMP wastewater can be used as substrate for bioelectricity production in MFCs (unpublished results).

6 Materials and methods

6.1 Hydrogen production via dark fermentation

6.1.1 Overview of the hydrogen production experiments

Two heat-treated inocula were compared in batch for their dark fermentative H₂ production from xylose at mesophilic (37°C), thermophilic (55°C) and hyperthermophilic (70°C) conditions (Paper I). The first one was fresh activated sludge, and the second was digested activated sludge, both of which originating from the same municipal wastewater treatment plant (Viinikanlahti, Tampere, Finland). Fresh activated sludge, which enabled higher H₂ production than the digested activated sludge, was then used as inoculum for the next experiments. The influence of inoculum pretreatments (pH and temperature shocks) on mesophilic (37 °C), thermophilic (55 °C) and hyperthermophilic (70 °C) dark fermentation of xylose, and on the composition of the active microbial communities, was studied in a batch assay (Paper II). A fluidized bed reactor (FBR) was inoculated with heat-treated fresh activated sludge to evaluate the continuous H₂ production from a synthetic xylose-containing feed stream in the temperature range 55-70 °C in comparison to a mesophilic (37 °C) FBR (Paper III). Finally, inoculum from the thermophilic FBR, acclimated to 70 °C, was used to study H₂ production from thermomechanical pulping (TMP) wastewater in a wide temperature range (37-80 °C) in anaerobic batch incubations using a temperature gradient incubator (Paper IV). The overall experimental design was as summarized in Table 6.1.

Table 6.1: Overview of the hydrogen production experiments showing the objective, experiment type and temperatures tested.

Objective	Experiment type	Temperatures tested	Paper
Inoculum selection	Batch	37, 55, 70	I
Inoculum pretreatment selection	Batch	37, 55, 70	II
H ₂ production from xylose-containing synthetic wastewater	Continuous	37, 55, 60, 65, 70	III
H ₂ production from TMP wastewater	Batch	37, 42, 48, 55, 59, 65, 70, 74, 80	IV

6.1.2 Inocula and their pretreatment

Fresh and digested activated sludge, widely used for studies on dark fermentation (Li and Fang 2007), were collected either from the Viinikanlahti (Tampere, Finland) or from the Mutton Island (Galway, Ireland) municipal wastewater treatment plant (Table 6.2). The fresh activated sludge from Viinikanlahti and Mutton Island were collected from the recirculation line between the aeration tank and the secondary settler, and from the secondary settler itself, respectively. The digested activated sludge from Viinikanlahti was collected from a mesophilic (35 °C) anaerobic reactor digesting waste activated sludge. The inoculum for the batch study on dark fermentation of TMP wastewater (Paper IV) was biofilm-coated activated carbon originating from the thermophilic, xylose-fed FBR. It was collected after 185 days of thermophilic (55-70 °C) FBR operation, the last 27 days at 70 °C (Paper III). All the inocula were dewatered by settling and removal of the supernatant (Paper I, III, IV) or filtering through a 0.1 mm mesh (Paper II). After dewatering, the inocula contained the total solids (TS) and volatile solids (VS) concentration specified in Table 6.2.

Table 6.2: Total and volatile solids concentration of the inocula used in the hydrogen production experiments.

Inoculum	Total solids (g L ⁻¹)	Volatile solids (g L ⁻¹)	Paper
Fresh activated sludge (Viinikanlahti)	17.3 (± 0.1)	8.8 (± 0.1)	I, III
Digested activated sludge (Viinikanlahti)	46.5 (± 0.4)	24.0 (± 0.1)	I
Fresh activated sludge (Mutton island)	28.1 (± 2.4)	22.7 (± 2.0)	II
Biofilm coated activated carbon from FBR	558 (± 14)	529 (± 14)	IV

The heat shock (Paper I, II and III) was conducted by exposing the fresh or digested activated sludge, placed in thin 15 mL tubes, to 90 °C for 15 min using a pre-heated water bath. Freezing and thawing (Paper II) was done by exposing the fresh activated sludge to -20 °C for 24 hours in 15 mL tubes and then defrosting the sludge in a water bath at 30 °C. The acidic shock (Paper II) was done by adjusting the pH of the fresh activated sludge to 3.0 with HCl, incubating at about 20 °C for 24 hours, and then increasing the pH back to 7.0 with NaOH. The alkaline shock (Paper II) was done by adjusting the pH to 10.0 with NaOH, incubating at about 20 °C for 24 hours, and then adjusting the pH back to 7.0 with HCl. Both HCl and NaOH were used at a concentration of 1 or 3 M and the sludge was continuously stirred by using a magnetic stirrer while adjusting the pH.

6.1.3 Experimental set-up of batch experiments

Batch assays to study H₂ production at 37, 55 and 70 °C were conducted in 120 mL serum bottles with 50 mL effective culture volume (Paper I, II). The cultivation medium used was DSMZ medium 144 (German Collection of Microorganisms and Cell Cultures, 2008), with the following modifications: the concentration of yeast extract was reduced to 0.3 g L⁻¹ (Nissilä et al. 2011), tryptone was not added and xylose (50 mM) was used as the carbon source instead of glucose (Paper I, II). The pH of the cultivation medium was adjusted either to 5.5 (Paper I) or to 7.0 (Paper II) with 1 M HCl.

Four consecutive batch cultures (6-8 days each) were performed to study H₂ production from xylose at 37, 55 and 70 °C (Paper I). In the first culture, the bottles were inoculated with either heat-treated fresh or digested activated sludge (2 g VS L⁻¹) and cultivation medium was added up to 50 mL. The following three batch cultures were done by adding 45 mL of fresh medium to 5 mL of cultivation from the previous batch culture in a clean serum bottle (Paper I). The effect of pretreatments on H₂ production at 37 and 55 °C

(Paper II), and 70 °C (unpublished results) was studied in single batch cultures (4-6 days) using serum bottles containing 5 mL of heat-shock-, freeze and thawing-, acidic shock- or alkaline shock-pretreated fresh activated sludge and 45 mL of medium (Paper II). In both the batch experiments (Paper I and II), the initial xylose concentration of the mixture of medium and inoculum was 50 mM. All the serum bottles were flushed with N₂ for 5-10 min before and after inoculation to ensure anaerobicity. All the batch cultures were conducted in triplicate. A control bottle without xylose (Paper I) or with untreated activated sludge (Paper II) was also prepared and incubated similarly as the other bottles.

Batch assays with the TMP wastewater were conducted in anaerobic tubes with a total volume of 26 mL, including 17 mL working volume and 9 mL headspace (Paper IV). Biofilm-coated activated carbon (2 mL) from the thermophilic FBR (Table 6.2) was added as inoculum to 15 mL of TMP wastewater (Table 6.3). All the tubes were flushed with N₂ for 5 min after inoculation. The initial pH was adjusted to 6.3 (\pm 0.1) using 1 M NaOH. The tubes were incubated in a temperature-gradient incubator (Test Tube Oscillator, Terratec Asia Pacific, Australia) at 200 rpm shaking and at 37, 42, 48, 55, 59, 65, 70, 74 or 80°C (duplicate tubes at each temperature) for a total of 111 hours (Paper IV).

Table 6.3: Composition of the thermomechanical pulping (TMP) wastewater used in Paper IV.

Parameter	Concentration (mg L ⁻¹)
Total solids (TS)	3771 \pm 10
Volatile solids (VS)	2452 \pm 8
Total COD	3352 \pm 82
Soluble COD	3289 \pm 54
Total nitrogen	< 10
Total PO ₄ ³⁻ -P	2.8
Acetate	< 30
Furfural	< 10
Glucose	43 (\pm 2)
Xylose	38 (\pm 0)

6.1.4 Experimental set-up of continuous hydrogen production experiment

The experiment was conducted using two FBRs (Figure 6.1) having 1 L effective volume each, containing 300 mL activated carbon as carrier material (Paper III). The activated carbon bed was expanded by 30% by applying a recirculation flow rate of about 1900 mL min⁻¹ using a peristaltic pump (Masterflex, USA). Both incubators (Labilo, Finland) and a water jacket (Julabo, Germany) were utilized to control the temperature of the FBRs. The xylose-containing synthetic wastewater was similar to the one used for the batch assays (Paper I and II), but the concentration of KH₂PO₄, K₂HPO₄ and yeast extract

was reduced 10 times. The pH inside the reactor was kept at $5.0 (\pm 0.1)$ by automatic titration (Metrohm, Switzerland).

The FBRs were inoculated with 50 mL of heat-treated ($90\text{ }^{\circ}\text{C}$, 15 min) fresh activated sludge. The FBRs were started-up in batch mode for two days, and then switched to continuous mode (day 0) and operated for 7 days at a hydraulic retention time (HRT) of 12 hours. On day 7, the HRT was decreased to 6 hours. The thermophilic FBR was initially operated at $55 (\pm 1)\text{ }^{\circ}\text{C}$. Temperature was then increased to $60 (\pm 1)\text{ }^{\circ}\text{C}$ on day 77, to $65 (\pm 1)\text{ }^{\circ}\text{C}$ on day 119, and to $70 (\pm 1)\text{ }^{\circ}\text{C}$ on day 158 and kept at $70\text{ }^{\circ}\text{C}$ until day 185. The mesophilic FBR was operated at $37 (\pm 1)\text{ }^{\circ}\text{C}$ for 185 days, and then at $55 (\pm 1)\text{ }^{\circ}\text{C}$ until day 228.

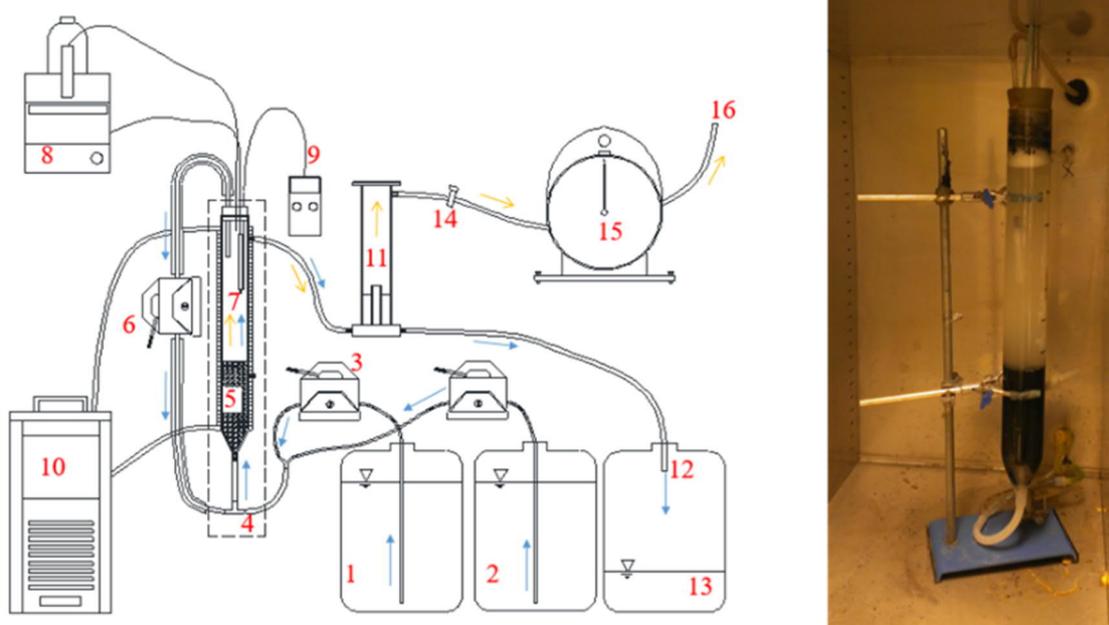


Figure 6.1: A schematic representation and a photograph of the fluidized bed reactor (FBR) set-up used in Paper III. Medium influent tank (1), xylose influent tank (2), peristaltic pumps for influent feeding (3), influent sampling point (4), activated carbon bed with the active biomass (5), peristaltic pump for recirculation (6), pH probe (7), automatic titrator (8), temperature control (9), water jacket (10), gas-liquid separator (11), effluent sampling point (12), effluent tank (13), gas sampling point (14), gas meter (15), and gas outlet (16). Liquid path (\rightarrow), gas path (\rightarrow). The dashed rectangle represents the FBR located inside the incubator.

6.2 Electricity production in microbial fuel cells

6.2.1 Overview of the experiments

A mesophilic (37 °C) and a thermophilic (55 °C) h-type, xylose-fed microbial fuel cell (MFC) (Figure 6.2a), were compared in terms of power production and composition of the anode-attached, membrane-attached and planktonic microbial communities (Paper V). Then, optimization strategies were applied in order to improve power production under thermophilic conditions (unpublished results). The studied strategies included start-up using a poised anode potential of -289 mV vs. an Ag/AgCl reference electrode, chemical elimination of methanogens using BESA and use of two different MFC designs, i.e. upflow MFC (Figure 6.2b) and cuboidal MFC (Figure 6.2c). Thermomechanical pulping (TMP) wastewater was tested as substrate for electricity production in a mesophilic (37 °C) upflow MFC (unpublished results). The experimental plan is summarized in Table 6.4. The detailed description of experimental set-up for the unpublished results is not included in this material and methods section.

Table 6.4: Overview of the MFC experiments showing the objective, experiment type and temperatures tested.

Objective	Experiment type	Temperatures tested	Paper
Study of the microbial communities	Batch	37, 55	V
Optimize thermophilic electricity production	Batch	55	Unpublished
Electricity production from TMP wastewater	Continuous	37	Unpublished

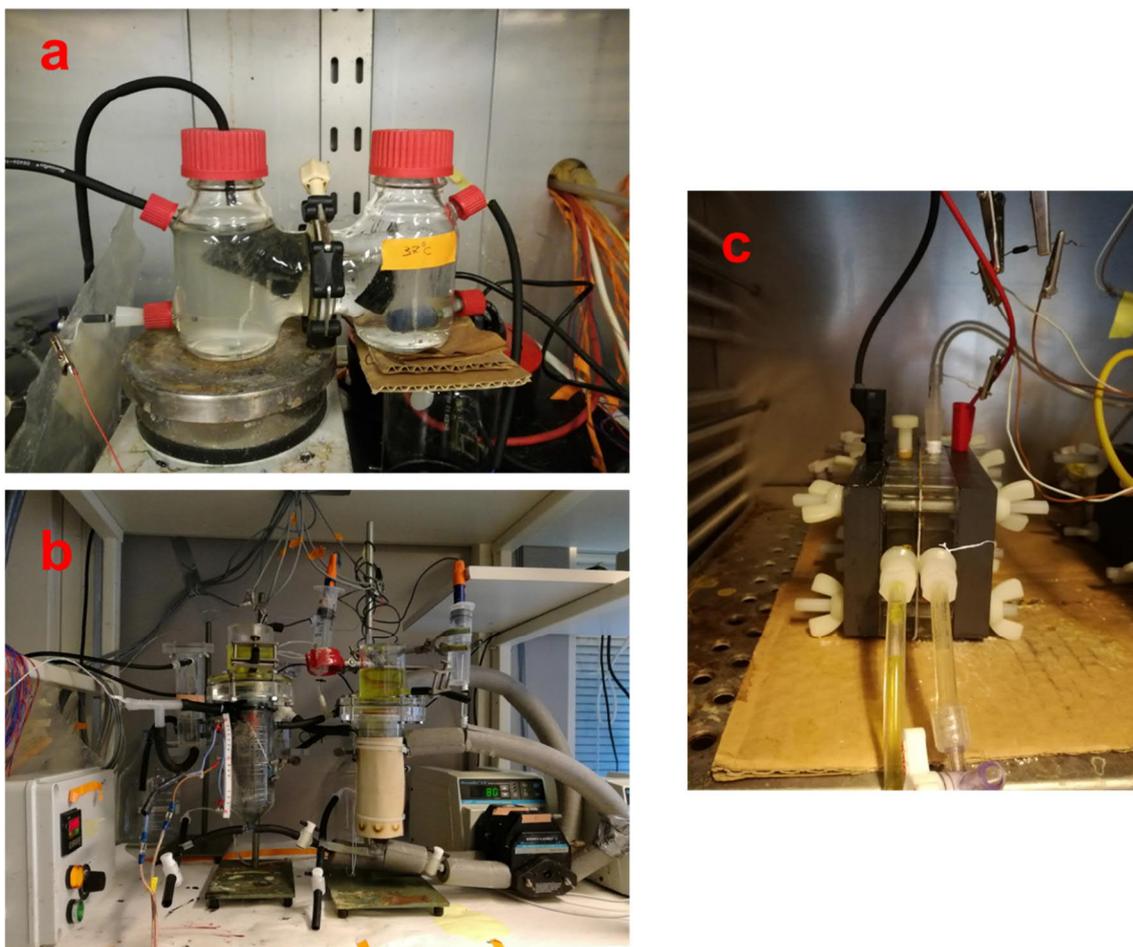


Figure 6.2: Photograph of the h-type (a), upflow (b) and cuboidal (c) two-chamber MFCs used to study mesophilic and thermophilic electricity production.

6.2.2 Inoculum preparation

Two inocula, 15 mL each, were supplied to each h-type MFC to ensure a wide microbial community, containing both fermentative and exoelectrogenic microorganisms, capable to live in a broad temperature range (Paper V). The first one was fresh activated sludge from Viinikanlahti wastewater treatment plant, which had previously shown potential for H_2 production via dark fermentation of xylose at various temperatures (Paper I and III). The second one was anodic medium from a xylose-fed mesophilic (37 °C) MFC (Haavisto et al. 2017). The TS content of the activated sludge and the anolyte was $19.5 (\pm 0.2)$ and $22.1 (\pm 0.4) \text{ g L}^{-1}$, respectively, whereas the VS content was $10.6 (\pm 0.2)$ and $8.4 (\pm 0.5) \text{ g L}^{-1}$, respectively. The mixture of the two inocula, in 1/1 volume ratio, was flushed with N_2 for 10 min before adding them to the MFCs.

6.2.3 Mesophilic and thermophilic bioelectricity production from xylose

The anodic and cathodic chamber of the h-type MFCs (Figure 6.2a), 350 mL each, were separated by an anion exchange membrane (AMI-7001, Membranes International Inc., USA) (Paper V). The anode was a carbon brush, while the cathode was a carbon cloth coated with 20 mg of a Pt-based catalyst (20% platinum on Vulcan carbon, E-TEK, USA). The external resistance connecting the two electrodes was 100 Ω . The anolyte was a xylose-containing medium modified from Mäkinen et al. (2012), and its conductivity was 14.6 mS cm⁻¹. Oxygen was pumped to the cathodic chamber, filled with 300 mL Milli-Q® water, at a flow rate of 130 mL min⁻¹, by using an aquarium air pump (Marina 50).

During start-up, the anodic chamber of both MFCs was filled with the anolyte solution (270 mL) and inoculum (30 mL), and during the operation the anolyte was continuously stirred using magnetic stirrers. The two MFCs were operated inside incubators (Mettler, Germany) in order to keep the temperature at 37 (\pm 2) °C or 55 (\pm 2) °C, respectively. The pH of the anolyte was adjusted to 7.0 with NaOH and kept at 7.0 (\pm 0.2) using phosphate buffer. Both MFCs were operated in fed-batch mode. The feeding steps were done every 7-8 days by replacing 10% of the anolyte with the feeding solution, which had the same composition as the original anolyte, but with a 10-times higher xylose concentration. The initial xylose concentration was 0.3 g L⁻¹ in the first six fed-batch cycles, and was then increased to 1.0 g L⁻¹ (day 0) for the following eleven fed-batch cycles.

6.2.4 Power and polarization curves

Power and polarization curves were obtained by operating the MFC in open circuit mode for 30 min and then closing the circuit using a resistor box (TENMA 72-7270, Taiwan) and decreasing the resistance stepwise from 15 k Ω to 5 Ω at 30-min intervals. Voltage was recorded just before switching the resistance and used to calculate current and power densities as specified in section 6.4.

6.3 Chemical and bioelectrochemical analyses

Analytical methods and instruments utilized in the experiments were as summarized in Table 6.5.

Table 6.5: Methods and equipment used for chemical and bioelectrochemical analyses in Papers I-V.

Parameter	Analytical technique, instrument model	Paper
Gas volume	Syringe method (Owen et al. 1979)	I, II, IV
	Gas meter, Ritter	III
Gas composition	Gas chromatography, Shimadzu GC-2014 thermal conductivity detector (TCD) and Porapak N column	I, II, III, IV
Sugar concentration	Colorimetric method (Dubois et al. 1956), Shimadzu Ordior UV-VIS spectrophotometer	I
	Liquid chromatography, Shimadzu HPLC with refractive index detector (RID) and Phenomenex RHM-monosaccharide column	II, III
	Liquid chromatography, Shimadzu HPLC with Phenomenex RPM-monosaccharide column	IV
VFA and alcohol concentration	Gas chromatography, Shimadzu GC-210 with flame ionization detector (FID) and Porapak N column	I, III
	Liquid chromatography, Shimadzu HPLC with RID and Phenomenex RHM-monosaccharide column	I, III, IV, V
	Liquid chromatography, Shimadzu HPLC with RID and Phenomenex ROA-Organic Acid H ⁺ column	II
pH	WTW 330 pH meter with Hamilton slim electrode	I,II, III, IV
COD, sCOD	Dichromate methods, Finnish standard SFS 5504	IV, V
TS, VS, Total N, PO ₃ ³⁻ -P	Standard procedures (APHA 1998)	IV
Furfural	Gas chromatography, Agilent GC with MS detector and Agilent HP-5MS capillary column	IV
Voltage	Data logger, Agilent 34970A	V
Conductivity	Conductivity meter, WTW inoLab	V
Dissolved oxygen	Muti-parameter meter, HQ40d meter with IntelliCAL optical probe	V

6.4 Microbial community analyses

6.4.1 Polymerase chain reaction-denaturing gradient gel electrophoresis

Microbial community samples for polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) (Table 6.6) were stored at -20 °C after collection. DNA extraction and PCR-DGGE were performed according to Mäkinen et al. (2012). The visible DGGE bands were cut, eluted in sterile water and re-amplified by PCR as described by Koskinen et al. (2006), prior to sending them to Macrogen (South Korea) for sequencing. The nucleotide sequences were analyzed by Bio-Edit software (Hall

1999) and compared with the sequences in the GenBank nucleotide collection database using the BLAST software (Altschul et al. 1990).

6.4.2 Illumina Miseq sequencing

Samples for Illumina Miseq sequencing (Table 6.6) were stored at -80 °C after collection. Nucleic acids were co-extracted using a method modified from Griffiths et al. (2000). No further procedures were done for DNA level analysis. For RNA level analysis, DNA was removed by adding 1 µL turbo DNase and 2.5 µL buffer (Invitrogen, Thermo Fisher, USA), followed by incubation at 37 °C for 30 min. DNase was then inactivated by addition of 2.5 µL DNase inactivator (Invitrogen) and, after centrifugation (10000xg, 1.5 min), the RNA-containing supernatant was transferred to a fresh tube. Complementary DNA (cDNA) was obtained from RNA using M-MuLV Reverse Transcriptase (New England, BioLabs, USA), following the instructions provided by the supplier. Samples of DNA and cDNA were sent to FISABIO (Valencia, Spain) for high-throughput sequencing of partial 16S rRNA genes on an Illumina MiSeq platform (Caporaso et al. 2011). Sequence screening, alignment to Silva database, clustering, chimeras removal and taxonomic classification (cut-off = 97%) were performed using Mothur (Schloss et al. 2009) as described by Kozich et al. (2013).

Table 6.6: List of samples collected, gene targeted and primers used for microbial community analysis in Papers I-V.

Sample origin and type	Analysis performed	Target	Primers	Paper
Settled sludge from batch bottles	PCR-DGGE, sequencing of selected bands	16S rRNA gene (DNA)	BacV3f, 907r	I
	MiSeq sequencing	16S rRNA ^a (RNA)	515f, 806r	II
Biofilm coated activated carbon from FBR	PCR-DGGE, sequencing of selected bands	16S rRNA gene (DNA)	BacV3f, 907r	III
	MiSeq sequencing	16S rRNA ^a (RNA)	515f, 806r	III
Biofilm coated activated carbon from batch vials	MiSeq sequencing	16S rRNA ^a (RNA)	515f, 806r	IV
Anodic biofilm, anolyte and membrane biofilm in MFCs	MiSeq sequencing	16S rRNA gene (DNA), 16S rRNA ^a (RNA)	515f, 806r	V

^a Transcribed to 16S rRNA gene (cDNA).

6.5 Calculations

After correcting the gas volume to standard temperature (0°C), cumulative H₂ and CO₂ production was calculated according to Logan et al. (2002):

$$V_{H,i} = V_{H,i-1} + C_{H,i}(V_{G,i} - V_{G,i-1}) + V(C_{H,i} - C_{H,i-1}) \quad (7)$$

where V_G , V_H and C_H are the current (i) or previous (i-1) cumulative gas volume, cumulative H₂ volume and fraction of H₂ in the headspace, respectively, and V is the volume of the headspace. The net energy gain (kJ L⁻¹) (Eq. 8) was estimated by subtracting the estimated energy requirement for heating the bioreactor (Eq. 9) from the energy recovered by combustion of the produced H₂ per L of wastewater treated (Eq. 10) (Perera et al. 2010):

$$\text{Net energy gain: } NE_G = E_G - E_L \quad (8)$$

$$\text{Energy loss: } E_L = C_W \cdot (T_F - T_I) \cdot \rho_W \quad (9)$$

$$\text{Energy gain: } E_G = Y_H \cdot MV_H \cdot C_X \cdot \rho_H \cdot LHV_H \quad (10)$$

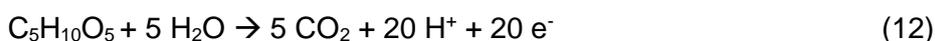
where Y_H is the H₂ yield, MV_H is the molar volume of H₂, C_X is the influent xylose concentration, ρ_H is the density of gaseous H₂, LHV_H is the lower heating value of H₂, C_W is the specific heat of water, T_F and T_I (K) is the temperature of the wastewater after and before heating, respectively, and ρ_W is the density of water.

The theoretical COD (Van Haandel and Van der Lubbe 2012) was calculated from the compounds detected by liquid chromatography:

$$COD_{tot} = 8 \cdot (4x + y - 2z) / (12x + y + 16z) \text{ g } COD_{tot} \text{ g}^{-1} \text{ C}_x\text{H}_y\text{O}_z \quad (11)$$

where x , y and z are the number of C, H and O atoms in the organic molecule, respectively.

CE of the MFCs was calculated according to Oh et al. (2004), accounting 20 mol of electrons exchanged per mol of xylose, according to the following equation:



Currents density (I) and power density (P) were calculated according from the Ohm's law using the following equations:

$$I = U / (R \cdot V) \quad (13)$$

$$P = U^2 / (R \cdot V) \quad (14)$$

where U is the voltage recorded in the data logger, R is the external resistance, and V is the analyte volume.

The IBM SPSS Statistics package was used to perform the statistical analyses to assess significant differences in the H₂ production (Paper II-IV), including one-way analysis of variance (ANOVA) and the Tukey test (Box et al. 1978) at p = 0.05.

7 Results and discussion

7.1 Dark fermentative hydrogen production

7.1.1 Comparison of hydrogen production potential of different inocula

H₂ production from xylose was detected with both fresh and digested activated sludge in batch bottle cultivations conducted at 37, 55 and 70 °C. At both 37 and 55 °C, the H₂ yield and xylose removal efficiency were higher with the fresh rather than the digested heat-treated activated sludge (Table 7.1). At 37 °C, the lowest H₂ yield obtained with the digested activated sludge was likely due to the presence of microorganisms competing with H₂ producing bacteria, such as lactate producers, which grow in the pH range 3.5-4.5 (Fujita et al. 2010), and were therefore favored by the low final pH (< 4) of the batch cultures inoculated with digested activated sludge (Table 7.1).

H₂ production and xylose removal efficiency in the hyperthermophilic (70 °C) batch cultures were low with both inocula studied (Table 7.1). The low final pH < 5 likely inhibited the hyperthermophilic H₂ producing microorganisms, which typically have an optimum pH close to 7 (Ogg and Patel 2009; Vipotnik et al. 2016). Kongjan et al. (2009) and Zhao et al. (2010) obtained H₂ production from xylose at 70 °C, but at a higher initial pH (6.5-7.5) and using an inoculum previously adapted for H₂ production at 70 °C.

Based on the results obtained, fresh activated sludge was selected as the inoculum for the next experiments focusing on the effect of pretreatments on dark fermentation of xylose and continuous H₂ production from xylose in FBRs (Paper II and III).

Table 7.1: H₂ yield, xylose removal efficiency, final pH and main metabolites detected in batch and continuous experiments performed using xylose (50 mM) as the substrate and the various inocula, inoculum pretreatment methods, initial pH and temperature.

Inoculum	Pretreatment	T (°C)	Initial pH	Operation mode	H ₂ yield (mol H ₂ mol ⁻¹ xylose _{added})	Xylose removal efficiency (%)	Final pH	Main metabolites (mM) ^a	Reference		
Digested activated sludge	Heat shock	37	5.5	Fed-batch (I)	0.9 (± 0.1)	98 (± 0)	4.7 (± 0.0)	Butyrate (30), Acetate (22)	Paper I		
				(II)	0.5 (± 0.0)	98 (± 0)	4.2 (± 0.1)	Acetate (18), Butyrate (16)			
				(III)	1.0 (± 0.2)	69 (± 7)	3.5 (± 0.1)	Butyrate (25), Acetate (16)			
				(IV)	0.1 (± 0.2)	20 (± 5)	3.6 (± 0.5)	Butyrate (5), Acetate (4)			
		55	Fed-batch (I)	0.2 (± 0.0)	93 (± 1)	4.1 (± 0.1)	Ethanol (20), Acetate (8), Butyrate (4)				
			(II)	0.8 (± 0.2)	72 (± 5)	4.0 (± 0.2)	Butyrate (17), Acetate (8), Ethanol (4)				
			(III)	0.6 (± 0.1)	51 (± 15)	3.5 (± 0.1)	Butyrate (14), Acetate (12)				
			(IV)	0.4 (± 0.0)	28 (± 5)	3.5 (± 0.1)	Acetate (8), Butyrate (8)				
		70	Fed-batch (I)	0.2 (± 0.1)	71 (± 4)	4.5 (± 0.0)	Ethanol (12), Acetate (11)				
			(II)	0.2 (± 0.1)	59 (± 16)	4.2 (± 0.0)	Ethanol (12), Acetate (11)				
			(III)	0.1 (± 0.0)	15 (± 5)	4.1 (± 0.1)	Acetate (9), Ethanol (8)				
			(IV)	0.1 (± 0.0)	12 (± 2)	4.1 (± 0.1)	Acetate (5)				
		Fresh activated sludge		37		Fed-batch (I)	0.3 (± 0.0)	98 (± 0)		4.9 (± 0.0)	Butyrate (30), Acetate (21)
						(II)	0.6 (± 0.0)	99 (± 0)		4.5 (± 0.1)	Butyrate (19), Acetate (17)
						(III)	1.2 (± 0.1)	97 (± 2)		4.1 (± 0.1)	Butyrate (28), Acetate (19)
						(IV)	1.2 (± 0.1)	99 (± 0)		3.6 (± 0.1)	Butyrate (31), Acetate (17)
55	Fed-batch (I)			0.6 (± 0.1)	98 (± 0)	5.3 (± 0.1)	Ethanol (37), Butyrate (15), Acetate (9)				
	(II)			0.9 (± 0.1)	98 (± 0)	4.3 (± 0.0)	Acetate (24), Butyrate (23), Ethanol (13), Propionate (3)				
	(III)			1.3 (± 0.1)	81 (± 14)	4.0 (± 0.0)	Butyrate (29), Acetate (19)				
	(IV)			1.1 (± 0.1)	67 (± 9)	3.6 (± 0.0)	Butyrate (23), Acetate (18)				

Table 7.1: Continued.

Inoculum	Pretreatment	T (°C)	Initial pH	Operation mode	H ₂ yield (mol H ₂ mol ⁻¹ xylose _{added})	Xylose removal efficiency (%)	Final pH	Main metabolites (mM) ^a	Reference
Fresh activated sludge	Heat shock	70	5.5	Fed-batch (I)	0.2 (± 0.0)	85 (± 5)	4.9 (± 0.2)	Ethanol (31), Acetate (10)	Paper I
				(II)	0.1 (± 0.0)	48 (± 4)	4.3 (± 0.1)	Ethanol (15), Acetate (10)	
				(III)	0.1 (± 0.0)	14 (± 6)	4.2 (± 0.0)	Ethanol (10), Acetate (8)	
				(IV)	0.1 (± 0.0)	21 (± 11)	3.9 (± 0.0)	Acetate (6), Ethanol (6), Lactate (3)	
	Untreated	37	7.0	Batch	0.1 (± 0.0)	94 (± 2)	4.0 (± 0.0)	Acetate (15), Lactate (10), Propionate (8), Ethanol (7), Butyrate (3)	Paper II
	Acidic shock				0.8 (± 0.0)	92 (± 2)	4.4 (± 0.1)	Butyrate (15), Acetate (14), Ethanol (13), Propionate (3)	
	Alkaline shock				0.5 (± 0.1)	94 (± 1)	4.0 (± 0.0)	Acetate (18), Lactate (10), Ethanol (8), Butyrate (6)	
	Heat shock				0.6 (± 0.1)	94 (± 1)	4.3 (± 0.1)	Ethanol (16), Acetate (13), Butyrate (9), Lactate (3)	
	Freezing and thawing				0.2 (± 0.0)	96 (± 1)	4.4 (± 0.0)	Ethanol (13), Acetate (10), Propionate (8), Butyrate (5)	
	Untreated	55			0.5 (± 0.1)	90 (± 1)	4.0 (± 0.1)	Acetate (15), Lactate (14), Butyrate (6)	
	Acidic shock				0.3 (± 0.0)	75 (± 5)	4.3 (± 0.0)	Lactate (8), Butyrate (4), Ethanol (3)	
	Alkaline shock				1.2 (± 0.1)	96 (± 0)	4.5 (± 0.0)	Butyrate (13), Acetate (6)	
	Heat shock				0.2 (± 0.0)	73 (± 3)	4.2 (± 0.0)	Lactate (8)	
	Freezing and thawing				0.6 (± 0.1)	96 (± 1)	4.2 (± 0.1)	Acetate (7), Butyrate (5), Ethanol (5), Lactate (5)	
Untreated	70			0.1 (± 0.1)	52 (± 16)	5.2 (± 0.4)	Acetate (7), Lactate (6), Ethanol (3)	Unpublished	
Acidic shock				0.0 (± 0.0)	54 (± 20)	6.0 (± 0.0)	-		

Table 7.1: Continued.

Inoculum	Pretreatment	T (°C)	Initial pH	Operation mode	H ₂ yield (mol H ₂ mol ⁻¹ xylose _{added})	Xylose removal efficiency (%)	Final pH	Main metabolites (mM) ^a	Reference
Fresh activated sludge	Alkaline shock	70	7.0	Batch	0.0 (± 0.0)	19 (± 11)	6.1 (± 0.1)	Acetate (3)	Unpublished
	Heat shock				0.2 (± 0.0)	29 (± 7)	5.3 (± 0.6)	Acetate (10), Lactate (7), Ethanol (6)	
	Freezing and thawing				0.1 (± 0.0)	33 (± 17)	5.9 (± 0.2)	-	
	Heat shock	37 ^b	5.0	Continuous (FBR)	0.5 (± 0.0)	100 (± 0)	5.0 ^d	Acetate (32), Butyrate (13)	Paper III
		55 ^b			1.0 (± 0.0)	100 (± 0)	5.0 ^d	Butyrate (22), Acetate (19)	
		55 ^c			1.2 (± 0.0)	99 (± 2)	5.0 ^d	Butyrate (25), Acetate (24)	
		60 ^c			0.8 (± 0.0)	100 (± 0)	5.0 ^d	Acetate (42), Butyrate (29)	
		65 ^c			0.8 (± 0.1)	100 (± 0)	5.0 ^d	Acetate (34), Butyrate (33)	
		70 ^c			1.2 (± 0.0)	99 (± 0)	5.0 ^d	Acetate (31), Butyrate (22)	

^a Metabolites produced with a concentration of at least 3 mM. ^b Mesophilic FBR. ^c Thermophilic FBR. ^d pH controlled by automatic titration

7.1.2 Inoculum pretreatments

The effect of temperature (heat shock or freezing and thawing) and pH (acidic or alkaline shock) pretreatment of the inoculum on dark fermentation of xylose by fresh activated sludge was evaluated at 37 and 55 °C (Paper II), and 70 °C (unpublished results). At 37 °C, xylose was consumed with an efficiency of above 90% regardless of the pretreatment applied, and the acidic shock resulted in the highest H₂ yield of 0.8 mol H₂ mol⁻¹ xylose_{added} (Table 7.1). Chang et al. (2011) also reported a higher H₂ yield from activated sludge pretreated by an acidic shock than aeration, alkaline shock, chemical treatment or heat shock. However, an acidic shock of activated sludge has also been reported to favor lactate production at the expense of H₂ (Ren et al. 2008b).

At 55 °C, the alkaline shock was the most effective pretreatment, resulting in a H₂ yield of 1.2 mol H₂ mol⁻¹ xylose_{added}, and a xylose removal efficiency of 96% (Table 7.1). The alkaline shock resulted in a lower pH decrease rate, and thus a higher final pH (Table 7.1) than the other pretreatments, which promoted H₂ production. To our knowledge, only two studies (O-Thong et al. 2009; Luo et al. 2010) compared the effect of pretreatments on thermophilic dark fermentative H₂ production, and none of them reported alkaline shock as the best pretreatment (Table 3.1). In fact, O-Thong et al. (2009) reported a loading shock (incubation of the inoculum with 83.25 g_{COD} L⁻¹ sucrose for two days), as the most effective pretreatment, whereas Luo et al. (2010) obtained higher H₂ yields using the untreated rather than the pretreated inoculum.

At 70 °C, H₂ production and xylose removal were low regardless of the pretreatment applied, likely because bacteria originating from a wastewater treatment process at < 20 °C did not have enough time to acclimatize to such high temperatures. Heat treatment resulted in a slightly higher H₂ yield compared to other pretreatments, but competitive pathways such as lactate and ethanol production limited the H₂ production (Table 7.1).

7.1.3 Continuous hydrogen production

Although a heat shock does not appear to be the most effective pretreatment after just one batch incubation (Paper II), the H₂ yield constantly increased in the four consecutive batch incubations (Paper I) (Table 7.1). Therefore, heat-treated activated sludge was used as inoculum for the FBRs (Paper III).

Continuous dark fermentation of xylose was evaluated in a thermophilic FBR, in which the temperature was stepwise increased from 55 to 70 °C at 5 °C intervals, and compared to a mesophilic (37 °C) FBR. A higher H₂ yield was generally obtained in the thermophilic FBR compared to the mesophilic FBR (Figure 7.1), likely due to the different

microbial communities developed in the two FBRs (see section 7.1.5). In fact, generally, thermophilic microorganisms yield more H₂ than mesophiles (Lee et al. 2011), due to the faster kinetics and the more favorable thermodynamics for H₂ production through the acetate pathway (Verhaart et al. 2010). H₂ production at 70 °C, which was negligible in the batch experiments (Paper I and II), was obtained in the FBR 10 days after increasing the temperature of the FBR from 65 to 70 °C, resulting in a stable H₂ yield of 1.2-1.3 mol H₂ mol⁻¹ xylose_{added} (Figure 7.1). This is likely due to the longer acclimation time compared to the previous batch experiments (Papers I and II). The HPR of 282.1 mL H₂ h⁻¹ L⁻¹ obtained at 70 °C (Paper III) is among the highest reported for dark fermentation of sugars by mixed cultures in bioreactors operated in continuous mode (for HPRs obtained in previous studies, see Table 3.3).

In both the mesophilic and thermophilic FBR, the xylose removal efficiency was constantly above 90% with the exception of an adaptation period that occurred in the thermophilic FBR after switching the temperature from 65 to 70 °C (days 158-172) (Paper III). In the mesophilic FBR, the H₂ production was more unstable than in the thermophilic FBR (Figure 7.1). On days 16, 74 and 89, the H₂ yield dropped to zero, and increased concentrations of acetate were detected in the effluent on the same days (Paper II), suggesting H₂ consumption by homoacetogenesis (see section 7.1.6). On days 91-185, the H₂ yield of the mesophilic FBR fluctuated i.e. increased and decreased due to the accumulation of VFAs, which negatively affected the mesophilic H₂ producing microbial community (Wang et al. 2008). When the operation temperature of the mesophilic FBR was increased from 37 to 55 °C, after about 15 days of adaptation, the H₂ yields increased to values comparable to the ones obtained in the thermophilic FBR, operated from the beginning at 55 °C (Figure 7.1). This suggests that operation conditions of a FBR can be turned successfully from mesophilic to thermophilic.

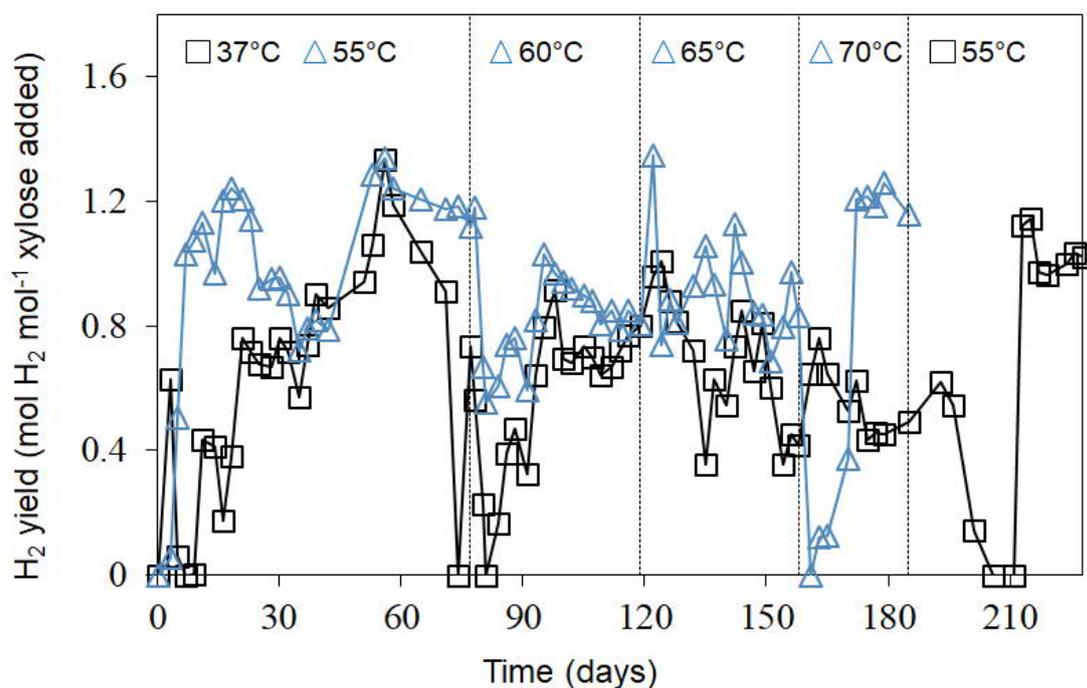


Figure 7.1: H₂ yield obtained in mesophilic (operated at 37 °C up to 185 days and at 55 °C in days 186-225, indicated by black line and squares) and thermophilic (operated at 55-70 °C, indicated by blue line and triangles) fluidized bed reactors (FBRs) treating xylose at pH 5 (details on liquid effluent composition are in Paper III).

7.1.4 Hydrogen production from thermomechanical pulping wastewater

The biofilm-coated activated carbon enriched for dark fermentation of xylose at 55-70 °C in the thermophilic FBR (Paper III) was used to evaluate H₂ production from thermomechanical pulping (TMP) wastewater at 37-80 °C in a batch experiment (Paper IV). H₂ was successfully produced from TMP wastewater in the temperature range 37-70 °C (Paper IV), with a maximum H₂ yield of 3.6 mmol H₂ g⁻¹ COD_{added} obtained at 70 °C (Table 7.2). This yield is about two times lower than the maximum yields of 1.2-1.3 mol H₂ mol⁻¹ xylose_{added} (7.5-8.1 mmol H₂ g⁻¹ COD_{added}) obtained in batch and continuous dark fermentation of xylose (Table 7.1), which is, nevertheless, a more easily degradable substrate than TMP wastewater. The H₂ yield obtained from TMP wastewater at 70 °C is of the same order of magnitude of the yields obtained by direct fermentation of industrial sugar-containing wastewater, such as starch wastewater, under thermophilic conditions (Xie et al. 2014; Khongkliang et al. 2017).

At temperatures < 70 °C, the produced H₂ was partially (at 37, 42, 59 and 65 °C) or totally (at 48 and 55 °C) consumed by homoacetogenesis (see section 7.1.6 focusing on

metabolic pathways), resulting in low final H₂ yields (after 111 hours of incubation). H₂ production from TMP wastewater was insignificant at both 74 and 80 °C (Paper IV), which was likely a too high temperature to keep active the H₂ producing microbial community dominated by *Thermoanaerobacterium* sp. (Ren et al. 2008a). Regardless of the incubation temperature, the total COD removal efficiency was surprisingly higher than the 30-40% expected for dark fermentation (Sharma and Li 2010). This was likely due to the adsorption of VFAs (mainly butyrate) on the activated carbon, as shown in a control experiment conducted with fresh activated carbon and a mixture of VFAs in Milli-Q® water (Paper IV).

Table 7.2: H₂ yield, COD removal efficiency, final pH and main metabolites detected in the batch cultures of TMP wastewater at various temperatures (Paper IV), inoculated with biofilm-coated activated carbon from a thermophilic FBR (Paper III) operated at 70 °C at the time of sampling.

T (°C)	H ₂ yield (mmol H ₂ g ⁻¹ COD)	COD removal (%)	Final pH ^a	Main metabolites (mM)
37	1.4 (± 0.1)	73 (± 0)	5.5 (± 0.1)	Acetate (4), Butyrate (1), Ethanol (1)
42 ^b	0.6	80 (± 8)	5.7 (± 0.1)	Acetate (5), Butyrate (1), Ethanol (1)
48	0.1 (± 0.0)	76 (± 1)	6.0 (± 0.0)	Acetate (7), Butyrate (1)
55	0.0 (± 0.0)	71 (± 5)	6.0 (± 0.1)	Acetate (9), Butyrate (1)
59	0.6 (± 0.3)	71 (± 1)	5.9 (± 0.0)	Acetate (8), Butyrate (1)
65	1.8 (± 0.2)	72 (± 1)	5.2 (± 0.1)	Acetate (5), Butyrate (1)
70	3.6 (± 0.1)	74 (± 4)	5.3 (± 0.0)	Acetate (4)
74	0.1 (± 0.0)	70 (± 2)	6.1 (± 0.0)	Acetate (1), Butyrate (1)
80	0.0 (± 0.0)	79 (± 2)	6.0 (± 0.0)	Acetate (1), Butyrate (1)

^a The initial pH of all batch cultures was 6.3. ^b H₂ was produced only in one of the duplicate batch cultures.

7.1.5 Microbial community composition

The family of *Clostridiaceae* was found in the batch incubations of activated sludge with xylose at 37 °C regardless of the inoculum pretreatment applied (Paper II). The family of *Clostridiaceae* includes many H₂ producing species widely reported to dominate mesophilic dark fermentative bioreactors (Liu et al. 2009; Si et al. 2015; Sivagurunathan et al. 2016b). *Clostridium* sp. dominated the microbial communities also after four consecutive batch incubations with xylose conducted to enrich H₂ producers from heat-treated activated sludge (Paper I) and during the FBR operation (Paper III). *Clostridium* sp. was the prevalent active microorganism also in the mesophilic batch cultures with TMP wastewater (Paper IV).

At 37 °C, the H₂ yield was often lowered by the proliferation of lactate producers such as *Sporolactobacillus* sp. (Paper I) and *Lactobacillus* sp. (Paper II), which produce lactate

at the expenses of H₂, and may also inhibit *Clostridium* sp. growth by excreting toxins (Noike et al. 2002). An acidic shock was the most effective pretreatment to enrich H₂ producers, resulting in a relative abundance of 71-75% *Clostridiaceae* and 8-17% *Lactobacillaceae* upon dark fermentation of xylose (Paper II). Similarly, Kim et al. (2014) reported a relative abundance of 70% and 20% for *Clostridium* sp. and *Lactobacillus* sp. after mesophilic dark fermentation of acidic shock-pretreated (pH 3, 12 hours) food waste. However, the relative abundance of *Lactobacillus* sp. further decreased when applying the acidic shock at pH 2 and pH 1 (Kim et al. 2014). This suggests that the H₂ yield can be further increased by optimizing the pretreatment conditions to minimize the relative abundance of lactate producers. Lactate producers were not detected at any time from the FBR (Paper III), indicating that they were likely out-competed by *Clostridium* sp. in the continuously-fed system. However, in a previous study, *Lactobacillus* was found among the biofilm community in a FBR operated in similar conditions (37 °C, pH 5.5, granular activated carbon as carrier material), but inoculated with heat-treated methanogenic granules and using glucose as the substrate (Cisneros-Pérez et al. 2017).

In the batch cultures at 55 °C, all the pretreatments applied, with the exception of the heat shock, resulted in the proliferation of *Clostridiaceae* as the dominating H₂ producing microorganisms (Paper II). The family of *Clostridiaceae* includes several H₂ producing species, such as *C. thermosaccharolyticum* (Islam et al. 2016) and *C. thermopalmarium* (Lawson Anani Soh et al. 1991). After heat shock, a microorganism closely related to *Thermoanaerobacterium thermosaccharolyticum* was detected with a high (up to 94%) relative abundance (Paper II). *T. thermosaccharolyticum* is a microorganism active in the pH range 5.5-7.0 (Ren et al. 2008a) able to produce H₂ from various monomeric sugars (Ren et al. 2008a; Abreu et al. 2012; Khamtib and Reungsang 2012) and even from polysaccharides such as cellulose and xylan (Cao et al. 2014). *T. thermosaccharolyticum*, as well as *Clostridium* sp., were also found after four batch cultures of heat-treated activated sludge at 55 °C (Paper I). *T. thermosaccharolyticum* was also the dominant microorganism in both the xylose-fed FBR (Paper III) and the batch cultures with TMP wastewater (Paper IV) in the temperature range 55-70 °C. This confirms the importance of *Thermoanaerobacterium* sp. for thermophilic dark fermentation.

7.1.6 Metabolic pathways

The various inoculum pretreatments resulted in a different composition of the metabolites produced by dark fermentation of xylose at 37 and 55 °C (Paper II), and 70 °C (unpublished data). In general, H₂ was produced through the acetate and the butyrate pathway, or both, whereas lactate and ethanol production were the main competitive pathways (Table 7.1). At 37 °C, acetate and butyrate, the main metabolites typically

produced by dark fermentation of monomeric sugars by *Clostridiaceae* such as *C. butyricum* (Seppälä et al. 2011) and *C. acetobutylicum* (Grupe and Gottschalk 1992), were detected regardless the inoculum pretreatment applied. Solventogenesis, which is a detoxification process occurring in case of accumulation of VFAs (Angenent et al. 2004), also occurred at 37 °C regardless of the inoculum pretreatment applied, resulting in ethanol production (Table 7.1). A mixed-type fermentation occurred also at 55 °C, but the alkaline shock repressed lactate production, one of the main pathways competing with H₂ producing pathways, resulting in the highest H₂ yield (Table 7.1). No butyrate was found after dark fermentation of xylose at 70 °C by the untreated and pretreated activated sludge (Table 7.1). H₂ was likely produced through the acetate pathway (Verhaart et al. 2010), but the low microbial activity at the high temperatures resulted in low xylose removal efficiencies and H₂ yields (Table 7.1).

H₂ producing pathways were successfully selected in longer cultivation times, both in consecutive batch cultures (Paper I) and during continuous FBR operation (Paper III). Acetate and butyrate were, in fact, the main metabolites produced from xylose after four consecutive mesophilic (37 °C) and thermophilic (55 °C) batch cultures inoculated with heat treated activated sludge, as well as during the FBR operation, regardless of the operation temperature (Table 7.1). At 55 °C, ethanol was the main metabolite produced by the heat-treated activated sludge in the first batch culture step, but then ethanol was gradually replaced by butyrate, the concentration of which linearly correlated with the H₂ yield (Paper I). Similarly, ethanol was produced in the FBRs for a few days after exposing the microbial communities to 55 °C, but ethanol production was then replaced by butyrate and H₂ production after longer FBR operation (Paper III). This was attributed to either a change in the microbial community or a switch in the metabolic pathway.

Homoacetogenesis was one of the main causes for the lower H₂ yield obtained in the mesophilic FBR compared to the thermophilic FBR (Figure 7.1). The genus *Clostridium*, which was dominating the mesophilic microbial communities (Paper III), includes many species of homoacetogenic bacteria (Ryan et al. 2008). This is in agreement with the study by Koskinen et al. (2006), which attributed the instability of H₂ production in a glucose-fed FBR to the adhesion of homoacetogenic microorganisms to the carrier material. The more stable H₂ production in the thermophilic FBR (Figure 7.1) implies a minor role of homoacetogenesis at the higher operation temperatures (55-70 °C), as also suggested by Luo et al. (2011).

Acetate was the main metabolite found in the batch cultures of biofilm-coated activated carbon in TMP wastewater at a wide temperature range (37-70 °C) (Table 7.2). Acetate production was likely due to either H₂ production through the acetate pathway, or H₂ consumption through homoacetogenesis. However, it should be noted that VFAs (mainly

butyrate, but also acetate) may be adsorbed to the activated carbon and thus not detectable in the liquid phase (Paper IV). Therefore, the contribution of the butyrate pathway on the final H₂ yield may be underestimated. H₂ consumption by homoacetogenesis occurred at temperatures < 70 °C, possibly after depletion of substrates suitable for heterotrophic metabolism (Oh et al. 2003). The highest acetate production obtained at 55 °C and the concomitant H₂ consumption, suggest that the dominant homoacetogenic microorganisms were thermophiles. *Moorella thermoacetica*, which was part of the active microbial community at 55 °C, is a known thermophilic homoacetogen (Drake et al. 2006). Several species of the genus *Clostridium* have been reported to perform autotrophic acetate production as well (Ryan et al. 2008). At 70 °C, the absence of homoacetogenesis resulted in the highest H₂ yield (Table 7.2).

7.2 Microbial fuel cells for biological electricity production

7.2.1 Bioelectricity production in mesophilic MFCs

A higher power production was obtained in the mesophilic rather than in the thermophilic h-type two-chamber MFC in all the eleven fed-batch cycles, likely due to the different composition of the microbial communities in the two MFCs (Paper V). However, the low CE in the mesophilic MFC (12%) resulted in a power density of only 1.0 W m⁻³ (Table 7.3). The low CE and power density was likely due to the MFC design. In fact, power production in air-cathode MFC is often limited by the low proton conduction through the membrane and by the slow rate of oxygen reduction (Rismani-Yazdi et al. 2008). A CE up to 82% was achieved in a xylose-fed, two-chamber MFC using 50 mM ferricyanide as the catholyte (Mäkinen et al. 2013), suggesting that oxygen reduction at the cathode was the main limiting factor. Furthermore, different catholytes can indirectly affect the anodic potential, and thus the anodic microbial community (Torres et al. 2009). Power up to 13 W m⁻³ was obtained in an air-cathode, xylose-fed MFC (Huang and Logan 2008). However, their anode and cathode were composed of four carbon brushes and Pt-coated carbon cloth with four polymeric diffusion layers, respectively, whereas a single carbon brush and Pt-coated carbon cloth were used as anode and cathode in Paper V.

Table 7.3: Power density and coulombic efficiency (CE) obtained in the various MFCs operated using different design, substrates and temperatures. The power production reported refers to the highest, stable power density obtained in the various MFCs. CE was calculated based on removed xylose or acetate for the MFCs fed with synthetic medium, and based on removed COD for the MFC fed with TMP wastewater.

Inoculum	MFC design and membrane	Cathode	Operation mode	T (°C)	Substrate	Power (W m ⁻³)	CE (%)	Reference
Activated sludge + MFC effluent	h-type, AEM	Air	Fed-batch	37	Xylose	1.0	12	Paper V
MFC effluent	Upflow, AEM	Ferricyanide	Continuous	37	TMP wastewater	0.07	2	Unpublished
	Upflow, CEM		Continuous	37		0.2	3	Unpublished
Activated sludge + MFC effluent	h-type, AEM	Air	Fed-batch	55	Xylose	0.03	3	Paper V
Compost and digestate	Upflow, AEM	Ferricyanide	Fed-batch	55	Acetate	0.2	15	Unpublished
Effluent from thermophilic upflow MFC	Cuboidal, AEM	Ferricyanide	Fed-batch	55	Acetate	0.8	9.6	Unpublished

An upflow MFC, with a composed anode electrode (activated carbon in a steel cage), an anion exchange membrane (AEM) and ferricyanide (50 mM) as catholyte, was used to study bioelectricity production from thermomechanical pulping wastewater (TMP) at 37 °C (unpublished results). The upflow MFC was inoculated with effluent from a similar upflow MFC enriched for electricity production from xylose (Haavisto et al. 2017). The highest, stable power density of 0.07 W m⁻³ was produced from TMP wastewater (3.0-3.5 g COD L⁻¹), amended with 2 g L⁻¹ of sodium hydrogen carbonate as pH buffer, at 1.8 days HRT (unpublished results). The power density obtained was lower than the maximum power density of about 3 W m⁻³ obtained from xylose (0.5 g L⁻¹) by Haavisto et al. (2017). Many reasons may have contributed to such low power production, including the complexity and recalcitrance of the substrate and the low conductivity of the TMP wastewater (2-3 mS cm⁻¹ after the addition of hydrogen carbonate), being 5 times lower than the conductivity of the xylose containing medium (14-15 mS cm⁻¹) used by Haavisto et al. (2017). Furthermore, the low concentration of anions, such as phosphates, in the wastewater was likely limiting proton transfer through the AEM: in fact, power production increased to 0.2 W m⁻³ after replacing the AEM with a cation exchange membrane (CEM) (Table 7.3).

7.2.2 Bioelectricity production in thermophilic MFCs

A power density of only 0.03 mW m⁻³ was obtained in the h-type, xylose-fed, thermophilic (55 °C) MFC (Table 7.3), which was likely lacking microbial species able to convert xylose and its degradation products to electricity (Paper V). An alternative start-up strategy was applied to enrich thermophilic exoelectrogenic microorganisms in a thermophilic (55 °C) upflow MFC (unpublished results). Acetate (1 g L⁻¹) was chosen as substrate with the aim to specifically enrich acetate-utilizing exoelectrogenic microorganisms, as acetate utilization was clearly limited in the thermophilic h-type MFC (Paper V). To further select exoelectrogenic microorganisms, the thermophilic upflow MFC was started-up with an applied potential of -289 mV vs. Ag/AgCl (-60 mV vs. SHE), which was successfully used to enrich the thermophilic exoelectrogenic microorganism *Thermincola ferriacetica* from acetate by Parameswaran et al. (2013). BESA (1 g L⁻¹) was also added to inhibit the growth of methanogens without affecting exoelectrogenic bacteria (Chae et al. 2010b). At 55 °C, a 6-7 times higher power density was obtained in the acetate-fed upflow MFC compared to the xylose-fed, h-type MFC (Table 7.3). In fact, the thermophilic upflow MFC produced a stable power of 0.2 W m⁻³ during six consecutive fed-batch cycles, which corresponds approximately to 80 days in total (unpublished results).

The analyte from the thermophilic upflow MFC was tested as inoculum for two cuboidal two-chamber MFCs (30 mL anodic and cathodic chamber volume) consisting of graphite plain electrodes connected through a 100 Ω resistor (unpublished results). The MFC was operated in fed-batch mode with acetate (5 g L⁻¹) as the substrate in the anolyte and ferricyanide (50 mM) as the terminal electron acceptor in the catholyte. Electricity was successfully produced in the cuboidal two-chamber MFCs three days after inoculation and power production increased in the following fed-batch cycles, reaching the highest power density of 0.8 W m⁻³, with a CE of about 10% (calculated based on removed acetate) obtained in one of the duplicate MFCs (Table 7.3), after about 20 days of operation (unpublished results). Although a power density of similar order of magnitude was obtained in the other duplicate MFC, the CE was only 4% due to a higher acetate consumption (see section 7.2.3).

7.2.3 Metabolic pathways

In the mesophilic h-type two chamber MFC, the prompt power increase after the addition of xylose indicates that electricity was produced by direct electron transfer (Marshall and May 2009). Xylose was firstly converted to VFAs by fermentative microorganisms, and then VFAs were oxidized to CO₂ and H₂O by exoelectrogens, resulting in a soluble COD removal efficiency of 80% (Paper V). After the depletion of VFAs, the power density remained stable for about 30 hours, suggesting that VFAs were accumulated inside the microbial cells and oxidized intracellularly (Snider et al. 2012). VFAs, mainly acetate, butyrate and propionate, were found in the effluent of the mesophilic upflow MFC with TMP wastewater as the substrate, resulting in a COD removal efficiency of 30-40% (unpublished results).

In the thermophilic h-type MFC, xylose was consumed in the first 12 hours after its addition to the MFC anode, but the resulting acetate produced was not consumed even after 144 hours (Paper V). A power density peak was obtained just after the xylose depletion, suggesting that the microbial community included exoelectrogenic thermophiles growing on xylose, but was lacking acetate-utilizing microorganisms. Acetate utilization was slow also in the thermophilic, acetate-fed upflow MFC: 12-14 days were required, in average, to consume 80% of the acetate introduced to the anode chamber in every fed-batch cycle (unpublished results). Surprisingly, a different acetate removal efficiency (55% and 97%) was obtained in the two duplicate cuboidal MFC inoculated with the effluent from the upflow MFC, resulting in a different CE (unpublished results). It is plausible that, despite the same inoculum source, more non-exoelectrogenic microorganisms were introduced to one of the duplicate MFC, or that exoelectrogenic and non-exoelectrogenic microorganisms shared the space differently in the two anodic biofilms. Microbial community analysis is required to confirm this hypothesis.

7.2.4 Structure and role of the attached and planktonic microbial communities

The microbial community analysis revealed a clear difference in the microbial communities not only when comparing the mesophilic and thermophilic h-type, xylose-fed MFCs, but also when comparing the anode-attached, membrane-attached and planktonic communities in the same MFC. Microorganisms of the *Geobacteraceae* family dominated the active microbial community (65% of the relative abundance) growing attached to the anode of the mesophilic xylose-fed MFC, and were associated to power generation (Paper V). The *Geobacteraceae* family includes many species of known exoelectrogenic microorganisms typically found in mesophilic MFCs, regardless of the substrate, inoculum source and MFC set-up (Gao et al. 2014; Lesnik and Liu 2014; Jiang et al. 2016; Haavisto et al. 2017). The planktonic community in the anolyte included 6-7 families with a similar relative abundance of 8-18% (Paper V). *Geobacter* sp. is known to transfer electrons to the anode by direct contact transfer or through conductive nanowires, but not by mediated transfer (Kumar et al. 2017a). In fact, its relative abundance in the active planktonic community was only 3% (Paper V).

In the thermophilic, h-type, xylose-fed MFC, the scarcity of the thermophilic exoelectrogenic species and the high relative abundance of competitors, mainly methanogens and H₂ oxidizers, resulted in a low power production (Paper V). Bacteria of the family *Thermodesulfobiaceae*, closely related to *Coprothermobacter* sp., were found among both the anode-attached and planktonic microbial communities with a relative abundance of 13 and 23%, respectively (Paper V). *Coprothermobacter* sp. can perform fermentation of organic substrates, syntrophic acetate oxidation and even long-range electron transfer (Gagliano et al. 2015). Its activity is enhanced by establishing a syntrophy with hydrogenotrophic methanogenic archaea such as *Methanothermobacteraceae* (Sasaki et al. 2011), which were indeed found with a high relative abundance (38%) among the active anode-attached families in the thermophilic MFC (Paper V). *Coprothermobacter* sp. has been detected from the anode-attached community also in previous studies utilizing thermophilic MFCs (Jong et al. 2006; Wrighton et al. 2008). Thus, despite the low acetate consumption in the thermophilic h-type MFC (Paper V), *Coprothermobacter* sp. is a possible acetate-utilizing exoelectrogenic microorganisms. However, its bioelectrochemical activity as a pure culture has not yet been investigated.

Methanobacteriaceae and *Hydrogenophilaceae* were the most abundant microorganisms competing with exoelectrogens in the thermophilic xylose-fed h-type MFC (Paper V). *Methanobacteraceae* are not able to perform acetoclastic methanogenesis as they lack cytochromes (Thauer et al. 2008), but may have consumed

electrons by performing hydrogenotrophic methanogenesis, decreasing the availability of electrons for electricity production, as reported previously by Chung and Okabe (2009). The family of *Hydrogenophilaceae* could have consumed a share of electrons by H₂ oxidation (Hayashi et al. 1999).

In both the mesophilic and the thermophilic h-type MFC, the biofilm growing on the part of the membrane facing the anode contained a diverse microbial community, including aerobic or facultative anaerobic microorganisms such as *Comamonadaceae* or *Armatimonadetes*. Although not directly involved in electricity generation, these microorganisms may be involved in the important role of consuming the small amount of oxygen crossing the AEM from the cathode to the anode (Kim et al. 2007), which can inhibit the strictly anaerobic exoelectrogens. However, aerobic metabolism reduces the quantity of substrate available for electricity generation (Kim et al. 2007). Membrane-attached microorganisms may reduce power output also by forming a thick biofilm which limits proton transfer from the anodic to the cathodic chamber (Miskan et al. 2016).

8 Conclusions

Despite its aerobic origin, heat-treated (90 °C, 15 min) fresh activated sludge was shown to be a better inoculum than digested activated sludge for both mesophilic (37 °C) and thermophilic (55 °C) dark fermentation of xylose (Paper I). In a single batch, fresh activated sludge pretreated by an acidic shock (pH 3, 24 hours) and an alkaline shock (pH 11, 24 hours) resulted in a higher H₂ yield than heat treatment for dark fermentation of xylose at 37 and 55 °C, respectively (Paper II). However, at both 37 and 55 °C, H₂ production from heat-treated fresh activated sludge constantly increased in three consecutive batch cultures, suggesting that dark fermentative microbial communities may develop in the long-term (Paper I) under growth conditions optimized for H₂ production.

Hyperthermophilic (70 °C) H₂ production from xylose by heat-treated fresh activated sludge, which failed in batch (Paper I), was obtained in a FBR adapting the microbial community by increasing the temperature from 55 °C to 70 °C stepwise at 5 °C intervals (Paper III). At 70 °C, a stable yield of 1.2 mol H₂ mol⁻¹ xylose_{added} was obtained, resulting in a H₂ production rate of 282.1 mL H₂ h⁻¹ L⁻¹ (Paper III), which is among the highest reported in continuous thermophilic dark fermentation of monomeric sugars by mixed cultures. Dark fermentation at 70 °C can be particularly potential for treatment of TMP wastewater as it is released from the production process at high temperature (50-80 °C) and could be treated on site, with minimal energy requirement for heating. Furthermore, dark fermentation of TMP wastewater at 70 °C was shown to repress homoacetogenesis, which can partially or totally consume the produced H₂ at lower temperatures, resulting in low H₂ yields (Paper IV).

Dark fermentative microbial communities were studied in depth at DNA and, for the first time, RNA level (Papers II-IV). *Clostridium* sp. dominated the mesophilic (37 °C) dark fermentative microbial communities, regardless of the inoculum pretreatment method

applied, operation mode (batch or continuous) and substrate (Papers I-IV). H₂ production at 37 °C was shown to be dependent on the relative abundance of *Clostridiaceae* among the active microbial community (Paper II). In the same way, *Thermoanaerobacterium* sp. was the prevalent thermophilic H₂ producing microorganism in the temperature range 55-70 °C (Papers III and IV). At 37 °C, *Lactobacillus* sp. was the most common microorganism competing with H₂ producers for the substrate (Paper II), and homoacetogenic microorganisms, likely belonging to the genus *Clostridium*, were associated to H₂ consumption (Papers I, III and IV). In general, a lower relative abundance of competing microorganisms was found under thermophilic conditions, particularly at 70 °C, in which the relative abundance of *Thermoanaerobacterium* sp. was above 90% (Paper III and IV).

A RNA approach was used, for the first time, to depict the role of the anode-attached, membrane-attached and planktonic microbial communities on electricity production in MFCs (Paper V). At 37 °C, power production was likely sustained by an anode-attached community of *Geobacteraceae*, whereas only a very low power production was obtained at 55 °C due to the high relative abundance of methanogenic and H₂ oxidizing microorganisms. However, a chemical inhibition of methanogens and imposing a negative anodic potential in the start-up phase can promote the growth of thermophilic exoelectrogenic microorganisms. Aerobic microorganisms, found among the membrane-attached community at both 37 and 55 °C, might be involved in consuming the oxygen diffusing from the cathodic to the anodic chamber through the anion exchange membrane. This favors the strictly anaerobic exoelectrogenic microorganisms, but at the same time aerobic metabolism in the membrane biofilm reduces the share of electrons available for electricity production.

In summary, this study demonstrated that both dark fermentation and microbial fuel cells can be implemented for energy recovery from treatment of sugar-containing wastewaters. Thermophilic dark fermentation of synthetic pentose sugar-containing wastewater resulted in higher H₂ yields than the mesophilic processes (Papers I-III). The highest H₂ yield from a synthetic, xylose-containing wastewater (1.2 mol H₂ mol⁻¹ xylose_{added}) and from thermomechanical pulping (TMP) wastewater (3.6 mmol H₂ g⁻¹ COD_{added}) were obtained at 70 °C. Conversely, a higher power density, with a maximum of 1.0 W m⁻³, was produced by the mesophilic (37 °C) than the thermophilic (55 °C) h-type two chamber MFC fed with a synthetic, xylose-containing medium. A mesophilic upflow MFC was also shown to produce electricity from TMP wastewater, in continuous, with a stable power density of 0.2 W m⁻³.

9 Recommendations for future research

This study demonstrated that dark fermentation of sugar-containing wastewaters may result in higher H₂ yields under thermophilic than mesophilic conditions. It was also shown, for the first time, that thermomechanical pulping (TMP) wastewater is suitable for H₂ production via dark fermentation, with an optimum temperature of 70 °C. However, this experiment was conducted in batch, and long-term experiments in bioreactors operated in continuous mode are required to understand the potential of dark fermentation for treatment of TMP wastewater. Due to its high mass transfer properties, FBRs might be particularly suitable for dark fermentation of a complex substrate such as TMP wastewater. Furthermore, high organic loading rates should be explored in continuous mode to increase H₂ production rates for possible industrial applications.

Although suitable for H₂ production from TMP wastewater, dark fermentation also produces an effluent rich in VFAs and alcohols that can be further exploited to obtain value-added products, according to a biorefinery concept. Many different strategies can be applied for recovery of energy of other valuable products from dark fermentative effluents, including production of H₂ or methane, bioelectricity and recovery of short and/or long chain VFAs. All those strategies need to be tested and compared in laboratory-scale experiments in order to select the best strategy for possible full-scale applications.

In this study, it was shown that the combination of thermophilic conditions (55 °C) and alkaline inoculum pretreatment (pH 11, 24 hours) can enhance H₂ yields by repressing competitors and H₂ consuming microorganisms such as lactate producers and homoacetogens. The effect of the alkaline pretreatment in long-term bioreactor operation must be elucidated and the H₂ yields must be compared to those obtained from inocula pretreated by the more commonly applied heat shocks.

Although H₂ overproduction, i.e. H₂ yields higher than the yield stoichiometrically attributable to the volatile fatty acids (VFAs) produced, has been reported in dark fermentation (particularly under thermophilic conditions), the causes have not yet been elucidated. Further research is suggested to explain this phenomenon and to enable exploitation of these unusual dark fermentative pathways to achieve high H₂ yields.

Further research is required to make the power output of thermophilic MFCs comparable to that obtained under ambient or mesophilic conditions. An in-depth study of the microbial communities can help in developing strategies to improve power production in thermophilic MFCs. MFC start-up imposing a negative potential can be a promising strategy to select exoelectrogenic microorganisms. Further research is suggested to determine the optimal applied potential and exposure time.

In this study, the DNA and RNA-level microbial community analysis revealed that species of the *Thermodesulfobiaceae* family can be involved in electricity production under thermophilic conditions. Experiments with pure cultures of *Thermodesulfobiaceae*, e.g. *Coprothermobacter* sp., are suggested to confirm this hypothesis and to elucidate their electron transfer mechanism.

References

- Abbassi-Guendouz A, Trably E, Hamelin J, Dumas C, Steyer JP, Delgenès JP, Escudié R (2013) Microbial community signature of high-solid content methanogenic ecosystems. *Bioresour Technol* 133:256–262.
- Abreu AA, Karakashev D, Angelidaki I, Sousa DZ, Alves MM (2012) Biohydrogen production from arabinose and glucose using extreme thermophilic anaerobic mixed cultures. *Biotechnol Biofuels* 5:6.
- Al-Mamun A, Ahmad W, Baawain MS, Khadem M, Dhar BR (2018) A review of microbial desalination cell technology: Configurations, optimization and applications. *J Clean Prod* 183:458–480.
- Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. *J Mol Biol* 215:403–410.
- Angenent LT, Karim K, Al-Dahhan MH, Wrenn BA, Domínguez-Espinosa R (2004) Production of bioenergy and biochemicals from industrial and agricultural wastewater. *Trends Biotechnol* 22:477–485.
- APHA (1998) *Standard Methods for the Examination of Water and Wastewater*, twentieth ed. American Public Health Association/American Water Works Association/Water Environment Federation, Washington DC.
- Appels L, Baeyens J, Degève J, Dewil R (2008) Principles and potential of the anaerobic digestion of waste-activated sludge. *Prog Energy Combust Sci* 34:755–781.
- Arends JBA, Patil SA, Roume H, Rabaey K (2017) Continuous long-term electricity-driven bioproduction of carboxylates and isopropanol from CO₂ with a mixed microbial community. *J CO₂ Util* 20:141–149.
- Argun H, Kargi F (2009) Effects of sludge pre-treatment method on bio-hydrogen production by dark fermentation of waste ground wheat. *Int J Hydrogen Energy* 34:8543–8548.
- Arimi MM, Knodel J, Kiprof A, Namango SS, Zhang Y, Geißen S-U (2015) Strategies for improvement of biohydrogen production from organic-rich wastewater: A review. *Biomass and Bioenergy* 75:101–118.
- Baghchehsaraee B, Nakhla G, Karamanev D, Margaritis A, Reid G (2008) The effect of heat pretreatment temperature on fermentative hydrogen production using mixed cultures. *Int J Hydrogen Energy* 33:4064–4073.
- Bakonyi P, Borza B, Orlovits K, Simon V, Nemestóthy N, Bélafi-Bakó K (2014) Fermentative hydrogen production by conventionally and unconventionally heat pretreated seed cultures: A comparative assessment. *Int J Hydrogen Energy* 39:5589–5596.

- Bakonyi P, Buitrón G, Valdez-Vazquez I, Nemestóthy N, Bélafi-Bakó K (2017) A novel gas separation integrated membrane bioreactor to evaluate the impact of self-generated biogas recycling on continuous hydrogen fermentation. *Appl Energy* 190:813–823.
- Barca C, Soric A, Ranava D, Giudici-Ortoni M-T, Ferrasse J-H (2015) Anaerobic biofilm reactors for dark fermentative hydrogen production from wastewater: A review. *Bioresour Technol* 185:386–398.
- Barros AR, Cavalcante de Amorim EL, Reis CM, Shida GM, Silva EL (2010) Biohydrogen production in anaerobic fluidized bed reactors: Effect of support material and hydraulic retention time. *Int J Hydrogen Energy* 35:3379–3388.
- Barros AR, Silva EL (2012) Hydrogen and ethanol production in anaerobic fluidized bed reactors: Performance evaluation for three support materials under different operating conditions. *Biochem Eng J* 61:59–65.
- Batlle-Vilanova P, Ganigué R, Ramió-Pujol S, Bañeras L, Jiménez G, Hidalgo M, Balaguer MD, Colprim J, Puig S (2017) Microbial electrosynthesis of butyrate from carbon dioxide: Production and extraction. *Bioelectrochemistry* 117:57–64.
- Beckers L, Masset J, Hamilton C, Delvigne F, Toye D, Crine M, Thonart P, Hiligsmann S (2015) Investigation of the links between mass transfer conditions, dissolved hydrogen concentration and biohydrogen production by the pure strain *Clostridium butyricum* CWBI1009. *Biochem Eng J* 98:18–28.
- Boghani HC, Kim JR, Dinsdale RM, Guwy AJ, Premier GC (2013) Control of power sourced from a microbial fuel cell reduces its start-up time and increases bioelectrochemical activity. *Bioresour Technol* 140:277–285.
- Bonanni SP, Schrott GD, Busalmen JP (2012) A long way to the electrode: How do *Geobacter* cells transport their electrons? *Biochem Soc Trans* 40:1274–1279.
- Borole AP, Hamilton CY, Vishnivetskaya TA, Leak D, Andras C, Morrell-Falvey J, Keller M, Davison B (2009) Integrating engineering design improvements with exoelectrogen enrichment process to increase power output from microbial fuel cells. *J Power Sources* 191:520–527.
- Borole AP, Reguera G, Ringeisen B, Wang Z-W, Feng Y, Kim BH (2011) Electroactive biofilms: Current status and future research needs. *Energy Environ Sci* 4:4813–4834.
- Box GEP, Hunter WG, Hunter JS (1978) *Statistics for experimenters: An introduction to design, data analysis, and model building*. John Wiley and sons.
- Bundhoo MAZ, Mohee R (2016) Inhibition of dark fermentative bio-hydrogen production: A review. *Int J Hydrogen Energy* 41:6713–6733.
- Bundhoo MAZ, Mohee R, Hassan MA (2015) Effects of pre-treatment technologies on dark fermentative biohydrogen production: A review. *J Environ Manage* 157:20–48.

- Bundhoo ZMA (2017) Coupling dark fermentation with biochemical or bioelectrochemical systems for enhanced bio-energy production: A review. *Int J Hydrogen Energy* 42:26667–26686.
- Busalmen JP, Esteve-Nuñez A, Feliu JM (2008) Whole cell electrochemistry of electricity-producing microorganisms evidence an adaptation for optimal exocellular electron transport. *Environ Sci Technol* 42:2445–2450.
- Butti SK, Velvizhi G, Sulonen MLK, Haavisto JM, Oguz Koroglu E, Yusuf Cetinkaya A, Singh S, Arya D, Annie Modestra J, Vamsi Krishna K, Verma A, Ozkaya B, Lakaniemi A-M, Puhakka JA, Venkata Mohan S (2016) Microbial electrochemical technologies with the perspective of harnessing bioenergy: Maneuvering towards upscaling. *Renew Sustain Energy Rev* 53:462–476.
- Cai T, Park SY, Li Y (2013) Nutrient recovery from wastewater streams by microalgae: Status and prospects. *Renew Sustain Energy Rev* 19:360–369.
- Cao G-L, Zhao L, Wang A-J, Wang Z-Y, Ren N-Q (2014) Single-step bioconversion of lignocellulose to hydrogen using novel moderately thermophilic bacteria. *Biotechnol Biofuels* 7:82.
- Caporaso JG, Lauber CL, Walters WA, Berg-Lyons D, Lozupone CA, Turnbaugh PJ, Fierer N, Knight R (2011) Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. *Proc Natl Acad Sci U S A* 108:4516–22.
- Carmona-Martínez AA, Harnisch F, Kuhlicke U, Neu TR, Schröder U (2013) Electron transfer and biofilm formation of *Shewanella putrefaciens* as function of anode potential. *Bioelectrochemistry* 93:23–29.
- Carver SM, Vuoriranta P, Tuovinen OH (2011) A thermophilic microbial fuel cell design. *J Power Sources* 196:3757–3760.
- Cavalcante de Amorim EL, Barros AR, Rissato Zamariolli Damianovic MH, Silva EL (2009) Anaerobic fluidized bed reactor with expanded clay as support for hydrogen production through dark fermentation of glucose. *Int J Hydrogen Energy* 34:783–790.
- Cavinato C, Giuliano A, Bolzonella D, Pavan P, Cecchi F (2012) Bio-hythane production from food waste by dark fermentation coupled with anaerobic digestion process: A long-term pilot scale experience. *Int J Hydrogen Energy* 37:11549–11555.
- Cetinkaya AY, Ozdemir OK, Koroglu EO, Hasimoglu A, Ozkaya B (2015) The development of catalytic performance by coating Pt-Ni on CMI7000 membrane as a cathode of a microbial fuel cell. *Bioresour Technol* 195:188–193.
- Chabert N, Amin Ali O, Achouak W (2015) All ecosystems potentially host electrogenic bacteria. *Bioelectrochemistry* 106:88–96.
- Chae K-J, Choi M-J, Kim K-Y, Ajayi FF, Park W, Kim C-W, Kim IS (2010a) Methanogenesis control by employing various environmental stress conditions in two-chambered microbial fuel cells. *Bioresour Technol* 101:5350–5357.

- Chae K-J, Choi M-J, Kim K-Y, Ajayi FF, Park W, Kim C-W, Kim IS (2010b) Methanogenesis control by employing various environmental stress conditions in two-chambered microbial fuel cells. *Bioresour Technol* 101:5350–5357.
- Chae K-J, Choi M-J, Lee J-W, Kim K-Y, Kim IS (2009) Effect of different substrates on the performance, bacterial diversity, and bacterial viability in microbial fuel cells. *Bioresour Technol* 100:3518–3525.
- Chaganti SR, Kim D-H, Lalman JA (2012) Dark fermentative hydrogen production by mixed anaerobic cultures: Effect of inoculum treatment methods on hydrogen yield. *Renew Energy* 48:117–121.
- Chang J-S, Lee K-S, Lin P-J (2002) Biohydrogen production with fixed-bed bioreactors. *Int J Hydrogen Energy* 27:1167–1174.
- Chang S, Li J-Z, Liu F (2011) Evaluation of different pretreatment methods for preparing hydrogen-producing seed inocula from waste activated sludge. *Renew Energy* 36:1517–1522.
- Chatellard L, Trably E, Carrère H (2016) The type of carbohydrates specifically selects microbial community structures and fermentation patterns. *Bioresour Technol* 221:541–549.
- Chen Y-M, Wang C-T, Yang Y-C, Chen W-J (2013) Application of aluminum-alloy mesh composite carbon cloth for the design of anode/cathode electrodes in *Escherichia coli* microbial fuel cell. *Int J Hydrogen Energy* 38:11131–11137.
- Cheng S, Logan BE (2007) Ammonia treatment of carbon cloth anodes to enhance power generation of microbial fuel cells. *Electrochem Commun* 9:492–496.
- Cheng SS, Chao YC, Yang KH, Bai M Der (2011) Process recovery of biohydrogenation in a pilot plant from methanogens invasion. *Int J Hydrogen Energy* 36:8779–8784.
- Cheong D-Y, Hansen CL (2006) Bacterial stress enrichment enhances anaerobic hydrogen production in cattle manure sludge. *Appl Microbiol Biotechnol* 72:635–643.
- Cherubini F (2010) The biorefinery concept: Using biomass instead of oil for producing energy and chemicals. *Energy Convers Manag* 51:1412–1421.
- Chung K, Okabe S (2009) Continuous power generation and microbial community structure of the anode biofilms in a three-stage microbial fuel cell system. *Appl Microbiol Biotechnol* 83:965–977.
- Ciranna A, Pawar SS, Santala V, Karp M, Van Niel EWJ (2014) Assessment of metabolic flux distribution in the thermophilic hydrogen producer *Caloramator celer* as affected by external pH and hydrogen partial pressure. *Microb Cell Fact* 13:48.
- Cisneros-Pérez C, Etchebehere C, Celis LB, Carrillo-Reyes J, Alatríste-Mondragón F, Razo-Flores E (2017) Effect of inoculum pretreatment on the microbial community structure and its performance during dark fermentation using anaerobic fluidized-

- bed reactors. *Int J Hydrogen Energy* 42:9589–9599.
- Collins MD, Lawson PA, Willems A, Cordoba JJ, Fernandez-Garayzabal J, Garcia P, Cai J, Hippe H, Farrow JAE (1994) The phylogeny of the genus *Clostridium*: Proposal of five new genera and eleven new species combinations. *Int J Syst Bacteriol* 44:812–826.
- Commault AS, Lear G, Packer MA, Weld RJ (2013) Influence of anode potentials on selection of *Geobacter* strains in microbial electrolysis cells. *Bioresour Technol* 139:226–234.
- Coronado J, Perrier M, Tartakovsky B (2013) Pulse-width modulated external resistance increases the microbial fuel cell power output. *Bioresour Technol* 147:65–70.
- Crabtree GW, Dresselhaus MS, Buchanan M V (2004) The hydrogen economy. *Phys Today* December:39–45.
- D'Ippolito G, Dipasquale L, Vella FM, Romano I, Gambacorta A, Cutignano A, Fontana A (2010) Hydrogen metabolism in the extreme thermophile *Thermotoga neapolitana*. *Int J Hydrogen Energy* 35:2290–2295.
- Das P, Tiwari P (2018) Valorization of packaging plastic waste by slow pyrolysis. *Resour Conserv Recycl* 128:69–77.
- De Vrieze J, Christiaens MER, Walraedt D, Devooght A, Ijaz UZ, Boon N (2017) Microbial community redundancy in anaerobic digestion drives process recovery after salinity exposure. *Water Res* 111:109–117.
- Demirel B, Yenigun O, Onay TT (2005) Anaerobic treatment of dairy wastewaters: A review. *Process Biochem* 40:2583–2595.
- Dincer I (2012) Green methods for hydrogen production. *Int J Hydrogen Energy* 37:1954–1971.
- Dong L, Zhenhong Y, Yongming S, Longlong M (2010) Evaluation of pretreatment methods on harvesting hydrogen producing seeds from anaerobic digested organic fraction of municipal solid waste (OFMSW). *Int J Hydrogen Energy* 35:8234–8240.
- Dos Reis CM, Silva EL (2011) Effect of upflow velocity and hydraulic retention time in anaerobic fluidized-bed reactors used for hydrogen production. *Chem Eng J* 172:28–36.
- Drake HL, Küsel K, Matthies C (2006) Acetogenic Prokaryotes. In: Springer (ed) *The Prokaryotes*. New York, pp 354–420.
- Du Z, Li H, Gu T (2007) A state of the art review on microbial fuel cells: A promising technology for wastewater treatment and bioenergy. *Biotechnol Adv* 25:464–482.
- Dubois M, Gilles K, Hamilton JK, Rebers P, Smith F (1956) Colorimetric method for determination of sugars and related substances. *Anal Chem* 28:350–356.

- Duppeti H, Chakraborty S, Das BS, Mallick N, Kotamreddy JNR (2017) Rapid assessment of algal biomass and pigment contents using diffuse reflectance spectroscopy and chemometrics. *Algal Res* 27:274–285.
- Ekstrand E-M, Larsson M, Truong X-B, Cardell L, Borgström Y, Björn A, Ejlertsson J, Svensson BH, Nilsson F, Karlsson A (2013) Methane potentials of the Swedish pulp and paper industry – A screening of wastewater effluents. *Appl Energy* 112:507–517.
- Elbeshbishy E, Hafez H, Dhar BR, Nakhla G (2011) Single and combined effect of various pretreatment methods for biohydrogen production from food waste. *Int J Hydrogen Energy* 36:11379–11387.
- Elbeshbishy E, Hafez H, Nakhla G (2010) Enhancement of biohydrogen producing using ultrasonication. *Int J Hydrogen Energy* 35:6184–6193.
- Etchebehere C, Castelló E, Wenzel J, Del Pilar Anzola-Rojas M, Borzacconi L, Buitrón G, Cabrol L, Carminato VM, Carrillo-Reyes J, Cisneros-Pérez C, Fuentes L, Moreno-Andrade I, Razo-Flores E, Filippi GR, Tapia-Venegas E, Toledo-Alarcón J, Zaiat M (2016) Microbial communities from 20 different hydrogen-producing reactors studied by 454 pyrosequencing. *Appl Microbiol Biotechnol* 100:3371–3384.
- European Commission (2014) A policy framework for climate and energy in the period from 2020 to 2030. <https://ec.europa.eu/energy/en/topics/energy-strategy/2030-energy-strategy> Brussels.
- Fazal T, Mushtaq A, Rehman F, Ullah Khan A, Rashid N, Farooq W, Rehman MSU, Xu J (2018) Bioremediation of textile wastewater and successive biodiesel production using microalgae. *Renew Sustain Energy Rev* 82:3107–3126.
- Fu Q, Fukushima N, Maeda H, Sato K, Kobayashi H (2015) Bioelectrochemical analysis of a hyperthermophilic microbial fuel cell generating electricity at temperatures above 80 °C. *Biosci Biotechnol Biochem* 79:1200–1206.
- Fu Q, Kobayashi H, Kawaguchi H, Vilcaez J, Wakayama T, Maeda H, Sato K (2013a) Electrochemical and phylogenetic analyses of current-generating microorganisms in a thermophilic microbial fuel cell. *J Biosci Bioeng* 115:268–271.
- Fu Q, Kobayashi H, Kawaguchi H, Wakayama T, Maeda H, Sato K (2013b) A thermophilic gram-negative nitrate-reducing bacterium, *Calditerrivibrio nitroreducens*, exhibiting electricity generation capability. *Environ Sci Technol* 47:12583–12590.
- Fujita R, Mochida K, Kato Y, Goto K (2010) *Sporolactobacillus putidus* sp. nov., an endospore-forming lactic acid bacterium isolated from spoiled orange juice. *Int J Syst Evol Microbiol* 60:1499–1503.
- Gadow SI, Jiang H, Hojo T, Li Y-Y (2013) Cellulosic hydrogen production and microbial community characterization in hyper-thermophilic continuous bioreactor. *Int J Hydrogen Energy* 38:7259–7267.

- Gadow SI, Li Y-Y, Liu Y (2012) Effect of temperature on continuous hydrogen production of cellulose. *Int J Hydrogen Energy* 37:15465–15472.
- Gagliano MC, Braguglia CM, Petruccioli M, Rossetti S (2015) Ecology and biotechnological potential of the thermophilic fermentative *Coprothermobacter* spp. *FEMS Microbiol Ecol* 91:5.
- Galperin MY (2013) Genome Diversity of Spore-Forming *Firmicutes*. *Microbiol Spectr* 1:1–27.
- Gao C, Wang A, Wu W-M, Yin Y, Zhao Y-G (2014) Enrichment of anodic biofilm inoculated with anaerobic or aerobic sludge in single chambered air-cathode microbial fuel cells. *Bioresour Technol* 167:124–132.
- Garcia-Nunez JA, Rodriguez DT, Fontanilla CA, Ramirez NE, Silva Lora EE, Frear CS, Stockle C, Amonette J, Garcia-Perez M (2016) Evaluation of alternatives for the evolution of palm oil mills into biorefineries. *Biomass and Bioenergy* 95:310–329.
- Geppert F, Liu D, van Eerten-Jansen M, Weidner E, Buisman C, Ter Heijne A (2016) Bioelectrochemical power-to-gas: State of the art and future perspectives. *Trends Biotechnol* 34:879–894.
- Griffiths RI, Whiteley AS, O'Donnell AG, Bailey MJ (2000) Rapid method for coextraction of DNA and RNA from natural environments for analysis of ribosomal DNA- and rRNA-based microbial community composition. *Appl Environ Microbiol* 66:5488–5491.
- Grupe H, Gottschalk G (1992) Physiological events in *Clostridium acetobutylicum* during the shift from acidogenesis to solventogenesis in continuous culture and presentation of a model for shift induction. *Appl Environ Microbiol* 58:3896–3902.
- Guo L, Lu M, Li Q, Zhang J, She Z (2015) A comparison of different pretreatments on hydrogen fermentation from waste sludge by fluorescence excitation-emission matrix with regional integration analysis. *Int J Hydrogen Energy* 40:197–208.
- Guwy AJ, Dinsdale RM, Kim JR, Massanet-Nicolau J, Premier G (2011) Fermentative biohydrogen production systems integration. *Bioresour Technol* 102:8534–8542.
- Ha PT, Lee TK, Rittmann BE, Park J, Chang IS (2012) Treatment of alcohol distillery wastewater using a bacteroidetes-dominant thermophilic microbial fuel cell. *Environ Sci Technol* 46:3022–3030.
- Haavisto JM, Kokko ME, Lay C-H, Puhakka JA (2017) Effect of hydraulic retention time on continuous electricity production from xylose in up-flow microbial fuel cell. *Int J Hydrogen Energy* 42:27494–27501.
- Haddadi S, Nabi-Bidhendi G-R, Mehrdadi N (2014) Evaluation of inoculation method and limiting conditions on bacterial activity in microbial electrochemical cells. *J Environ Chem Eng* 2:612–618.
- Hall T (1999) BioEdit: A user-friendly biological sequence alignment editor and analysis

- program for Windows 95/98/NT. Nucleic Acids Symp. Ser. 41:95–98.
- Hassan Khan MB, Gueguim Kana EB (2016) Design, implementation and assessment of a novel bioreactor for fermentative biohydrogen process development. *Int J Hydrogen Energy* 41:10136–10144.
- Hasyim R, Imai T, Reungsang A, O-Thong S (2011) Extreme-thermophilic biohydrogen production by an anaerobic heat treated digested sewage sludge culture. *Int J Hydrogen Energy* 36:8727–8734.
- Hayashi NR, Ishida T, Yokota A, Kodama T, Igarashi Y (1999) *Hydrogenophilus thermoluteolus* gen. nov., sp. nov., a thermophilic, facultatively chemolithoautotrophic, hydrogen-oxidizing bacterium. *Int J Syst Bacteriol* 49:783–786.
- Hernández-Fernández FJ, Pérez De Los Ríos A, Salar-García MJ, Ortiz-Martínez VM, Lozano-Blanco LJ, Godínez C, Tomás-Alonso F, Quesada-Medina J (2015) Recent progress and perspectives in microbial fuel cells for bioenergy generation and wastewater treatment. *Fuel Process Technol* 138:284–297.
- Hilgsmann S, Beckers L, Masset J, Hamilton C, Thonart P (2014) Improvement of fermentative biohydrogen production by *Clostridium butyricum* CWBI1009 in sequenced-batch, horizontal fixed bed and biodisc-like anaerobic reactors with biomass retention. *Int J Hydrogen Energy* 39:6899–6911.
- Hilgsmann S, Masset J, Hamilton C, Beckers L, Thonart P (2011) Comparative study of biological hydrogen production by pure strains and consortia of facultative and strict anaerobic bacteria. *Bioresour Technol* 102:3810–3818.
- Holladay JD, Hu J, King DL, Wang Y (2009) An overview of hydrogen production technologies. *Catal Today* 139:244–260.
- Hu B, Chen S (2007) Pretreatment of methanogenic granules for immobilized hydrogen fermentation. *Int J Hydrogen Energy* 32:3266–3273.
- Huang L, Logan BE (2008) Electricity production from xylose in fed-batch and continuous-flow microbial fuel cells. *Appl Microbiol Biotechnol* 80:655–664.
- Hussain A, Mehta P, Raghavan V, Wang H, Guiot SR, Tartakovsky B (2012) The performance of a thermophilic microbial fuel cell fed with synthesis gas. *Enzyme Microb Technol* 51:163–170.
- International Energy Agency (2017) Energy Statistics.
- IPCC (2014) Climate change 2014: Synthesis report. Contribution of working groups I, II and III to the fifth assessment report of the intergovernmental panel on climate change [Core writing team, R.K. Pachauri and L.A. Meyer (eds.)]. IPCC, Geneva, Switzerland, 151 pp.
- Islam S, Zhang C, Sui K, Guo C, Liu C (2016) Coproduction of hydrogen and volatile fatty acid via thermophilic fermentation of sweet sorghum stalk from co-culture of

- Clostridium thermocellum* and *Clostridium thermosaccharolyticum*. Int J Hydrogen Energy 42:830–837.
- Ivanova G, Rákhely G, Kovács KL (2009) Thermophilic biohydrogen production from energy plants by *Caldicellulosiruptor saccharolyticus* and comparison with related studies. Int J Hydrogen Energy 34:3659–3670.
- Jeong D-Y, Cho S-K, Shin H-S, Jung K-W (2013) Application of an electric field for pretreatment of a seeding source for dark fermentative hydrogen production. Bioresour Technol 139:393–396.
- Jiang Q, Xing D, Sun R, Zhang L, Feng Y, Ren N (2016) Anode biofilm communities and the performance of microbial fuel cells with different reactor configurations. RSC Adv 6:85149–85155.
- Jong BC, Kim BH, Chang IS, Liew PWY, Choo YF, Kang GS (2006) Enrichment, performance, and microbial diversity of a thermophilic mediatorless microbial fuel cell. Environ Sci Technol 40:6449–6454.
- Kamali M, Khodaparast Z (2015) Review on recent developments on pulp and paper mill wastewater treatment. Ecotoxicol Environ Saf 114:326–342.
- Kanai T, Imanaka H, Nakajima A, Uwamori K, Omori Y, Fukui T, Atomi H, Imanaka T (2005) Continuous hydrogen production by the hyperthermophilic archaeon, *Thermococcus kodakaraensis* KOD1. J Biotechnol 116:271–282.
- Karadag D, Puhakka JA (2010) Effect of changing temperature on anaerobic hydrogen production and microbial community composition in an open-mixed culture bioreactor. Int J Hydrogen Energy 35:10954–10959.
- Kargi F, Eren NS, Ozmihci S (2012) Bio-hydrogen production from cheese whey powder (CWP) solution: Comparison of thermophilic and mesophilic dark fermentations. Int J Hydrogen Energy 37:8338–8342.
- Karthikeyan R, Wang B, Xuan J, Wong JWC, Lee PKH, Leung MKH (2015) Interfacial electron transfer and bioelectrocatalysis of carbonized plant material as effective anode of microbial fuel cell. Electrochim Acta 157:314–323.
- Kaur A, Boghani HC, Michie I, Dinsdale RM, Guwy AJ, Premier GC (2014) Inhibition of methane production in microbial fuel cells: Operating strategies which select electrogens over methanogens. Bioresour Technol 173:75–81.
- Kelly PT, He Z (2014) Nutrients removal and recovery in bioelectrochemical systems: A review. Bioresour Technol 153:351–360.
- Keskin T, Aksöyek E, Azbar N (2011) Comparative analysis of thermophilic immobilized biohydrogen production using packed materials of ceramic ring and pumice stone. Int J Hydrogen Energy 36:15160–15167.
- Keskin T, Giusti L, Azbar N (2012) Continuous biohydrogen production in immobilized biofilm system versus suspended cell culture. Int J Hydrogen Energy 37:1418–1424.

- Khamtib S, Reungsang A (2012) Biohydrogen production from xylose by *Thermoanaerobacterium thermosaccharolyticum* KKU19 isolated from hot spring sediment. *Int J Hydrogen Energy* 37:12219–12228.
- Khongkliang P, Kongjan P, Utarapichat B, Reungsang A, O-Thong S (2017) Continuous hydrogen production from cassava starch processing wastewater by two-stage thermophilic dark fermentation and microbial electrolysis. *Int J Hydrogen Energy* 42:27584–27592.
- Kiely PD, Regan JM, Logan BE (2011) The electric picnic: Synergistic requirements for exoelectrogenic microbial communities. *Curr Opin Biotechnol* 22:378–385.
- Kim D-H, Han S-K, Kim S-H, Shin H-S (2006) Effect of gas sparging on continuous fermentative hydrogen production. *Int J Hydrogen Energy* 31:2158–2169.
- Kim D-H, Jang S, Yun Y-M, Lee M-K, Moon C, Kang W-S, Kwak S-S, Kim M-S (2014) Effect of acid-pretreatment on hydrogen fermentation of food waste: Microbial community analysis by next generation sequencing. *Int J Hydrogen Energy* 39:16302–16309.
- Kim JR, Cheng S, Oh S-E, Logan BE (2007) Power generation using different cation, anion, and ultrafiltration membranes in microbial fuel cells. *Environ Sci Technol* 41:1004–1009.
- Kim JR, Premier GC, Hawkes FR, Dinsdale RM, Guwy AJ (2009) Development of a tubular microbial fuel cell (MFC) employing a membrane electrode assembly cathode. *J Power Sources* 187:393–399.
- Kim JR, Premier GC, Hawkes FR, Rodríguez J, Dinsdale RM, Guwy AJ (2010) Modular tubular microbial fuel cells for energy recovery during sucrose wastewater treatment at low organic loading rate. *Bioresour Technol* 101:1190–1198.
- Kinnunen V, Ylä-Outinen A, Rintala J (2015) Mesophilic anaerobic digestion of pulp and paper industry biosludge—long-term reactor performance and effects of thermal pretreatment. *Water Res* 87:105–111.
- Kongjan P, Min B, Angelidaki I (2009) Biohydrogen production from xylose at extreme thermophilic temperatures (70 °C) by mixed culture fermentation. *Water Res* 43:1414–1424.
- Koroglu EO, Cetinkaya AY, Ozkaya B, Demir A (2016) Simultaneous production of bioelectricity and treatment of membrane concentrate in multitube microbial fuel cell. *J Biosci Bioeng* 122:594–600.
- Koskinen PEP, Kaksonen AH, Puhakka JA (2006) The relationship between instability of H₂ production and compositions of bacterial communities within a dark fermentation fluidized-bed bioreactor. *Biotechnol Bioeng* 97:742–758.
- Koskinen PEP, Lay C-H, Puhakka JA, Lin P-J, Wu S-Y, Örlygsson J, Lin C-Y (2008) High-efficiency hydrogen production by an anaerobic, thermophilic enrichment culture from an icelandic hot spring. *Biotechnol Bioeng* 101:665–678.

- Kotsopoulos TA, Zeng RJ, Angelidaki I (2006) Biohydrogen production in granular up-flow anaerobic sludge blanket (UASB) reactors with mixed cultures under hyperthermophilic temperature (70°C). *Biotechnol Bioeng* 94:296–302.
- Kozich JJ, Westcott SL, Baxter NT, Highlander SK, Schloss PD (2013) Development of a dual-index sequencing strategy and curation pipeline for analyzing amplicon sequence data on the Miseq illumina sequencing platform. *Appl Environ Microbiol* 79:5112–5120.
- Kumar A, Hsu LH-H, Kavanagh P, Barrière F, Lens PNL, Lapinsonnière L, Lienhard V JH, Schröder U, Jiang X, Leech D (2017a) The ins and outs of microorganism–electrode electron transfer reactions. *Nat Rev Chem* 1:24.
- Kumar G, Sivagurunathan P, Sen B, Mudhoo A, Davila-Vazquez G, Wang G, Kim S-H (2017b) Research and development perspectives of lignocellulose-based biohydrogen production. *Int Biodeterior Biodegradation* 119:225–238.
- Kumar M, Sundaram S, Gnansounou E, Larroche C, Thakur IS (2018) Carbon dioxide capture, storage and production of biofuel and biomaterials by bacteria: A review. *Bioresour Technol* 247:1059–1068.
- Kumar R, Singh L, Wahid ZA, Din MFM (2015) Exoelectrogens in microbial fuel cells toward bioelectricity generation: A review. *Int J energy Res* 39:1048–1067.
- Kumar R, Singh L, Zularisam AW (2016) Exoelectrogens: Recent advances in molecular drivers involved in extracellular electron transfer and strategies used to improve it for microbial fuel cell applications. *Renew Sustain Energy Rev* 56:1322–1336.
- Laurinavichene T, Tekucheva D, Laurinavichius K, Tsygankov A (2018) Utilization of distillery wastewater for hydrogen production in one-stage and two-stage processes involving photofermentation. *Enzyme Microb Technol* 110:1–7.
- Lawson Anani Soh A, Ralambotiana H, Ollivier B, Prensier G, Tine E, Garcia J-L (1991) *Clostridium thermopalmarium* sp. nov., a moderately thermophilic butyrate-producing bacterium isolated from palm wine in Senegal. *Syst Appl Microbiol* 14:135–139.
- Lee D-J, Show K-Y, Su A (2011) Dark fermentation on biohydrogen production: Pure culture. *Bioresour Technol* 102:8393–8402.
- Lee K-S, Tseng T-S, Liu Y-W, Hsiao Y-D (2012) Enhancing the performance of dark fermentative hydrogen production using a reduced pressure fermentation strategy. *Int J Hydrogen Energy* 37:15556–15562.
- Lee Y-W, Chung J (2010) Bioproduction of hydrogen from food waste by pilot-scale combined hydrogen/methane fermentation. *Int J Hydrogen Energy* 35:11746–11755.
- Lesnik KL, Liu H (2014) Establishing a core microbiome in acetate-fed microbial fuel cells. *Appl Microbiol Biotechnol* 98:4187–4196.

- Li C, Fang HHP (2007) Fermentative hydrogen production from wastewater and solid wastes by mixed cultures. *Crit Rev Environ Sci Technol* 37:1–39.
- Li W-W, Sheng G-P, Liu X-W, Yu H-Q (2011) Recent advances in the separators for microbial fuel cells. *Bioresour Technol* 102:244–252.
- Li Y, Li H (2014) Type IV pili of *Acidithiobacillus ferrooxidans* can transfer electrons from extracellular electron donors. *J Basic Microbiol* 54:226–231.
- Lin C-N, Wu S-Y, Chang J-S (2006) Fermentative hydrogen production with a draft tube fluidized bed reactor containing silicone-gel-immobilized anaerobic sludge. *Int J Hydrogen Energy* 31:2200–2210.
- Lin P-J, Chang J-S, Yang L-H, Lin C-Y, Wu S-Y, Lee K-S (2011) Enhancing the performance of pilot-scale fermentative hydrogen production by proper combinations of HRT and substrate concentration. *Int J Hydrogen Energy* 36:14289–14294.
- Liobikienė G, Butkus M (2017) The European Union possibilities to achieve targets of Europe 2020 and Paris agreement climate policy. *Renew Energy* 106:298–309.
- Liu H, Logan BE (2004) Electricity generation using an air-cathode single chamber microbial fuel cell in the presence and absence of a proton exchange membrane. *Environ Sci Technol* 38:4040–4046.
- Liu H, Wang G, Zhu D, Pan G (2009) Enrichment of the hydrogen-producing microbial community from marine intertidal sludge by different pretreatment methods. *Int J Hydrogen Energy* 34:9696–9701.
- Liu X-W, Li W-W, Yu H-Q (2014) Cathodic catalysts in bioelectrochemical systems for energy recovery from wastewater. *Chem Soc Rev* 43:7718–7745.
- Lobato J, Cañizares P, Fernández FJ, Rodrigo MA (2012) An evaluation of aerobic and anaerobic sludges as start-up material for microbial fuel cell systems. *N Biotechnol* 29:415–420.
- Logan BE (2009) Exoelectrogenic bacteria that power microbial fuel cells. *Nat Rev Microbiol* 7:375–381.
- Logan BE, Hamelers B, Rozendal R, Schröder U, Keller J, Freguia S, Aelterman P, Verstraete W, Rabaey K (2006) Microbial fuel cells: Methodology and technology. *Environ Sci Technol* 40:5181–5192.
- Logan BE, Oh S-E, Kim IS, Van Ginkel S (2002) Biological hydrogen production measured in batch anaerobic respirometers. *Environ Sci Technol* 36:2530–2535.
- Lu L, Ren ZJ (2016) Microbial electrolysis cells for waste biorefinery: A state of the art review. *Bioresour Technol* 215:254–264.
- Lu M, Chen S, Babanova S, Phadke S, Salvacion M, Mirhosseini A, Chan S, Carpenter K, Cortese R, Bretschger O (2017) Long-term performance of a 20-L continuous

- flow microbial fuel cell for treatment of brewery wastewater. *J Power Sources* 356:274–287.
- Lu M, Fong S, Li Y (2012) Cathode reactions and applications in microbial fuel cells: A review. *Crit Rev Environ Sci Technol* 42:2504–2525.
- Luo G, Karakashev D, Xie L, Zhou Q, Angelidaki I (2011) Long-term effect of inoculum pretreatment on fermentative hydrogen production by repeated batch cultivations: Homoacetogenesis and methanogenesis as competitors to hydrogen production. *Biotechnol Bioeng* 108:1816–1827.
- Luo G, Xie L, Zou Z, Wang W, Zhou Q (2010) Evaluation of pretreatment methods on mixed inoculum for both batch and continuous thermophilic biohydrogen production from cassava stillage. *Bioresour Technol* 101:959–964.
- Lusk BG, Khan QF, Parameswaran P, Hameed A, Ali N, Rittmann BE, Torres CI (2015) Characterization of electrical current-generation capabilities from thermophilic bacterium *Thermoanaerobacter pseudethanolicus* using xylose, glucose, cellobiose, or acetate with fixed anode potentials. *Environ Sci Technol* 49:14725–14731.
- Lutpi NA, Md Jahim J, Mumtaz T, Harun S, Abdul PM (2016) Batch and continuous thermophilic hydrogen fermentation of sucrose using anaerobic sludge from palm oil mill effluent via immobilisation technique. *Process Biochem* 51:297–307.
- Machani M, Nourelfath M, D'Amours S (2014) A mathematically-based framework for evaluating the technical and economic potential of integrating bioenergy production within pulp and paper mills. *Biomass and Bioenergy* 63:126–139.
- Makarova KS, Slesarev A, Wolf Y, Sorokin A, Mirkin B, Koonin E V., Pavlov A, Pavlova N, Karamychev V, Polouchine N, Shakhova V, Grigoriev I, Lou Y, Rohksar D, Lucas S, Huang K, Goodstein DM, Hawkins T, Plengvidhya V, Welker DL, Hughes JE, Goh Y, Benson A, Baldwin KA, Lee J-H, Díaz-Muñoz I, Dosti B, Smeianov V V., Wechter W, Barabote R, Lorca GL, Altermann E, Barrangou R, Ganesan B, Xie Y, Rawsthorne H, Tamir D, Parker C, Breidt F, Broadbent JR, Hutkins R, O'Sullivan D, Steele JL, Unlu G, Saier MH, Klaenhammer TR, Richardson P, Kozyavkin S, Weimer BC, Mills DA (2006) Comparative genomics of the lactic acid bacteria. *Proc Natl Acad Sci USA* 103:15611–15616.
- Mäkinen AE, Lay C-H, Nissilä ME, Puhakka J a. (2013) Bioelectricity production on xylose with a compost enrichment culture. *Int J Hydrogen Energy* 38:15606–15612.
- Mäkinen AE, Nissilä ME, Puhakka JA (2012) Dark fermentative hydrogen production from xylose by a hot spring enrichment culture. *Int J Hydrogen Energy* 37:12234–12240.
- Mapelli C, Mapelli V, Olsson L, Mombelli D, Gruttadauria A, Barella S (2013) Viability study of the use of cast iron open cell foam as microbial fuel cell electrodes. *Adv Eng Mater* 15:112–117.
- Marshall CW, May HD (2009) Electrochemical evidence of direct electrode reduction by a thermophilic Gram-positive bacterium, *Thermincola ferriacetica*. *Energy Environ*

Sci 2:699–705.

- Masi F, Rizzo A, Regelsberger M (2018) The role of constructed wetlands in a new circular economy, resource oriented, and ecosystem services paradigm. *J Environ Manage* 216:275–284.
- Masset J, Hiligsmann S, Hamilton C, Beckers L, Franck F, Thonart P (2010) Effect of pH on glucose and starch fermentation in batch and sequenced-batch mode with a recently isolated strain of hydrogen-producing *Clostridium butyricum* CWBI1009. *Int J Hydrogen Energy* 35:3371–3378.
- Mathis BJ, Marshall CW, Milliken CE, Makkar RS, Creager HD, May HD (2008) Electricity generation by thermophilic microorganisms from marine sediment. *Appl Microbiol Biotechnol* 78:147–155.
- Mehdinia A, Dejaloud M, Jabbari A (2013) Nanostructured polyaniline-coated anode for improving microbial fuel cell power output. *Chem Pap* 67:1096–1102.
- Meruane G, Vargas T (2003) Bacterial oxidation of ferrous iron by *Acidithiobacillus ferrooxidans* in the pH range 2.5–7.0. *Hydrometallurgy* 71:149–158.
- Meyer T, Edwards EA (2014) Anaerobic digestion of pulp and paper mill wastewater and sludge. *Water Res* 65:321–349.
- Miceli JF, Parameswaran P, Kang D-W, Krajmalnik-Brown R, Torres CI (2012) Enrichment and analysis of anode-respiring bacteria from diverse anaerobic inocula. *Environ Sci Technol* 46:10349–10355.
- Miskan M, Ismail M, Ghasemi M, Md Jahim J, Nordin D, Abu Bakar MH (2016) Characterization of membrane biofouling and its effect on the performance of microbial fuel cell. *Int J Hydrogen Energy* 41:543–552.
- Misturini Rossi D, Berne da Costa J, Aquino de Souza E, Ruaro Peralba M do C, Samios D, Záchia Ayub MA (2011) Comparison of different pretreatment methods for hydrogen production using environmental microbial consortia on residual glycerol from biodiesel. *Int J Hydrogen Energy* 36:4814–4819.
- Modestra JA, Mohan SV (2017) Microbial electrosynthesis of carboxylic acids through CO₂ reduction with selectively enriched biocatalyst: Microbial dynamics. *J CO₂ Util.* 20:190–199.
- Mohammadi P, Ibrahim S, Annuar MSM (2012) Comparative study on the effect of various pretreatment methods on the enrichment of hydrogen producing bacteria in anaerobic granulated sludge from brewery wastewater. *Korean J Chem Eng* 29:1347–1351.
- Mohd Yusoff MZ, Hu A, Feng C, Maeda T, Shirai Y, Hassan MA, Yu CP (2013) Influence of pretreated activated sludge for electricity generation in microbial fuel cell application. *Bioresour Technol* 145:90–96.
- Motte J-C, Sambusiti C, Dumas C, Barakat A (2015) Combination of dry dark

- fermentation and mechanical pretreatment for lignocellulosic deconstruction: An innovative strategy for biofuels and volatile fatty acids recovery. *Appl Energy* 147:67–73.
- Mu Y, Yu H-Q, Wang G (2007) Evaluation of three methods for enriching H₂-producing cultures from anaerobic sludge. *Enzyme Microb Technol* 40:947–953.
- Muñoz-Páez KM, Ruiz-Ordáz N, García-Mena J, Ponce-Noyola MT, Ramos-Valdivia AC, Robles-González IV, Villa-Tanaca L, Barrera-Cortés J, Rinderknecht-Seijas N, Poggi-Varaldo HM (2013) Comparison of biohydrogen production in fluidized bed bioreactors at room temperature and 35 °C. *Int J Hydrogen Energy* 38:12570–12579.
- Nancharaiah Y V., Venkata Mohan S, Lens PNL (2015) Metals removal and recovery in bioelectrochemical systems: A review. *Bioresour Technol* 195:102–114.
- Nguyen T-AD, Han SJ, Kim JP, Kim MS, Sim SJ (2010) Hydrogen production of the hyperthermophilic eubacterium, *Thermotoga neapolitana* under N₂ sparging condition. *Bioresour Technol* 101:S38–S41.
- Nikolaidis P, Poullikkas A (2017) A comparative overview of hydrogen production processes. *Renew Sustain Energy Rev* 67:597–611.
- Nissilä ME, Tähti HP, Rintala JA, Puhakka JA (2011) Thermophilic hydrogen production from cellulose with rumen fluid enrichment cultures: Effects of different heat treatments. *Int J Hydrogen Energy* 36:1482–1490.
- Nizami AS, Rehan M, Waqas M, Naqvi M, Ouda OKM, Shahzad K, Miandad R, Khan MZ, Syamsiro M, Ismail IMI, Pant D (2017) Waste biorefineries: Enabling circular economies in developing countries. *Bioresour Technol* 241:1101–1117.
- Noike T, Takabatake H, Mizuno O, Ohba M (2002) Inhibition of hydrogen fermentation of organic wastes by lactic acid bacteria. *Int J Hydrogen Energy* 27:1367–1371.
- Ogg CD, Patel BKC (2009) *Caloramator australicus* sp. nov., a thermophilic, anaerobic bacterium from the Great Artesian Basin of Australia. *Int J Syst Evol Microbiol* 59:95–101.
- Oh S-E, Min B, Logan BE (2004a) Cathode performance as a factor in electricity generation in microbial fuel cells. *Environ Sci Technol* 38:4900–4904.
- Oh S-E, Van Ginkel S, Logan BE (2003) The relative effectiveness of pH control and heat treatment for enhancing biohydrogen gas production. *Environ Sci Technol* 37:5186–90.
- Oh Y-K, Kim SH, Kim M-S, Park S (2004b) Thermophilic biohydrogen production from glucose with trickling biofilter. *Biotechnol Bioeng* 88:690–698.
- O-Thong S, Prasertsan P, Birkeland N (2009) Evaluation of methods for preparing hydrogen-producing seed inocula under thermophilic condition by process performance and microbial community analysis. *Bioresour Technol* 100:909–918.

- Ottaviano LM, Ramos LR, Botta LS, Amâncio Varesche MB, Silva EL (2017) Continuous thermophilic hydrogen production from cheese whey powder solution in an anaerobic fluidized bed reactor: Effect of hydraulic retention time and initial substrate concentration. *Int J Hydrogen Energy* 42:4848–4860.
- Owen WF, Stuckey DC, Healy Jr. JB, Young LY, McCarty PL (1979) Bioassay for monitoring biochemical methane potential and anaerobic toxicity. *Water Res* 13:485–492.
- Özdenkçi K, De Blasio C, Muddassar HR, Melin K, Oinas P, Koskinen J, Sarwar G, Järvinen M (2017) A novel biorefinery integration concept for lignocellulosic biomass. *Energy Convers Manag* 149:974–987.
- Pant D, Singh A, Van Bogaert G, Irving Olsen S, Singh Nigam P, Diels L, Vanbroekhoven K (2012) Bioelectrochemical systems (BES) for sustainable energy production and product recovery from organic wastes and industrial wastewaters. *RSC Adv* 2:1248–1263.
- Parajuli R, Dalgaard T, Jørgensen U, Adamsen APS, Knudsen MT, Birkved M, Gylling M, Schjørring JK (2015) Biorefining in the prevailing energy and materials crisis: A review of sustainable pathways for biorefinery value chains and sustainability assessment methodologies. *Renew Sustain Energy Rev* 43:244–263.
- Parameswaran P, Bry T, Popat SC, Lusk BG, Rittmann BE, Torres CI (2013) Kinetic, electrochemical, and microscopic characterization of the thermophilic, anode-respiring bacterium *Thermincola ferriacetica*. *Environ Sci Technol* 47:4934–4940.
- Pendyala B, Chaganti SR, Lalman JA, Shanmugam SR, Heath DD, Lau PCK (2012) Pretreating mixed anaerobic communities from different sources: Correlating the hydrogen yield with hydrogenase activity and microbial diversity. *Int J Hydrogen Energy* 37:12175–12186.
- Perera KRJ, Ketheesan B, Gadhamshetty V, Nirmalakhandan N (2010) Fermentative biohydrogen production: Evaluation of net energy gain. *Int J Hydrogen Energy* 35:12224–12233.
- Pham TH, Boon N, Aelterman P, Clauwaert P, De Schampelaire L, Vanhaecke L, De Mayer K, Höfte M, Verstraete W, Rabaey K (2008) Metabolites produced by *Pseudomonas* sp. enable a Gram-positive bacterium to achieve extracellular electron transfer. *Appl Microbiol Biotechnol* 77:1119–1129.
- Pokhrel D, Viraraghavan T (2004) Treatment of pulp and paper mill wastewater—A review. *Sci Total Environ* 333:37–58.
- Premier GC, Kim JR, Michie I, Dinsdale RM, Guwy AJ (2011) Automatic control of load increases power and efficiency in a microbial fuel cell. *J Power Sources* 196:2013–2019.
- Qiu C, Wen J, Jia X (2011) Extreme-thermophilic biohydrogen production from lignocellulosic bioethanol distillery wastewater with community analysis of hydrogen-producing microflora. *Int J Hydrogen Energy* 36:8243–8251.

- Rabaey K, Clauwaert P, Aelterman P, Verstraete W (2005) Tubular microbial fuel cells for efficient electricity generation. *Environ Sci Technol* 39:8077–8082.
- Rafieenia R, Lavagnolo MC, Pivato A (2018) Pre-treatment technologies for dark fermentative hydrogen production: Current advances and future directions. *Waste Manag* 71:734–748.
- Ratkowsky DA, Olley J, McMeekin TA, Ball A (1982) Relationship between temperature and growth rate of bacterial cultures. *J Bacteriol* 149:1–5.
- Reilly M, Dinsdale R, Guwy A (2014) Mesophilic biohydrogen production from calcium hydroxide treated wheat straw. *Int J Hydrogen Energy* 39:16891–16901.
- Ren N, Cao G, Wang A, Lee D, Guo W, Zhu Y (2008a) Dark fermentation of xylose and glucose mix using isolated *Thermoanaerobacterium thermosaccharolyticum* W16. *Int J Hydrogen Energy* 33:6124–6132.
- Ren N, Guo W-Q, Wang X-J, Xiang W-S, Liu B-F, Wang X-Z, Ding J, Chen Z-B (2008b) Effects of different pretreatment methods on fermentation types and dominant bacteria for hydrogen production. *Int J Hydrogen Energy* 33:4318–4324.
- Ren N, Li J, Li B, Wang Y, Liu S (2006) Biohydrogen production from molasses by anaerobic fermentation with a pilot-scale bioreactor system. *Int J Hydrogen Energy* 31:2147–2157.
- Rintala JA, Lepistö SS (1992) Anaerobic treatment of thermomechanical pulping whitewater at 35-70°C. *Water Res* 26:1297–1305.
- Rintala JA, Puhakka JA (1994) Anaerobic treatment in pulp- and paper-mill waste management: A review. *Bioresour Technol* 47:1–18.
- Rismani-Yazdi H, Carver SM, Christy AD, Tuovinen OH (2008) Cathodic limitations in microbial fuel cells: An overview. *J Power Sources* 180:683–694.
- Rismani-Yazdi H, Carver SM, Christy AD, Yu Z, Bibby K, Peccia J, Tuovinen OH (2013) Suppression of methanogenesis in cellulose-fed microbial fuel cells in relation to performance, metabolite formation, and microbial population. *Bioresour Technol* 129:281–288.
- Rodenas Motos P, Molina G, ter Heijne A, Sleutels T, Saakes M, Buisman C (2017) Prototype of a scaled-up microbial fuel cell for copper recovery. *J Chem Technol Biotechnol* 92:2817–2824.
- Ryan P, Forbes C, Colleran E (2008) Investigation of the diversity of homoacetogenic bacteria in mesophilic and thermophilic anaerobic sludges using the formyltetrahydrofolate synthetase gene. *Water Sci Technol* 57:675–680.
- Saady NMC (2013) Homoacetogenesis during hydrogen production by mixed cultures dark fermentation: Unresolved challenge. *Int J Hydrogen Energy* 38:13172–13191.
- Santoro C, Arbizzani C, Erable B, Ieropoulos I (2017) Microbial fuel cells: From

- fundamentals to applications. A review. *J Power Sources* 356:225–244.
- Santos SC, Rosa PRF, Sakamoto IK, Amâncio Varesche MB, Silva EL (2014a) Hydrogen production from diluted and raw sugarcane vinasse under thermophilic anaerobic conditions. *Int J Hydrogen Energy* 39:9599–9610.
- Santos SC, Rosa PRF, Sakamoto IK, Varesche MBA, Silva EL (2014b) Organic loading rate impact on biohydrogen production and microbial communities at anaerobic fluidized thermophilic bed reactors treating sugarcane stillage. *Bioresour Technol* 159:55–63.
- Sasaki K, Morita M, Sasaki D, Nagaoka J, Matsumoto N, Ohmura N, Shinozaki H (2011) Syntrophic degradation of proteinaceous materials by the thermophilic strains *Coprothermobacter proteolyticus* and *Methanothermobacter thermautotrophicus*. *J Biosci Bioeng* 112:469–472.
- Sawicka JE, Robador A, Hubert C, Jørgensen BB, Brüchert V (2010) Effects of freeze–thaw cycles on anaerobic microbial processes in an Arctic intertidal mud flat. *ISME J* 4:585–594.
- Schloss PD, Westcott SL, Ryabin T, Hall JR, Hartmann M, Hollister EB, Lesniewski RA, Oakley BB, Parks DH, Robinson CJ, Sahl JW, Stres B, Thallinger GG, Van Horn DJ, Weber CF (2009) Introducing mothur: Open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Appl Environ Microbiol* 75:7537–7541.
- Schröder U (2007) Anodic electron transfer mechanisms in microbial fuel cells and their energy efficiency. *Phys Chem Chem Phys* 9:2619–2629.
- Sen B, Suttar RR (2012) Mesophilic fermentative hydrogen production from sago starch-processing wastewater using enriched mixed cultures. *Int J Hydrogen Energy* 37:15588–15597.
- Seppälä JJ, Puhakka JA, Yli-Harja O, Karp MT, Santala V (2011) Fermentative hydrogen production by *Clostridium butyricum* and *Escherichia coli* in pure and cocultures. *Int J Hydrogen Energy* 36:10701–10708.
- Sharma Y, Li B (2010) Optimizing energy harvest in wastewater treatment by combining anaerobic hydrogen producing biofermentor (HPB) and microbial fuel cell (MFC). *Int J Hydrogen Energy* 35:3789–3797.
- Shaw JA, Jenney Jr. FE, Adams MWW, Lynd LR (2008) End-product pathways in the xylose fermenting bacterium, *Thermoanaerobacterium saccharolyticum*. *Enzyme Microb Technol* 42:453–458.
- Shen H-B, Yong X-Y, Chen Y-L, Liao Z-H, Si R-W, Zhou J, Wang S-Y, Yong Y-C, OuYang P-K, Zheng T (2014) Enhanced bioelectricity generation by improving pyocyanin production and membrane permeability through sophorolipid addition in *Pseudomonas aeruginosa*-inoculated microbial fuel cells. *Bioresour Technol* 167:490–494.

- Show K-Y, Lee D-J, Chang J-S (2011) Bioreactor and process design for biohydrogen production. *Bioresour Technol* 102:8524–8533.
- Si B, Liu Z, Zhang Y, Li J, Xing X-H, Li B, Duan N, Lu H (2015) Effect of reaction mode on biohydrogen production and its microbial diversity. *Int J Hydrogen Energy* 40:3191–3200.
- Sigurbjornsdottir MA, Orlygsson J (2012) Combined hydrogen and ethanol production from sugars and lignocellulosic biomass by *Thermoanaerobacterium* AK54, isolated from hot spring. *Appl Energy* 97:785–791.
- Singh R, Krishna BB, Mishra G, Kumar J, Bhaskar T (2016) Strategies for selection of thermo-chemical processes for the valorisation of biomass. *Renew Energy* 98:226–237.
- Sivagurunathan P, Kumar G, Bakonyi P, Kim S-H, Kobayashi T, Xu KQ, Lakner G, Tóth G, Nemestóthy N, Bélafi-Bakó K (2016a) A critical review on issues and overcoming strategies for the enhancement of dark fermentative hydrogen production in continuous systems. *Int J Hydrogen Energy* 41:3820–3836.
- Sivagurunathan P, Kumar G, Park J-H, Park J-H, Park H-D, Yoon J-J, Kim S-H (2016b) Feasibility of enriched mixed cultures obtained by repeated batch transfer in continuous hydrogen fermentation. *Int J Hydrogen Energy* 41:4393–4403.
- Snider RM, Strycharz-Glaven SM, Tsoi SD, Erickson JS, Tender LM (2012) Long-range electron transport in *Geobacter sulfurreducens* biofilms is redox gradient-driven. *Proc Natl Acad Sci USA* 109:15467–15472.
- Soboh B, Linder D, Hedderich R (2004) A multisubunit membrane-bound [NiFe] hydrogenase and an NADH-dependent Fe-only hydrogenase in the fermenting bacterium *Thermoanaerobacter tengcongensis*. *Microbiology* 150:2451–2463.
- Solomon S, Plattner G-K, Knutti R, Friedlingstein P (2009) Irreversible climate change due to carbon dioxide emissions. *Proc Natl Acad Sci USA* 106:1704–1709.
- Sonawane JM, Yadav A, Ghosh PC, Adeloju SB (2017) Recent advances in the development and utilization of modern anode materials for high performance microbial fuel cells. *Biosens Bioelectron* 90:558–576.
- Song Z-X, Dai Y, Fan Q, Li X, Fan Y-T, Hou H (2012) Effects of pretreatment method of natural bacteria source on microbial community and bio-hydrogen production by dark fermentation. *Int J Hydrogen Energy* 37:5631–5636.
- Sulonen MLK, Kokko ME, Lakaniemi AM, Puhakka JA (2015) Electricity generation from tetrathionate in microbial fuel cells by acidophiles. *J Hazard Mater* 284:182–189.
- Sulonen MLK, Kokko ME, Lakaniemi A-M, Puhakka JA (2018) Simultaneous removal of tetrathionate and copper from simulated acidic mining water in bioelectrochemical and electrochemical systems. *Hydrometallurgy* 176:129–138.
- Sun D, Chen J, Huang H, Liu W, Ye Y, Cheng S (2016) The effect of biofilm thickness

- on electrochemical activity of *Geobacter sulfurreducens*. *Int J Hydrogen Energy* 41:16523–16528.
- Suryawanshi PC, Chaudhari AB, Kothari RM (2010) Thermophilic anaerobic digestion: The best option for waste treatment. *Crit Rev Biotechnol* 30:31–40.
- Suvilampi J, Lepistö R, Rintala J (2001) Biological treatment of pulp and paper mill process and wastewaters under thermophilic conditions – A review. *Pap Timber* 83:320–325.
- Tang X, Guo K, Li H, Du Z, Tian J (2011) Bioresource Technology Electrochemical treatment of graphite to enhance electron transfer from bacteria to electrodes. *Bioresour Technol* 102:3558–3560.
- Tao R, Lakaniemi A-M, Rintala JA (2017) Cultivation of *Scenedesmus acuminatus* in different liquid digestates from anaerobic digestion of pulp and paper industry biosludge. *Bioresour Technol* 245:706–713.
- Thauer RK, Jungermann K, Decker K (1977) Energy conservation in chemotrophic anaerobic bacteria. *Bacteriol Rev* 41:100–180.
- Thauer RK, Kaster A-K, Seedorf H, Buckel W, Hedderich R (2008) Methanogenic archaea: Ecologically relevant differences in energy conservation. *Nat Rev Microbiol* 6:579–591.
- Toczyłowska-Mamińska R (2017) Limits and perspectives of pulp and paper industry wastewater treatment – A review. *Renew Sustain Energy Rev* 78:764–772.
- Torres CI, Krajmalnik-Brown R, Parameswaran P, Marcus AK, Wanger G, Gorby YA, Rittmann BE (2009) Selecting anode-respiring bacteria based on anode potential: Phylogenetic, electrochemical, and microscopic characterization. *Environ Sci Technol* 43:9519–9524.
- Ucar D, Zhang Y, Angelidaki I (2017) An overview of electron acceptors in microbial fuel cells. *Front Microbiol* 8:643.
- Van Haandel A, Van der Lubbe J (2012) *Handbook of biological wastewater treatment: Design and Optimisation of Activated Sludge Systems*. Quist Publishing, Leidschendam, The Netherlands.
- Van Niel EWJ, Budde MAW, De Haas GG, Van der Wal FJ, Claassen PAM, Stams AJM (2002) Distinctive properties of high hydrogen producing extreme thermophiles, *Caldicellulosiruptor saccharolyticus* and *Thermotoga elfii*. *Int J Hydrogen Energy* 27:1391–1398.
- Venkata Mohan S, Lalit Babu V, Sarma PN (2008) Effect of various pretreatment methods on anaerobic mixed microflora to enhance biohydrogen production utilizing dairy wastewater as substrate. *Bioresour Technol* 99:59–67.
- Venkata Mohan S, Nikhil GN, Chiranjeevi P, Nagendranatha Reddy C, Rohit M V., Kumar AN, Sarkar O (2016) Waste biorefinery models towards sustainable circular

- bioeconomy: Critical review and future perspectives. *Bioresour Technol* 215:2–12.
- Venkata Mohan S, Rohit M V., Chiranjeevi P, Chandra R, Navaneeth B (2015) Heterotrophic microalgae cultivation to synergize biodiesel production with waste remediation: Progress and perspectives. *Bioresour Technol* 184:169–178.
- Venkata Mohan S, Velvizhi G, Annie Modestra J, Srikanth S (2014) Microbial fuel cell: Critical factors regulating bio-catalyzed electrochemical process and recent advancements. *Renew Sustain Energy Rev* 40:779–797.
- Verhaart MRA, Bielen AAM, Van der Oost J, Stams AJM, Kengen SWM (2010) Hydrogen production by hyperthermophilic and extremely thermophilic bacteria and archaea: Mechanisms for reductant disposal. *Environ Technol* 31:993–1003.
- Vicari F, Mateo S, Fernandez-Morales FJ, Cañizares P, Galia A, Scialdone O, Rodrigo MA (2017) Influence of the methodology of inoculation in the performance of air-breathing microbial fuel cells. *J Electroanal Chem* 803:81–88.
- Vipotnik Z, Jessen JE, Scully SM, Orlygsson J (2016) Effect of culture conditions on hydrogen production by *Thermoanaerobacter* strain AK68. *Int J Hydrogen Energy* 41:181–189.
- Wang B, Wan W, Wang J (2008) Inhibitory effect of ethanol, acetic acid, propionic acid and butyric acid on fermentative hydrogen production. *Int J Hydrogen Energy* 33:7013–7019.
- Wang H, Fang M, Fang Z, Bu H (2010) Effects of sludge pretreatments and organic acids on hydrogen production by anaerobic fermentation. *Bioresour Technol* 101:8731–8735.
- Wang J, Wan W (2008) Comparison of different pretreatment methods for enriching hydrogen-producing bacteria from digested sludge. *Int J Hydrogen Energy* 33:2934–2941.
- Wang J, Wan W (2009) Factors influencing fermentative hydrogen production: A review. *Int J Hydrogen Energy* 34:799–811.
- Wang J, Yin Y (2017) Principle and application of different pretreatment methods for enriching hydrogen-producing bacteria from mixed cultures. *Int J Hydrogen Energy* 42:4804–4823.
- Wang Y-Y, Ai P, Hu C-X, Zhang Y-L (2011) Effects of various pretreatment methods of anaerobic mixed microflora on biohydrogen production and the fermentation pathway of glucose. *Int J Hydrogen Energy* 36:390–396.
- Wei L, Han H, Shen J (2012) Effects of cathodic electron acceptors and potassium ferricyanide concentrations on the performance of microbial fuel cell. *Int J Hydrogen Energy* 37:12980–12986.
- Willis K, Maureaud C, Wilcox C, Hardesty BD (2017) How successful are waste abatement campaigns and government policies at reducing plastic waste into the

- marine environment? *Mar Policy*. *In press*. doi: 10.1016/j.marpol.2017.11.037
- Wong YM, Juan JC, Ting A, Wu TY (2014) High efficiency bio-hydrogen production from glucose revealed in an inoculum of heat-pretreated landfill leachate sludge. *Energy* 72:628–635.
- Wrighton KC, Agbo P, Warnecke F, Weber KA, Brodie EL, DeSantis TZ, Hugenholtz P, Andersen GL, Coates JD (2008) A novel ecological role of the Firmicutes identified in thermophilic microbial fuel cells. *ISME J* 2:1146–1156.
- Wrighton KC, Thrash JC, Melnyk RA, Bigi JP, Byrne-Bailey KG, Remis JP, Schichnes D, Auer M, Chang CJ, Coates JD (2011) Evidence for direct electron transfer by a Gram-positive bacterium isolated from a microbial fuel cell. *Appl Environ Microbiol* 77:7633–7639.
- Xie L, Dong N, Wang L, Zhou Q (2014) Thermophilic hydrogen production from starch wastewater using two-phase sequencing batch fermentation coupled with UASB methanogenic effluent recycling. *Int J Hydrogen Energy* 39:20942–20949.
- Xue Y, Xu Y, Liu Y, Ma Y, Zhou P (2001) *Thermoanaerobacter tengcongensis* sp. nov., a novel anaerobic, saccharolytic, thermophilic bacterium isolated from a hot spring in Tengcong, China. *Int J Syst Evol Microbiol* 51:1335–1341.
- Yin Y, Hu J, Wang J (2014) Enriching hydrogen-producing bacteria from digested sludge by different pretreatment methods. *Int J Hydrogen Energy* 39:13550–13556.
- Yokoyama H, Waki M, Moriya N, Yasuda T, Tanaka Y, Haga K (2007) Effect of fermentation temperature on hydrogen production from cow waste slurry by using anaerobic microflora within the slurry. *Appl Microbiol Biotechnol* 74:474–483.
- Yong Y-C, Dong X-C, Chan-Park MB, Song H, Chen P (2012) Macroporous and monolithic anode based on polyaniline hybridized three-dimensional graphene for high-performance microbial fuel cells. *ACS Nano* 6:2394–2400.
- Zeidan AA, Van Niel EWJ (2010) A quantitative analysis of hydrogen production efficiency of the extreme thermophile *Caldicellulosiruptor owensensis* OLT^T. *Int J Hydrogen Energy* 35:1128–1137.
- Zhang E, Zhai W, Luo Y, Scott K, Wang X, Diao G (2016) Acclimatization of microbial consortia to alkaline conditions and enhanced electricity generation. *Bioresour Technol* 211:736–742.
- Zhang F, Chen Y, Dai K, Zeng RJ (2014) The chemostat study of metabolic distribution in extreme-thermophilic (70 °C) mixed culture fermentation. *Appl Microbiol Biotechnol* 98:10267–10273.
- Zhang L, Li J, Zhu X, Ye D, Fu Q, Liao Q (2017a) Startup performance and anodic biofilm distribution in continuous-flow microbial fuel cells with serpentine flow fields: Effects of external resistance. *Ind Eng Chem Res* 56:3767–3774.
- Zhang Q, Zhang Z, Wang Y, Lee D-J, Li G, Zhou X, Jiang D, Xu B, Lu C, Li Y, Ge X

- (2018) Sequential dark and photo fermentation hydrogen production from hydrolyzed corn stover: A pilot test using 11 m³ reactor. *Bioresour Technol* 253:382–386.
- Zhang S, You J, Kennes C, Cheng Z, Ye J, Chen D, Chen J, Wang L (2017b) Current advances of VOCs degradation by bioelectrochemical systems: A review. *Chem Eng J* 334:2625–2637.
- Zhang Z-P, Show K-Y, Tay J-H, Liang DT, Lee D-J (2008) Biohydrogen production with anaerobic fluidized bed reactors—A comparison of biofilm-based and granule-based systems. *Int J Hydrogen Energy* 33:1559–1564.
- Zhang Z-P, Tay J-H, Show K-Y, Yan R, Liang DT, Lee D-J, Jiang W-J (2007) Biohydrogen production in a granular activated carbon anaerobic fluidized bed reactor. *Int J Hydrogen Energy* 32:185–191.
- Zhao C, Karakashev D, Lu W, Wang H, Angelidaki I (2010) Xylose fermentation to biofuels (hydrogen and ethanol) by extreme thermophilic (70 °C) mixed culture. *Int J Hydrogen Energy* 35:3415–3422.
- Zheng H, Zeng RJ, Angelidaki I (2008) Biohydrogen production from glucose in upflow biofilm reactors with plastic carriers under extreme thermophilic conditions (70°C). *Biotechnol Bioeng* 100:1034–1038.
- Zheng H-S, Guo W-Q, Yang S-S, Feng X-C, Du J-S, Zhou X-J, Chang J-S, Ren N-Q (2014) Thermophilic hydrogen production from sludge pretreated by thermophilic bacteria: Analysis of the advantages of microbial community and metabolism. *Bioresour Technol* 172:433–437.
- Zhu H, Béland M (2006) Evaluation of alternative methods of preparing hydrogen producing seeds from digested wastewater sludge. *Int J Hydrogen Energy* 31:1980–1988.
- Zhu X, Logan BE (2014) Copper anode corrosion affects power generation in microbial fuel cells. *J Chem Technol Biotechnol* 89:471–474.
- Zhu X, Yates MD, Hatzell MC, Ananda Rao H, Saikaly PE, Logan BE (2014) Microbial community composition is unaffected by anode potential. *Environ Sci Technol* 48:1352–1358.

I

**BIOHYDROGEN PRODUCTION FROM XYLOSE BY FRESH AND
DIGESTED ACTIVATED SLUDGE AT 37, 55 AND 70 °C**

by

Dessi, P., Lakaniemi, A.-M., Lens, P.N.L., 2017

Water Research 115:120-129

Reproduced with kind permission by Elsevier



Biohydrogen production from xylose by fresh and digested activated sludge at 37, 55 and 70 °C



Paolo Dessì^{a,*}, Aino-Maija Lakaniemi^a, Piet N.L. Lens^{a,b}

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

^b UNESCO–IHE, Institute for Water Education, Westvest 7, 2611AX Delft, The Netherlands

ARTICLE INFO

Article history:

Received 23 August 2016

Received in revised form

20 February 2017

Accepted 26 February 2017

Available online 28 February 2017

Keywords:

Biohydrogen

Inocula

Temperature

Xylose

Butyrate

Dark fermentation

ABSTRACT

Two heat-treated inocula, fresh and digested activated sludge from the same municipal wastewater treatment plant, were compared for their H₂ production via dark fermentation at mesophilic (37 °C), thermophilic (55 °C) and hyperthermophilic (70 °C) conditions using xylose as the substrate. At both 37 and 55 °C, the fresh activated sludge yielded more H₂ than the digested sludge, whereas at 70 °C, neither of the inocula produced H₂ effectively. A maximum yield of 1.85 mol H₂ per mol of xylose consumed was obtained at 55 °C. H₂ production was linked to acetate and butyrate production, and there was a linear correlation ($R^2 = 0.96$) between the butyrate and H₂ yield for the fresh activated sludge inoculum at 55 °C. Approximately 2.4 mol H₂ per mol of butyrate produced were obtained against a theoretical maximum of 2.0, suggesting that H₂ was produced via the acetate pathway prior to switching to the butyrate pathway due to the increased H₂ partial pressure. *Clostridia* sp. were the prevalent species at both 37 and 55 °C, irrespectively of the inoculum type. Although the two inocula originated from the same plant, different thermophilic microorganisms were detected at 55 °C. *Thermoanaerobacter* sp., detected only in the fresh activated sludge cultures, may have contributed to the high H₂ yield obtained with such an inoculum.

© 2017 Elsevier Ltd. All rights reserved.

1. Introduction

The intensive use of fossil fuels results in their rapid depletion and increased emission of greenhouse gases, in particular CO₂. Therefore, energy production is expected to shift towards renewable and more eco-friendly alternatives in the coming decades. Energy recovery from wastewaters can be a good strategy to pursue the double objective of sustainability and emission reduction. Many industries, such as the pulp and paper industry, produce wastewaters rich in organic compounds, which must be treated prior to discharge, but yet have a high potential for energy recovery (Rajeshwari et al., 2000). Traditional aerobic treatment is expensive, due to the huge amount of oxygen required to oxidize the organic compounds. In contrast, anaerobic processes allow coupling of wastewater treatment and energy production in the form of biogas (Kamali and Khodaparast, 2015).

Methane production from organic compounds is a well-developed technology, but hydrogen (H₂) production is a

promising alternative as well because its heating value per gram is the highest among fuels, and because it does not release CO₂ to the atmosphere upon combustion (Dincer and Acar, 2015). Studies on biological H₂ production have focused on bio-photolysis of water, water gas-shift reaction, photo-fermentation and dark fermentation of organic compounds (Bundhoo and Mohee, 2016). The main advantages of dark fermentation over the other technologies are its high H₂ production rate, the simple operation (the reactor configurations are the same of the already well-established anaerobic digestion), and lower energy requirement (Show et al., 2012). Its main drawbacks are the relative low H₂ yield (mol H₂ per mol of substrate) and the formation of by-products, such as CO₂, volatile fatty acids and alcohols (Rittmann and Herwig, 2012).

Dark fermentation is a biological process in which fermentative bacteria produce H₂ to dispose of excessive electrons generated in the oxidation of organic compounds through a hydrogenase enzyme and electron carriers such as nicotinamide adenine dinucleotide (NADH) or reduced ferredoxin (Lee et al., 2011). The maximum H₂ yield by dark fermentation is reached if acetate is the only by-product of the oxidative process. The overall H₂ production is strongly affected by the inoculum and the operating

* Corresponding author.

E-mail address: paolo.dessi@tut.fi (P. Dessì).

conditions, such as temperature, pH, substrate concentration and H₂ partial pressure (Li and Fang, 2007). Depending on the operating conditions, part of the electrons can be directed to producing compounds more reduced than acetate, such as butyrate or ethanol, resulting in a lower H₂ yield (Li and Fang, 2007).

Temperature is a crucial parameter for most biotechnological processes, because different temperatures can reshape the microbial communities involved in the bioprocess (Karadag and Puhakka, 2010). Furthermore, increasing temperature positively affects both the kinetics and thermodynamics of the process (Verhaart et al., 2010). Thermophilic microorganisms are generally characterized by faster growth and reaction rates than mesophilic species. A direct conversion of sugars to acetate, which yields the maximum amount of H₂, is thermodynamically not favorable at low temperature, but becomes more favorable as the temperature increases, thus making proton reduction to H₂ coupled to NADH oxidation exergonic (Verhaart et al., 2010). Another advantage of high temperature processes is the reduced contamination by pathogens and H₂ consuming bacteria (Van Groenestijn et al., 2002). Industries produce wastewaters at various temperatures, and treating them at their original temperature, without heating or cooling, seems a cost-effective approach. For example, pulp and paper industries typically produce wastewaters with elevated temperatures (50–70 °C), which are often cooled down to 30–40 °C prior to biological treatment (Suvilampi et al., 2001).

Selection of the inoculum is also a key for a successful biohydrogen production process. From the industrial point of view, dark fermentation with mixed cultures is preferable over pure cultures because of easier operation and control, not requiring sterilization, and possibility to use a wide range of feedstocks, as several different microorganisms are often required to degrade completely complex substrates (Wang and Wan, 2009). However, mixed cultures may contain species that degrade organic compounds by other pathways than H₂ production. Hydrogenotrophic methanogens, propionate-producers, homoacetogens, and even sulfate and nitrate reducing bacteria consume H₂ as a part of their metabolism (Bundhoo and Mohee, 2016). Though most H₂ consuming bacteria are non-sporulating and can be removed by pretreating the inoculum, their complete elimination cannot be ensured. For example, the thermophilic homoacetogenic bacterium *Moorella glycerini* is a spore-forming microorganism (Slobodkin et al., 1997) and may resist the pretreatment.

Heat treatment is the most common pretreatment used to select spore-forming, hydrogen-producing microorganisms (Bundhoo et al., 2015). Many heat-treated inocula have been tested in dark fermentation, including sewage sludge (Baghchehsaraee et al., 2008; Hasyim et al., 2011; Lin et al., 2008), aerobic and anaerobic sludge from different plants treating organic waste (Bakonyi et al., 2014; Cavalcante de Amorim et al., 2009), landfill leachate (Wong et al., 2014), hot spring cultures (Koskinen et al., 2008), and compost (Cao et al., 2014). Despite the abundance of data available in the literature, both on H₂ production and the microorganisms involved, the studies often differ in their operating conditions, making it difficult to evaluate and distinguish the effect of the inoculum on the process (Table 1). Although the combined effect of inoculum and temperature on dark fermentation is of both scientific and practical interest, to our knowledge, a direct comparison of the potential of two inocula for H₂ production at mesophilic, thermophilic and hyperthermophilic conditions, keeping the other initial conditions stable, has not yet been performed.

This study aimed to compare two heat-treated inocula, activated sludge and digester sludge from the same municipal wastewater treatment plant, for biohydrogen production under mesophilic (37 °C), thermophilic (55 °C) and hyperthermophilic (70 °C) conditions. Xylose, a pentose sugar commonly present in pulp and paper wastewater, was used as the substrate. The correlations between H₂ and soluble compounds produced via dark fermentation of xylose by the activated sludge inoculum were then determined in order to understand the metabolic pathways at 55 °C, the temperature at which the H₂ yield was the highest.

2. Materials and methods

2.1. Source of biomass

The two sludge types used as inoculum were collected in July 2015 from the Viinikanlahti municipal wastewater treatment plant (Tampere, Finland). The first sludge type was fresh activated sludge from the recirculation line between the outdoor aeration tank and the secondary settler. The average outdoor temperature in Tampere usually ranges between −6.7 °C in February and +17.4 °C in July, although winter temperatures below −20 °C are also possible (Finnish Meteorological Institute, see: en.ilmatieteenlaitos.fi/statistics-from-1961-onwards). The second type was digester

Table 1

H₂ yields obtained in various batch studies conducted at different temperatures and using different initial pH and xylose concentrations. The reported H₂ yield refer to the highest one obtained in the cited studies.

Inoculum	Pre-treatment	T (°C)	Initial pH	Initial xylose (mM)	H ₂ yield ^a (mol per mol xylose)	Reference
Activated sludge	Heat treatment	35	6.5	124.9	1.30	Lin et al. (2006)
Activated sludge	Heat treatment	35	5.5	66.6	1.88	De Sá et al. (2013)
Activated sludge	Heat treatment	35	6.5	124.9	2.25	Lin and Cheng (2006)
<i>Clostridium butyricum</i>	— ^b	37	7.5	124.9	0.73	Lo et al. (2008)
Granulated sludge	Heat treatment	37	5.5	23.9	0.80	Maintinguer et al. (2011)
Digested activated sludge	—	37	6.7	33.3	2.64	Chaganti et al. (2012)
<i>Clostridium beijerinckii</i>	—	40	8	66.6	2.31	An et al. (2014)
Activated sludge	Heat treatment	40	7.1	124.9	1.30	Lin et al. (2008)
Mixed culture compost	—	55	5	13.3	1.70	Calli et al. (2008)
<i>Thermoanaerobacter thermosaccharolyticum</i>	—	60	6.7	33.3	2.07	Zhang et al. (2011)
<i>Thermoanaerobacter thermosaccharolyticum</i>	—	60	6.5	66.6	2.09	Khamtib and Reungsang (2012)
<i>Thermoanaerobacter thermosaccharolyticum</i>	—	60	6.5	66.6	2.19	Ren et al. (2008)
<i>Thermoanaerobacter thermosaccharolyticum</i>	—	60	7.0	33.3	1.72	Cao et al. (2014)
Geothermal spring	—	60	7.9	66.6	1.65	Zeidan and Van Niel (2009)
Biomass from H ₂ producing reactor	—	70	7.0–8.0	3.3	1.62	Kongjan et al. (2009)
Biomass from H ₂ producing reactor	—	70	7.0	13.3	1.84	Zhao et al. (2010)

^a Highest H₂ yield obtained in the experiment.

^b Not applied.

sludge from a mesophilic (35 °C) anaerobic digester treating waste activated sludge. After settling and removing the supernatant, both sludge samples were divided in 10 mL batches to thin 15 mL anaerobic tubes, and heat treated at 90 °C for 15 min (Maintinguer et al., 2011) by incubation in a pre-heated water bath prior to use as inoculum for the H₂ production experiments.

2.2. Batch experimental set-up

Batch assays were conducted in 120 mL serum bottles with a total working volume of 50 mL. The growth medium was DSMZ 144 with the following modifications: tryptone was not added, the concentration of yeast extract was reduced to 0.3 g L⁻¹ (Nissilä

et al., 2011) and xylose (7.50 g L⁻¹, 50 mM) was used as the substrate instead of glucose. The pH of the growth medium was adjusted to 5.5 with 1 M HCl.

In the first culture, the bottles were inoculated with 11.4 mL activated sludge (8.8 ± 0.1 g VS L⁻¹) or 4.2 mL of digester sludge (24.0 ± 0.1 g VS L⁻¹), resulting in an inoculum concentration of about 2 g VS L⁻¹, and medium was added up to 50 mL. The initial xylose concentration of the mixture (medium and inoculum) was 50 mM. The following three batch cultures were inoculated by transferring 5 mL of cultivation from the previous batch culture to 45 mL of fresh medium with 55.6 mM of xylose, in order to reach a final xylose concentration of 50 mM. To ensure anaerobic conditions, the serum bottles were flushed with N₂ for 5–10 min before

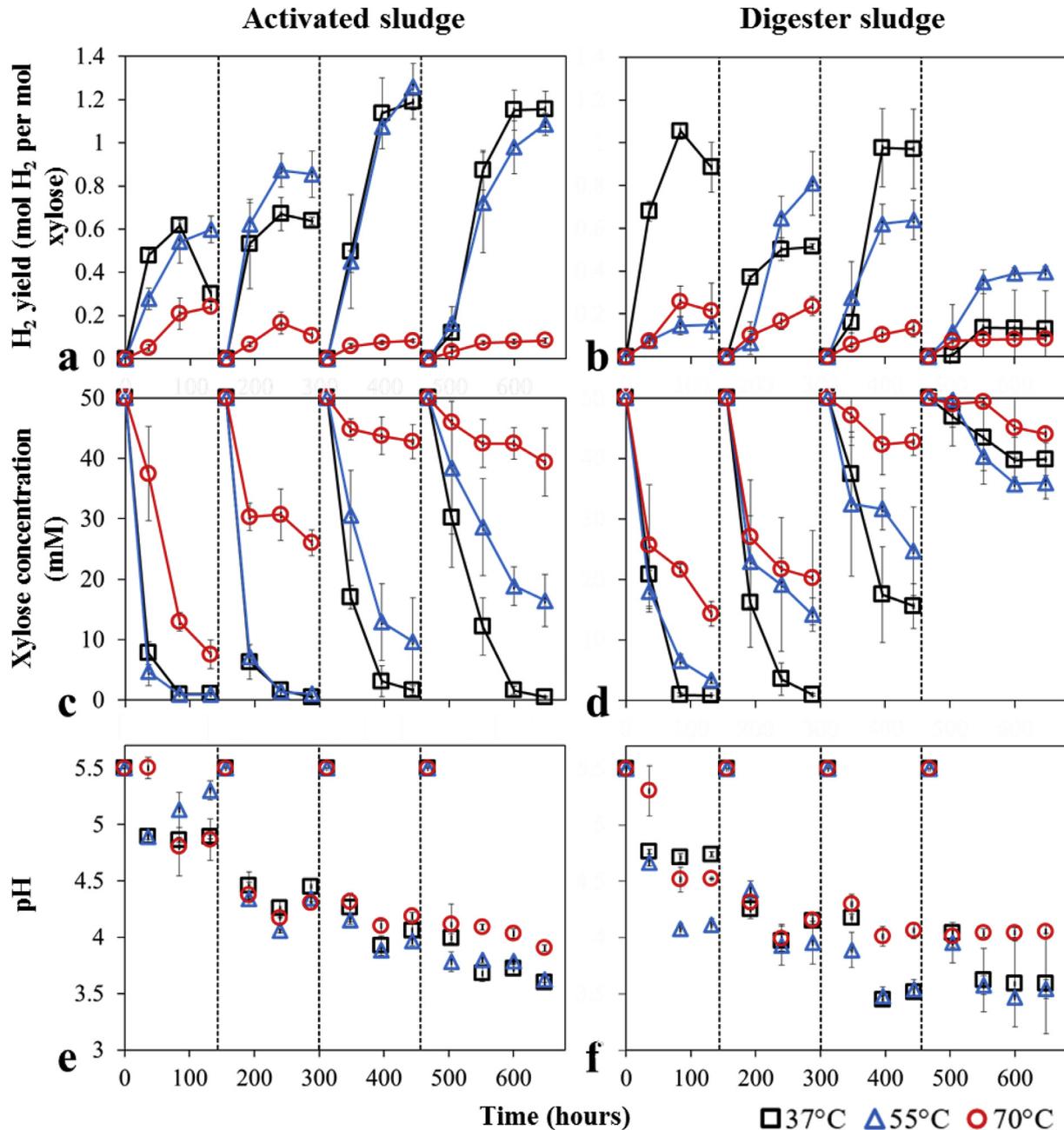


Fig. 1. H₂ yield (mol H₂ per mol of xylose added), residual xylose and pH trend with the activated and the digester sludge at 37, 55 and 70 °C. Every point shown in the graphs is calculated as the average of three independent batch cultures, error bars indicate the standard deviation of the triplicates. The dotted lines refer to the end of every batch culture and start of a new one.

and after inoculation. To avoid interference in the gas measurement due to the N₂ flushing, the pressure in the headspace was equilibrated to atmospheric pressure by removing the excessive gas with a syringe before starting the incubation. The bottles were incubated at 37, 55 and 70 °C for 6–8 days. All the batch cultures were conducted in triplicate. A control bottle without xylose for all the triplicates was also prepared in all steps.

2.3. Microbial community analyses

Samples for microbial community analysis were collected at the end of the last batch culture and stored at –20 °C. DNA extraction and polymerase chain reaction–denaturing gradient gel electrophoresis (PCR–DGGE) were performed according to Mäkinen et al. (2012). The forward primer for PCR was GC–BacV3f, while the reverse primer was 907r resulting in a PCR product of approximately 550 base pairs. All the analyses were done in duplicate. The visible bands were cut using a surgical blade, eluted in sterile water and re–amplified by PCR (primers BacV3f and 907r) as described by Koskinen et al. (2006). The product quality was checked by running the PCR products on a 1% agarose gel before sending the samples to

Macrogen (South Korea) for sequencing. The nucleotide sequences obtained were analyzed by Bio-Edit software (version 7.2.5) (Hall, 1999), in order to remove primer sequences, and compared with the sequences in the GenBank nucleotide collection database using BLAST software (Altschul et al., 1990) (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>).

2.4. Analytical methods

The overpressure of the bottles was measured using a syringe method, which consisted of collecting the produced gas in a graduated syringe until the pressure inside the bottle reached atmospheric pressure and subsequent reading the produced gas volume (Owen et al., 1979). Gas samples from the headspace of the bottles (0.2 mL) were analyzed with a Shimadzu gas chromatograph GC–2014 equipped with a Poropak N column (80/100 mesh) and a thermal conductivity detector (TCD). The temperature of the oven, injector and detector were at 80, 110 and 110 °C, respectively. Nitrogen was used as the carrier gas. The gas volume was corrected to standard temperature (0 °C). Cumulative H₂ and CO₂ production was calculated with the following equation (Logan et al., 2002):

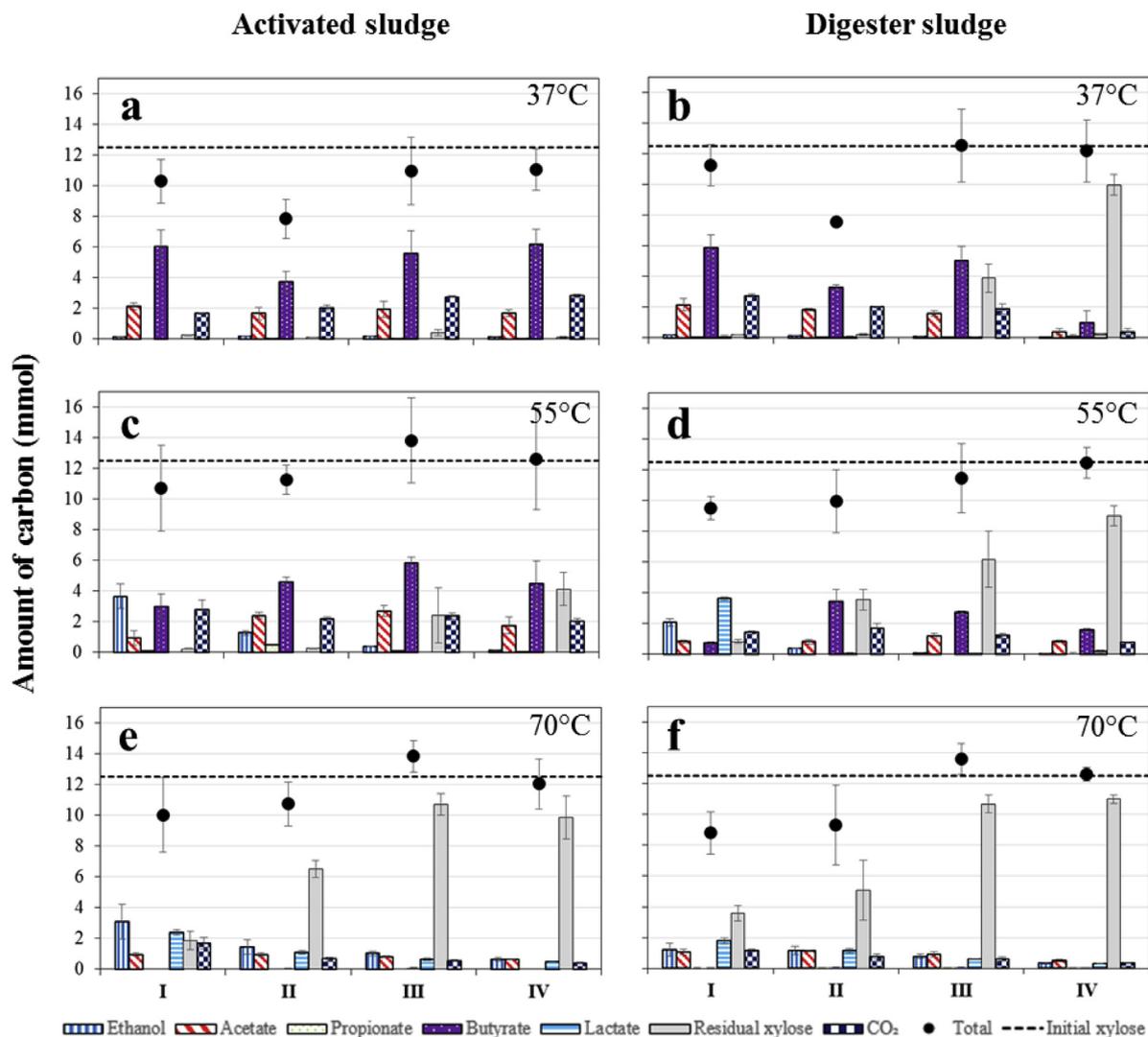


Fig. 2. Carbon distribution at the end of each batch culture. The columns refer to the mmol of carbon found in the different metabolites at the end of every batch cultures and the black dots represent their sum. The dotted line refers to the 12.5 mmol of carbon introduced as xylose at the beginning of each incubation. Every column or point shown in the graphs is calculated as the average of three independent batch cultures, error bars indicate the standard deviation of the triplicates.

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

where V_G , V_H and C_H are the current (i) or previous (i-1) measurement of cumulative gas volume, cumulative H_2 volume and fraction of H_2 in the headspace of serum bottles, respectively, and V is the volume of the headspace.

Xylose in the liquid phase was determined by using a colorimetric phenol–sulphuric acid method (DuBois et al., 1956) with a Shimadzu Ordior UV–1700 Pharmaspec UV–VIS spectrophotometer at 485 nm wavelength. Acetate, propionate, isobutyrate, butyrate, valerate, ethanol and butanol were measured by a gas chromatograph equipped with flame ionization detector (GC–FID) according to Kinnunen et al. (2015). Lactate and formate were measured with a Shimadzu high–performance liquid chromatograph (HPLC) equipped with a Rezex RHM–monosaccharide column (Phenomenex, USA) held at 40 °C and a refractive index detector (Shimadzu, Japan). The mobile phase was 5 mM H_2SO_4 and flow rate was 0.6 mL min^{-1} .

3. Results

3.1. Dark fermentation of xylose by the activated and the digester sludge

At 37 and 55 °C, the H_2 yield with the activated sludge inoculum constantly increased during the first three batch cultures (Fig. 1a), reaching a maximum of 1.19 (± 0.08) and 1.26 (± 0.11) mol H_2 per mol of xylose (added) at 37 and 55 °C, respectively. At 37 °C, the H_2 yield was similar at the end of the third and fourth batch culture, but at 55 °C, it decreased by approximately 13% at the end of the fourth batch culture compared to the third one. The digester sludge started to produce H_2 effectively from the first batch culture at

37 °C, reaching a maximum yield of 1.05 (± 0.04) mol H_2 per mol of xylose (added) after 84 h (Fig. 1b). In the third batch culture, the yield was similar to the first one, but decreased by 50% and 90% in the second and fourth batch culture, respectively. At 55 °C, digester sludge started to produce H_2 effectively after 192 h, reaching a maximum of 0.81 (± 0.15) mol H_2 per mol of xylose (added) at the end of the second batch culture. However, the yield consistently decreased in the following two batch cultures, resulting in a 50% lower yield at the end of the fourth batch culture compared to the second one. Clear consumption of H_2 was observed (H_2 yield dropped) only in the first batch culture at 37 °C (Fig. 1a and b), regardless of the inoculum. At 70 °C, H_2 yield was lower compared to both 37 and 55 °C, with a maximum of only 0.22 (± 0.07) mol H_2 per mol of xylose (added) in the first batch culture with digester sludge inoculum (Fig. 1a and b). Methane in batch cultures was always below the detection limit of the GC–TCD, as well as H_2 , CO_2 , and methane in the control bottles without substrate.

At 37 °C, xylose was consumed (>97%) in all four batch cultures with the activated sludge inoculum, while at 55 °C, its removal efficiency began to decrease from the third batch culture onwards and was only 67% after the fourth batch culture (Fig. 1c). At 70 °C, xylose was efficiently consumed (85%) during the first batch culture, but its removal efficiency decreased and was only 15–20% at the end of the third and fourth batch culture (Fig. 1c). Batch cultures with the digester sludge inoculum followed the same trend at 55 and 70 °C, with a decrease in xylose removal efficiencies from approximately 93% and 71% at the end of the first batch culture to 28% and 12% at the end of the fourth batch culture, respectively (Fig. 1d). Unlike the batch cultures with the fresh activated sludge, the xylose removal efficiency decreased drastically also at 37 °C in the batch cultures with the digester sludge, being >97% at the end of the second batch culture and only 20% at the end of the fourth batch culture.

In every batch culture of both inoculum types, the pH started to decrease as soon as the xylose degradation started, and the pH was remarkably below the initial value of 5.5 after 36 h incubation (Fig. 1f and g). At both 37 and 55 °C, during the incubations, the final pH decreased consistently, being below 4.0 at the end of the fourth batch culture. At 70 °C, pH was somewhat higher (about 4.0) at the end of the fourth batch culture.

3.2. Carbon distribution and metabolites concentration

H_2 production from xylose at the different temperatures resulted in the production of soluble carbon–based compounds in different proportions (Fig. 2). Part of the carbon was removed from the liquid phase mainly as CO_2 , while some of it remained in the solution as xylose or was converted to volatile fatty acids (mainly acetate, butyrate and lactate) or alcohols (mainly ethanol). Generally, a higher percentage of xylose was consumed in the batch cultures with the activated sludge inoculum compared to the batch cultures with the digester sludge. Acetate was produced by both inocula at all the temperatures studied (Fig. 2). Butyrate was produced by both inocula at 37 and 55 °C, whereas it was not detected at 70 °C but ethanol was produced instead. At 55 °C, ethanol production was high (about 37 mM) in the first batch culture with both inocula, but its concentration decreased in the following batch cultures (Fig. 2; Table S1 in supplementary material). Lactate was also detected at 70 °C with the activated sludge inoculum and at all the studied temperatures in the batch cultures with the digester sludge inoculum (Fig. 2). A small concentration of acetate (<1 mmol of carbon) was detected in the control bottles only in the first batch cultures, regardless of the inoculum and temperature.

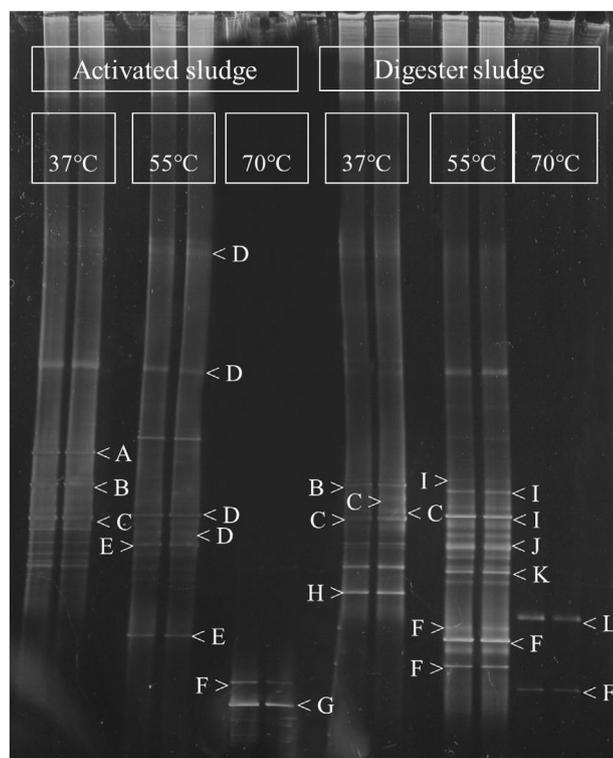


Fig. 3. Bacterial community composition analyzed by PCR–DGGE from the batch cultures with the fresh activated and digester sludge inocula after the four batch cultures at 37, 55 and 70 °C. The band labels refer to Table 2.

3.3. Microbial community analysis

The microbial community composition shown by DGGE (number and location of the bands) after four successive batch cultures was different with the different inocula and incubation temperatures (Fig. 3). At 37 °C, the enriched microbial communities were dominated by bacteria having 91–100% similarity to *Clostridia* sp., based on the partial 16S rRNA sequencing. More specifically, sequencing of the selected bands indicated the presence of microorganisms having 98–100% similarity to *Clostridium butyricum* and *Clostridium acetobutylicum* in the batch cultures with both inocula (Table 2). At 37 °C, genes possibly related to *Sporolactobacillus* sp. (92% similarity to *Sporolactobacillus putidus*) were detected only with the digester sludge inoculum. At 55 °C, *Thermoanaerobacter thermosaccharoliticum* (98% similarity) and *Caloramator australicus* (97–99% similarity) were present in the batch cultures with the fresh activated and digester sludge inoculum, respectively. At 70 °C, *Caloramator australicus* (97–99% similarity) was detected in the batch cultures with both inocula, while genes related to *Thermoanaerobacter* sp. (100% similarity) and *Caldanaerobius* sp. (99% similarity) were found in the batch cultures with the fresh activated and the digester sludge, respectively (Table 2).

3.4. H₂ production pathways by the activated sludge inoculum at 37 and 55 °C

Although a similar H₂ production was obtained at both 37 and 55 °C in the batch cultures with activated sludge (Fig. 1a), approximately 97% of the xylose was consumed at 37 °C, whereas only 67% in the fourth batch culture at 55 °C (Fig. 1c), indicating a higher H₂ yield per mol of xylose consumed at 55 °C (Fig. 4a). Therefore, the microbial community at 55 °C has the potential to yield more H₂ compared to the community at 37 °C, and this is probably related to a different biodegradation pathway. At 37 °C, the H₂ yield stabilized to 1.20 (±0.10) mol H₂ per mol of xylose consumed, while at 55 °C, it constantly increased reaching a maximum of 1.85 (±0.51) mol H₂ per mol of xylose consumed after the first 84 h of the fourth batch culture, before decreasing to 1.64 (±0.19) mol H₂ per mol of xylose consumed at the end of the experiment (Fig. 4a). At 55 °C, both acetate and butyrate followed the same trend as the H₂ production (Fig. 4b). The acetate and

butyrate yields constantly increased during the consecutive batch cultures reaching a maximum of approximately 0.7 and 0.8 mol per mol of xylose consumed for acetate and butyrate, respectively, 84 h after initiating the fourth batch culture. Then, the yields decreased to 0.5 and 0.7 mol per mol of xylose consumed, respectively, at the end of the experiment. Ethanol production was high in the first batch culture (0.7 mol ethanol per mol of xylose consumed) and consistently decreased in the following cultures, becoming negligible in the fourth culture (Fig. 4b).

A linear correlation ($R^2 = 0.96$) was found between the H₂ and butyrate yield at 55 °C (Fig. 4c). Based on the linear regression, approximately 2.4 mol H₂ per mol of butyrate were produced. Conversely, the H₂ yield and ethanol yield seem to be inversely proportional (Fig. 4b).

4. Discussion

4.1. Dark fermentation of xylose by the activated sludge and the digester sludge

At both 37 and 55 °C, the activated sludge inoculum yielded more H₂ than the digester sludge. Although both inocula originated from the same wastewater treatment plant, different microbial communities developed after four batch cultures at all three incubation temperatures. Except for the first culture at 37 °C, the H₂ produced was never consumed (Fig. 1), which confirmed that the heat treatment effectively eliminated most H₂ consuming microorganisms. In the first culture at 37 °C, H₂ consumption was likely attributed to homoacetogenesis, as methane was not detected. Few species of spore forming homoacetogenic bacteria may resist heat treatment (Slobodkin et al., 1997), but their growth is hindered in the pH range (3.5–5.5) of this experiment (Fig. 1e and f). However, *Clostridium acetobutylicum*, present in the batch cultures at 37 °C with both inocula (Table 2) can switch its metabolism from acidogenesis (and H₂ production) to solventogenesis (and H₂ consumption) in case of low pH (<4.5) and high H₂ partial pressure (Kim and Zeikus, 1992). Simultaneous production and consumption of H₂ can thus not be excluded, and the presented results are the net H₂ production (difference between H₂ produced and consumed). Furthermore, only the dominant microorganisms can be detected by PCR–DGGE and thus, the contribution of some

Table 2

Identification of the DGGE bands obtained after four successive batch cultures at 37, 55 and 70 °C based on the comparison of their 16S rRNA gene sequences to those collected in the GenBank and their presence (+) or absence (–) in the different batch cultures.

BM ^a	Microorganism ^b	Access number	Matching sequence length ^c	Similarity (%) ^d	Activated sludge			Digester sludge		
					37	55	70	37	55	70
A	<i>Clostridium</i> sp.	FJ361757	477	99	+	–	–	–	–	–
B	<i>Clostridium acetobutylicum</i>	KP410577 KP410579	457–515	99	+	–	–	+	–	–
C	<i>Clostridium butyricum</i>	CP013352 KT072767	418–492	98–100	+	–	–	+	–	–
D	<i>Clostridium</i> sp.	KR052807	381–490	92–100	–	+	–	–	–	–
E	<i>Thermoanaerobacter thermosaccharoliticum</i>	KT274717	426	98	–	+	–	–	–	–
F	<i>Caloramator australicus</i>	HM228391	385–449	97–99	–	–	+	–	+	+
G	<i>Thermoanaerobacter</i> sp.	KR007668	452	100	–	–	+	–	–	–
H	<i>Sporolactobacillus putidus</i>	NR_112774	486	92	–	–	–	+	–	–
I	<i>Clostridium</i> sp.	AB504378 AB537983	433–451	91–98	–	–	–	–	+	–
J	<i>Clostridium thermopalmarium</i>	KM036191	428	98	–	–	–	–	+	–
K	<i>Clostridium isatidis</i>	NR_026347	425	93	–	–	–	–	+	–
L	<i>Caldanaerobius</i> sp.	JX984966	429	99	–	–	–	–	–	+

^a Band mark in Fig. 3.

^b Closest species in GenBank.

^c Number of nucleotide pairs used in the sequence comparison.

^d Percentage of identical nucleotide pairs between the 16S rRNA gene sequence and the closest species in GenBank.

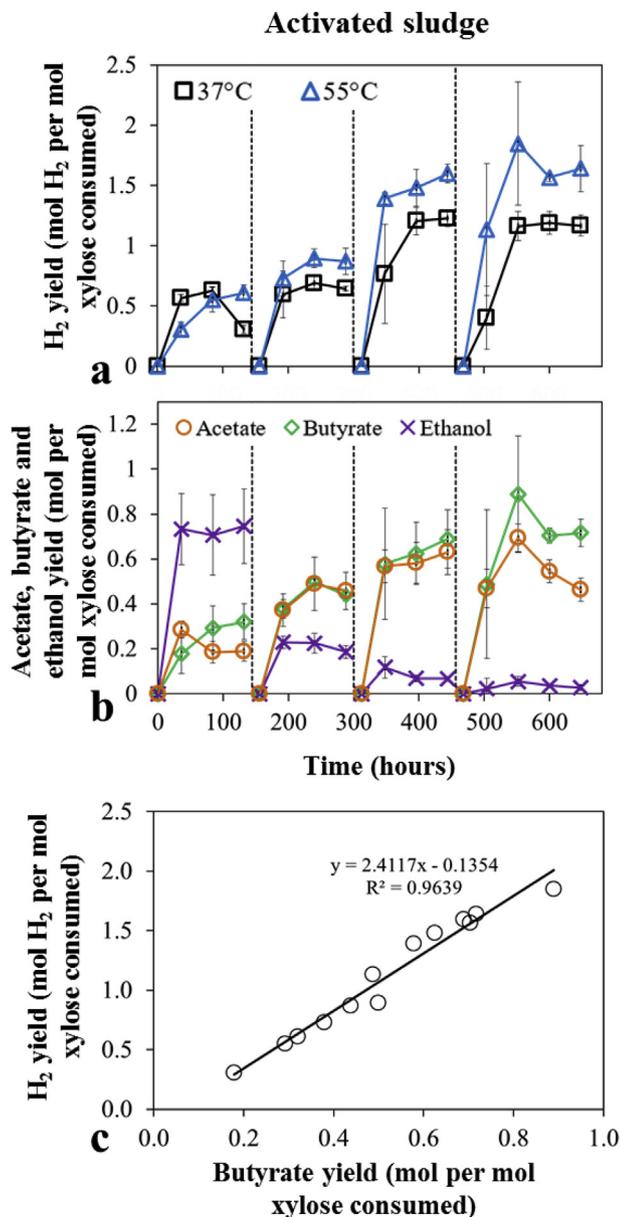


Fig. 4. H₂ yield (mol H₂ per mol of xylose consumed) obtained with the activated sludge at 37 and 55 °C (a) and the acetate, butyrate and ethanol yields obtained with the activated sludge inoculum at 55 °C (b) with respect to time. H₂ yield was shown to be directly proportional to butyrate (c) when activated sludge was used as inoculum at 55 °C. Every point shown in the graphs is calculated as the average of three independent batch cultures, error bars show the standard deviation of the triplicates.

species which might had a role in either H₂ production or consumption could be missing.

For both inocula, and all the temperatures investigated, the pH profile (Fig. 1e and f) does not correlate well with the xylose concentration profiles (Fig. 1c and d). This is especially evident in the last two batch cultures of the digester inoculum, in which the pH dropped to <4 even when xylose consumption was lower than in the previous batch cultures. One possible explanation is that, from the first batch culture, bacteria accumulated undissociated volatile fatty acids, which then dissociated inside the cell due to the neutral cytosolic pH, causing an intracellular overload of protons which were subsequently forced out from the cytoplasm (Jönsson et al., 2013), causing the pH drop observed in the last two batch cultures. This might also explain the decreased xylose degradation rate

in the last two batch cultures of both inocula. Excretion of the protons outside the cells costs energy, e.g. in the form of adenosine triphosphate (ATP), thus limiting the energy available for microbial growth (Bundhoo and Mohee, 2016). Also the carbon balances support this hypothesis: in the first two batch cultures of both inocula, and for all temperatures investigated, up to 30% of the carbon introduced as xylose was not detected as CO₂ or soluble metabolites (Fig. 2). It is plausible that part of the carbon was retained inside the cells in the form of volatile fatty acids, alcohols or storage products. Conversely, in the third and fourth batch culture, the sum of carbon detected as CO₂ and soluble metabolites sometimes exceeded (by 10% at the most) the amount of carbon provided as xylose. Accordingly, the accumulated volatile fatty acids inhibited the H₂ producing bacteria (Van Ginkel and Logan, 2005), possibly inducing their death and cell lysis, thus releasing the cell content and causing an overestimation of carbon detected in the medium. Also acids in the dissociated form, which cannot penetrate the cell membrane, can cause cell lysis by increasing the ionic strength of the medium (Van Niel et al., 2003). It should be noted that the contribution of growth of microorganisms, dissolved CO₂, and yeast extract has not been considered in the carbon balance, and further investigation is required to confirm their role in the carbon balance.

4.2. Comparative H₂ production by the activated sludge and the digester sludge at 37 °C

At 37 °C, the microbial community was dominated by *Clostridia* species (Table 2). Due to the high percentages of acetate and butyrate in the liquid phase, *Clostridium butyricum* and *Clostridium acetobutylicum*, detected at 37 °C with both inocula, were likely associated with H₂ production. *Clostridium butyricum* produces H₂ by dark fermentation via the acetate and butyrate pathway, and it is active at a pH as low as 4.4 (Seppälä et al., 2011). *Clostridium acetobutylicum* produces H₂, acetate and butyrate via acidogenesis at a pH as low as 4.7, before switching the metabolic pathway to solventogenesis (Grube and Gottschalk, 1992). However, as evidenced by the low (<2.2 mM) ethanol concentration in the liquid phase of batch cultures at 37 °C (Table S1 in supplementary material), solventogenesis did not occur even at the lowest pH values achieved in the batch cultures. This is likely due to the insufficient butyrate concentration in the medium, as a butyrate concentration of 2 g L⁻¹ is required to trigger solventogenesis (Cheng et al., 2012). The highest butyrate concentration detected in this study was about 30 mM (2.6 g L⁻¹) at the end of the first and fourth batch culture with the activated sludge inoculum at 37 °C (Table S1 in supplementary material), but most of the xylose was already consumed at that point (Fig. 1c).

The low pH likely gave good conditions for the growth of *Sporolactobacillus* sp., a lactic acid-producing mesophilic bacterium growing in the pH range 3.5–5.5, with an optimum of pH 4.5 (Fujita et al., 2010), which was found only in the batch cultures at 37 °C with the digester sludge inoculum. At 37 °C, lactate (about 2 mM) was found only in the fourth batch culture of the digester sludge (Table S1 in supplementary material), when the low pH of 3.5 could have reduced the substrate competition among the H₂ producing microorganisms. In the batch cultures with the activated sludge inoculum, the absence of lactate may indicate a low concentration of *Sporolactobacillus* sp. in the microbial community. This bacterium is likely one of the causes for the low H₂ yield obtained in the fourth batch culture of the digester sludge at 37 °C (Fig. 1b), as part of the electrons were directed to reduce pyruvate to lactate via NADH oxidation instead of reducing protons to molecular H₂. Furthermore, lactic acid bacteria can excrete bacteriocins, which are toxic to other bacteria, including *Clostridium* (Noike et al.,

2002). However, a protein and enzyme–level study is required to assess the inhibitory effect of bacteriocins on H₂ producing bacteria, which is out of the scope of this paper.

At 37 °C, in batch cultures with activated sludge, the H₂ yield per mol of xylose consumed was lower than the one obtained at 55 °C (Fig. 4a). H₂ yields by mesophilic mixed cultures are generally lower than by thermophilic cultures (Table 1), but yields of 2.25 and 2.64 mol H₂ per mol of xylose have been obtained by Lin and Cheng (2006) and Chaganti et al. (2012) at 35 and 37 °C, respectively, using a similar inoculum to the ones used in this study. However, Lin and Cheng (2006) worked at an initial pH of 6.5 and substrate concentration of 124.9 mM, whereas Chaganti et al. (2012) used a statistical approach to optimize several chemical and physical parameters, such as pH, oleic acid concentration and biomass concentration.

4.3. Comparative H₂ production by the activated sludge and the digester sludge at 55 °C

Clostridia species were also detected at 55 °C with both inocula (Table 2) and associated with H₂ production via the acetate and butyrate pathway. *Clostridium thermopalmarium*, found in batch cultures with the digester inoculum at 55 °C, mainly ferments sugars to butyrate, producing H₂, CO₂ and small amounts of acetate, lactate and ethanol (Lawson Anani Soh et al., 1991). At 55 °C, the different activity of *Clostridium* sp. with the activated and the digester sludge can be attributed to the different pH. During the third and fourth batch culture of the digester sludge, as happened at 37 °C, the pH dropped to as low as 3.5 (Fig. 1f), resulting in low xylose degradation. Xylose degradation was low also in the fourth batch culture of the activated sludge, in which the pH dropped below 4.0 (Fig. 1e). *Thermoanaerobacter thermosaccharoliticum*, found at 55 °C with the activated sludge inoculum, has been used to ferment a variety of monomeric sugars, including 33.3 mM xylose (Cao et al., 2014), resulting in the total degradation of the substrate and the production of 1.7 mol H₂ per mol of xylose with acetate and butyrate as the main soluble end products (Cao et al., 2014). However, the initial pH of their experiment was set to 7.0, whereas in this study the initial pH was 5.5. *T. thermosaccharoliticum* effectively produces H₂ from xylose in a pH range 5–7, whereas its H₂ yield dramatically decreases at lower pH values (Ren et al., 2008).

The highest H₂ yield of 1.85 mol H₂ per mol of xylose consumed was obtained in this study during the fourth batch culture of activated sludge at 55 °C (Fig. 4a). This is in line with the results obtained by Calli et al. (2008) who reported a maximum yield of 1.7 mol H₂ per mol xylose at 55 °C (Table 1). Interestingly, even if the compost used as inoculum by Calli et al. (2008) was not pre-treated, methane was not detected, confirming that thermophilic conditions reduce the risk of contamination by methanogens. A similar H₂ yield (1.65 mol H₂ per mol xylose) was obtained at 65 °C with a geothermal spring inoculum (Zeidan and Van Niel, 2009). A slightly higher H₂ yield of 2.07–2.19 mol H₂ per mol of xylose has been reported in thermophilic (60 °C) batch incubations (Table 1) by using a pure culture of *T. thermosaccharoliticum* (Khamtib and Reungsang, 2012; Ren et al., 2008; Zhang et al., 2011). This bacterium may have a significant contribution to the H₂ yield by activated sludge at 55 °C.

4.4. Comparative H₂ production by the activated sludge and the digester sludge at 70 °C

At 70 °C, hyperthermophilic bacteria were found present even in the activated sludge, despite the temperature in Finland seldom exceeds 25 °C in summer. In the wastewater treatment plant where the sludge was collected, the aeration basins are exposed to

ambient temperatures. All the hyperthermophilic species detected after four batch cultures with the activated and digester sludge, including *Caldanaerobius* sp., *Caloramator australicus* and *Thermoanaerobacter* sp., generate H₂ from carbohydrates producing acetate and ethanol as the end product at a pH optimum of 7 or even slightly higher (Lee et al., 2008; Ogg and Patel, 2009; Vipotnik et al., 2016). The low xylose degradation and H₂ yield (Fig. 1), the presence of ethanol and acetate in the medium and the absence of butyrate (Fig. 2) indicate that the bacteria were barely active at the beginning of the batch cultures at 70 °C, when the pH was >5, before being completely inhibited after a further pH decrease.

H₂ production at 70 °C was achieved by Kongjan et al. (2009) and Zhao et al. (2010) with a maximum yield of 1.62 and 1.84 mol H₂ per mol of xylose, respectively, but the experiments were conducted at a higher initial pH and lower substrate concentration compared to this study (Table 1). Furthermore, in both cases, the inoculum was previously enriched for H₂ production at 70 °C.

4.5. H₂ production pathways in the fresh activated sludge inoculum at 55 °C

The linear regression between the H₂ and butyrate yield at 55 °C with the fresh activated sludge inoculum (Fig. 4c) shows a production of approximately 2.4 mol H₂ per mol of butyrate. However, only 2.0 mol H₂ per mol of butyrate is theoretically obtainable (2), suggesting that H₂ was produced also through the acetate pathway (3).



A direct conversion of xylose to acetate, despite being thermodynamically more favorable under thermophilic than mesophilic conditions, is strongly affected by the H₂ partial pressure. At 55 °C, H₂ production through the acetate pathway is thermodynamically feasible only at H₂ partial pressures of far less than 1 kPa, and then the pathway shifts to butyrate production (Verhaart et al., 2010). Based on our calculations done using the ideal gas law (Fig. S1 in supplementary material), 1 kPa was reached during the first 36 h in batch cultures of activated sludge at 55 °C (despite overpressure removal during each sampling). It is, therefore, plausible that H₂ first evolved through the acetate pathway, and then the metabolic pathway shifted to butyrate production due to the accumulation of H₂ in the headspace. This would explain the higher total H₂ yield than the theoretical production through the butyrate pathway. Furthermore, according to Valdez-Vazquez et al. (2006), a H₂ partial pressure of 0.75 atm (74 kPa) or even lower is sufficient to inhibit thermophilic H₂ producing microorganisms. In this study, the highest H₂ partial pressures reached are in the range of 60–85 kPa (Fig. S1 in supplementary material), suggesting that the H₂ partial pressure, as well as low pH, could have negatively affected the process at 55 °C.

Although acetate production followed a similar trend to butyrate (Fig. 4b), no correlation with H₂ yield was found, suggesting that acetate was produced also through other pathways with no H₂ production. The correlation between butyrate and H₂ yield was not at all found at 37 °C (data not shown), probably due to a more diverse microbial community and thus, a wider variety of metabolic pathways.

Ethanol was the main metabolite produced during the first batch culture at 55 °C (Fig. 4b). In the subsequent cultures, its yield decreased while the butyrate and H₂ yield increased. This suggests that butyrate (2) and ethanol (4) production were competitive pathways.



The shift from ethanol to butyrate fermentation can be attributed to either a change in microbial community or a shift in the metabolic pathway of the active microbial species during the four successive batch cultures. The metabolic shift is confirmed by the fact that, in the first batch culture with the activated sludge at 55 °C, gas composition was approximately 65% CO₂ and only 35% H₂ (Fig. S2c in supplementary material), but the share of H₂ constantly increased in the subsequent batch cultures being about 57% of the total gas at the end of third and fourth batch culture.

This study demonstrated that activated sludge can be used as inoculum for thermophilic H₂ production from xylose containing wastewaters. However, a further study with a continuously fed bioreactor is required to evaluate the potential and stability of this process for full-scale applications.

5. Conclusions

- Using heat treated activated sludge as the inoculum, xylose containing wastewaters can be treated at 55 °C obtaining higher H₂ yields than at 37 °C
- The highest H₂ yield of 1.85 mol H₂ per mol of xylose consumed was obtained with activated sludge during the fourth batch culture at 55 °C. At the beginning of every culture, H₂ production was likely associated with the acetate pathway and then shifted towards the butyrate pathway due to the increased H₂ partial pressure
- At 55 °C, ethanol was produced in the first batch culture. In the following cultures, ethanol production steadily decreased while butyrate and H₂ production steadily increased, indicating a clear shift in the xylose degradation pathway towards dark fermentation. This suggests that for non-adapted inocula, a start-up period may be required prior to obtaining high H₂ yields.
- H₂ production at 70 °C was negligible, possibly because the pH was below the optimum for the detected hyperthermophiles present in the inoculum.

Funding

This work was supported by the Marie Skłodowska-Curie European Joint Doctorate (EJD) in Advanced Biological Waste-To-Energy Technologies (ABWET) funded from Horizon 2020 under grant agreement no. 643071.

Acknowledgements

The authors gratefully thank Mira Sulonen (Tampere University of Technology, Finland) for the support with the microbial community analyses, Timo Lepistö (Tampere University of Technology, Finland) for improving the grammar of the manuscript and the Viinikanlahti municipal wastewater treatment plant (Tampere, Finland) for providing the activated and digester sludge.

Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.watres.2017.02.063>.

References

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J., 1990. Basic local alignment search tool. *J. Mol. Biol.* 215, 403–410.

An, D., Li, Q., Wang, X., Yang, H., Guo, L., 2014. Characterization on hydrogen production performance of a newly isolated *Clostridium beijerinckii* YA001 using

xylose. *Int. J. Hydrogen Energy* 39, 19928–19936.

Baghchehsaraee, B., Nakhla, G., Karamanev, D., Margaritis, A., Reid, G., 2008. The effect of heat pretreatment temperature on fermentative hydrogen production using mixed cultures. *Int. J. Hydrogen Energy* 33, 4064–4073.

Bakonyi, P., Borza, B., Orlovits, K., Simon, V., Nemesőthy, N., Bélafi-Bakó, K., 2014. Fermentative hydrogen production by conventionally and unconventionally heat pretreated seed cultures: a comparative assessment. *Int. J. Hydrogen Energy* 39, 5589–5596.

Bundhoo, M.A.Z., Mohee, R., 2016. Inhibition of dark fermentative bio-hydrogen production: a review. *Int. J. Hydrogen Energy* 41, 6713–6733.

Bundhoo, M.A.Z., Mohee, R., Hassan, M.A., 2015. Effects of pre-treatment technologies on dark fermentative biohydrogen production: a review. *J. Environ. Manag.* 157, 20–48.

Calli, B., Schoenmaekers, K., Vanbroekhoven, K., Diels, L., 2008. Dark fermentative H₂ production from xylose and lactose — effects of on-line pH control. *Int. J. Hydrogen Energy* 33, 522–530.

Cao, G.-L., Zhao, L., Wang, A.-J., Wang, Z.-Y., Ren, N.-Q., 2014. Single-step bioconversion of lignocellulose to hydrogen using novel moderately thermophilic bacteria. *Biotechnol. Biofuels* 7, 82.

Cavalcante de Amorim, E.L., Barros, A.R., Rissato Zamarioli Damianovic, M.H., Silva, E.L., 2009. Anaerobic fluidized bed reactor with expanded clay as support for hydrogen production through dark fermentation of glucose. *Int. J. Hydrogen Energy* 34, 783–790.

Chaganti, S.R., Kim, D.H., Lalman, J.A., Shewa, W.A., 2012. Statistical optimization of factors affecting biohydrogen production from xylose fermentation using inhibited mixed anaerobic cultures. *Int. J. Hydrogen Energy* 37, 11710–11718.

Cheng, C.L., Che, P.Y., Chen, B.Y., Lee, W.J., Chien, L.J., Chang, J.S., 2012. High yield biobutanol production by solvent-producing bacterial microflora. *Bioreour. Technol.* 113, 58–64.

De Sá, L.R.V., Cammarota, M.C., De Oliveira, T.C., Oliveira, E.M.M., Matos, A., Ferreira-Leitão, V.S., 2013. Pentoses, hexoses and glycerin as substrates for biohydrogen production: an approach for Brazilian biofuel integration. *Int. J. Hydrogen Energy* 38, 2986–2997.

Dincer, I., Acar, C., 2015. Review and evaluation of hydrogen production methods for better sustainability. *Int. J. Hydrogen Energy* 40, 11094–11111.

DuBois, M., Gilles, K., Hamilton, J.K., Rebers, P., Smith, F., 1956. Colorimetric method for determination of sugars and related substances. *Anal. Chem.* 28, 350–356.

Fujita, R., Mochida, K., Kato, Y., Goto, K., 2010. *Sporolactobacillus putidus* sp. nov., an endospore-forming lactic acid bacterium isolated from spoiled orange juice. *Int. J. Syst. Evol. Microbiol.* (60), 1499–1503.

Grupe, H., Gottschalk, G., 1992. Physiological events in *Clostridium acetobutylicum* during the shift from acidogenesis to solventogenesis in continuous culture and presentation of a model for shift induction. *Appl. Environ. Microbiol.* 58, 3896–3902.

Hall, T.A., 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucl. Acids Symp. Ser.* 41, 95–98.

Hasyim, R., Imai, T., Reungsang, A., O-Thong, S., 2011. Extreme-thermophilic biohydrogen production by an anaerobic heat treated digested sewage sludge culture. *Int. J. Hydrogen Energy* 36, 8727–8734.

Jönsson, L.J., Alriksson, B., Nilvebrant, N.-O., 2013. Bioconversion of lignocellulose: inhibitors and detoxification. *Biotechnol. Biofuels* 6, 16.

Kamali, M., Khodaparast, Z., 2015. Review on recent developments on pulp and paper mill wastewater treatment. *Ecotoxicol. Environ. Saf.* 114, 326–342.

Karadag, D., Puhakka, J.A., 2010. Effect of changing temperature on anaerobic hydrogen production and microbial community composition in an open-mixed culture bioreactor. *Int. J. Hydrogen Energy* 35, 10954–10959.

Khamtib, S., Reungsang, A., 2012. Biohydrogen production from xylose by *Thermoanaerobacterium thermosaccharolyticum* KCU19 isolated from hot spring sediment. *Int. J. Hydrogen Energy* 37, 12219–12228.

Kim, B.H., Zeikus, G.J., 1992. Hydrogen metabolism in *Clostridium acetobutylicum* fermentation. *J. Microbiol. Biotechnol.* 2, 248–254.

Kinnunen, V., Ylä-Outinen, A., Rintala, J., 2015. Mesophilic anaerobic digestion of pulp and paper industry biosludge—long-term reactor performance and effects of thermal pretreatment. *Water Res.* 87, 105–111.

Kongjan, P., Min, B., Angelidaki, I., 2009. Biohydrogen production from xylose at extreme thermophilic temperatures (70 °C) by mixed culture fermentation. *Water Res.* 43, 1414–1424.

Koskinen, P.E.P., Kaksonen, A.H., Puhakka, J.A., 2006. The relationship between instability of H₂ production and compositions of bacterial communities within a dark fermentation fluidized-bed bioreactor. *Biotechnol. Bioeng.* 97, 742–758.

Koskinen, P.E.P., Lay, C.-H., Puhakka, J.A., Lin, P.-J., Wu, S.-Y., Örylgsson, J., Lin, C.-Y., 2008. High-efficiency hydrogen production by an anaerobic, thermophilic enrichment culture from an Icelandic hot spring. *Biotechnol. Bioeng.* 101, 665–678.

Lawson Anani Soh, A., Ralambotiana, H., Ollivier, B., Prensier, G., Tine, E., Garcia, J.-L., 1991. *Clostridium thermopalmarium* sp. nov., a moderately thermophilic butyrate-producing bacterium isolated from palm wine in Senegal. *Syst. Appl. Microbiol.* 14, 135–139.

Lee, D.J., Show, K.Y., Su, A., 2011. Dark fermentation on biohydrogen production: pure culture. *Bioreour. Technol.* 102, 8393–8402.

Lee, Y.J., Mackie, R.L., Cann, I.K.O., Wiegand, J., 2008. Description of *Caldanaerobius fijiensis* gen. nov., sp. nov., an inulin-degrading, ethanol-producing, thermophilic bacterium from a Fijian hot spring sediment, and reclassification of *Thermoanaerobacterium polysaccharolyticum* and *Thermoanaerobacterium zeae* as *Caldanaerobius polysaccharolyticus* comb. nov. and *Caldanaerobius zeae* comb.

- nov. Int. J. Syst. Evol. Microbiol. 58, 666–670.
- Li, C., Fang, H.H.P., 2007. Fermentative hydrogen production from wastewater and solid wastes by mixed cultures. Crit. Rev. Environ. Sci. Technol. 37, 1–39.
- Lin, C.-Y., Cheng, C.-H., 2006. Fermentative hydrogen production from xylose using anaerobic mixed microflora. Int. J. Hydrogen Energy 31, 832–840.
- Lin, C.-Y., Hung, C.-H., Chen, C.-H., Chung, W.-T., Cheng, L.-H., 2006. Effects of initial cultivation pH on fermentative hydrogen production from xylose using natural mixed cultures. Process Biochem. 41, 1383–1390.
- Lin, C.-Y., Wu, C.-C., Wu, J.-H., Chang, F.-Y., 2008. Effect of cultivation temperature on fermentative hydrogen production from xylose by a mixed culture. Biomass Bioenergy 32, 1109–1115.
- Lo, Y.-C., Chen, W.-M., Hung, C.-H., Chen, S.-D., Chang, J.-S., 2008. Dark H₂ fermentation from sucrose and xylose using H₂-producing indigenous bacteria: feasibility and kinetic studies. Water Res. 42, 827–842.
- Logan, B.E., Oh, S.E., Kim, I.S., Van Ginkel, S., 2002. Biological hydrogen production measured in batch anaerobic respirometers. Environ. Sci. Technol. 36, 2530–2535.
- Maintinguer, S.I., Fernandes, B.S., Duarte, I.C.S., Ka, N., Adorno, M.A.T., Varesche, M.B.A., 2011. Fermentative hydrogen production with xylose by *Clostridium* and *Klebsiella* species in anaerobic batch reactors. Int. J. Hydrogen Energy 36, 13508–13517.
- Mäkinen, A.E., Nissilä, M.E., Puhakka, J.A., 2012. Dark fermentative hydrogen production from xylose by a hot spring enrichment culture. Int. J. Hydrogen Energy 37, 12234–12240.
- Nissilä, M.E., Tähti, H.P., Rintala, J.A., Puhakka, J.A., 2011. Thermophilic hydrogen production from cellulose with rumen fluid enrichment cultures: effects of different heat treatments. Int. J. Hydrogen Energy 36, 1482–1490.
- Noike, T., Takabatake, H., Mizuno, O., Ohba, M., 2002. Inhibition of hydrogen fermentation of organic wastes by lactic acid bacteria. Int. J. Hydrogen Energy 27, 1367–1371.
- Ogg, C.D., Patel, B.K.C., 2009. *Caloramator australicus* sp. nov., a thermophilic, anaerobic bacterium from the Great Artesian Basin of Australia. Int. J. Syst. Evol. Microbiol. 59, 95–101.
- Owen, W.F., Stuckey, D.C., Healy Jr., J.B., Young, L.Y., McCarty, P.L., 1979. Bioassay for monitoring biochemical methane potential and anaerobic toxicity. Water Res. 13, 485–492.
- Rajeshwari, K.V., Balakrishnan, M., Kansal, A., Lata, K., Kishore, V.V.N., 2000. State-of-the-art of anaerobic digestion technology for industrial wastewater treatment. Renew. Sustain. Energy Rev. 4, 135–156.
- Ren, N., Cao, G., Wang, A., Lee, D., Guo, W., Zhu, Y., 2008. Dark fermentation of xylose and glucose mix using isolated *Thermoanaerobacterium thermosaccharolyticum* W16. Int. J. Hydrogen Energy 33, 6124–6132.
- Rittmann, S., Herwig, C., 2012. A comprehensive and quantitative review of dark fermentative biohydrogen production. Microb. Cell Fact. 11, 115.
- Seppälä, J.J., Puhakka, J.A., Yli-Harja, O., Karp, M.T., Santala, V., 2011. Fermentative hydrogen production by *Clostridium butyricum* and *Escherichia coli* in pure and cocultures. Int. J. Hydrogen Energy 36, 10701–10708.
- Show, K.Y., Lee, D.J., Tay, J.H., Lin, C.Y., Chang, J.S., 2012. Biohydrogen production: current perspectives and the way forward. Int. J. Hydrogen Energy 37, 15616–15631.
- Slobodkin, A., Reysenbach, A., Mayer, F., Wiegel, J., 1997. Isolation and characterization of the homoacetogenic thermophilic bacterium *Moorella glycerini* sp. nov. Int. J. Syst. Bacteriol. 47, 969–974.
- Suvilampi, J., Lepistö, R., Rintala, J., 2001. Biological treatment of pulp and paper mill process and wastewaters under thermophilic conditions – a review. Pap. Timber 83, 320–325.
- Valdez-Vazquez, I., Rios-Leal, E., Muñoz-Pez, K.M., Poggi-Varaldo, H.M., 2006. Improvement of Biohydrogen production from solid wastes by intermittent venting and gas flushing of batch reactors headspace. Environ. Sci. Technol. 40, 3409–3415.
- Van Ginkel, S., Logan, B.E., 2005. Inhibition of biohydrogen production by undissociated acetic and butyric acids. Environ. Sci. Technol. 39, 9351–9356.
- Van Groenestijn, J.W., Hazewinkel, J.H.O., Nienoord, M., Bussmann, P.J.T., 2002. Energy aspects of biological hydrogen production in high rate bioreactors operated in the thermophilic temperature range. Int. J. Hydrogen Energy 27, 1141–1147.
- Van Niel, E.W.J., Claassen, P.A.M., Stams, A.J.M., 2003. Substrate and product inhibition of hydrogen production by the extreme thermophile, *Caldicellulosiruptor saccharolyticus*. Biotechnol. Bioeng. 81, 255–262.
- Verhaart, M.R., Bielen, A.A., van der Oost, J., Stams, A.J., Kengen, S.W., 2010. Hydrogen production by hyperthermophilic and extremely thermophilic bacteria and archaea: mechanisms for reductant disposal. Environ. Technol. 31, 993–1003.
- Vipotnik, Z., Jessen, J.E., Scully, S.M., Orlygsson, J., 2016. Effect of culture conditions on hydrogen production by *Thermoanaerobacter* strain AK68. Int. J. Hydrogen Energy 41, 181–189.
- Wang, J., Wan, W., 2009. Factors influencing fermentative hydrogen production: a review. Int. J. Hydrogen Energy 34, 799–811.
- Wong, Y.M., Juan, J.C., Ting, A., Wu, T.Y., 2014. High efficiency bio-hydrogen production from glucose revealed in an inoculum of heat-pretreated landfill leachate sludge. Energy 72, 628–635.
- Zeidan, A.A., Van Niel, E.W.J., 2009. Developing a thermophilic hydrogen-producing co-culture for efficient utilization of mixed sugars. Int. J. Hydrogen Energy 34, 4524–4528.
- Zhang, K., Ren, N., Cao, G., Wang, A., 2011. Biohydrogen production behavior of moderately thermophile *Thermoanaerobacterium thermosaccharolyticum* W16 under different gas-phase conditions. Int. J. Hydrogen Energy 36, 14041–14048.
- Zhao, C., Karakashev, D., Lu, W., Wang, H., Angelidaki, I., 2010. Xylose fermentation to biofuels (hydrogen and ethanol) by extreme thermophilic (70 °C) mixed culture. Int. J. Hydrogen Energy 35, 3415–3422.

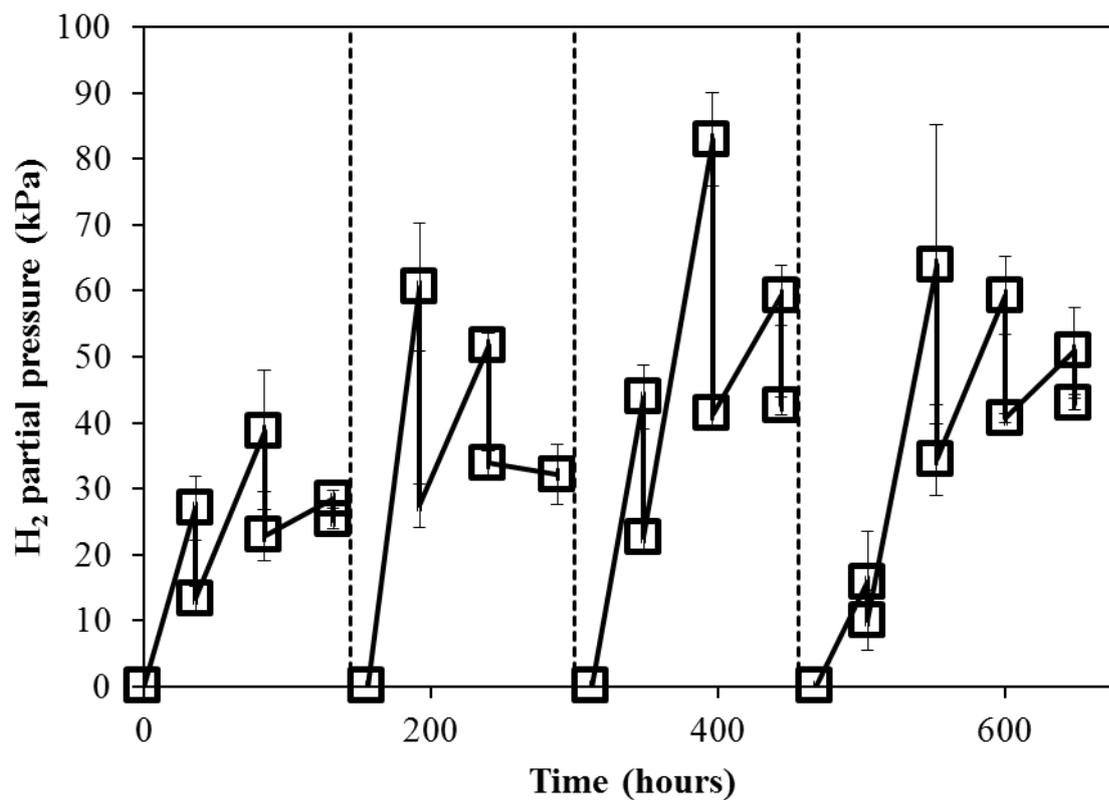


Figure S1. Evolution of H₂ partial pressure with time, assuming equilibrium conditions between the liquid and gas phase. The two points represented for every sampling time refer to the H₂ partial pressure before (the highest) and after (the lower) removing the overpressure. Every point shown in the graphs is calculated as the average of three independent batch cultures, error bars show the standard deviation of the triplicates. The dotted lines refer to the end of a batch culture and start of a new batch culture.

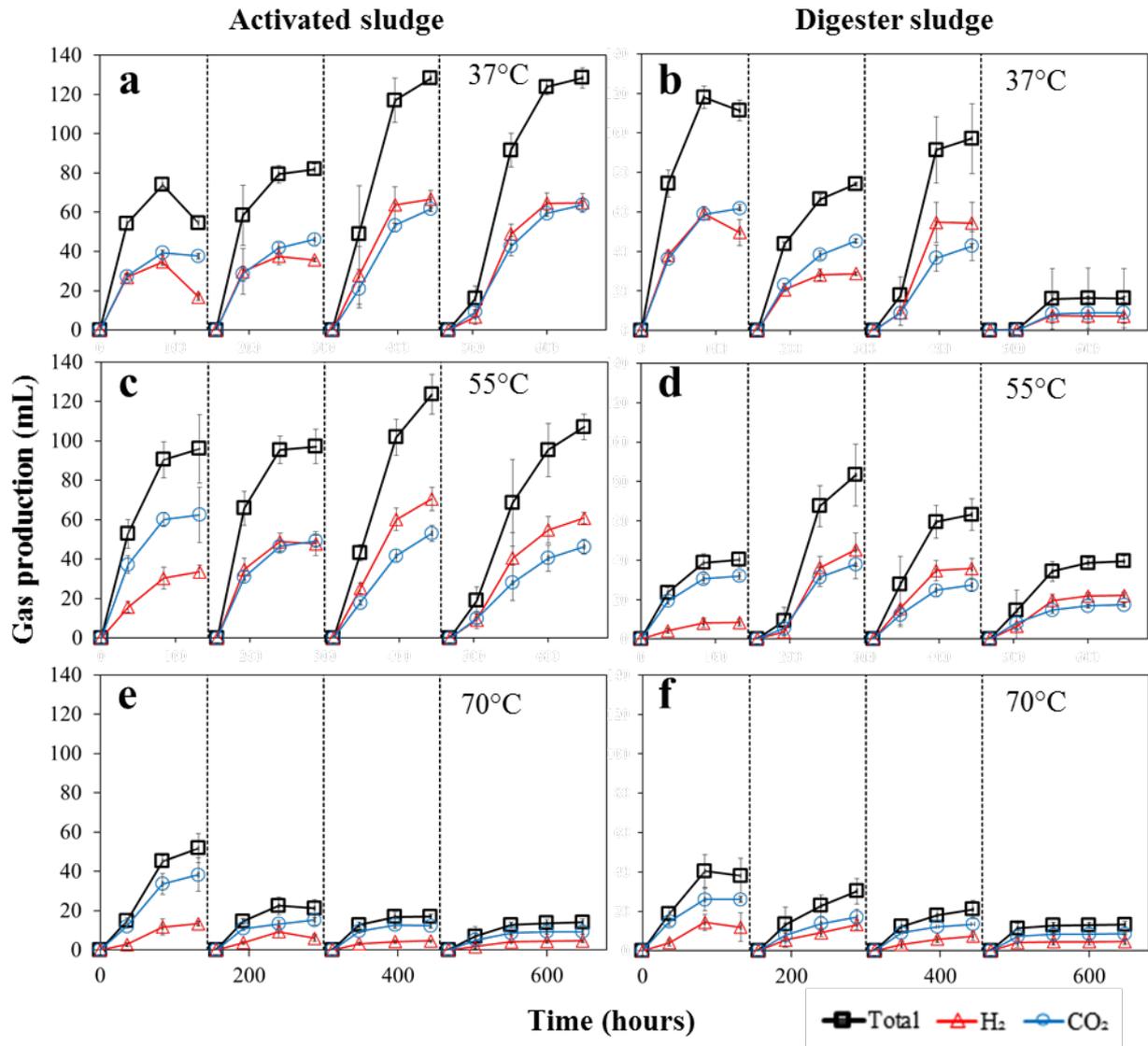


Figure S2. Cumulative gas production obtained with the fresh activated and the digester sludge inoculum. Every point shown in the graphs is calculated as the average of three independent batch cultures, error bars indicate the standard deviation of the triplicates. The dotted lines refer to the end of every batch culture and start of a new culture.

Table S1. Concentration of most abundant metabolites found at the end of the four consecutive batch cultures.

Inoculum	T (°C)	Batch culture	Concentration (mM)			
			Acetate	Butyrate	Ethanol	Lactate
Activated sludge	37	I	21.4 (± 2.2)	30.2 (± 5.2)	1.6 (± 0.3)	-
		II	16.8 (± 4.1)	18.6 (± 3.4)	2.0 (± 0.0)	-
		III	19.5 (± 5.0)	28.0 (± 7.2)	2.1 (± 0.1)	-
		IV	16.8 (± 2.1)	31.0 (± 4.9)	1.6 (± 0.0)	-
	55	I	9.3 (± 4.7)	15.0 (± 4.0)	36.6 (± 8.1)	-
		II	24.2 (± 1.8)	23.0 (± 1.6)	12.8 (± 1.4)	-
		III	27.0 (± 3.4)	29.2 (± 1.9)	3.9 (± 0.3)	-
		IV	17.6 (± 5.6)	22.5 (± 7.3)	1.3 (± 0.3)	-
	70	I	9.7 (± 0.9)	-	30.8 (± 11.3)	15.8 (± 1.2)
		II	9.5 (± 1.4)	-	14.5 (± 4.7)	7.3 (± 0.8)
		III	8.2 (± 0.4)	0.5 (± 0.2)	10.2 (± 1.3)	4.3 (± 0.4)
		IV	6.3 (± 0.1)	-	6.3 (± 1.2)	3.2 (± 0.0)
Digester sludge	37	I	21.6 (± 4.0)	29.5 (± 4.0)	2.2 (± 0.3)	-
		II	18.3 (± 0.8)	16.4 (± 0.9)	1.6 (± 0.1)	-
		III	15.8 (± 1.8)	25.3 (± 4.5)	0.9 (± 0.2)	-
		IV	4.2 (± 2.0)	5.1 (± 3.9)	-	1.7 (± 0.0)
	55	I	8.2 (± 0.8)	3.6 (± 0.3)	20.7 (± 2.5)	24.2 (± 0.7)
		II	8.2 (± 1.5)	17.1 (± 3.9)	3.9 (± 0.4)	-
		III	12.1 (± 1.7)	13.6 (± 0.6)	1.1 (± 0.1)	-
		IV	8.4 (± 0.6)	7.9 (± 0.6)	0.6 (± 0.1)	1.2 (± 0.7)
	70	I	10.7 (± 1.9)	-	12.0 (± 4.1)	11.9 (± 1.3)
		II	11.5 (± 0.6)	-	11.6 (± 2.6)	7.6 (± 0.9)
		III	9.1 (± 1.8)	-	7.8 (± 1.3)	4.1 (± 0.0)
		IV	5.3 (± 0.7)	-	3.4 (± 0.4)	2.1 (± 0.3)

II

**INOCULUM PRETREATMENT DIFFERENTIALLY AFFECTS THE ACTIVE
MICROBIAL COMMUNITY PERFORMING MESOPHILIC AND
THERMOPHILIC DARK FERMENTATION OF XYLOSE**

by

Dessì, P., Porca, E., Frunzo, L., Lakaniemi, A.-M., Collins, G., Esposito, G., Lens, P.N.L.,
2018

International Journal of Hydrogen Energy 43: 9233-9245

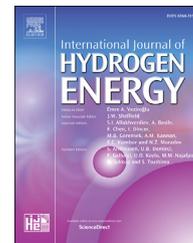
Reproduced with kind permission by Elsevier



ELSEVIER

Available online at www.sciencedirect.com

ScienceDirect

journal homepage: www.elsevier.com/locate/he

Inoculum pretreatment differentially affects the active microbial community performing mesophilic and thermophilic dark fermentation of xylose

Paolo Dessì ^{a,*}, Estefania Porca ^b, Luigi Frunzo ^c, Aino–Maija Lakaniemi ^a, Gavin Collins ^b, Giovanni Esposito ^d, Piet N.L. Lens ^{a,e}

^a Tampere University of Technology, Faculty of Natural Sciences, P.O. Box 541, FI-33101, Tampere, Finland

^b Microbial Communities Laboratory, School of Natural Sciences, National University of Ireland Galway, University Road, Galway, H91 TK33, Ireland

^c Department of Mathematics and Applications “Renato Caccioppoli”, University of Naples Federico II, Via Cintia, Monte S. Angelo, Naples, Italy

^d Department of Civil and Mechanical Engineering, University of Cassino and Southern Lazio, Via di Biasio, 43, Cassino, FR, Italy

^e UNESCO–IHE, Institute for Water Education, Westvest 7, 2611AX, Delft, The Netherlands

ARTICLE INFO

Article history:

Received 13 November 2017

Received in revised form

12 March 2018

Accepted 18 March 2018

Available online 19 April 2018

Keywords:

Biohydrogen

pH shock

Temperature shock

Clostridium

Lactobacillus

MiSeq

ABSTRACT

The influence of different inoculum pretreatments (pH and temperature shocks) on mesophilic (37 °C) and thermophilic (55 °C) dark fermentative H₂ production from xylose (50 mM) and, for the first time, on the composition of the active microbial community was evaluated. At 37 °C, an acidic shock (pH 3, 24 h) resulted in the highest yield of 0.8 mol H₂ mol⁻¹ xylose. The H₂ and butyrate yield correlated with the relative abundance of Clostridiaceae in the mesophilic active microbial community, whereas Lactobacillaceae were the most abundant non-hydrogenic competitors according to RNA-based analysis. At 55 °C, Clostridium and Thermoanaerobacterium were linked to H₂ production, but only an alkaline shock (pH 10, 24 h) repressed lactate production, resulting in the highest yield of 1.2 mol H₂ mol⁻¹ xylose. This study showed that pretreatments differentially affect the structure and productivity of the active mesophilic and thermophilic microbial community developed from an inoculum.

© 2018 Hydrogen Energy Publications LLC. Published by Elsevier Ltd. All rights reserved.

Introduction

The increasing energy demand results in the depletion of fossil fuel reserves and in the emission of enormous quantities of greenhouse gases to the atmosphere. Due to its high

energy content and carbon neutrality, hydrogen (H₂) is a promising green alternative to fossil fuels [1]. Among H₂ production technologies (for a review, see Nikolaidis and Poulkikas [2]), biological methods have the advantage of coupling H₂ generation with the treatment of organic carbon-containing wastes and wastewaters. Biological H₂ production

* Corresponding author.

E-mail address: paolo.dessi@tut.fi (P. Dessì).

<https://doi.org/10.1016/j.ijhydene.2018.03.117>

0360-3199/© 2018 Hydrogen Energy Publications LLC. Published by Elsevier Ltd. All rights reserved.

technologies include photo and dark fermentation, direct and indirect photolysis, and the water gas-shift reaction [3]. The high H₂ production rate, the simple reactor technology (similar to the well-established anaerobic digestion), and the abundance of microorganisms capable to produce H₂ from a wide range of substrates may promote the establishment of dark fermentation in industrial applications [4].

Lignocellulosic materials, including agricultural crops, wood and their processing and waste residues, are among the most abundant raw materials in nature [5]. Such materials are suitable for dark fermentation as their hydrolysis produces monomeric sugars (e.g. hexoses such as glucose and pentoses such as xylose) which can be biologically converted to H₂ [6]. Most of studies on dark fermentation of monomeric sugars have been conducted on glucose, whereas xylose, the second most abundant sugar released during hydrolysis of lignocellulosic biomass [7], has not yet attained much attention.

Promising H₂ yields have been obtained through dark fermentation of monomeric sugars by pure cultures or synthetic co-cultures of H₂ producing microorganisms (for reviews, see Lee et al. [8] and Elsharnouby et al. [9]). Despite the high H₂ yields obtained with pure cultures, a diverse microbial consortium is often required for dark fermentation of more complex substrates, such as waste-derived carbohydrate mixtures. Furthermore, the easier and economic process control as well as the lack of requirements for sterilisation makes mixed cultures more suitable for industrial waste treatment applications than pure cultures [10].

The main drawback of mixed cultures and unsterile conditions is the possible development of microorganisms competing with H₂ producers for the substrates or even H₂ consuming microorganisms in the dark fermentation reactor, which can drastically reduce the overall H₂ yield. Homoacetogenic bacteria and hydrogenotrophic methane producing archaea are the two most common H₂ consuming microorganisms, but also propionate producers as well as sulphate and nitrate reducers use H₂ in their metabolism [3]. Lactate producing bacteria, typically found in dark fermentation systems [11], not only compete with H₂ producers for the substrates, but can even inhibit the growth of H₂ producers by either causing acidification or excreting bacteriocins [12].

Inoculum pretreatment technologies have been widely studied on mixed cultures to select H₂ producing microorganisms at the expense of H₂ consumers. When exposed to harsh conditions, some H₂ producers, such as *Clostridium*, *Bacillus* and *Thermoanaerobacterium*, produce endospores as a defence mechanism [13,14]. This increases their survival chances compared to non-spore-forming H₂ consumers as the spore-formers are able to regeminate once the environmental conditions become favourable. Temperature and pH shocks are the most widely applied pretreatment methods to enhance the share of H₂ producers in a mixed microbial community [15]. High temperature disrupts the cell wall of non-spore-forming microorganisms, causing cell lysis and protein denaturation [16]. Low temperature can cause protein gelling, intracellular formation of ice crystals and membrane lipid stiffening [17]. Acidic and alkaline shocks affect the electric charge of the cell membrane, may inactivate key enzymes, and may cause a change of the intracellular pH, which could lead to cell wall disruption [18]. Other pretreatment

methods include substrate loading shock, chemical treatment (e.g. using 2-bromoethansulphonate acid or chloroform, which inhibit methanogenic archaea), aeration, electric shocks, ionising irradiation, microwaves, or ultrasonication (for a review, see Wang and Yin [15]).

Many studies have compared the effect of the various pretreatments, and their combinations, on mesophilic [19–26] and thermophilic [27,28] dark fermentative H₂ production. However, due to the different inoculum, substrate, and operating conditions, many results appear controversial. Most of the studies compared the various pretreatments in terms of H₂ production, rather than focusing on the microbial community. Analysing the response of the microbial communities to the different inoculum pretreatments is crucial in order to define a strategy for optimization of dark fermentative H₂ production. In a few cases, DNA-based analysis using low sensitivity techniques such as denaturing gradient gel electrophoresis (DGGE) have been applied to detect the dominant species of the microbial community [19–21,25–27]. More novel microbial techniques, such as next generation high-throughput sequencing, likely yield more detailed information on how the different species in the microbial community are affected by various pretreatments. RNA-based approaches provide even more useful information than DNA-based ones [29], enabling detection of the microbial species that remain active after the pretreatment, and thus determination of the species that are involved in H₂ production and possible competitive pathways. Therefore, this study pursues the double aim of (i) finding out how different inoculum pretreatments shape the active microbial communities and (ii) how such microbial communities evolve and produce H₂ during mesophilic (37 °C) and thermophilic (55 °C) dark fermentation of xylose.

Materials and methods

Source and pretreatment of inoculum

Activated sludge was selected as the parent inoculum, because it enabled higher H₂ production from xylose than anaerobic digester sludge from the same wastewater treatment plant in a previous study [30]. The activated sludge used in this study was collected from a secondary settler of a municipal wastewater treatment plant (Mutton Island, Galway, Ireland). It was dewatered by filtration through a 0.1 mm diameter mesh. After filtration, total solids, volatile solids, and pH were 28.1 (±2.4) g L⁻¹, 22.7 (±2.0) g L⁻¹, and 6.9, respectively. The activated sludge was stored at 4 °C for approximately one week prior to being used in the experiment.

Temperature (heat treatment, freezing and thawing) and pH (acidic, alkaline) shocks, widely used to select H₂ producing organisms from several inocula [15], were chosen among the possible pretreatment methods. The heat shock was conducted by exposing the inoculum, collected in thin 15 mL tubes, to 90 °C for 15 min using a pre-heated water bath (Clifton, UK). Freezing and thawing was done by incubating sludge samples at -20 °C for 24 h in thin 15 mL tubes and then defrosting them at 30 °C in a water bath. The acidic shock was given by adjusting the pH of the sludge to 3.0 with HCl,

incubating at room temperature (about 20 °C) for 24 h, and then increasing the pH back to 7.0 with NaOH. The alkaline shock was done by adjusting the pH to 10.0 with NaOH, incubating at room temperature for 24 h, and then decreasing the pH back to 7.0 with HCl. Both HCl and NaOH were used at a concentration of 1 or 3 M. The sludge was continuously stirred by using a magnetic stirrer while adjusting the pH.

Incubation and sampling

Batch cultures were performed in glass serum bottles (120 mL volume), filled with 45 mL of a modified DSMZ medium n.144 containing xylose (50 mM) as carbon source [30], and inoculated with 5 mL of pretreated activated sludge. Anaerobic batch cultures with untreated sludge were also prepared as negative controls. All batch cultures were conducted as triplicates. The initial pH was adjusted to 7.0 with 1 M NaOH, and bottles were flushed with N₂ for 5 min prior to incubation. Thermostatic conditions (37 or 55 °C) and shaking at 60 rpm were provided by shaker incubators (Thermo Scientific, USA). The batch cultures lasted until cumulative H₂ production was not observed for 48 h in any of the triplicate bottles (96–144 h in total).

Gas samples (5 mL) were collected daily, and stored into 5.9 mL EXETAINER[®] hydrogen-tight gas sampling tubes (Labco, UK). Liquid samples were also collected daily and stored at –20 °C in 1.5 mL Eppendorf tubes for analysis. Sludge samples were collected before being inoculated into the serum bottles (0 h samples) and from the bottom of serum bottles at the end of the batch culture at 37 °C or 55 °C. They were then stored at –80 °C in RNA-free 1.5 mL Eppendorf tubes until microbiological analysis.

Microbial community analyses based on 16S cDNA

Microbiological samples were pelleted by centrifugation (10,000 *xg*, 10 min). Nucleic acid co-extraction was done using a method modified from Griffiths et al. [31]. DNA inhibition, cDNA synthesis, high-throughput sequencing (Illumina MiSeq) and bioinformatics analysis of partial 16S rRNA gene sequences using primers 515f and 805r were performed as reported previously [32], but using a more recent version of Mothur (1.39.5) and the Silva database (v128). The total number of sequences was 5,494,444 and they were reduced to 3,294,730 (2,276,495 unique sequences) after a quality check. The hierarchical clustering dendrogram was done using the "Pvclust" package of "R" software [33].

Analytical methods

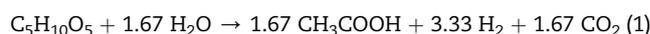
Gas produced was quantified by a syringe method [34] and its composition was determined by gas chromatography as reported previously [30]. Cumulative H₂ and CO₂ production was calculated using a H₂ mass balance equation [35], and corrected to standard temperature (0 °C). Total solids and volatile solids were determined according to the standard methods [36]. pH was measured using a pH meter (WTW inoLab) equipped with a Slimtrode electrode (Hamilton). The composition of the liquid phase (xylose, volatile fatty acids and

alcohols) was determined by high-performance liquid chromatograph (Shimadzu) equipped with a Rezex[™] ROA-Organic Acid H⁺ (8%) column (Phenomenex, USA), held at 70 °C, and a refractive index detector (Shimadzu, Japan). The mobile phase and flow rate were 0.013 N H₂SO₄ and 0.6 mL min⁻¹, respectively.

Calculations

The following H₂ production or consumption pathways were considered the most likely to occur based on their low ΔG⁰ values (Eqs. (1)–(4)) [37]:

Acetate fermentation (ΔG⁰ = –250.8 kJ):



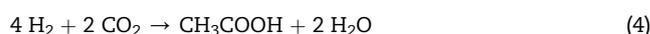
Butyrate fermentation (ΔG⁰ = –307.9 kJ):



Propionate fermentation (ΔG⁰ = –373.7 kJ):



Homoacetogenesis (ΔG⁰ = –75.5 kJ):



The theoretical H₂ yield (mol H₂ mol⁻¹ xylose_{consumed}) was estimated based on the volatile fatty acids detected by HPLC, according to Eqs. (1)–(4), as follows (Eq. (5)) [38]:

$$HY_T = 2*AY + 2*BY - PY \quad (5)$$

where AY, BY and PY are the acetate, butyrate and propionate yield in mol mol⁻¹ xylose_{consumed}, respectively.

The discrepancy value D (mol H₂ mol⁻¹ xylose_{consumed}) was calculated as the difference between the measured (HY_M) and the theoretical (HY_T) H₂ yield (Eq. (6)):

$$D = HY_M - HY_T \quad (6)$$

The contribution of acetate fermentation (AY_H) and homoacetogenesis (AY_A) on the total acetate production (AY = AY_H + AY_A) was estimated by attributing a share of the total measured acetate to the two acetate producing pathways (Eqs. (1) and (4)) in order to minimise D (Eq. (7)):

$$D = HY_M - (2*AY_H + 2*BY - PY - 4 AY_A) \quad (7)$$

Statistical analysis

To assess significant differences in the effect of the tested pretreatments on H₂ yield, one-way analysis of variance (ANOVA) and the Tukey test [39] at p = 0.05 were conducted using the IBM SPSS Statistics package. The output of the statistical analysis is provided in the [supporting material \(File S1\)](#).

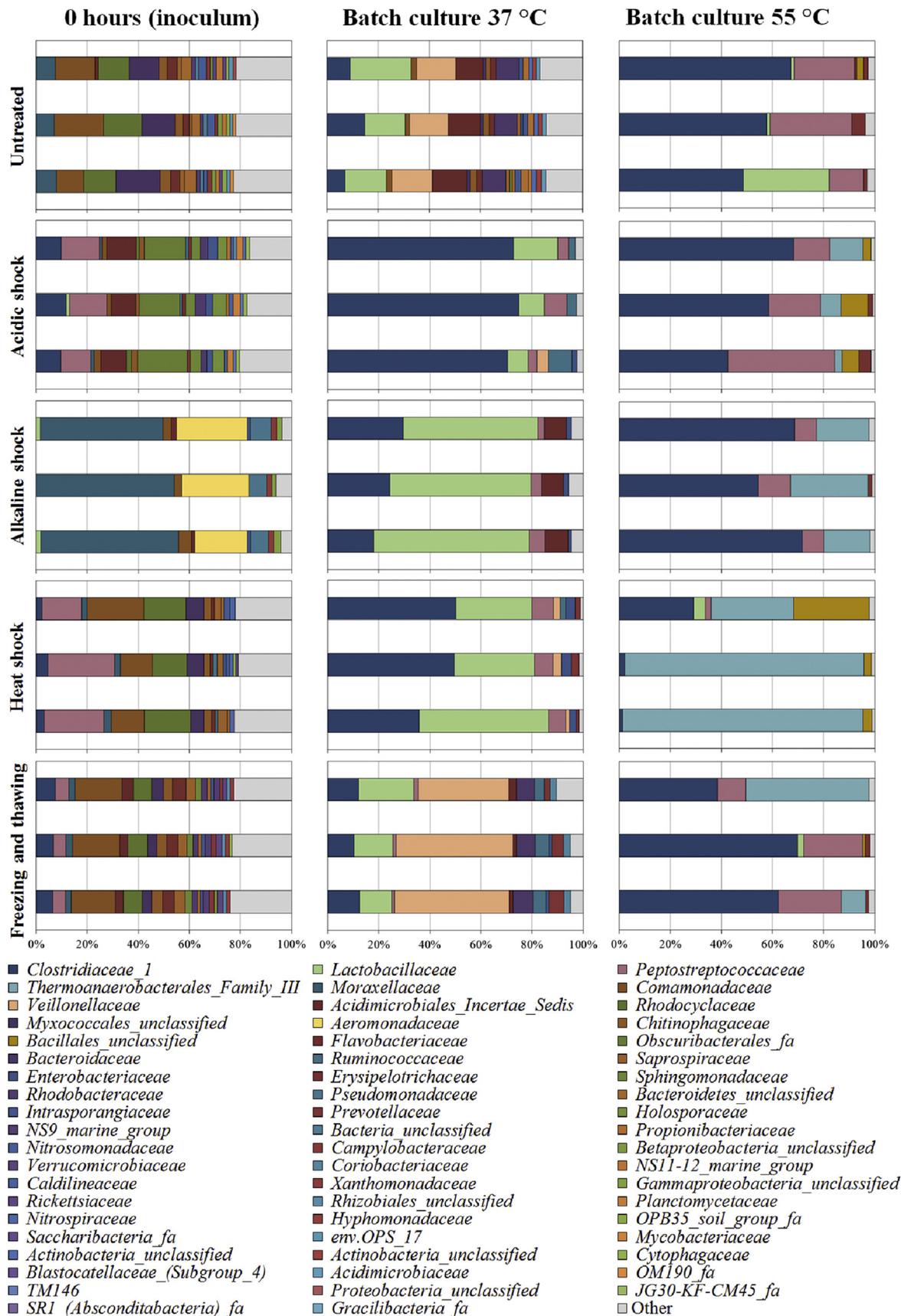


Fig. 1 – Relative abundance of the active families resulting from MiSeq sequencing of the partial 16S rRNA on microbiological samples collected before and after batch cultures with the untreated and pretreated sludge at 37 and 55 °C. Microbial community of each replicate sample is shown separately. “Other” refers to the sum of families with relative abundance <1%.

Results and discussion

Effect of the inoculum pretreatments on the active microbial community

No dominant microorganisms were detected in the untreated activated sludge, which was characterised by a presence of various families of microorganisms with relative abundance below 20% (Fig. 1), mainly belonging to the phylum of *Proteobacteria* and *Bacteroidetes* (Fig. S1).

Despite the aerobic origin of the sludge, heat shock, freezing and thawing, as well as acidic shock favoured the establishment of families of spore forming anaerobic microorganisms such as *Clostridiaceae* and *Peptostreptococcaceae* (Fig. 1). Some non-spore-forming microorganisms, such as *Comamonadaceae* sp. [40], were able to resist the heat shock and the freezing and thawing treatment, with a relative abundance of 12–22% and 17–19%, respectively, but their relative abundance was <5% after the acidic or alkaline shock (Fig. 1).

After the alkaline shock, the active microbial community was very different than the one obtained after the other pretreatments, as shown by the hierarchical clustering dendrogram (Fig. 2). The community was dominated by *Proteobacteria* belonging to the families of *Moraxellaceae* (relative abundance of 48–54%) and *Aeromonadaceae* (21–28%), and more specifically by microorganisms closely related to *Acinetobacter* sp. and *Aeromonas* sp. (Fig. 1; Table 1). Although being non-spore forming [41,42], both *Acinetobacter* and *Aeromonas* survived the alkaline pretreatment, but their relative abundance decreased to <1% after applying the other pretreatments. This suggests that microorganisms are differently affected by the different pretreatments, and that also some non-spore-forming microorganisms could survive the temperature and pH stress applied. The alkaline shock pretreatment repressed more microbial families than the other pretreatments, as confirmed by the low diversity of the active microbial community, according to the diversity indexes (Table 2).

Effect of pretreatments on mesophilic (37 °C) dark fermentation of xylose

Effect of pretreatments on H₂ yield at 37 °C

Based on H₂ yields, the acidic shock was the most effective pretreatment for mesophilic (37 °C) dark fermentation, resulting in a yield of 0.8 mol H₂ mol⁻¹ xylose_{added} (Fig. 3). No statistically significant differences were found between the alkaline and heat shock, which resulted in a yield of 0.5–0.6 mol H₂ mol⁻¹ xylose_{added}, and in both cases H₂ production stopped within 48 h. Freezing and thawing was the least effective pretreatment method, resulting in a yield of only 0.2 mol H₂ mol⁻¹ xylose_{added}, which was similar to the yield obtained with the untreated sludge (Fig. 3).

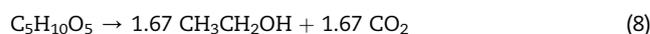
Chang et al. [22] compared various pretreatment methods (including acidic and alkaline shock, heat shock, aeration, and chemical treatment) on waste activated sludge and reported that an acidic shock (pH 3, 24 h) was the most effective pretreatment, yielding 1.5 mol H₂ mol⁻¹ glucose_{consumed} at 35 °C and at an initial pH of 7. Furthermore, Chang et al. [22] reported a 20% increase of the H₂ yield from glucose after five

consecutive mesophilic batch cultures with acidic shock-pretreated activated sludge (without repeating the acidic shock) suggesting that the pretreatment effect can be sustained for extended time periods. An acidic shock was also reported as the most efficient inoculum pretreatment by Liu et al. [25] and Cheong et al. [43] when performing dark fermentation of glucose using marine intertidal sludge and sludge from a cattle manure treatment plant, respectively. The effectiveness of inoculum pretreatment for H₂ production is, nevertheless, case-specific, as it depends on the inoculum, substrate and operating conditions. For example, heat shock (100 °C) of digested sludge resulted in a higher H₂ yield than acidic shock (pH 3) in mesophilic (35–37 °C) dark fermentation of rice and lettuce powder [44] and glucose [45]. Chemical treatment of granular and digested activated sludge with 2-bromoethanesulfonic acid (BESA) was the most effective pretreatment (over heat shock and acidic shock among the others) for dark fermentation of glucose [46]. Pretreatment of cow dung compost by infrared rays resulted in the highest H₂ production from sucrose [47], whereas an electric shock applied to digested sludge promoted H₂ production from glucose [21]. In both cases [21,47], a heat shock resulted in a lower H₂ yield compared to either infrared rays or electric shock.

Effect of pretreatments on the xylose fermentation pathways at 37 °C

In all the mesophilic batch cultures, more than 90% of the xylose was consumed (Fig. 4). Acetate was always produced, regardless of the pretreatment applied, with a final concentration ranging between 10 and 21 mM. In dark fermentation, the acetate pathway yields the highest amount of 3.3 mol H₂ mol⁻¹ xylose (Eq. (1)). However, the H₂ partial pressure may cause a shift of the dark fermentation pathway to butyrate production [48], which yields only 1.67 mol H₂ mol⁻¹ xylose (Eq. (2)). This seems to be also the case in this study, as butyrate was found in all the batch cultures (Fig. 4) with a concentration proportional to the H₂ yield (Fig. 5a). The H₂ yield obtained from the mesophilic batch cultures with the acidic shock-, alkaline shock-, and heat shock-pretreated activated sludge is, however, too high to be entirely associated to the butyrate pathway, which stoichiometrically yields 2 mol H₂ mol⁻¹ butyrate (Eq. (2)). This suggests that H₂ was produced through both the acetate and the butyrate pathway (Table 3).

At 37 °C, ethanol was produced in all cultures regardless of the pretreatment, with a maximum of 16 mM in the batch cultures with the heat shock-pretreated activated sludge (Fig. 4). Solventogenesis can occur as a detoxification process in case of low pH (<5) and accumulation of undissociated volatile fatty acids [49]. In fact, regardless of the pretreatment applied, the pH of the batch medium decreased to below 5.0 after 24–48 h incubation (Fig. 4). According to the ΔG⁰ values [37], the most thermodynamically favourable pathway for biological ethanol production (Eq. (8), ΔG⁰ = -286.7 kJ) does not involve directly H₂, but it decreases the amount of substrate (e.g. xylose) available for H₂ production:



The low pH was likely associated to lactate production. In fact, the highest lactate concentration of 14 and 10 mM was

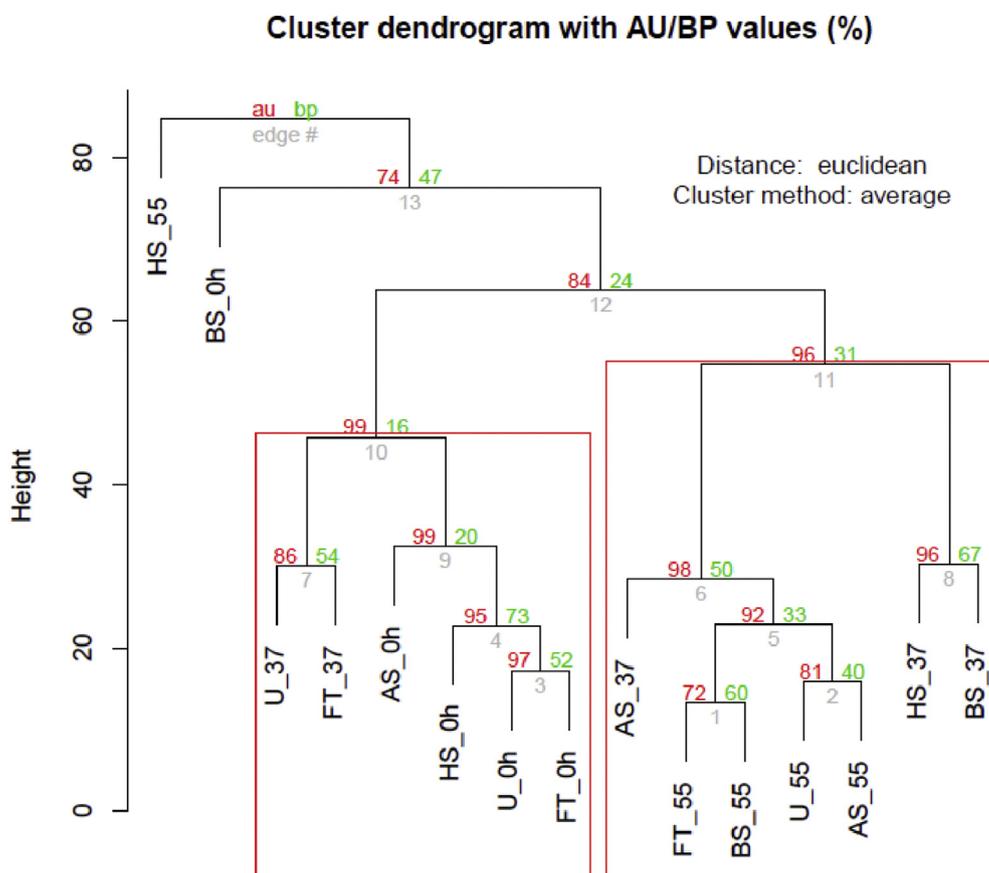


Fig. 2 – Hierarchical clustering of active microbial communities from untreated (U) and pretreated (AS, acidic shock; BS, alkaline shock; HS, heat shock; FT, freezing and thawing) inoculum before (0 h) or after mesophilic (37 °C) or thermophilic (55 °C) dark fermentation of xylose. The values reported are the approximately unbiased (au) p-value, bootstrap probability (bp), and cluster label (edge #). Clusters with au >95% are indicated in the rectangles. The analysis was conducted on the average relative abundance of three replicate samples.

obtained in the batch cultures with the untreated and alkaline shock-pretreated activated sludge, respectively (Fig. 4), resulting in the lowest observed pH of 4.0. Chaganti et al. [23] also reported lactate production from glucose at 37 °C when using untreated and alkaline shock-pretreated (pH 11, 24 h) anaerobic sludge as inoculum. Similarly to ethanol, the most common lactate-yielding biological pathway is independent from H₂ production or consumption (Eq. (9), ΔG⁰ = −56.2 kJ), but it decreases the substrate available for H₂ production:



In all the mesophilic batch cultures, the H₂ yield estimated from the volatile fatty acids produced by dark fermentation of xylose (according to Eqs. (1)–(4)) exceeds the measured H₂ yield (Table 3). This indicates that not all the acetate was produced through the acetate fermentation pathway (Eq. (1)), but also through non-hydrogenic or even H₂ consuming pathways. Homoacetogenesis (Eq. (4)) is one of the most common acetogenic pathways lowering the net H₂ production in dark fermentation [37]. Homoacetogenesis likely occurred in the mesophilic batch cultures regardless of the pretreatment applied, and was estimated to produce 20–35% of the

total amount of acetate (0.04–0.08 mol acetate mol^{−1} xylose_{consumed}) based on stoichiometric calculations (Table 3).

Active microbial communities after batch culture at 37 °C

Cultivation at 37 °C resulted in a lower diversity of the active microbial community from pretreated than untreated activated sludge, according to the diversity indexes (Table 2). Methane was not detected in the mesophilic batch cultures, including the untreated control, suggesting the absence or inactivity of methanogenic archaea. In fact, the relative abundance of active archaea was <1% in all the batch cultures. Similarly, Yin et al. [24] and Chang et al. [22] did not detect any methane in their mesophilic batch cultures with the pretreated and untreated activated sludge. However, Ren et al. [26] reported methanogenic activity in mesophilic batch cultures with alkaline shock-pretreated (pH 11, 24 h) activated sludge.

Based on the data of this study, mesophilic H₂ production correlated reversely with the diversity of the active microbial community (Fig. 5b). All mesophilic batch cultures with pretreated activated sludge were dominated by microorganisms belonging to the phylum *Firmicutes*, present with a relative abundance of 76–98% (Fig. S1). Yin and Wang [50] pretreated anaerobically digested sewage sludge by gamma irradiation,

Table 1 – Association of a representative 16S rRNA gene sequence of the most abundant 12 microbial families obtained in this study (Fig. 1) to those collected in the GenBank.

Family ^a	Genus and species ^b	Accession number	Matching sequence ^c	Similarity (%) ^d
Clostridiaceae	<i>Clostridium</i> sp.	HF566199	458–749	99
Lactobacillaceae	<i>Lactobacillus mucosae</i>	MF425117	490–781	99
Peptostreptococcaceae	<i>Romboutsia ilealis</i>	LN555523	2,071,187–2,071,477	99
Thermoanaerobacteriales Family III	<i>Thermoanaerobacterium thermosaccharolyticum</i>	MF405082	414–705	99
Moraxellaceae	<i>Acinetobacter</i> sp.	KY818302	465–756	99
Comamonadaceae	<i>Acidovorax</i> sp.	LC279183	425–716	99
Veillonellaceae ^e	<i>Megamonas</i> sp.	LT628480	522–812	99
Acidimicrobiales Incertae Sedis	<i>Candidatus Microthrix parvicella</i>	KM052469	91–383	99
Rhodocyclaceae ^f	<i>Dechloromonas</i> sp.	KY029047	486–777	99
Myxococcales unclassified ^g	–	–	–	–
Aeromonadaceae	<i>Aeromonas</i> sp.	MF461171	478–769	100
Chitinophagaceae	<i>Terrimonas arctica</i>	NR_134213	454–745	99

^a The families refer to Fig. 1.

^b Closest cultured species in GenBank.

^c Position (in bp) in which the sequence overlaps the reference sequence.

^d Percentage of identical nucleotide pairs between the 16S rRNA gene sequence and the closest cultured species in GenBank.

^e Classified as *Selenomonadaceae* in the GenBank.

^f Classified as *Azonexaceae* in the GenBank.

^g No match at family, genus and species level.

obtaining >99% of *Firmicutes* in the microbial community after 36 h of mesophilic (33 °C) batch incubation with glucose (94.4 mM) as the substrate for dark fermentation. This suggests that *Firmicutes* are commonly found with high relative abundance among the mesophilic dark fermentative microbial communities, regardless of the pretreatment applied to the inoculum.

The family of *Clostridiaceae* was found regardless of the pretreatment applied, and even in the batch cultures with the untreated activated sludge (Fig. 1). The relative abundance of *Clostridiaceae* had a positive correlation with both the H₂ and butyrate yield (Fig. 5a). In fact, the acidic shock, which was the most effective pretreatment in terms of H₂ yield (Fig. 3), promoted a high relative abundance of *Clostridiaceae*, which accounted for 71–75% of the active microbial community after 144 h incubation at 37 °C (Fig. 1). *Clostridium* sp. have been widely reported to dominate mesophilic dark fermentative microbial communities, both in batch systems and continuous bioreactors [30,51,52]. Liu et al. [25] applied similar pretreatments to those applied in this study to an intertidal marine sediment to perform mesophilic (37 °C) dark fermentation of glucose. They reported similar results to this study in terms of pretreatment efficacy and dominant microbial communities. However, their DNA-level analysis, performed by PCR-DGGE, only allowed the detection of the dominant species present in the system, whereas the RNA-level approach used in this study describes more accurately the active microbial communities involved in the dark fermentative process. It is important to note, however, that despite being the prevalent H₂ producing microorganisms in mesophilic batch cultures, several *Clostridium* sp. are known homoacetogenic microorganisms able to switch their metabolism from H₂ fermentation to homoacetogenesis [37,53].

Lactate producers of the *Lactobacillaceae* family were the main competitors to H₂ producing microorganisms in all

mesophilic batch cultures of this study (Fig. 1). Despite the relatively high H₂ yield, these microorganisms were found even in the batch cultures with the acidic shock-pretreated activated sludge, with a relative abundance of 8–17%. Kim et al. [54] sequenced DNA from the microbial community developed upon mesophilic (35 °C) dark fermentation of acidic shock-pretreated (pH 3, 12 h) food waste, without an external inoculum. They reported a relative abundance of 70% of *Clostridium* and 20% *Lactobacillus*, similar to the relative abundances obtained in this study. Furthermore, Kim et al. [54] reported a higher relative abundance of *Clostridium* (up to 90%) and a higher H₂ yield after performing an acidic shock at pH 2 and 1 for 12 h, suggesting that the pretreatment conditions used in this study can be further optimised to increase the H₂ yield. In contrast, an acidic shock (pH 3, 24 h) of activated sludge has been also reported to inhibit H₂ producing microorganisms and favour the establishment of lactic acid producing bacteria [26]. In the batch cultures with the alkaline shock-pretreated activated sludge, the low final pH of 4.0 (Fig. 4) likely inhibited H₂ producers and favoured lactic acid bacteria, resulting in the highest relative abundance of *Lactobacillaceae* (53–61% of the relative abundance), which produced lactate at the expenses of H₂. Lactic acid bacteria may also inhibit *Clostridium* by excreting toxins [12].

Freezing and thawing resulted in a high relative abundance of *Proteobacteria* (Fig. S1), such as *Veillonellaceae* (36–46%), which were found also in the batch cultures with the untreated sludge (Fig. 1). The representative sequence of *Veillonellaceae* matched the genus *Megamonas* (Table 1), which has been previously reported to produce propionate and acetate from the fermentation of glucose [55]. Both propionate (8 mM) and acetate (10 mM) were indeed detected in the batch cultures with the freezing and thawing-pretreated activated sludge (Fig. 4). Both propionate production and the low relative abundance (12%) of *Clostridium* were likely the main causes for the low H₂ yield obtained in these batch cultures (Fig. 1).

Table 2 – Diversity of the active microbial community (cDNA) after various pretreatments, and after incubation at 37 or 55 °C, measured by the Shannon, Simpson and Pielou's J' index. The number in parenthesis is the standard deviation of the triplicate batch cultures.

Sample ^a	Pretreatment	Shannon diversity	Simpson diversity	J' Evenness
0 h (inoculum)	Untreated	3.10 (±0.06)	0.92 (±0.01)	0.73 (±0.02)
	Acidic shock	3.08 (±0.02)	0.92 (±0.00)	0.72 (±0.01)
	Alkaline shock	1.56 (±0.07)	0.66 (±0.02)	0.41 (±0.01)
	Heat shock	2.86 (±0.02)	0.88 (±0.00)	0.66 (±0.01)
	Freezing and thawing	3.36 (±0.03)	0.94 (±0.00)	0.78 (±0.01)
Batch culture 37 °C	Untreated	2.81 (±0.07)	0.90 (±0.01)	0.66 (±0.02)
	Acidic shock	1.01 (±0.10)	0.45 (±0.03)	0.30 (±0.03)
	Alkaline shock	1.21 (±0.21)	0.56 (±0.06)	0.35 (±0.04)
	Heat shock	1.30 (±0.07)	0.64 (±0.02)	0.46 (±0.04)
	Freezing and thawing	2.02 (±0.11)	0.77 (±0.03)	0.50 (±0.02)
Batch culture 55 °C	Untreated	1.12 (±0.08)	0.56 (±0.06)	0.30 (±0.02)
	Acidic shock	1.14 (±0.11)	0.58 (±0.06)	0.38 (±0.02)
	Alkaline shock	1.10 (±0.20)	0.56 (±0.08)	0.34 (±0.02)
	Heat shock	0.68 (±0.53)	0.31 (±0.29)	0.23 (±0.16)
	Freezing and thawing	1.01 (±0.08)	0.54 (±0.06)	0.30 (±0.03)

^a All samples were subsampled to the size of the smallest sample (8654 sequences).

Effect of pretreatments on thermophilic (55 °C) dark fermentation

Effect of pretreatments on H₂ yield at 55 °C

At 55 °C, the alkaline shock resulted in the highest yield of 1.2 mol H₂ mol⁻¹ xylose_{added}, two times higher than the freezing and thawing pretreatment (Fig. 3), which resulted in

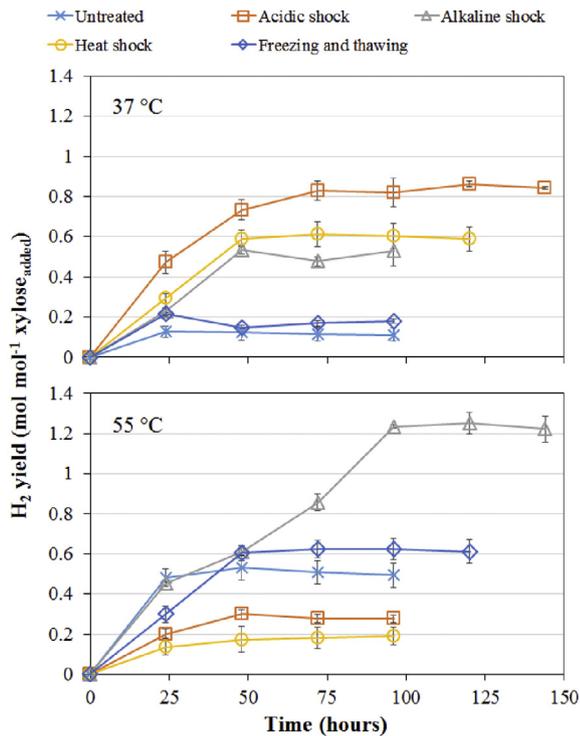


Fig. 3 – H₂ yield from mesophilic (37 °C) and thermophilic (55 °C) dark fermentation of xylose by the untreated or pretreated activated sludge. The results shown are the average of three independent batch cultures. The error bars represent the standard deviation of the triplicate batch cultures.

similar H₂ yield as the untreated activated sludge (0.5–0.6 mol H₂ mol⁻¹ xylose_{added}). Both the acidic shock and heat shock resulted in the lowest H₂ yield of 0.2–0.3 mol H₂ mol⁻¹ xylose_{added}. Interestingly, H₂ was continuously produced for 96 h in the thermophilic batch cultures with the alkaline shock-pretreated activated sludge, whereas it ceased in the first 24–48 h in the other batch cultures. To our knowledge, only two studies have been performed to compare the effect of pretreatments on thermophilic dark fermentation [27,28], and none of them reported alkaline shock as the most efficient pretreatment method. However, in the previous studies, granular anaerobic sludge [28] or anaerobic digester sludge from a palm oil mill plant [27] was used as inoculum, whereas activated sludge treating municipal wastewater was used in this study. O-Thong et al. [27] reported a higher thermophilic (60 °C) H₂ yield from sucrose (1.96 mol H₂ mol⁻¹ hexose) with loading shock-pretreated (2 days incubation with 83.25 g_{COD} L⁻¹ sucrose) than acidic shock-, alkaline shock-, chemical treatment-, and heat shock-pretreated anaerobic sludge. Luo et al. [28] obtained a higher thermophilic (60 °C) H₂ yield from cassava stillage by untreated than pretreated anaerobic granular sludge. This suggests that, depending on the origin of the inoculum, thermophilic conditions can be sufficient to favour H₂ producing bacteria over competitors. Luo et al. [28] also claimed that the effect of pretreatments was not significant in the long term, as the H₂ yield was similar after 28 days of continuous dark fermentation of the cassava stillage regardless the pretreatment applied.

Effect of pretreatments on the xylose oxidation pathways at 55 °C

Thermophilic batch cultures with the untreated and alkaline shock- or freezing and thawing-pretreated activated sludge resulted in a higher (>90%) xylose consumption than batch cultures with the acidic shock- (75%) and heat shock- (66%) pretreated activated sludge (Fig. 4). Interestingly, the alkaline shock was the only pretreatment which suppressed lactate production, as most of the xylose was converted to acetate and butyrate up to a final concentration of 6 and 13 mM, respectively

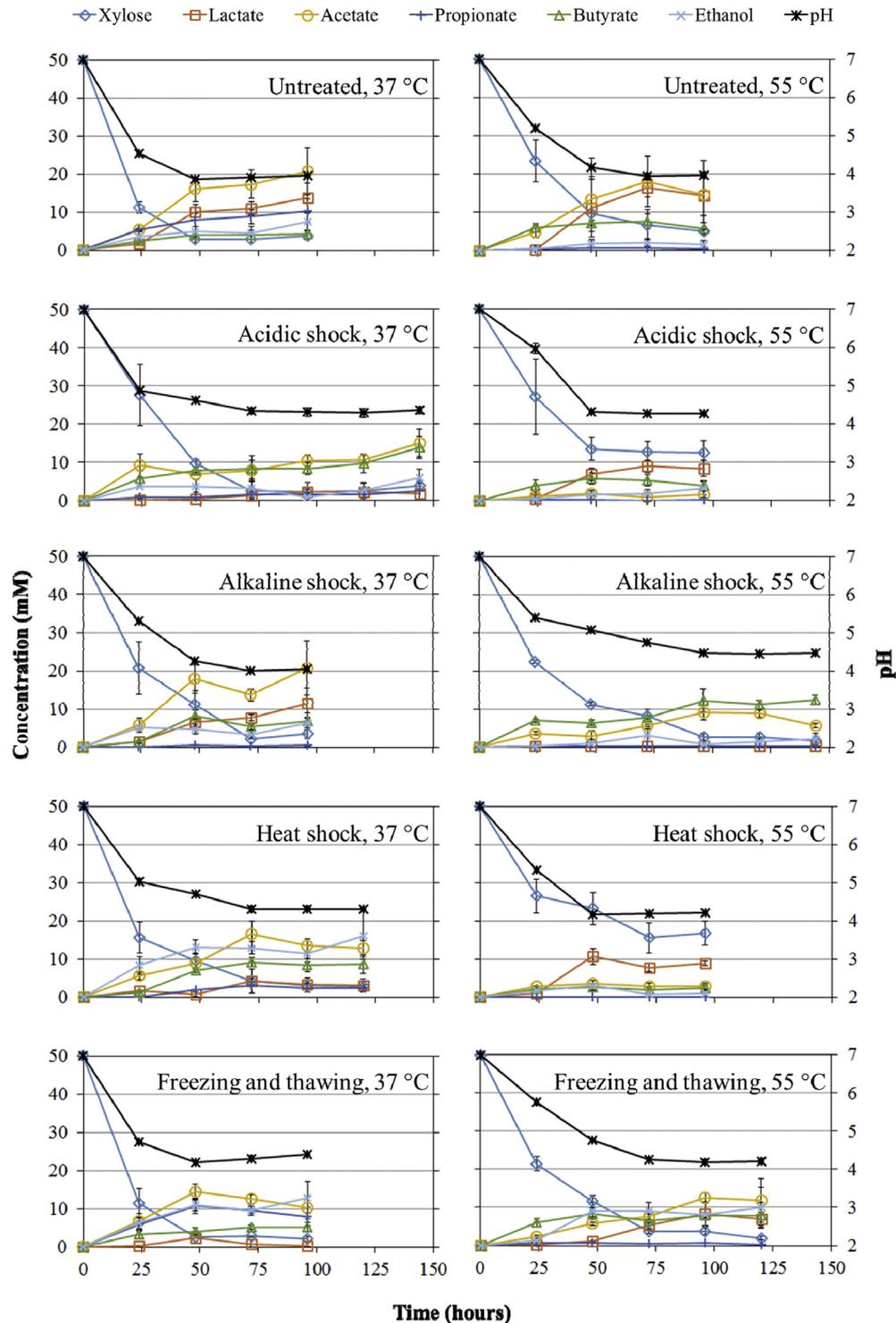


Fig. 4 – Xylose, metabolite concentration and pH profiles from mesophilic (37 °C) and thermophilic (55 °C) dark fermentation of xylose by the untreated or pretreated activated sludge. The results shown are the average of three independent batch cultures. The error bars represent the standard deviation of the triplicate batch cultures.

(Fig. 4). This was likely favoured by the pH decrease at a lower rate than in the other batch cultures, resulting in the highest final pH (4.4), which could have favoured H₂ production through the acetate and butyrate pathways.

In all the thermophilic batch cultures, with the exception of the untreated activated sludge, the measured H₂ yield was

higher than the yield estimated from volatile fatty acids production (Table 3). This suggests a minor (or even negligible) contribution of homoacetogenesis on the total acetate production in the thermophilic batch cultures of pretreated activated sludge (Table 3), as well as the occurrence of unexpected H₂ producing pathways. This is particularly evident in the batch

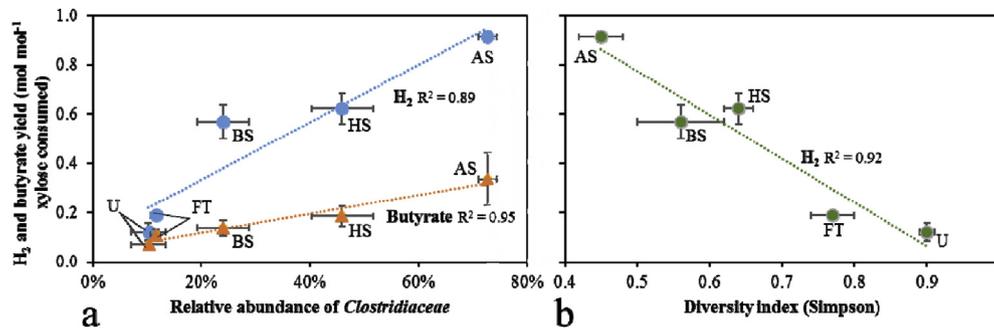


Fig. 5 – Correlation between the relative abundance of active *Clostridiaceae* and H₂ or butyrate yield (a) and between the diversity index (Simpson) and the H₂ yield (b) after mesophilic (37 °C) dark fermentation of xylose by the untreated (U) and pretreated (AS, acidic shock; BS, alkaline shock; HS, heat shock; FT, freezing and thawing) inoculum.

cultures of alkaline shock-pretreated activated sludge, in which the measured yield of 1.3 mol H₂ mol⁻¹ xylose_{consumed} was 30% higher than the estimated yield of 0.8 mol H₂ mol⁻¹ xylose_{consumed} (Table 3). H₂ overproduction has been previously reported in thermophilic dark fermentation [56], and attributed to an unusual H₂ production pathway, such as acetate oxidation. However, in the batch cultures with alkaline-shock pretreated activated sludge, the acetate concentration decreased from 9 to 6 mM only in the last 48 h of incubation (Fig. 4), while the cumulative H₂ yield remained stable during that time (Fig. 3), suggesting that acetate oxidation did not occur. Also Zheng et al. [57] obtained an average of 14% H₂ overproduction from glucose by anaerobic sludge and excluded the acetate oxidation pathway by use of an isotope feeding assay with ¹³C-labeled acetate. Zheng et al. [57] concluded that an unknown H₂ producing metabolic pathway was likely taking place in the initial stages, requiring further investigation.

In the thermophilic batch cultures with the freezing and thawing-pretreated inoculum, lactate (8 mM) and ethanol (10 mM) were produced together with acetate (12 mM) and butyrate (8 mM), suggesting that none of the metabolic pathways was prevailing over the other. In the batch cultures with untreated activated sludge, xylose was initially fermented to acetate and butyrate, which both reached a concentration of 5–6 mM after 24 h incubation. Then, a switch from the butyrate to the lactate pathway occurred, and lactate was produced together with acetate in the subsequent 48 h, resulting in a final concentration of 15 mM lactate and acetate (Fig. 4). In the batch cultures with the acidic shock- and heat shock-pretreated sludge, lactate was the main metabolite, with a concentration of 8 and 9 mM, respectively (Fig. 4), resulting in the lowest H₂ yield (Fig. 3). Lactate production resulted in a fast pH decrease: the final pH was 4.2–4.3 in the batch cultures with the acidic shock-, heat shock- and freezing and thawing-pretreated activated sludge, and even lower (3.9) in the batch cultures with the untreated activated sludge, in which lactate production was the highest (Fig. 4). Such a low pH likely inhibited thermophilic H₂ production [58].

Active microbial communities after batch culture at 55 °C

After incubation at 55 °C, regardless of the pretreatment applied, >98% of the active microbial community was

composed of microorganisms of the phylum *Firmicutes* (Fig. S1). In general, the thermophilic active microbial communities were less diverse than the mesophilic ones (Table 2). This is in agreement with Qiu et al. [59] who reported a lower diversity of the microbial community when performing dark fermentation of xylose at 55 than 37 °C. The active microbial communities from thermophilic batch cultures with the untreated and acidic shock-, alkaline shock-, and freezing and thawing-pretreated activated sludge, were all similar, and dominated by the families of *Clostridiaceae* and *Peptostreptococcaceae* (Fig. 1).

A different active microbial community developed in the thermophilic batch culture with the heat shock-pretreated activated sludge (Fig. 1), in which a family of *Thermoanaerobacteriales* (Fig. 1), closely related to *T. thermosaccharolyticum* (Table 1), was prevailing with a relative abundance up to 94%. Similarly, O-Thong et al. (2009) compared various pretreatments on thermophilic (60 °C) dark fermentation of sucrose in batch experiments, and reported the predominance of *T. thermosaccharolyticum* from heat shock-pretreated anaerobic sludge, whereas *Clostridium* became dominant from both acidic and alkaline shock-pretreated anaerobic sludge. The family of *Clostridiaceae* includes thermophilic species, such as *C. thermosaccharolyticum* [60] and *C. thermopalmarium* [61], able to convert sugars to H₂, acetate and butyrate. *T. thermosaccharolyticum* effectively produces H₂ from xylose at 55–60 °C and in the pH range 5.5–7.0 [62]. In the batch cultures with the heat shock-pretreated activated sludge of this study, *T. thermosaccharolyticum* was thus likely inhibited by the low pH, which decreased to <4.5 within 48 h (Fig. 4), resulting in a low H₂ yield (Fig. 3) and poor xylose utilization (Fig. 4).

Bacillales, found in the thermophilic batch cultures with the heat shock- (3–30%) and acidic shock- (3–11%) pretreated activated sludge (Fig. 1) might be responsible for lactate production. In fact, the order of *Bacillales* includes lactic acid producers such as *Sporolactobacillus*, a spore-forming acidophilic microorganism previously reported in thermophilic dark fermentation of sugars [63].

Practical implications

The start-up strategy applied, including selection and pretreatment of the inoculum, influences the H₂ production even for the long-term operation of continuous dark fermentative

Table 3 – Comparison between the measured H₂ yield and the yield estimated stoichiometrically from the volatile fatty acids produced in the batch cultures of untreated and pretreated activated sludge at 37 or 55 °C. The contribution of acetate fermentation and homoacetogenesis was also estimated in order to minimise the discrepancy between measured and estimated H₂ yields. All values in the table are reported in mol mol⁻¹ xylose_{consumed}, and represent average values of the triplicate batch cultures.

T (°C)	Pretreatment	Volatile fatty acid yield			H ₂ production or consumption (mol H ₂ mol ⁻¹ xylose consumed)		H ₂ yield		Discrepancy	Estimated contribution of different pathways on total acetate production		
		Acetate	Butyrate	Propionate	Linked to acetate	Linked to butyrate	Linked to propionate	Estimated		Measured	Acetate fermentation	Homoacetogenesis
37	Untreated	0.32	0.07	0.16	0.63	0.14	-0.16	0.61	0.12	0.49	0.23	0.08
	Acidic shock	0.31	0.34	0.06	0.62	0.67	-0.06	1.24	0.91	0.33	0.26	0.05
	Alkaline shock	0.39	0.14	0.01	0.77	0.28	-0.01	1.04	0.57	0.47	0.31	0.08
	Heat shock	0.27	0.18	0.05	0.54	0.37	-0.05	0.86	0.62	0.24	0.23	0.04
	Freezing and thawing	0.22	0.11	0.17	0.43	0.22	-0.17	0.48	0.19	0.30	0.17	0.05
55	Untreated	0.32	0.13	0.01	0.65	0.26	-0.01	0.89	0.55	0.34	0.27	0.06
	Acidic shock	0.05	0.11	0.00	0.09	0.21	0.00	0.30	0.37	-0.07	0.05	0.00
	Alkaline shock	0.12	0.26	0.01	0.24	0.52	-0.01	0.75	1.27	-0.52	0.12	0.00
	Heat shock	0.06	0.05	0.00	0.13	0.11	0.00	0.23	0.29	-0.06	0.06	0.00
	Freezing and thawing	0.15	0.11	0.01	0.31	0.21	-0.01	0.51	0.64	-0.13	0.15	0.00

bioreactors [64]. The results of this study indicate that an acidic shock may help in limiting, but not fully eliminating, lactate production in mesophilic dark fermentation. At 55 °C, lactate production was suppressed by an alkaline shock, which is thus a suitable pretreatment for starting-up thermophilic H₂ producing bioreactors. Inoculum pretreatment appears necessary for mesophilic dark fermentation, whereas in thermophilic bioreactors less invasive strategies, such as finding a suitable pH, organic loading rate (OLR) and hydraulic retention time (HRT) can be used to select H₂ producing microorganisms at the expense of competing microorganisms [65]. Optimal pH, OLR and HRT values are, nevertheless, case-specific as they depend on the microbial community present in the inoculum.

After selection of the pretreatment method, the H₂ yield can be further improved by optimising the pretreatment conditions (e.g. pH, temperature, and exposure time) [54,66]. In addition, it should be noted that, even if a low H₂ yield is obtained in a batch culture, an efficient H₂ producing microbial community may develop in the long term. For example, Dessì et al. [30] reported a 320% H₂ yield increase when performing four consecutive thermophilic (55 °C) batch cultures of heat shock-pretreated (90 °C, 15 min) activated sludge due to a metabolic shift from the ethanol to the butyrate pathway. Therefore, to assess the potential of a pretreated inoculum for dark fermentation, additional long-term experiments need to accompany the initial screening assays.

Conclusions

A RNA-level approach was used for the first time to evaluate the impact of four different inoculum pretreatment methods on mesophilic and thermophilic dark fermentation of xylose. H₂ production at 37 °C depended on the relative abundance of *Clostridiaceae* in the microbial community, and an acidic shock favoured their establishment. At 55 °C, an alkaline shock was the most effective pretreatment method for favouring the establishment of H₂ producing microorganisms at the expense of competitors. This study supports the selection of start-up strategies in order to obtain a high and stable H₂ yield in continuous dark fermentative bioreactors.

Funding

This work was supported by the Marie Skłodowska-Curie European Joint Doctorate (EJD) in Advanced Biological Waste-To-Energy Technologies (ABWET) funded from Horizon 2020 under grant agreement no. 643071.

Acknowledgements

The authors gratefully thank Leena Ojanen (Tampere University of Technology, Finland) for analysing the gas samples, Camilla Thorn (National University of Ireland Galway, Ireland) for helping with the microbial community analysis, and the Mutton island wastewater treatment plant (Galway, Ireland) for providing the activated sludge inoculum.

Appendix A. Supplementary data

Supplementary data related to this article can be found at <https://doi.org/10.1016/j.ijhydene.2018.03.117>.

REFERENCES

- [1] Palomo-Briones R, Razo-Flores E, Bernet N, Trably E. Dark-fermentative biohydrogen pathways and microbial networks in continuous stirred tank reactors: novel insights on their control. *Appl Energy* 2017;198:77–87.
- [2] Nikolaidis P, Poullikkas A. A comparative overview of hydrogen production processes. *Renew Sustain Energy Rev* 2017;67:597–611.
- [3] Bundhoo MAZ, Mohee R. Inhibition of dark fermentative biohydrogen production: a review. *Int J Hydrogen Energy* 2016;41:6713–33.
- [4] Arimi MM, Knodel J, Kiprop A, Namango SS, Zhang Y, Geißen S-U. Strategies for improvement of biohydrogen production from organic-rich wastewater: a review. *Biomass Bioenergy* 2015;75:101–18.
- [5] Kumar G, Bakonyi P, Periyasamy S, Kim SH, Nemestóthy N, Bélafi-Bakó K. Lignocellulose biohydrogen: practical challenges and recent progress. *Renew Sustain Energy Rev* 2015;44:728–37.
- [6] Das D, Khanna N, Dasgupta CN. *Biohydrogen production: fundamentals and technology advances*. CRC Press; 2014.
- [7] Li W, Li M, Zheng L, Liu Y, Zhang Y, Yu Z, et al. Simultaneous utilization of glucose and xylose for lipid accumulation in black soldier fly. *Biotechnol Biofuels* 2015;8:117.
- [8] Lee DJ, Show KY, Su A. Dark fermentation on biohydrogen production: pure culture. *Bioresour Technol* 2011;102:8393–402.
- [9] Elsharnouby O, Hafez H, Nakhla G, El Naggar MH. A critical literature review on biohydrogen production by pure cultures. *Int J Hydrogen Energy* 2013;38:4945–66.
- [10] Li C, Fang HHP. Fermentative hydrogen production from wastewater and solid wastes by mixed cultures. *Crit Rev Environ Sci Technol* 2007;37:1–39.
- [11] Etchebehere C, Castelló E, Wenzel J, Del Pilar Anzola-Rojas M, Borzacconi L, Buitrón G, et al. Microbial communities from 20 different hydrogen-producing reactors studied by 454 pyrosequencing. *Appl Microbiol Biotechnol* 2016;100:3371–84.
- [12] Noike T, Takabatake H, Mizuno O, Ohba M. Inhibition of hydrogen fermentation of organic wastes by lactic acid bacteria. *Int J Hydrogen Energy* 2002;27:1367–71.
- [13] Collins MD, Lawson PA, Willems A, Cordoba JJ, Fernandez-Garayzabal J, Garcia P, et al. The phylogeny of the genus *Clostridium*: proposal of five new genera and eleven new species combinations. *Int J Syst Bacteriol* 1994;44:812–26.
- [14] Galperin MY. Genome diversity of spore-forming Firmicutes. *Microbiol Spectr* 2013;1:1–27.
- [15] Wang J, Yin Y. Principle and application of different pretreatment methods for enriching hydrogen-producing bacteria from mixed cultures. *Int J Hydrogen Energy* 2017;42:4804–23.
- [16] Appels L, Baeyens J, Degève J, Dewil R. Principles and potential of the anaerobic digestion of waste-activated sludge. *Prog Energy Combust Sci* 2008;34:755–81.
- [17] Sawicka JE, Robador A, Hubert C, Jørgensen BB, Brüchert V. Effects of freeze–thaw cycles on anaerobic microbial processes in an Arctic intertidal mud flat. *ISME J* 2010;4:585–94.
- [18] Rafeenia R, Lavagnolo MC, Pivato A. Pre-treatment technologies for dark fermentative hydrogen production: current advances and future directions. *Waste Manag* 2017. <https://doi.org/10.1016/j.wasman.2017.05.024>.
- [19] Misturini Rossi D, Berne da Costa J, Aquino de Souza E, Ruaro Peralba M do C, Samios D, Záchia Ayub MA. Comparison of different pretreatment methods for hydrogen production using environmental microbial consortia on residual glycerol from biodiesel. *Int J Hydrogen Energy* 2011;36:4814–9.
- [20] Guo L, Lu M, Li Q, Zhang J, She Z. A comparison of different pretreatments on hydrogen fermentation from waste sludge by fluorescence excitation-emission matrix with regional integration analysis. *Int J Hydrogen Energy* 2015;40:197–208.
- [21] Jeong D-Y, Cho S-K, Shin H-S, Jung K-W. Application of an electric field for pretreatment of a seeding source for dark fermentative hydrogen production. *Bioresour Technol* 2013;139:393–6.
- [22] Chang S, Li J-Z, Liu F. Evaluation of different pretreatment methods for preparing hydrogen-producing seed inocula from waste activated sludge. *Renew Energy* 2011;36:1517–22.
- [23] Chaganti SR, Kim D-H, Lalman JA. Dark fermentative hydrogen production by mixed anaerobic cultures: effect of inoculum treatment methods on hydrogen yield. *Renew Energy* 2012;48:117–21.
- [24] Yin Y, Hu J, Wang J. Enriching hydrogen-producing bacteria from digested sludge by different pretreatment methods. *Int J Hydrogen Energy* 2014;39:13550–6.
- [25] Liu H, Wang G, Zhu D, Pan G. Enrichment of the hydrogen-producing microbial community from marine intertidal sludge by different pretreatment methods. *Int J Hydrogen Energy* 2009;34:9696–701.
- [26] Ren N, Guo W-Q, Wang X-J, Xiang W-S, Liu B-F, Wang X-Z, et al. Effects of different pretreatment methods on fermentation types and dominant bacteria for hydrogen production. *Int J Hydrogen Energy* 2008;33:4318–24.
- [27] O-Thong S, Prasertsan P, Birkeland N. Evaluation of methods for preparing hydrogen-producing seed inocula under thermophilic condition by process performance and microbial community analysis. *Bioresour Technol* 2009;100:909–18.
- [28] Luo G, Xie L, Zou Z, Wang W, Zhou Q. Evaluation of pretreatment methods on mixed inoculum for both batch and continuous thermophilic biohydrogen production from cassava stillage. *Bioresour Technol* 2010;101:959–64.
- [29] De Vrieze J, Regueiro L, Props R, Vilchez-Vargas R, Jáuregui R, Pieper DH, et al. Presence does not imply activity: DNA and RNA patterns differ in response to salt perturbation in anaerobic digestion. *Biotechnol Biofuels* 2016;9:244.
- [30] Dessì P, Lakaniemi A-M, Lens PNL. Biohydrogen production from xylose by fresh and digested activated sludge at 37, 55 and 70 °C. *Water Res* 2017;115:120–9.
- [31] Griffiths RI, Whiteley AS, O'Donnell AG, Bailey MJ. Rapid method for coextraction of DNA and RNA from natural environments for analysis of ribosomal DNA- and rRNA-based microbial community composition. *Appl Environ Microbiol* 2000;66:5488–91.
- [32] Dessì P, Porca E, Waters NR, Lakaniemi A-M, Collins G, Lens PNL. Thermophilic versus mesophilic dark fermentation in xylose-fed fluidised bed reactors: biohydrogen production and active microbial community. *Int J Hydrogen Energy* 2018;43:5473–85.
- [33] Suzuki R, Shimodaira H. Pvclust: an R package for assessing the uncertainty in hierarchical clustering. *Bioinformatics* 2006;22:1540–2.
- [34] Owen WF, Stuckey DC, Healy Jr JB, Young LY, McCarty PL. Bioassay for monitoring biochemical methane potential and anaerobic toxicity. *Water Res* 1979;13:485–92.
- [35] Logan BE, Oh S-E, Kim IS, Van Ginkel S. Biological hydrogen production measured in batch anaerobic respirometers. *Environ Sci Technol* 2002;36:2530–5.

- [36] APHA. Standard methods for the examination of water and wastewater. 20th ed. Washington DC: American Public Health Association/American Water Works Association/Water Environment Federation; 1998.
- [37] Saady NMC. Homoacetogenesis during hydrogen production by mixed cultures dark fermentation: unresolved challenge. *Int J Hydrogen Energy* 2013;38:13172–91.
- [38] Bakonyi P, Buitrón G, Valdez-Vazquez I, Nemestóthy N, Bélafi-Bakó K. A novel gas separation integrated membrane bioreactor to evaluate the impact of self-generated biogas recycling on continuous hydrogen fermentation. *Appl Energy* 2017;190:813–23.
- [39] Box GEP, Hunter WG, Hunter JS. Statistics for experimenters: an introduction to design, data analysis, and model building. John Wiley and sons; 1978.
- [40] Willems A, De Ley J, Gillis M, Kersters K. *Comamonadaceae*, a new family encompassing the *Acidovorans* rRNA complex, including *Variovorax paradoxus* gen. nov., comb. nov., for *Alcaligenes paradoxus* (Davis 1969). *Int J Syst Evol Microbiol* 1991;41:445–50.
- [41] Bouvet PJM, Grimont PAD. Taxonomy of the genus *Acinetobacter* with the recognition of *Acinetobacter baumannii* sp. nov., *Acinetobacter haemolyticus* sp. nov., *Acinetobacter johnsonii* sp. nov., and *Acinetobacter junii* sp. nov. and emended descriptions of *Acinetobacter calcoaceticus* and *Acinetobacter lwoffii*. *Int J Syst Bacteriol* 1986;36:228–40.
- [42] Alperi A, Martínez-Murcia AJ, Ko W-C, Monera A, Saavedra MJ, Figueras MJ. *Aeromonas taiwanensis* sp. nov. and *Aeromonas sanarellii* sp. nov., clinical species from Taiwan. *Int J Syst Evol Microbiol* 2010;60:2048–55.
- [43] Cheong D-Y, Hansen CL. Bacterial stress enrichment enhances anaerobic hydrogen production in cattle manure sludge. *Appl Microbiol Biotechnol* 2006;72:635–43.
- [44] Dong L, Zhenhong Y, Yongming S, Longlong M. Evaluation of pretreatment methods on harvesting hydrogen producing seeds from anaerobic digested organic fraction of municipal solid waste (OFMSW). *Int J Hydrogen Energy* 2010;35:8234–40.
- [45] Wang J, Wan W. Comparison of different pretreatment methods for enriching hydrogen-producing bacteria from digested sludge. *Int J Hydrogen Energy* 2008;33:2934–41.
- [46] Pendyala B, Chaganti SR, Lalman JA, Shanmugam SR, Heath DD, Lau PCK. Pretreating mixed anaerobic communities from different sources: correlating the hydrogen yield with hydrogenase activity and microbial diversity. *Int J Hydrogen Energy* 2012;37:12175–86.
- [47] Song Z, Dai Y, Fan Q, Li X, Fan Y, Hou H. Effects of pretreatment method of natural bacteria source on microbial community and bio-hydrogen production by dark fermentation. *Int J Hydrogen Energy* 2012;37:5631–6.
- [48] Angenent LT, Karim K, Al-Dahhan MH, Wrenn BA, Domínguez-Espinosa R. Production of bioenergy and biochemicals from industrial and agricultural wastewater. *Trends Biotechnol* 2004;22:477–85.
- [49] Van Ginkel S, Logan BE. Inhibition of Biohydrogen production by undissociated acetic and butyric acids. *Environ Sci Technol* 2005;39:9351–6.
- [50] Yin Y, Wang J. Changes in microbial community during biohydrogen production using gamma irradiated sludge as inoculum. *Bioresour Technol* 2016;200:217–22.
- [51] Si B, Liu Z, Zhang Y, Li J, Xing X-H, Li B, et al. Effect of reaction mode on biohydrogen production and its microbial diversity. *Int J Hydrogen Energy* 2015;40:3191–200.
- [52] Sivagurunathan P, Kumar G, Park J-H, Park J-H, Park H-D, Yoon J-J, et al. Feasibility of enriched mixed cultures obtained by repeated batch transfer in continuous hydrogen fermentation. *Int J Hydrogen Energy* 2016;41:4393–403.
- [53] Ryan P, Forbes C, Collieran E. Investigation of the diversity of homoacetogenic bacteria in mesophilic and thermophilic anaerobic sludges using the formyltetrahydrofolate synthetase gene. *Water Sci Technol* 2008;57:675–80.
- [54] Kim D-H, Jang S, Yun Y-M, Lee M-K, Moon C, Kang W-S, et al. Effect of acid-pretreatment on hydrogen fermentation of food waste: microbial community analysis by next generation sequencing. *Int J Hydrogen Energy* 2014;39:16302–9.
- [55] Chevrot R, Carlotti A, Sopena V, Marchand P, Rosenfeld E. *Megamonas rupellensis* sp. nov., an anaerobe isolated from the caecum of a duck. *Int J Syst Evol Microbiol* 2008;58:2921–4.
- [56] Kotsopoulos TA, Zeng RJ, Angelidaki I. Biohydrogen production in granular up-flow anaerobic sludge blanket (UASB) reactors with mixed cultures under hyper-thermophilic temperature (70°C). *Biotechnol Bioeng* 2006;94:296–302.
- [57] Zheng H, Zeng RJ, O'Sullivan C, Clarke WP. Critical analysis of hydrogen production from mixed culture fermentation under thermophilic condition (60 °C). *Appl Microbiol Biotechnol* 2016;100:5165–76.
- [58] Zhang T, Liu H, Fang HHP. Biohydrogen production from starch in wastewater under thermophilic condition. *J Environ Manag* 2003;69:149–56.
- [59] Qiu C, Zheng Y, Zheng J, Liu Y, Xie C, Sun L. Mesophilic and thermophilic biohydrogen production from xylose at various initial pH and substrate concentrations with microflora community analysis. *Energy Fuels* 2016;30:1013–9.
- [60] Islam S, Zhang C, Sui K, Guo C, Liu C. Coproduction of hydrogen and volatile fatty acid via thermophilic fermentation of sweet sorghum stalk from co-culture of *Clostridium thermocellum* and *Clostridium thermosaccharolyticum*. *Int J Hydrogen Energy* 2016;42:830–7.
- [61] Lawson Anani Soh A, Ralambotiana H, Ollivier B, Prensier G, Tine E, Garcia J-L. *Clostridium thermopalmarium* sp. nov., a moderately thermophilic butyrate-producing bacterium isolated from palm wine in Senegal. *Syst Appl Microbiol* 1991;14:135–9.
- [62] Ren N, Cao G, Wang A, Lee D, Guo W, Zhu Y. Dark fermentation of xylose and glucose mix using isolated *Thermoanaerobacterium thermosaccharolyticum* W16. *Int J Hydrogen Energy* 2008;33:6124–32.
- [63] Abreu AA, Karakashev D, Angelidaki I, Sousa DZ, Alves MM. Biohydrogen production from arabinose and glucose using extreme thermophilic anaerobic mixed cultures. *Biotechnol Biofuels* 2012;5:6.
- [64] Bakonyi P, Nemestóthy N, Simon V, Bélafi-Bakó K. Review on the start-up experiences of continuous fermentative hydrogen producing bioreactors. *Renew Sustain Energy Rev* 2014;40:806–13.
- [65] Sivagurunathan P, Kumar G, Bakonyi P, Kim SH, Kobayashi T, Xu KQ, et al. A critical review on issues and overcoming strategies for the enhancement of dark fermentative hydrogen production in continuous systems. *Int J Hydrogen Energy* 2016;41:3820–36.
- [66] Bakonyi P, Borza B, Orlovits K, Simon V, Nemestóthy N, Bélafi-Bakó K. Fermentative hydrogen production by conventionally and unconventionally heat pretreated seed cultures: a comparative assessment. *Int J Hydrogen Energy* 2014;39:5589–96.

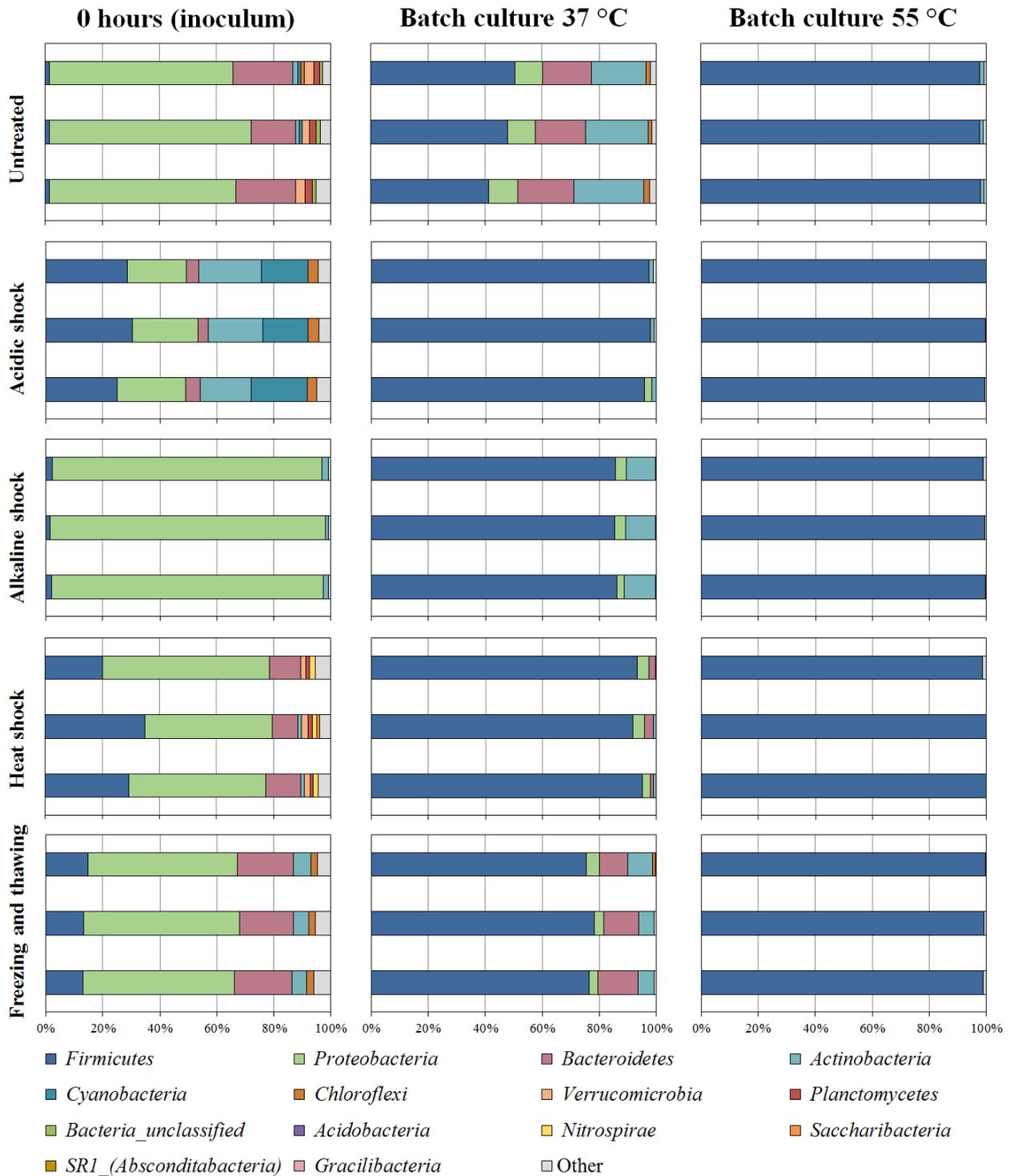


Figure S1. Relative abundance of the active phyla resulting from MiSeq sequencing of the partial 16S rRNA on microbiological samples collected before and after batch cultures with the untreated and pretreated activated sludge at 37 and 55 °C. The results are reported in triplicate. “Other” refers to the sum of phyla with relative abundance < 1%.

File S1. Statistical analysis (ANOVA and Tukey test) conducted at $p = 0.05$ to assess significant differences in H_2 yield from xylose in mesophilic (37 °C) and thermophilic (55 °C) batch cultures of untreated or pretreated (acidic shock, alkaline shock, heat shock, and freezing and thawing) activated sludge.

Batch cultures 37 °C

Descriptives

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean (p=0.05)		Minimum	Maximum
					Lower Bound	Upper Bound		
Untreated	3	0.1100	0.04000	0.02309	0.0106	0.2094	0.07	0.15
Acidic shock	3	0.8400	0.01000	0.00577	0.8152	0.8648	0.83	0.85
Alkaline shock	3	0.5333	0.09074	0.05239	0.3079	0.7587	0.45	0.63
Heat shock	3	0.5900	0.07000	0.04041	0.4161	0.7639	0.51	0.64
Freezing and thawing	3	0.1800	0.01000	0.00577	0.1552	0.2048	0.17	0.19
Total	15	0.4507	0.28427	0.07340	0.2932	0.6081	0.07	0.85

ANOVA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1.101	4	0.275	92.195	0.000
Within Groups	0.030	10	0.003		
Total	1.131	14			

Multiple Comparisons

Dependent Variable: H_2 yield 37 °C

Tukey HSD

(I) Pretreat	(J) Pretreat	Mean Difference	Std. Error	p-value	95% Confidence Interval (p=0.05)	
		(I-J)			Lower Bound	Upper Bound
Untreated	Acidic shock	-0.73000*	0.04462	0.000	-0.8769	-0.5831
	Alkaline shock	-0.42333*	0.04462	0.000	-0.5702	-0.2765
	Heat shock	-0.48000*	0.04462	0.000	-0.6269	-0.3331
	Freezing and thawing	-0.07000	0.04462	0.546	-0.2169	0.0769

Acidic shock	Untreated	0.73000*	0.04462	0.000	0.5831	0.8769
	Alkaline shock	0.30667*	0.04462	0.000	0.1598	0.4535
	Heat shock	0.25000*	0.04462	0.002	0.1031	0.3969
	Freezing and thawing	0.66000*	0.04462	0.000	0.5131	0.8069
Alkaline shock	Untreated	0.42333*	0.04462	0.000	0.2765	0.5702
	Acidic shock	-0.30667*	0.04462	0.000	-0.4535	-0.1598
	Heat treatment	-0.05667	0.04462	0.714	-0.2035	0.0902
	Freezing and thawing	0.35333*	0.04462	0.000	0.2065	0.5002
Heat shock	Untreated	0.48000*	0.04462	0.000	0.3331	0.6269
	Acidic shock	-0.25000*	0.04462	0.002	-0.3969	-0.1031
	Alkaline shock	0.05667	0.04462	0.714	-0.0902	0.2035
	Freezing and thawing	0.41000*	0.04462	0.000	0.2631	0.5569
Freezing and thawing	Untreated	0.07000	0.04462	0.546	-0.0769	0.2169
	Acidic shock	-0.66000*	0.04462	0.000	-0.8069	-0.5131
	Alkaline shock	-0.35333*	0.04462	0.000	-0.5002	-0.2065
	Heat treatment	-0.41000*	0.04462	0.000	-0.5569	-0.2631

*. The mean difference is significant at the 0.05 level if p-value is < 0.05

Tukey HSD^a

Pretreat	N	Subset for α^b = 0.05		
		1	2	3
Untreated	3	0.1100		
Freezing and thawing	3	0.1800		
Alkaline shock	3		0.5333	
Heat shock	3		0.5900	
Acidic shock	3			0.8400
Sig.		0.546	0.714	1.000

Means for groups in homogeneous subsets are displayed.

- Uses Harmonic Mean Sample Size = 3.000.
- Threshold value.

Acidic shock > Heat shock ~ Alkaline shock > Freezing and thawing ~ Untreated

Batch cultures 55 °C

Descriptives

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean (p=0.05)		Minimum	Maximum
					Lower Bound	Upper Bound		
Untreated	3	0.4967	0.07371	0.04256	0.3136	0.6798	0.44	0.58
Acidic shock	3	0.2767	0.02309	0.01333	0.2193	0.3340	0.25	0.29
Alkaline shock	3	1.2233	0.07572	0.04372	1.0352	1.4114	1.17	1.31
Heat shock	3	0.1900	0.05000	0.02887	0.0658	0.3142	0.14	0.24
Freezing and thawing	3	0.6133	0.07371	0.04256	0.4302	0.7964	0.53	0.67
Total	15	0.5600	0.38092	0.09835	0.3491	0.7709	0.14	1.31

ANOVA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1.992	4	0.498	126.834	0.000
Within Groups	0.039	10	0.004		
Total	2.031	14			

Multiple Comparisons

Dependent Variable: H₂ yield 55 °C

Tukey HSD

(I) Pretreat	(J) Pretreat	Mean Difference (I-J)	Std. Error	p- value	95% Confidence Interval	
					Lower Bound	Upper Bound
Untreated	Acidic shock	0.22000*	0.05116	0.011	0.0516	0.3884
	Alkaline shock	-0.72667*	0.05116	0.000	-0.8951	-0.5583
	Heat shock	0.30667*	0.05116	0.001	0.1383	0.4751
	Freezing and thawing	-0.11667	0.05116	0.228	-0.2851	0.0517
Acidic shock	Untreated	-0.22000*	0.05116	0.011	-0.3884	-0.0516
	Alkaline shock	-0.94667*	0.05116	0.000	-1.1151	-0.7783
	Heat shock	0.08667	0.05116	0.478	-0.0817	0.2551
	Freezing and thawing	-0.33667*	0.05116	0.000	-0.5051	-0.1683
Untreated	Untreated	0.72667*	0.05116	0.000	0.5583	0.8951

Alkaline shock	Acidic shock	0.94667*	0.05116	0.000	0.7783	1.1151
	Heat shock	1.03333*	0.05116	0.000	0.8649	1.2017
	Freezing and thawing	0.61000*	0.05116	0.000	0.4416	0.7784
Heat shock	Untreated	-0.30667*	0.05116	0.001	-0.4751	-0.1383
	Acidic shock	-0.08667	0.05116	0.478	-0.2551	0.0817
	Alkaline shock	-1.03333*	0.05116	0.000	-1.2017	-0.8649
	Freezing and thawing	-0.42333*	0.05116	0.000	-0.5917	-0.2549
Freezing and thawing	Untreated	0.11667	0.05116	0.228	-0.0517	0.2851
	Acidic shock	0.33667*	0.05116	0.000	0.1683	0.5051
	Alkaline shock	-0.61000*	0.05116	0.000	-0.7784	-0.4416
	Heat shock	0.42333*	0.05116	0.000	0.2549	0.5917

*. The mean difference is significant at the 0.05 level if p-value is < 0.05

Tukey HSD^a

Pretreat	N	Subset for α^b = 0.05		
		1	2	3
Heat shock	3	0.1900		
Acidic shock	3	0.2767		
Untreated	3		0.4967	
Freezing and thawing	3		0.6133	
Alkaline shock	3			1.2233
Sig.		0.478	0.228	1.000

Means for groups in homogeneous subsets are displayed.

- Uses Harmonic Mean Sample Size = 3,000.
- Threshold value.

Alkaline shock > Freezing and thawing ~ Untreated > Acidic shock ~ Heat shock



**THERMOPHILIC VERSUS MESOPHILIC DARK FERMENTATION
IN XYLOSE-FED FLUIDISED BED REACTORS: BIOHYDROGEN
PRODUCTION AND ACTIVE MICROBIAL COMMUNITY**

by

Dessì, P., Porca, E., Waters, N.R., Lakaniemi, A.-M., Collins, G., Lens, P.N.L.,
2018

International Journal of Hydrogen Energy 43:5473-5485

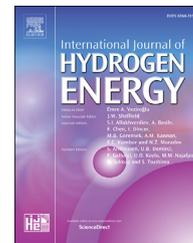
Reproduced with kind permission by Elsevier



ELSEVIER

Available online at www.sciencedirect.com

ScienceDirect

journal homepage: www.elsevier.com/locate/he

Thermophilic versus mesophilic dark fermentation in xylose-fed fluidised bed reactors: Biohydrogen production and active microbial community

Paolo Dessì ^{a,*}, Estefania Porca ^b, Nicholas R. Waters ^{b,c},
Aino-Maija Lakaniemi ^a, Gavin Collins ^b, Piet N.L. Lens ^{a,d}

^a Tampere University of Technology, Faculty of Natural Sciences, P.O. Box 541, FI-33101, Tampere, Finland

^b Microbial Communities Laboratory, School of Natural Sciences, National University of Ireland Galway, University Road, Galway, H91 TK33, Ireland

^c Information and Computational Sciences, The James Hutton Institute, Invergowrie, Dundee, DD2 5DA, Scotland, UK

^d UNESCO-IHE, Institute for Water Education, Westvest 7, 2611AX, Delft, The Netherlands

ARTICLE INFO

Article history:

Received 4 September 2017

Received in revised form

21 December 2017

Accepted 26 January 2018

Available online 2 March 2018

Keywords:

Active community

Biohydrogen

FBR

MiSeq

Thermoanaerobacterium

Thermophilic

ABSTRACT

Dark fermentative biohydrogen production in a thermophilic, xylose-fed (50 mM) fluidised bed reactor (FBR) was evaluated in the temperature range 55–70 °C with 5-degree increments and compared with a mesophilic FBR operated constantly at 37 °C. A significantly higher ($p = 0.05$) H₂ yield was obtained in the thermophilic FBR, which stabilised at about 1.2 mol H₂ mol⁻¹ xylose (36% of the theoretical maximum) at 55 and 70 °C, and at 0.8 mol H₂ mol⁻¹ xylose at 60 and 65 °C, compared to the mesophilic FBR (0.5 mol H₂ mol⁻¹ xylose). High-throughput sequencing of the reverse-transcribed 16S rRNA, done for the first time on biohydrogen producing reactors, indicated that *Thermoanaerobacterium* was the prevalent active microorganism in the thermophilic FBR, regardless of the operating temperature. The active microbial community in the mesophilic FBR was mainly composed of *Clostridium* and *Ruminiclostridium* at 37 °C. Thermophilic dark fermentation was shown to be suitable for treatment of high temperature, xylose-containing wastewaters, as it resulted in a higher energy output compared to the mesophilic counterpart.

© 2018 Hydrogen Energy Publications LLC. Published by Elsevier Ltd. All rights reserved.

Introduction

H₂ is a carbon free fuel considered as a promising candidate to replace fossil fuels in the near future [1]. Although hydrocarbons are currently the main feedstock for H₂ production, biomass is a renewable and environmentally friendly alternative feedstock [2]. Dark fermentation is the most studied among the biological H₂ production technologies because the

variety of usable organic substrates and the high achievable conversion rates may promote the scale-up of the process [3]. However, due to the thermodynamics of the reactions involved, which are more favourable at high temperature and low H₂ partial pressure, operation and optimisation of full-scale dark fermentation is more challenging than traditional anaerobic digestion [4].

Many pathways are possible for dark fermentation, depending on the microbial species, operating parameters,

* Corresponding author.

E-mail address: paolo.dessi@tut.fi (P. Dessì).

<https://doi.org/10.1016/j.ijhydene.2018.01.158>

0360-3199/© 2018 Hydrogen Energy Publications LLC. Published by Elsevier Ltd. All rights reserved.

and the substrate used. Glycolysis is the most common route for degradation of hexoses by *Clostridium* [5] and most thermophiles, including *Thermoanaerobacter* [6]. Glucose is oxidized to pyruvate, resulting in the generation of reduced nicotinamide adenine dinucleotide (NADH) and energy in the form of adenosine triphosphate [7]. Pyruvate may be further oxidized to acetylcoenzyme A through reduction of ferredoxin and then to volatile fatty acids (VFAs), or alcohols [7]. Metalloenzymes called hydrogenases use NADH or reduced ferredoxin as electron donor for proton oxidation [8], resulting in the formation of molecular H₂. The oxidation of glucose to H₂ and CO₂ yields 12 mol H₂ mol⁻¹ glucose. However, the dehydrogenation of acetate to CO₂ is endergonic, and the spontaneous oxidative process will thus end with acetate production, yielding only 4 mol H₂ mol⁻¹ glucose [9]. This thermodynamic limitation is also valid for pentose sugars such as xylose, which will then yield a maximum of 3.33 mol H₂ mol⁻¹ xylose.

In practice, the H₂ yield by mixed cultures varies from 14% to 70% of the theoretical limit [10]. For high H₂ partial pressures (>60 Pa), proton reduction by NADH is not thermodynamically favourable and the reaction switches, e.g. to the butyrate pathway [11], resulting in a lower H₂ yield. This can be mitigated by operating dark fermentation in well mixed systems, such as fluidised bed reactors (FBRs), in which the turbulent mixing regime favours the stripping of the produced H₂ [12]. Temperature and pH also strongly affect the microbial community and thus, the substrate degradation pathway [13]. Butyrate accumulation can trigger solventogenesis [14], which does not yield H₂. Some microorganisms, including various *Clostridium* sp., are facultative autotrophic and can reduce CO₂ with H₂ forming acetate [10]. Other known H₂-consuming microorganisms include hydrogenotrophic methanogens, propionate producers, and sulphate or nitrate reducing microorganisms [15]. Enhancing the growth of H₂ producers while avoiding the growth of H₂ consuming microorganisms in dark fermentative bioreactors is still an open challenge [16].

H₂ production at high temperatures can be advantageous in terms of H₂ yield and production rates [17,18]. High temperature positively affects the kinetics of the oxidative reactions and the growth of microorganisms [19]. Furthermore, the direct conversion of sugars to acetate becomes thermodynamically more favourable as the temperature increases, thus resulting in a high H₂ yield [20]. Thermophilic anaerobic microorganisms such as *Thermotoga* and *Thermoanaerobacterium* are excellent H₂ producers, as they use most of the reductants produced during glycolysis to form H₂, allowing yields between 3 and 4 mol H₂ mol⁻¹ hexose [20]. Although H₂ yields from pure cultures are typically higher [5], mixed cultures are preferable for industrial application, as they offer more stability and versatility, and sterilisation is not required [13]. Most studies on H₂ production at high temperature by mixed cultures have been conducted at 55, 60, or 70 °C, generally obtaining higher H₂ yields than in mesophilic trials with a similar inoculum and substrate [18,21,22]. Sources of inoculum used for thermophilic dark fermentation include e.g. sewage sludge [18,23], anaerobic sludge or digestates [21,24–28], animal dung or slurry [29,30], hot spring sediment [31,32], and biomass from previous laboratory-scale H₂ production experiments [33–39].

Handling and processing of organic substrates and the low H₂ yield are two of the main deterrents for the establishment of dark fermentation at commercial scale [40]. Despite the higher H₂ yield obtained at high temperatures, the net energy gain (the difference between the energy input needed to heat the reactor and output) seems to be indirectly proportional to the operation temperature [41]. However, some industrial wastewaters, such as thermomechanical pulping wastewaters, are produced at high (50–70 °C) temperatures [42] and could be treated on site avoiding cooling and minimising energy loss. Such wastewaters contain readily fermentable sugars, both hexoses (e.g. glucose) and pentoses (e.g. xylose), suitable for H₂ production by dark fermentation. Continuous dark fermentation of glucose has been widely studied at various temperatures [35,37,39,43,44] while much less attention was given to dark fermentation of xylose, especially at high temperature. In a previous study, H₂ production from xylose was compared in batch cultures at 37, 55 and 70 °C using heat treated (90 °C, 15 min) activated sludge from a wastewater treatment plant as the inoculum [18]. That study showed effective H₂ production at 55 °C, but not at 70 °C. However, the effect of temperature in the 55–70 °C temperature range is worth investigating, as a difference of a few degrees can affect the microbial community inside the reactor and thus, the H₂ production efficiency [44].

Recently, the establishment of next-generation sequencing techniques has improved the knowledge on H₂-producing microbial communities. Etchebehere et al. [45] performed 454 pyrosequencing on microbiological samples from 20 H₂ producing lab-scale bioreactors operated within a temperature range of 25–37 °C. Although the microbial communities were diverse due to the different operating conditions of the bioreactors, the authors observed a predominance of *Firmicutes* and distinguished high-yield H₂ producers (*Clostridium*, *Kosmotoga*, and *Enterobacter*), low-yield H₂ producers (*Veillonellaceae*) and competitors (*Lactobacillus*). Nitipan et al. [46] reported *Thermoanaerobacterium* as the dominant genus in a thermophilic (60 °C) sequencing batch reactor producing H₂ from palm oil mill effluent. Zhang et al. [47] showed that *Firmicutes* such as *Caldanaerobius*, *Caldicellulosiruptor* and *Thermoanaerobacter* became dominant in a hyperthermophilic (70 °C) H₂ producing, glucose-fed chemostat. To date, analysis of dark fermentative microbial communities by next-generation sequencing has been mainly based on the presence of 16S rRNA genes, which provides information on the structure of the microbial community. However, an analysis based on the expression of 16S rRNA genes describes more accurately the composition of the microbial community actively involved in dark fermentation [48].

This study aims to evaluate, for the first time, how dark fermentative H₂ production and the composition of the active microbial community are affected by a stepwise (5 °C) temperature increase in the 55–70 °C temperature range in a xylose-fed FBR inoculated with heat treated activated sludge. A second FBR was operated in parallel with the same inoculum, but at a lower temperature (37 °C) in order to compare its performance to the thermophilic counterpart, prior to increasing the temperature to 55 °C to observe the response of this mesophilic microbial community to the temperature shift.

Materials and methods

Source of biomass

Activated sludge was collected from the recirculation line between the aeration tank and the secondary settler in the Viinikanlahti municipal wastewater treatment plant (Tampere, Finland). It was settled, and, after removing the supernatant, heat-treated at 90 °C for 15 min as described by Dessi et al. [18] before being used as inoculum for the FBRs.

Composition of synthetic wastewater

Both FBRs were fed with a synthetic wastewater based on the DSMZ 144 medium with xylose (50 mM) as electron donor instead of glucose. However, tryptone, resazurin and Na₂S were omitted and the concentration of KH₂PO₄, K₂HPO₄, and yeast extract was reduced 10 times (to 0.55 mM, 0.86 mM, and 0.3 g l⁻¹, respectively).

FBR configuration

Two FBRs (Fig. 1), 1 L volume each, were operated with 300 mL activated carbon as carrier material for biomass adhesion. A recirculation flow rate of about 1900 mL min⁻¹ was applied by using a peristaltic pump (Masterflex, USA) to expand the activated carbon bed by 30% (the flow rate of minimum

fluidization was 1400 mL min⁻¹). To maintain a constant expansion, the recirculation flow was increased up to a maximum of 20% due to the adhesion of the biomass, which made the bed heavier. To achieve a stable temperature, the FBRs were operated inside incubators (Labilo, Finland). A water jacket (Julabo, Germany) was also installed, as the heat provided by the incubator was not enough to reach 70 °C inside the FBR.

To avoid microbial growth, the xylose solution was prepared in a different tank than the medium containing the nutrients and trace elements. Both solutions were flushed with N₂ in the feeding tanks, and their pH was adjusted to 5.0–5.5 with HCl prior to being fed to the FBRs through peristaltic pumps (Masterflex, USA). To minimise the growth of methanogenic archaea [49], the pH inside the reactor was kept at 5 (±0.1) by automatic titration (Metrohm, Switzerland). The FBRs were sealed on the top, and both liquid and gas were directed to a gas-liquid separator. The liquid was directed to an effluent tank, while the gas was directed to a gas meter (Ritter, Germany) before being released into a fume hood (Fig. 1).

FBR operation and sampling

Both FBRs were inoculated by 50 mL of heat-treated activated sludge (8.8 g VS L⁻¹). After two days of start-up in batch mode, the FBRs were switched to continuous mode (day 0) and

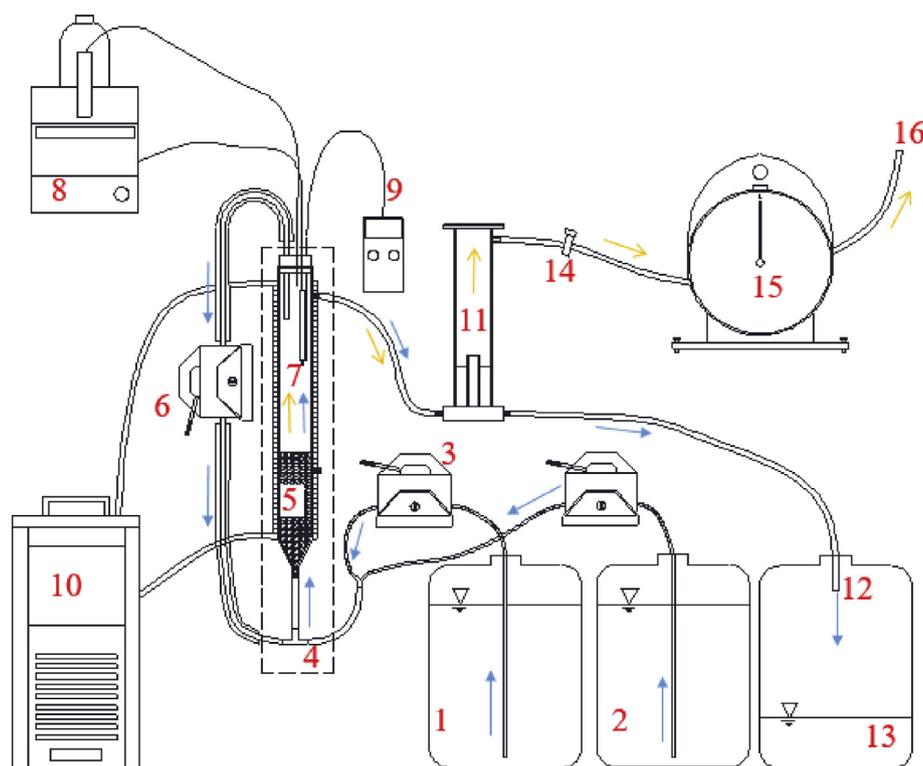


Fig. 1 – Overview of the experimental set-up used in this study. Medium influent tank (1), xylose influent tank (2), peristaltic pumps for influent feeding (3), influent sampling point (4), activated carbon bed with the active biomass (5), peristaltic pump for recirculation (6), pH probe (7), automatic titrator (8), temperature control (9), water bath (10), gas-liquid separator (11), effluent sampling point (12), effluent tank (13), gas sampling point (14), gas meter (15) and gas outlet (16). Liquid path (→), gas path (↗). The dashed rectangle represents the part of the set-up located inside the incubator.

operated for 7 days at a hydraulic retention time (HRT) of 12 h. On day 7, the HRT was decreased to 6 h. The thermophilic FBR was operated in continuous mode for a total of 185 days. It was initially operated at 55 (± 1) °C. Temperature was then increased to 60 (± 1) °C on day 77, to 65 (± 1) °C on day 119, and to 70 (± 1) °C on day 158 until day 185. The mesophilic FBR was operated at 37 (± 1) °C for 185 days, and then at 55 (± 1) °C until day 228.

Influent, effluent and gas samples were collected from the sampling points specified in Fig. 1. Biofilm-containing activated carbon was collected from the thermophilic FBR before every change in operating conditions (plus a mid-term sample collected on day 53), and stored at -20 °C for DNA analysis. Samples from the mesophilic FBR were also collected on the same days. An additional sample of carrier material was collected from the thermophilic FBR on day 119 (60 °C), 158 (65 °C) and 185 (70 °C) and, from the mesophilic reactor on day 185 (37 °C) and 228 (55 °C), and stored at -80 °C for RNA-level analysis. The collection system consisted of a syringe connected to a tube, which was used to suck 4–6 mL of biomass out of the FBR bed. While collecting the biomass samples, the FBRs were open from the top but flushed with N_2 to avoid exposure to air.

Chemical analysis

Influent and effluent composition was determined either with GC-FID according to Kinnunen et al. [50], or with HPLC as reported by Dessi et al. [18]. Gas samples were analysed for H_2 , CH_4 , and CO_2 with a Shimadzu gas chromatograph GC-2014 equipped with a Porapak N column (80/100 mesh) and a thermal conductivity detector. Oven, injector and detector temperatures were at 80, 110 and 110 °C, respectively.

Microbiological analysis

DNA extraction and polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) of the partial 16S rRNA gene were performed according to Mäkinen et al. [32]. The forward and reverse primers for PCR were GC-BacV3f and 907r, respectively. The visible bands in the polyacrylamide gel were cut by using a surgical blade, eluted in sterile water and re-amplified by PCR (primers BacV3f and 907r) before sending them to Macrogen (South Korea) for sequencing as described by Koskinen et al. [51]. The nucleotide sequences obtained were analysed using Bio-Edit [52] software version 7.2.5 and compared with the sequences in the GenBank nucleotide collection database using the BLAST software [53].

Nucleic acids for MiSeq analysis were co-extracted from the biofilm-containing activated carbon samples according to Griffiths et al. [54], but 3 M sodium acetate (1/10 of sample volume) and cold (-20 °C) 100% isopropanol (1 sample volume) were added for precipitation instead of polyethylene glycol. Furthermore, nucleic acids were re-suspended in sterile water instead of tris-EDTA buffer. DNA and RNA were quantified by a Nanodrop spectrophotometer (Nanodrop Technologies, USA). Their quality was assessed by measuring the absorbance ratio at 260/280 nm and 260/230 nm wavelength. Nucleic acid samples were diluted to a final concentration of

25 ng mL $^{-1}$. DNA was removed by adding 1 μ L turbo DNase and 2.5 μ L turbo DNase buffer (Invitrogen, Thermo Fisher, USA), followed by incubation at 37 °C for 30 min. DNase was then inactivated by addition of 2.5 μ L DNase inactivator (Invitrogen). After centrifugation (10000 \times g, 1.5 min), the RNA-containing supernatant was transferred to a fresh RNase free tube. The absence of DNA was confirmed by 16S rRNA gene PCR (primers 338f and 805r) followed by electrophoresis in 1% agarose gel (no bands obtained).

Complementary DNA (cDNA) was obtained from RNA using M-MuLV Reverse Transcriptase (New England, BioLabs, USA), following the instructions provided by the supplier. The success of the reverse transcription was confirmed by 16S rRNA gene PCR (primers 338f and 805r) and electrophoresis in 1% agarose gels (bands appeared). Samples of cDNA were sent to FISABIO (Valencia, Spain) for high-throughput sequencing of partial 16S rRNA genes on an Illumina MiSeq platform. Forward and reverse primers for PCR were 515f and 806r, respectively [55]. A total of 427,163 raw sequences was obtained from 5 samples. Sequence screening, alignment to Silva (v123) database, clustering, chimeras removal and taxonomic classification (cut-off = 97%) were performed using Mothur v1.39.3 [56], following the procedure described by Kozich et al. [57].

Net energy gain calculation

The net energy gain NE_C (kJ L $^{-1}$) (Equation (1)) was estimated by the difference between the energy E_C recovered by combustion of the H_2 produced per L of wastewater treated (Equation (2)) and the energy E_L required to heat the FBR to the desired temperature (Equation (3)) [41]:

$$\text{Net energy gain: } NE_C = EG - EL \quad (1)$$

$$\text{Energy gain: } EG = Y_H \cdot MV_H \cdot C_X \cdot \rho_H \cdot LHV_H \quad (2)$$

$$\text{Energy loss: } EL = C_W \cdot (T_F - T_I) \cdot \rho_W \quad (3)$$

where Y_H is the H_2 yield (mol H_2 mol $^{-1}$ xylose), MV_H is the molar volume of H_2 (22.414 L mol $^{-1}$), C_X is the influent xylose concentration (50 mol L $^{-1}$), ρ_H is the density of gaseous H_2 (8.9 $\times 10^{-5}$ kg L $^{-1}$), LHV_H is the lower heating value of H_2 (120 $\times 10^3$ kJ kg $^{-1}$), C_W is the specific heat of water (4.2 kJ kg $^{-1}$ K $^{-1}$), T_F and T_I (K) is the temperature of the wastewater after and before heating, respectively, and ρ_W is the density of water (1 kg L $^{-1}$).

Statistical analysis

To assess significant differences in H_2 yield at the various temperatures investigated, analysis of variance (ANOVA) and the Tukey test [58] at $p = 0.05$ were conducted using the IBM SPSS Statistics package. The statistical analysis was conducted on the last 4 sampling points collected at the various temperatures investigated in both the mesophilic and thermophilic FBR (8–10 operation days), which were considered a subsample representative of the FBR performance at each temperature. The output of the statistical analysis is provided in the supporting material (File S1).

Results

H₂ production in the thermophilic FBR

For all the temperatures investigated (55–70 °C), the thermophilic FBR yielded more H₂ than the mesophilic (37 °C) FBR (Fig. 2a versus 2b). At 55 °C (days 1–77), the H₂ yield increased steadily in the first days of operation, reaching 1.2 mol H₂ mol⁻¹ xylose on day 18, and remaining relatively stable on days 18–25 (Fig. 2a). In the following days, due to a problem with the liquid-gas separator (foam produced by the FBR partially clogged the gas line and part of the gas was likely lost with the effluent), the H₂ yield decreased to a minimum of 0.7 mol H₂ mol⁻¹ xylose on day 35. On day 52, after solving the issue by washing the liquid-gas separator, the H₂ yield increased sharply reaching a maximum of about 1.3 mol H₂ mol⁻¹ xylose on day 56 and then stabilising to 1.2 mol H₂ mol⁻¹ xylose on days 65–77 (Fig. 2a). On days 7–77, xylose was detected in the effluent at a concentration <2.5 mM (>95% removal). Acetate and butyrate were the main metabolites produced. Their concentration in the effluent increased on days 1–21, reaching a concentration of about 30 and 35 mM for acetate and butyrate, respectively (Fig. 2c). Ethanol was produced during the first days of operation, reaching a maximum concentration of about 10 mM on day 7 before decreasing to <2 mM on day 27. On days 65–77, the acetate concentration ranged between 20 and 24 mM, while the butyrate concentration ranged between 19 and 25 mM (Fig. 2c).

After increasing the temperature to 60 °C on day 77, the H₂ yield remained stable at 1.2 mol H₂ mol⁻¹ xylose for one day and then decreased to a minimum of 0.6 mol H₂ mol⁻¹ xylose on day 81 (Fig. 2a). H₂ remained low on days 81–91 and started to increase again, reaching a maximum of about 1.0 mol H₂ mol⁻¹ xylose on day 95. From day 98, the H₂ yield slightly decreased again and stabilised to 0.8 mol H₂ mol⁻¹ xylose on days 112–119 (Fig. 2a). The increase in temperature caused an increase in the VFAs concentration in the effluent (Fig. 2c). The acetate concentration almost doubled, ranging between 29 and 42 mM on days 88–119. The butyrate concentration increased as well, ranging between 25 and 35 mM on days 88–119 (Fig. 2c).

At 65 °C (days 120–158), the average H₂ yield was comparable to the yield obtained at 60 °C, but the production was more unstable compared to both 55 °C and 60 °C, ranging between 0.7 and 1.3 mol H₂ mol⁻¹ xylose (Fig. 2a). Increasing the temperature to 65 °C had minimal impact on the VFA concentration when compared to the 60 °C condition (Fig. 2c).

After increasing the temperature further to 70 °C, H₂ production ceased on day 161, but increased again from day 163, stabilising to values comparable to those obtained at 55 °C (1.2 mol H₂ mol⁻¹ xylose) on days 172–185 (Fig. 2a). A high concentration of xylose was detected in the effluent on days 163–174, with a peak of about 50 mM (0% removal) on day 163. On days 163–174, the acetate and butyrate concentrations decreased and slowly increased again, reaching values comparable to the ones obtained at 60 and 65 °C

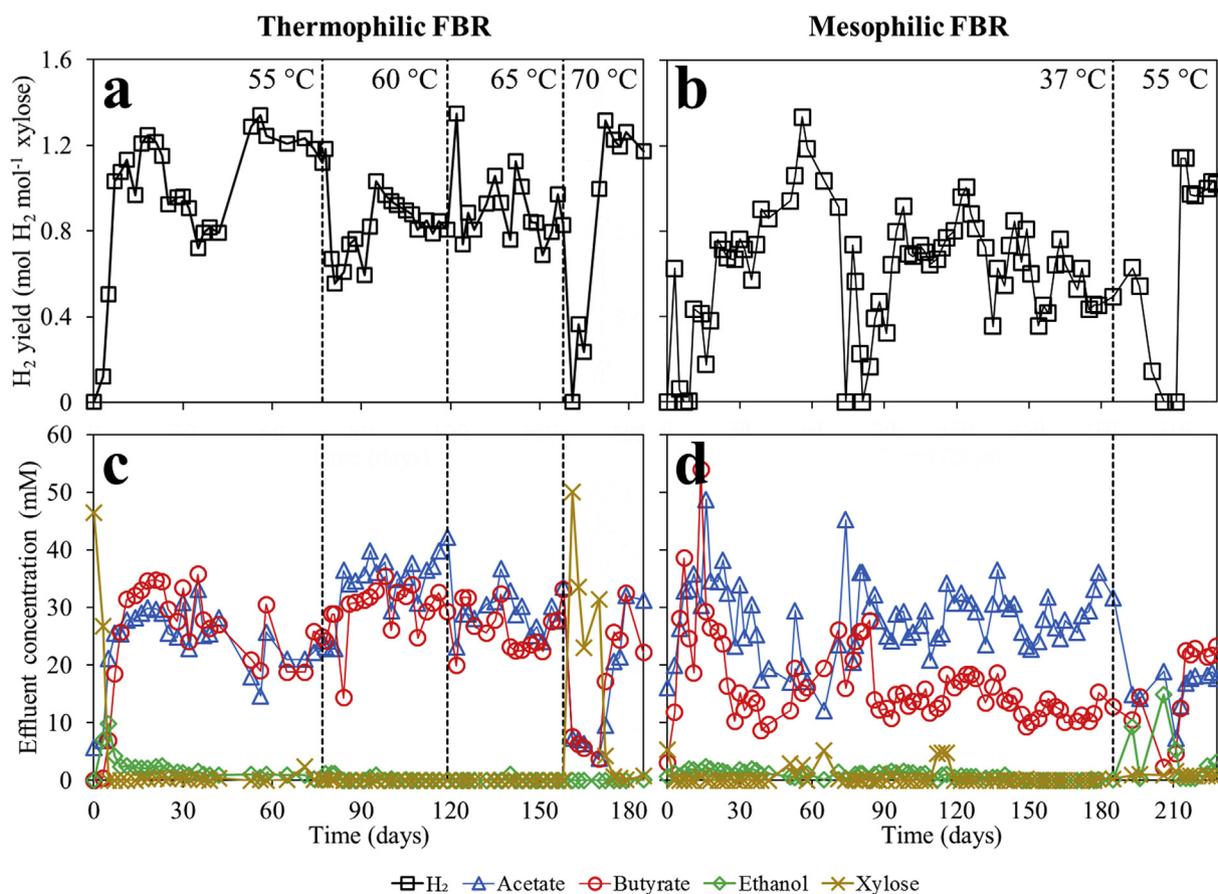


Fig. 2 – H₂ yield (mol H₂ mol⁻¹ xylose added) (a, b) and acetate, butyrate, ethanol and xylose concentration (mM) in the effluent (c, d) of the thermophilic (a, c) and mesophilic (b, d) FBR. The vertical dotted lines indicate a change of temperature.

(21–32 mM acetate and 22–32 mM butyrate) on days 175–185 (Fig. 2c).

H₂ production in the mesophilic FBR

The mesophilic FBR had a fluctuating H₂ production, especially during days 1–90 (Fig. 2b). The H₂ yield was above 1.0 mol H₂ mol⁻¹ xylose only on days 53–65. On day 7, 74 and 81, H₂ production ceased and increased again within 1–3 days. On days 90–185, the H₂ production fluctuated between 0.3 and 1.0 mol H₂ mol⁻¹ xylose. Between days 0–185, the xylose concentration in the effluent was <5 mM (>90% removal efficiency).

Similar to the H₂ yield, acetate and butyrate production was unstable during days 1–90 (Fig. 2d). The acetate concentration was often close to 40 mM and was even higher on day 18 and 76. Interestingly, the peaks of acetate (Fig. 2d) seem to mirror the drops in H₂ yield (Fig. 2b). On days 90–185, the butyrate concentration ranged between 10 and 19 mM, while the acetate concentration was more unstable, ranging between 20 and 36 mM (Fig. 2d).

After increasing the temperature to 55 °C (day 186), H₂ production ceased on day 206 (Fig. 2b), and then increased sharply to values comparable to those obtained in the thermophilic FBR at 55 °C on days 219–228 (1.0 mol H₂ mol⁻¹ xylose). After raising the temperature from 37 to 55 °C, xylose was still consumed efficiently (>97%). Ethanol was detected in the effluent on days 195–213, with a maximum of 15 mM on day 208 (Fig. 2d). However, the liquid phase composition was similar to the one obtained in the thermophilic FBR at 55 °C on days 217–228 (about 18 and 22 mM acetate and butyrate, respectively).

Microbial community composition

Different eubacterial community profiles from the thermophilic and mesophilic FBR were obtained by PCR-DGGE

analysis from the biofilm-containing activated carbon (Fig. 3; Table 1). In the thermophilic FBR, *Clostridium acetobutylicum* (100% similarity) was found at 55 °C on day 0 (after two days of start-up in batch mode), but was not detected in the subsequent samples (Fig. 3). On days 7–185, regardless the temperature, *Thermoanaerobacterium thermosaccharolyticum* (98–99% similarity) was the dominant microorganism in the thermophilic FBR (Fig. 3). *Alicyclobacillus* sp. (96–97% similarity) was found on day 7, after which its concentration was below detection limit at 55 °C on day 77 and at 60 °C on day 119, but it was detected again when temperature was further increased to 65 and 70 °C (Fig. 3).

A wider eubacterial community was found in the mesophilic FBR compared to the thermophilic FBR. On day 0 and day 7, nucleotide sequences with 99–100% similarity to *Clostridium pasteurianum* were detected. Species with 99–100% similarity to *Clostridium acetobutylicum* were detected on all sampling days (days 7–185). On day 77, nucleotide sequences close to *Pseudomonas* and *Delftia* sp. were also found. On days 119, 158, and 185, the DGGE profiles of the mesophilic community were similar to each other and dominated by *Clostridium* sp. (Fig. 3).

Based on the high-throughput sequencing of the reverse-transcribed 16S rRNA (Fig. 4), species belonging to the *Thermoanaerobacterium* genus dominated the active microbial community (>99% of the relative abundance) of the thermophilic FBR at both 60 and 70 °C (days 119 and 185). At 65 °C (day 158), about 22% of the relative abundance matched the genus *Clostridium* (Fig. 4). In the mesophilic FBR, microorganisms of the genus *Clostridium* and *Ruminiclostridium* dominated the active microbial community at 37 °C (Fig. 4). Increasing the FBR temperature to 55 °C resulted in a shift of the active microbial community towards the *Thermoanaerobacterium* genus (>90% of the relative abundance).

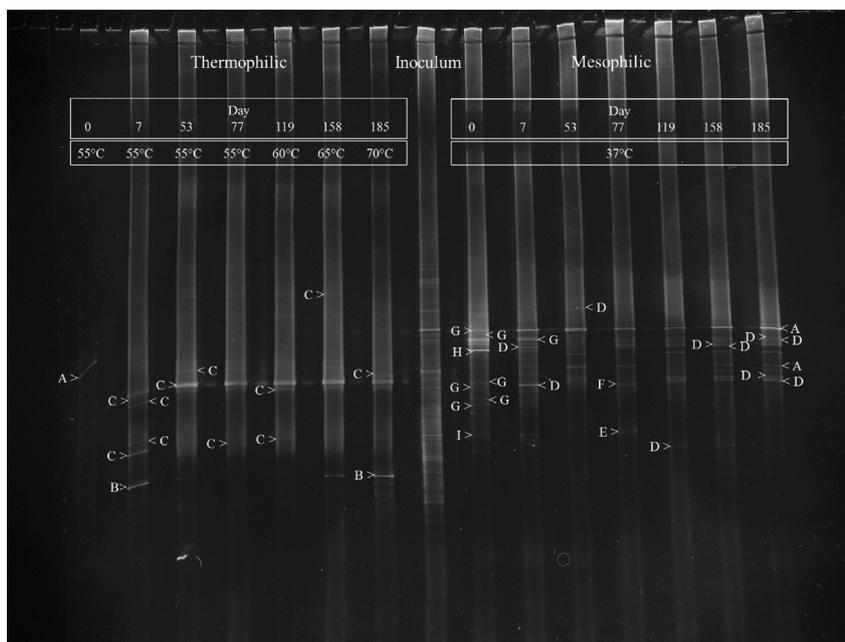


Fig. 3 – Microbial community profiles obtained by PCR–DGGE from biofilm-containing activated carbon carrier samples collected from the thermophilic and the mesophilic H₂ producing FBR. The band labels refer to Table 1.

Table 1 – Association of 16S rRNA gene sequences of DGGE bands to those collected in the GenBank.

BL ^a	Affiliation ^b	Accession number	Matching sequence length ^c	Similarity (%) ^d
A	<i>Clostridium acetobutylicum</i>	KP410577 KP410579	446–472	99–100
B	<i>Alicyclobacillus</i> sp.	JX505129	485–524	96–97
C	<i>Thermoanaerobacterium thermosaccharolyticum</i>	JX984968 KJ831072	405–499	98–99
D	<i>Clostridium</i> sp.	AF247003 EU887966 EU887970 KU886097 LC020495	367–491	94–99
E	<i>Pseudomonas</i> sp.	GQ903481	442	97
F	<i>Delftia</i> sp.	KT865666	465	99
G	<i>Clostridium pasteurianum</i>	KX378861	440–506	99–100
H	<i>Ralstonia</i> sp.	KT183537	496	99
I	<i>Lysobacter</i> sp.	JQ791571	429	93

^a Band label in Fig. 3a.

^b Closest cultured species in GenBank.

^c Number of nucleotide pairs used in the sequence comparison.

^d Percentage of identical nucleotide pairs between the 16S rRNA gene sequence and the closest species in GenBank.

Discussion

Thermophilic H₂ production and microbial community dynamics

The significantly higher and more stable H₂ yield obtained when performing dark fermentation of xylose under thermophilic than under mesophilic conditions (Fig. 2a versus 2b)

was mainly due to the composition of the microbial community (Fig. 3; Table 1), particularly the active microbial community (Fig. 4). Generally, thermophilic microorganisms yield more H₂ than mesophilic species [5]. A thermophilic community dominated by *Thermoanaerobacterium*, the prevalent active genus in the thermophilic FBR regardless of the temperature applied (Fig. 4), has also previously been reported to yield more H₂ than a mesophilic community dominated by

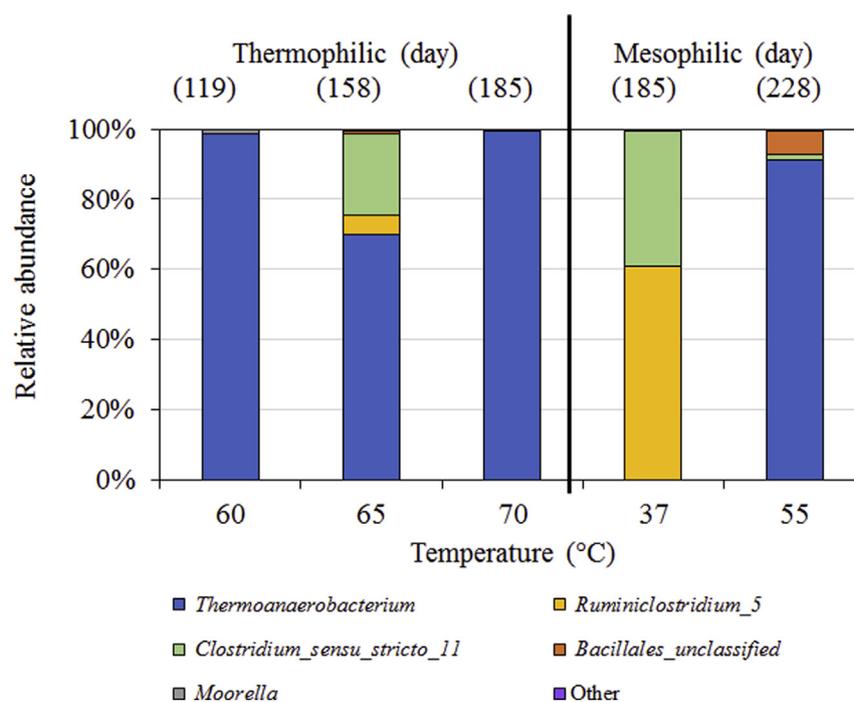


Fig. 4 – Relative abundance of the active microbial community, classified at genus level, obtained by MiSeq sequencing analysis of the 16S rRNA from biofilm-containing activated carbon samples, reverse transcribed to 16S cDNA. “Other” refers to the sum of genus with a relative abundance <1%.

Clostridium [49]. Furthermore, thermophilic conditions are not favourable for most H₂ consuming microorganisms [27].

For all the temperatures investigated (55–70 °C), the thermophilic microbial community was dominated by microorganisms closely related to *Thermoanaerobacterium thermosaccharolyticum* (Fig. 3; Table 1). Accordingly, the genus *Thermoanaerobacterium* was prevailing in the active thermophilic microbial community at 60, 65 and 70 °C (Fig. 4). In the thermophilic FBR, although the sample at 55 °C is missing in the active microbial community analysis (Fig. 4), the DGGE profile (Fig. 3) and the reactor performance (Fig. 2a) suggest that *Thermoanaerobacterium* was also the prevalent active genus at 55 °C. In fact, even in the mesophilic FBR, *Thermoanaerobacterium* became dominant upon increasing the temperature from 37 to 55 °C (Fig. 4). *T. thermosaccharolyticum* has been utilised in batch reactors to produce H₂ from xylose at various temperatures, obtaining a maximum yield of about 2.2 mol H₂ mol⁻¹ xylose at 60 °C [59,60]. However, in this study, the highest H₂ yield was only about 1.2 mol H₂ mol⁻¹ xylose at 55 °C and even 30% lower at 60 °C (Fig. 2a), although DGGE profiles appear similar (Fig. 3). One explanation is that, in this study, the pH was set to 5.0, whereas the optimum pH for *T. thermosaccharolyticum* is 6.5 [60]. Furthermore, Koskinen et al. [51] showed that in a mesophilic H₂-producing FBR, the attached microbial community is slightly different from the suspended one, which was not analysed in this study. It is thus plausible that the contribution of some H₂ producing or H₂ consuming microorganisms on the net H₂ yield was not considered.

In the thermophilic FBR, sequences belonging to the *Clostridium* genus were detected only at 65 °C, accounting for 22% of the active microbial community (Fig. 4). However, at 65 °C, *Clostridium* was not detected by PCR-DGGE. A possible explanation is that the concentration of *Clostridium* was under the detection limit of PCR-DGGE, but these microorganisms were particularly active at 65 °C and thus detected through the RNA-level sequencing analysis [48]. The activity of *Clostridium* is likely linked to the unstable H₂ yield obtained at 65 °C, as some *Clostridium* sp., such as the thermophilic *C. thermoacetium*, may oxidize sugars through competitive pathways to H₂ production or can even carry out homoacetogenesis [61]. The role of *Clostridium* in the xylose degradation at 65 °C could be further detailed by a proteomics analysis. At 65 °C, a bacterium closely related to *Alicyclobacillus* sp. appeared in the DGGE profile (Fig. 3). Genera with high similarity (99%) to *Alicyclobacillus* sp. have been found in a thermophilic FBR used for dark fermentation of cheese whey [62], but further studies are required to understand the role of this microorganism in the anaerobic processes.

The drop and subsequent increase in H₂ yield that occurred during the first days of operation at 70 °C, as well as the decreased xylose removal efficiency during these days (Fig. 2a and c) can be attributed to the acclimation of the microbial community to the higher temperature. Meng et al. [63] performed a proteomic study on *Thermoanaerobacter tengcongensis*, cultured at 55, 75 and 80 °C, and showed the existence of temperature-dependent protein complexes, which may affect the H₂ yield. The stable H₂ yield obtained at 70 °C after the acclimation (Fig. 2a) is due to the homogeneity of the active microbial community growing on the carrier material at that

temperature: >99% of the total cDNA sequences matched the genus *Thermoanaerobacterium* (Fig. 4).

According to the statistical analysis, the highest yield of 1.2 mol H₂ mol⁻¹ xylose was obtained at both 55 and 70 °C, whereas the H₂ yield was significantly lower (0.8 mol H₂ mol⁻¹ xylose) at 60 and 65 °C. Similarly, Yokoyama et al. [30] studied, in batch, dark fermentation of cow waste in the temperature range 37–85 °C, obtaining the highest H₂ production at 60 °C and 75 °C, and a lower H₂ production at 67 °C. However, their cultures were dominated by *Clostridium* sp. and *Caldanaerobacter* sp. at 60 and 75 °C, respectively, in contrast to this study with *Thermoanaerobacterium* sp. as the dominant microorganism at both 55 and 70 °C.

Mesophilic H₂ production and microbial community dynamics

The lower H₂ yield obtained under mesophilic conditions (Fig. 2b) was attributed to the microbial community. The DGGE profiles obtained on days 119, 158, and 185 were similar to each other and mainly composed of *Clostridium* (Fig. 3; Table 1), which was also shown to be the dominant active genus together with the closely related *Ruminiclostridium* genus (Fig. 4). Similarly, Si et al. [64] studied the microbial diversity of their mesophilic H₂ producing reactors by MiSeq sequencing, reporting *Clostridiaceae* as the most abundant family. Chatellard et al. [65] showed that *Clostridium*, in particular, dominated mesophilic microbial communities fermenting pentose-based substrates, as was also the case in this study.

Mesophilic H₂ production was unstable (Fig. 2b), likely due to the accumulation of fermentation products, mainly acetate and butyrate, which were produced at a too high rate to be flushed out with the effluent (Fig. 2d). At high concentrations, undissociated VFAs penetrate the cell membrane, lowering the internal pH and inhibiting H₂ production [15]. In fact, the H₂ yield (as well as the acetate and butyrate concentrations) cyclically increased and decreased in the mesophilic FBR between days 91–185, and the H₂ yield was higher when the concentration of VFAs was lower (Fig. 2b and d). Wang et al. [66] reported a decrease in H₂ yield at acetate concentrations higher than 50 mM. In this study, a drop in H₂ yield occurred at acetate concentrations of 40 mM, and the H₂ yield started to increase again when the acetate concentration was about 20–25 mM.

The significantly higher H₂ yield obtained after increasing the temperature of the mesophilic FBR from 37 to 55 °C (Fig. 2b) is clearly due to the shift in the active microbial community from the *Clostridium* and *Ruminiclostridium* genus to the *Thermoanaerobacterium* genus (Fig. 4). Interestingly, the H₂ yield at 55 °C remained comparable to the one obtained at 37 °C for 13 days, suggesting that the change in the dominant active community did not occur immediately upon the temperature change. The production of ethanol upon exposing the microbial community to 55 °C, and the subsequent shift to acetate, butyrate and H₂ production, was observed also in the first days of operation in the thermophilic FBR (days 1–20, Fig. 2c) and in a previous batch study with the same inoculum [18]. This can be attributed to either a change in the microbial community or a gradual shift from ethanol to butyrate fermentation as a response to the temperature shift [18].

Table 2 – Highest stable H₂ production rate (HPR) obtained by dark fermentation of simple sugars in various continuous studies conducted at high temperature (T ≥ 55 °C) and using different inocula, reactor types, pH and hydraulic retention time (HRT).

Inoculum	Pre-treatment	Reactor type ^a	Substrate (g COD L ⁻¹)	T (°C)	pH ^b	HRT (h)	HPR (mL H ₂ h ⁻¹ L ⁻¹)	Reference
Biomass from H ₂ producing CSTR immobilised on ceramic balls	–	UASB	Sucrose (11.2)	55	4.5–5.0 (nc)	3	112.5	[33]
Sludge from anaerobic digester immobilised on ceramic rings	Heat treatment (105 °C, 5 min)	UASB	Sucrose (11.2)	55	5.0–5.5 (nc)	1.5	124.2	[24]
Sludge from H ₂ producing reactor	Heat treatment (80 °C, 60 min)	FBR	Sucrose (5.0)	60	5.5 (i)	12	60.5	[34]
Biomass from H ₂ producing CSTR <i>Thermoanaerobacterium thermosaccharolyticum</i> immobilised on methanogenic granules	–	TBR	Glucose (7.3)	60	5.5 (c)	2	980.6	[35]
	Heat treatment (121 °C, 30 min)	UASB	Sucrose (22.5)	60	5.0–5.5 (nc)	1	3470	[70]
Biomass from H ₂ producing CSTR	–	CSTR	Xylose (1.1)	70	6.7 (nc)	72	2.6	[36]
Biomass from H ₂ producing CSTR	–	UASB	Glucose (2.1)	70	7.0 (i)	24	12.7	[37]
Methanogenic biomass from CSTR	Chemical treatment (BES) ^c	UASB	Glucose (4.8)	70	7.2 (i)	27	47.3	[38]
Biomass from H ₂ producing CSTR	–	CSTR	Glucose (4.6)	70	5.5 (c)	21.6	91.7	[39]
Activated sludge	Heat treatment (90 °C, 15 min)	FBR	Xylose (8.0)	70	5.0 (c)	6	282.1	This study
<i>Caldicellulosiruptor saccharolyticus</i>	–	TBR	Sucrose (11.5)	73	6.5 (c)	3–5	493.1 ^d	[71]

^a Continuous stirred tank reactor (CSTR), fluidised bed reactor (FBR), trickling bed reactor (TBR), upflow anaerobic sludge blanket (UASB).

^b The reported pH refers either to the initial pH (i) or the operation pH, which can be either controlled to a stable value (c) or not controlled (nc).

^c Added to the reactor feeding.

^d Per L of filtering bed.

The role of homoacetogenesis on mesophilic and thermophilic H₂ yield

A decrease in H₂ yield to below 0.2 mol H₂ mol⁻¹ xylose occurred in the mesophilic FBR on days 9, 16, 74, and 89 (Fig. 2b). On the same days, the CO₂ concentration also decreased (File S2 in the supporting material) and a peak of 35–50 mM acetate was detected within 1–2 days from the H₂ decrease (Fig. 2d), suggesting the occurrence of homoacetogenesis. H₂ and CO₂ were likely consumed to produce acetate autotrophically. Several *Clostridium* sp., which dominated the active mesophilic microbial community, are known homoacetogens [67]. The causes that trigger microorganisms from heterotrophic to the less energy yielding autotrophic metabolism are controversial. A H₂ partial pressure >500 Pa can favour homoacetogenesis [10] and H₂ can be simultaneously produced and consumed, but radioactive label tracking techniques would be required to distinguish the two processes [10]. Oh et al. [68] argued that in mixed culture fermentation, the switch to autotrophic metabolism occurs only after substrate depletion. This hypothesis seems less probable in the studied FBR, as xylose was fed continuously. However, it is plausible that H₂ producing microorganisms quickly consume the xylose, inducing the facultative bacteria to shift to autotrophic metabolism.

Koskinen et al. [51] studied the microbial community dynamics over time in a mesophilic (35 °C) FBR reactor inoculated with digested activated sludge and concluded that the adhesion of H₂ consuming microorganisms, including homoacetogens, to the carrier material may cause an unstable H₂ production. Similarly to this study, Dinamarca and Bakke [69]

reported a decrease from 1.5 to below 0.25 mol H₂ mol⁻¹ glucose after 57 days of reactor operation at 35 °C. The authors concluded that homoacetogenesis is directly correlated with the HRT and dependent on biomass density and sludge age [69]. Also Luo et al. [49] argued that, even if the inoculum is pre-treated, methanogenic and homoacetogenic microorganisms could develop again during long-term operation. Methanogens are typically inhibited by a pH below 6, while homoacetogenic bacteria are also inhibited by a pH below 6, but only under thermophilic conditions [49].

In the thermophilic FBR, excluding day 161 on which a decrease in H₂ yield was attributed to the increased temperature, sudden drops in H₂ yield were not observed (Fig. 2a) and the acetate concentration was more stable (Fig. 2c). This indicates a minor role of homoacetogenesis at thermophilic conditions. This is in agreement with Luo et al. [49], who reported no homoacetogenesis in thermophilic (55 °C) batch incubations at pH 5.5. Their thermophilic microbial community was dominated by *Thermoanaerobacterium* sp., as was the case in this study.

Comparison of H₂ production with previous studies

At 55 °C, despite the HRT of only 6 h, the H₂ yield per mol xylose added is consistent with a previous batch study with the same inoculum and substrate [18]. At 70 °C, however, H₂ was effectively produced during FBR operation, but not in the batch incubations [18], likely due to the longer time for acclimation of the biomass to the high temperature. The maximum H₂ production rate (HPR) of 282.1 mL H₂ h⁻¹ L⁻¹ obtained at 70 °C is among the highest reported in continuous

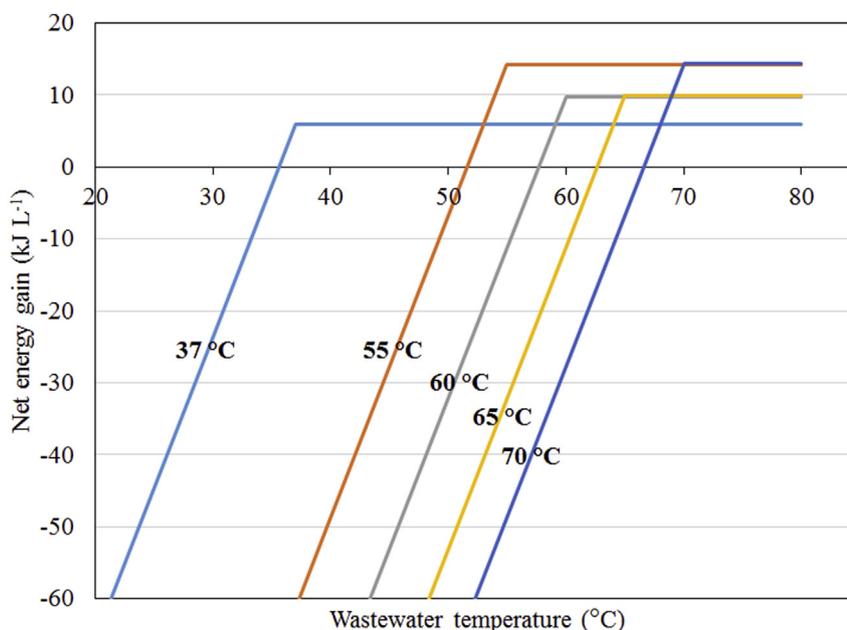


Fig. 5 – Effect of fermentation temperature on net energy gain from a xylose containing wastewater. X-axis shows the hypothetical temperature at which the wastewater is released. The coloured lines represent the net energy gain obtainable per litre of xylose (50 mM) containing wastewater treated under mesophilic (37 °C) or thermophilic (55, 60, 65, 70 °C) conditions. The net energy gain was calculated according to Perera et al. [41] from the average H₂ yields of the last 4 sampling points collected at the various temperatures investigated in both the mesophilic and thermophilic FBR (8–10 operation days). (For interpretation of the references to color/colour in this figure legend, the reader is referred to the Web version of this article.)

studies on thermophilic dark fermentation of sugars by mixed cultures (Table 2). This is likely due to the composition of the active microbial community dominated by an effective H₂ producer such as *Thermoanaerobacterium* (Fig. 4). A 12-times higher HPR (3470 mL H₂ h⁻¹ L⁻¹) has been obtained with a pure culture of *T. thermosaccharolyticum* immobilized on heat-treated methanogenic granules [70], but the sugar concentration (in terms of chemical oxygen demand) was almost 3 times higher than the concentration used in this study. Furthermore, O-Thong et al. [70] obtained the maximum HPR at an HRT of only 1 h, whereas in this study the HRT was set to 6 h.

Practical implications

Organic carbon-rich wastewaters are produced by industries at various temperatures. The energy gain from combustion of the H₂ produced and the energy required to heat the FBR to the desired temperature are two important aspects to take into consideration for a proper economic analysis. For a rough estimation of the effect of operation and feed wastewater temperature on energy gain of a H₂ producing system, a correlation between the wastewater temperature, fermentation temperature and net energy gain is presented in Fig. 5.

Despite the comparatively low H₂ yield obtained (Fig. 2b), dark fermentation at 37 °C is still the best option to treat wastewaters produced at temperatures up to about 50 °C based on the net energy gain. Thermophilic treatment at 55 °C is to be preferred for wastewaters produced at above 50 °C, and the net energy gain obtained at 70 °C is comparable to the one obtained at 55 °C for wastewaters produced at temperatures exceeding 70 °C (Fig. 5). Thermophilic processes could thus be advantageous to treat wastewaters produced at high temperature. However, many other aspects can influence the net energy gain both in a positive (heat recovery from hot wastewater, energy content of the effluent and removal of pathogens) or negative (heat losses, H₂ to energy conversion efficiency and maintenance and operation costs of the FBRs) way.

The data presented in this study, both on FBR performance and composition of the active microbial community, helps understanding the process and selecting the operation temperature in H₂ producing bioreactors depending on the temperature of the waste stream to be treated. However, more research is required to further increase the net energy gain. Post-treatment of the dark fermentation effluent for recovery of value-added products such as VFAs and alcohols or for further energy harvesting (e.g., H₂ production through photo-fermentation or microbial electrolysis cells, methane production through anaerobic digestion or bioelectricity production using microbial fuel cells) is a key factor towards process scale-up [7,72].

Conclusions

Thermoanaerobacterium dominated the thermophilic active microbial community, resulting in a higher and more stable H₂ yield in the thermophilic FBR compared to the mesophilic FBR dominated by *Clostridium*. Treating high temperature, xylose-

containing wastewaters by thermophilic dark fermentation can thus lead to a higher energy output compared to the mesophilic counterpart. Temperatures of 55 and 70 °C resulted in the net maximum H₂ yield of 1.2 mol H₂ mol⁻¹ xylose, whereas the competition by *Clostridium* caused unstable H₂ production at 65 °C. This study contributes to the understanding of dark fermentation of xylose in FBRs, and the microorganisms actively involved in the mesophilic or thermophilic process, which supports the development of high rate H₂ producing bioreactors.

Funding

This work was supported by the Marie Skłodowska-Curie European Joint Doctorate (EJD) in Advanced Biological Waste-To-Energy Technologies (ABWET) funded from Horizon 2020 under grant agreement no. 643071.

Acknowledgements

The authors thank Antti Nuottajärvi (TUT, Finland) for helping in the preparation of reactor set-up and Viinikanlahti municipal wastewater treatment plant (Tampere, Finland) for providing the activated sludge.

Appendix A. Supplementary data

Supplementary data related to this article can be found at <https://doi.org/10.1016/j.ijhydene.2018.01.158>.

REFERENCES

- [1] Hallenbeck PC, Abo-Hashesh M, Ghosh D. Strategies for improving biological hydrogen production. *Bioresour Technol* 2012;110:1–9.
- [2] Nikolaidis P, Poullikkas A. A comparative overview of hydrogen production processes. *Renew Sustain Energy Rev* 2017;67:597–611.
- [3] Arimi MM, Knodel J, Kiprop A, Namango SS, Zhang Y, Geißen S-U. Strategies for improvement of biohydrogen production from organic-rich wastewater: a review. *Biomass Bioenergy* 2015;75:101–18.
- [4] Van Groenestijn JW, Hazewinkel JHO, Nienoord M, Bussmann PJT. Energy aspects of biological hydrogen production in high rate bioreactors operated in the thermophilic temperature range. *Int J Hydrogen Energy* 2002;27:1141–7.
- [5] Lee D-J, Show K-Y, Su A. Dark fermentation on biohydrogen production: pure culture. *Bioresour Technol* 2011;102:8393–402.
- [6] Vipotnik Z, Jessen JE, Scully SM, Orlygsson J. Effect of culture conditions on hydrogen production by *Thermoanaerobacter* strain AK68. *Int J Hydrogen Energy* 2016;41:181–9.
- [7] Ghimire A, Frunzo L, Pirozzi F, Trably E, Escudie R, Lens PNL, et al. A review on dark fermentative biohydrogen production from organic biomass: process parameters and use of by-products. *Appl Energy* 2015;144:73–95.
- [8] Pawar SS, van Niel EWJ. Thermophilic biohydrogen production: how far are we? *Appl Microbiol Biotechnol* 2013;97:7999–8009.

- [9] Thauer RK, Jungermann K, Decker K. Energy conservation in chemotrophic anaerobic bacteria. *Bacteriol Rev* 1977;41:100–80.
- [10] Saady NMC. Homoacetogenesis during hydrogen production by mixed cultures dark fermentation: unresolved challenge. *Int J Hydrogen Energy* 2013;38:13172–91.
- [11] Angenent LT, Karim K, Al-dahhan MH, Wrenn BA, Domi R. Production of bioenergy and biochemicals from industrial and agricultural wastewater. *Trends Biotechnol* 2004;22:477–85.
- [12] Barca C, Soric A, Ranava D, Giudici-Orticoni M-T, Ferrasse J-H. Anaerobic biofilm reactors for dark fermentative hydrogen production from wastewater: a review. *Bioresour Technol* 2015;185:386–98.
- [13] Li C, Fang HHP. Fermentative hydrogen production from wastewater and solid wastes by mixed cultures. *Crit Rev Environ Sci Technol* 2007;37:1–39.
- [14] Husemann MHW, Papoutsakis ET. Solventogenesis in *Clostridium acetobutylicum* fermentations related to carboxylic acid and proton concentrations. *Biotechnol Bioeng* 1988;32:843–52.
- [15] Bundhoo MAZ, Mohee R. Inhibition of dark fermentative biohydrogen production: a review. *Int J Hydrogen Energy* 2016;41:6713–33.
- [16] Bundhoo MAZ, Mohee R, Hassan MA. Effects of pretreatment technologies on dark fermentative biohydrogen production: a review. *J Environ Manag* 2015;157:20–48.
- [17] Pradhan N, Dipasquale L, d'Ippolito G, Panico A, Lens PNL, Esposito G, et al. Hydrogen production by the thermophilic bacterium *Thermotoga neapolitana*. *Int J Mol Sci* 2015;16:12578–600.
- [18] Dessi P, Lakaniemi A-M, Lens PNL. Biohydrogen production from xylose by fresh and digested activated sludge at 37, 55 and 70 °C. *Water Res* 2017;115:120–9.
- [19] Ratkowsky DA, Olley J, McMeekin TA, Ball A. Relationship between temperature and growth rate of bacterial cultures. *J Bacteriol* 1982;149:1–5.
- [20] Verhaart MRA, Bielen AAM, van der Oost J, Stams AJM, Kengen SWM. Hydrogen production by hyperthermophilic and extremely thermophilic bacteria and archaea: mechanisms for reductant disposal. *Environ Technol* 2010;31:993–1003.
- [21] Kargi F, Eren NS, Ozmihci S. Bio-hydrogen production from cheese whey powder (CWP) solution: comparison of thermophilic and mesophilic dark fermentations. *Int J Hydrogen Energy* 2012;37:8338–42.
- [22] Zheng H-S, Guo W-Q, Yang S-S, Feng X-C, Du J-S, Zhou X-J, et al. Thermophilic hydrogen production from sludge pretreated by thermophilic bacteria: analysis of the advantages of microbial community and metabolism. *Bioresour Technol* 2014;172:433–7.
- [23] Lin C-Y, Wu C-C, Wu J-H, Chang F-Y. Effect of cultivation temperature on fermentative hydrogen production from xylose by a mixed culture. *Biomass Bioenergy* 2008;32:1109–15.
- [24] Keskin T, Aksöyek E, Azbar N. Comparative analysis of thermophilic immobilized biohydrogen production using packed materials of ceramic ring and pumice stone. *Int J Hydrogen Energy* 2011;36:15160–7.
- [25] Gokfiliz P, Karapinar I. The effect of support particle type on thermophilic hydrogen production by immobilized batch dark fermentation. *Int J Hydrogen Energy* 2017;42:2553–61.
- [26] Ghimire A, Luongo V, Frunzo L, Pirozzi F, Lens PNL, Esposito G. Continuous biohydrogen production by thermophilic dark fermentation of cheese whey: use of buffalo manure as buffering agent. *Int J Hydrogen Energy* 2017;42:4861–9.
- [27] Hasyim R, Imai T, Reungsang A, O-Thong S. Extreme-thermophilic biohydrogen production by an anaerobic heat treated digested sewage sludge culture. *Int J Hydrogen Energy* 2011;36:8727–34.
- [28] Mahmud SS, Jahim JM, Abdul PM. Pretreatment conditions of palm oil mill effluent (POME) for thermophilic biohydrogen production by mixed culture. *Int J Hydrogen Energy* 2017;42:27512–22.
- [29] Fangkum A, Reungsang A. Biohydrogen production from mixed xylose/arabinose at thermophilic temperature by anaerobic mixed cultures in elephant dung. *Int J Hydrogen Energy* 2011;36:13928–38.
- [30] Yokoyama H, Waki M, Moriya N, Yasuda T, Tanaka Y, Haga K. Effect of fermentation temperature on hydrogen production from cow waste slurry by using anaerobic microflora within the slurry. *Appl Microbiol Biotechnol* 2007;74:474–83.
- [31] Puhakka JA, Karadag D, Nissilä ME. Comparison of mesophilic and thermophilic anaerobic hydrogen production by hot spring enrichment culture. *Int J Hydrogen Energy* 2012;37:16453–9.
- [32] Mäkinen AE, Nissilä ME, Puhakka JA. Dark fermentative hydrogen production from xylose by a hot spring enrichment culture. *Int J Hydrogen Energy* 2012;37:12234–40.
- [33] Keskin T, Giusti L, Azbar N. Continuous biohydrogen production in immobilized biofilm system versus suspended cell culture. *Int J Hydrogen Energy* 2012;37:1418–24.
- [34] Lutpi NA, Md Jahim J, Mumtaz T, Harun S, Abdul PM. Batch and continuous thermophilic hydrogen fermentation of sucrose using anaerobic sludge from palm oil mill effluent via immobilisation technique. *Process Biochem* 2016;51:297–307.
- [35] Oh Y-K, Kim SH, Kim M-S, Park S. Thermophilic biohydrogen production from glucose with trickling biofilter. *Biotechnol Bioeng* 2004;88:690–8.
- [36] Kongjan P, Min B, Angelidaki I. Biohydrogen production from xylose at extreme thermophilic temperatures (70 °C) by mixed culture fermentation. *Water Res* 2009;43:1414–24.
- [37] Zheng H, Zeng RJ, Angelidaki I. Biohydrogen production from glucose in upflow biofilm reactors with plastic carriers under extreme thermophilic conditions (70 °C). *Biotechnol Bioeng* 2008;100:1034–8.
- [38] Kotsopoulos TA, Zeng RJ, Angelidaki I. Biohydrogen production in granular up-flow anaerobic sludge blanket (UASB) reactors with mixed cultures under hyperthermophilic temperature (70 °C). *Biotechnol Bioeng* 2006;94:296–302.
- [39] Zhang F, Chen Y, Dai K, Zeng RJ. The chemostat study of metabolic distribution in extreme-thermophilic (70 °C) mixed culture fermentation. *Appl Microbiol Biotechnol* 2014;98:10267–73.
- [40] Kumar G, Sivagurunathan P, Sen B, Mudhoo A, Davila-Vazquez G, Wang G, et al. Research and development perspectives of lignocellulose-based biohydrogen production. *Int Biodeterior Biodegrad* 2017;119:225–38.
- [41] Perera KRJ, Ketheesan B, Gadhamshetty V, Nirmalakhandan N. Fermentative biohydrogen production: evaluation of net energy gain. *Int J Hydrogen Energy* 2010;35:12224–33.
- [42] Suvilampi J, Lepistö R, Rintala J. Biological treatment of pulp and paper mill process and wastewaters under thermophilic conditions – a review. *Pap Timber* 2001;83:320–5.
- [43] Cavalcante de Amorim EL, Barros AR, Rissato Zamariolli Damianovic MH, Silva EL. Anaerobic fluidized bed reactor with expanded clay as support for hydrogen production through dark fermentation of glucose. *Int J Hydrogen Energy* 2009;34:783–90.

- [44] Karadag D, Puhakka JA. Effect of changing temperature on anaerobic hydrogen production and microbial community composition in an open-mixed culture bioreactor. *Int J Hydrogen Energy* 2010;35:10954–9.
- [45] Etchebehere C, Castelló E, Wenzel J, del Pilar Anzola-Rojas M, Borzacconi L, Buitrón G, et al. Microbial communities from 20 different hydrogen-producing reactors studied by 454 pyrosequencing. *Appl Microbiol Biotechnol* 2016;100:3371–84.
- [46] Nitipan S, Mamimin C, Intrasungkha N, Birkeland NK, O-Thong S. Microbial community analysis of thermophilic mixed culture sludge for biohydrogen production from palm oil mill effluent. *Int J Hydrogen Energy* 2014;39:19285–93.
- [47] Zhang F, Yang J-H, Dai K, Ding Z-W, Wang L-G, Li Q-R, et al. Microbial dynamics of the extreme-thermophilic (70°C) mixed culture for hydrogen production in a chemostat. *Int J Hydrogen Energy* 2016;41:11072–80.
- [48] De Vrieze J, Regueiro L, Props R, Vilchez-Vargas R, Jáuregui R, Pieper DH, et al. Presence does not imply activity: DNA and RNA patterns differ in response to salt perturbation in anaerobic digestion. *Biotechnol Biofuels* 2016;9:244.
- [49] Luo G, Karakashev D, Xie L, Zhou Q, Angelidaki I. Long-term effect of inoculum pretreatment on fermentative hydrogen production by repeated batch cultivations: homoacetogenesis and methanogenesis as competitors to hydrogen production. *Biotechnol Bioeng* 2011;108:1816–27.
- [50] Kinnunen V, Ylä-Outinen a, Rintala J. Mesophilic anaerobic digestion of pulp and paper industry biosludge—long-term reactor performance and effects of thermal pretreatment. *Water Res* 2015;87:105–11.
- [51] Koskinen PEP, Kaksonen AH, Puhakka JA. The relationship between instability of H₂ production and compositions of bacterial communities within a dark fermentation fluidized-bed bioreactor. *Biotechnol Bioeng* 2006;97:742–58.
- [52] Hall T. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symp Ser* 1999;41:95–8.
- [53] Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. *J Mol Biol* 1990;215:403–10. [https://doi.org/10.1016/S0022-2836\(05\)80360-2](https://doi.org/10.1016/S0022-2836(05)80360-2).
- [54] Griffiths RI, Whiteley AS, O'Donnell AG, Bailey MJ. Rapid method for coextraction of DNA and RNA from natural environments for analysis of ribosomal DNA- and rRNA-based microbial community composition. *Appl Environ Microbiol* 2000;66:5488–91.
- [55] Caporaso JG, Lauber CL, Walters WA, Berg-Lyons D, Lozupone CA, Turnbaugh PJ, et al. Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. *Proc Natl Acad Sci U S A* 2011;108:4516–22.
- [56] Schloss PD, Westcott SL, Ryabin T, Hall JR, Hartmann M, Hollister EB, et al. Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Appl Environ Microbiol* 2009;75:7537–41.
- [57] Kozich JJ, Westcott SL, Baxter NT, Highlander SK, Schloss PD. Development of a dual-index sequencing strategy and curation pipeline for analyzing amplicon sequence data on the Miseq illumina sequencing platform. *Appl Environ Microbiol* 2013;79:5112–20.
- [58] Box GEP, Hunter WG, Hunter JS. *Statistics for experimenters: an introduction to design, data analysis, and model building*. John Wiley and sons; 1978.
- [59] Khamtib S, Reungsang A. Biohydrogen production from xylose by *Thermoanaerobacterium thermosaccharolyticum* KKU19 isolated from hot spring sediment. *Int J Hydrogen Energy* 2012;37:12219–28.
- [60] Ren N, Cao G, Wang A, Lee D, Guo W, Zhu Y. Dark fermentation of xylose and glucose mix using isolated *Thermoanaerobacterium thermosaccharolyticum* W16. *Int J Hydrogen Energy* 2008;33:6124–32.
- [61] Koesnandar, Nishio N, Nagai S. . Effects of trace metal ions on the growth, homoacetogenesis and corrinoid production by *Clostridium thermoaceticum*. *J Ferment Bioeng* 1991;71:181–5.
- [62] Ottaviano LM, Ramos LR, Botta LS, Amâncio Varesche MB, Silva EL. Continuous thermophilic hydrogen production from cheese whey powder solution in an anaerobic fluidized bed reactor: effect of hydraulic retention time and initial substrate concentration. *Int J Hydrogen Energy* 2017;42:4848–60.
- [63] Meng B, Qian Z, Wei F, Wang W, Zhou C, Wang Z, et al. Proteomic analysis on the temperature-dependent complexes in *Thermoanaerobacter tengcongensis*. *Proteomics* 2009;9:3189–200.
- [64] Si B, Liu Z, Zhang Y, Li J, Xing X-H, Li B, et al. Effect of reaction mode on biohydrogen production and its microbial diversity. *Int J Hydrogen Energy* 2015;40:3191–200.
- [65] Chatellard L, Trably E, Carrère H. The type of carbohydrates specifically selects microbial community structures and fermentation patterns. *Bioresour Technol* 2016;221:541–9.
- [66] Wang B, Wan W, Wang J. Inhibitory effect of ethanol, acetic acid, propionic acid and butyric acid on fermentative hydrogen production. *Int J Hydrogen Energy* 2008;33:7013–9.
- [67] Ryan P, Forbes C, Collieran E. Investigation of the diversity of homoacetogenic bacteria in mesophilic and thermophilic anaerobic sludges using the formyltetrahydrofolate synthetase gene. *Water Sci Technol* 2008;57:675–80.
- [68] Oh S-E, Van Ginkel S, Logan BE. The relative effectiveness of pH control and heat treatment for enhancing biohydrogen gas production. *Environ Sci Technol* 2003;37:5186–90.
- [69] Dinamarca C, Bakke R. Apparent hydrogen consumption in acid reactors: observations and implications. *Water Sci Technol* 2009;59:1441–7.
- [70] O-Thong S, Prasertsan P, Karakashev D, Angelidaki I. High-rate continuous hydrogen production by *Thermoanaerobacterium thermosaccharolyticum* PSU-2 immobilized on heat-pretreated methanogenic granules. *Int J Hydrogen Energy* 2008;33:6498–508.
- [71] Van Groenestijn JW, Geelhoed JS, Goorissen HP, Meesters KPM, Stams AJM, Claassen PAM. Performance and population analysis of a non-sterile trickle bed reactor inoculated with *Caldicellulosiruptor saccharolyticus*, a thermophilic hydrogen producer. *Biotechnol Bioeng* 2009;102:1361–7.
- [72] Sharma Y, Li B. Optimizing energy harvest in wastewater treatment by combining anaerobic hydrogen producing biofermentor (HPB) and microbial fuel cell (MFC). *Int J Hydrogen Energy* 2010;35:3789–97.

File S1. Statistical analysis (ANOVA and Tukey test) conducted at $p = 0.05$ to assess significant differences in H_2 yield at the various temperatures investigated in the mesophilic (37 °C) and thermophilic (55 °C) FBR.

Descriptives

FBR	T (°C)	N	Mean H_2 yield	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
						Mesophilic	37		
	55	4	1.0040	0.02961	0.01481	0.9568	1.0511	0.97	1.03
Thermophilic	55	4	1.1855	0.04847	0.02424	1.1083	1.2626	1.12	1.23
	60	4	0.8221	0.02957	0.01479	0.7751	0.8692	0.79	0.85
	65	4	0.8197	0.11724	0.05862	0.6332	1.0063	0.69	0.97
	70	4	0.2139	0.03945	0.01973	1.1511	1.2767	1.17	1.26
	Total	24	0.9173	0.26748	0.05460	0.8043	1.0302	0.43	1.26

ANOVA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1.586	5	0.317	95.077	0.000
Within Groups	0.060	18	0.003		
Total	1.646	23			

Multiple comparisons

Multiple Comparisons

Dependent Variable: Yield

Tukey HSD

(I) Temperature	(J) Temperature	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
37 (M) **	55 (M)	-0.54537*	0.04084	0.000	-0.6751	-0.4156
	55 (T)	-0.72689*	0.04084	0.000	-0.8567	-0.5971
	60 (T)	-0.36356*	0.04084	0.000	-0.4933	-0.2338
	65 (T)	-0.36115*	0.04084	0.000	-0.4909	-0.2314
	70 (T)	-0.75530*	0.04084	0.000	-0.8851	-0.6255
55 (M)	37 (M)	0.54537*	0.04084	0.000	0.4156	0.6751
	55 (T)	-0.18152*	0.04084	0.004	-0.3113	-0.0517
	60 (T)	0.18181*	0.04084	0.004	0.0520	0.3116
	65 (T)	0.18422*	0.04084	0.003	0.0544	0.3140
	70 (T)	-0.20993*	0.04084	0.001	-0.3397	-0.0801
55 (T)	37 (M)	0.72689*	0.04084	0.000	0.5971	0.8567
	55 (M)	0.18152*	0.04084	0.004	0.0517	0.3113
	60 (T)	0.36333*	0.04084	0.000	0.2335	0.4931
	65 (T)	0.36574*	0.04084	0.000	0.2360	0.4955
	70 (T)	-0.02841	0.04084	0.980	-0.1582	0.1014
60.00 (T)	37 (M)	0.36356*	0.04084	0.000	0.2338	0.4933
	55 (M)	-0.18181*	0.04084	0.004	-0.3116	-0.0520
	55 (T)	-0.36333*	0.04084	0.000	-0.4931	-0.2335
	65 (T)	0.00242	0.04084	1.000	-0.1274	0.1322
	70 (T)	-0.39173*	0.04084	0.000	-0.5215	-0.2620
65.00 (T)	37 (M)	0.36115*	0.04084	0.000	0.2314	0.4909
	55 (M)	-0.18422*	0.04084	0.003	-0.3140	-0.0544
	55 (T)	-0.36574*	0.04084	0.000	-0.4955	-0.2360
	60 (T)	-0.00242	0.04084	1.000	-0.1322	0.1274
	70 (T)	-0.39415*	0.04084	0.000	-0.5239	-0.2644
70.00 (T)	37 (M)	0.75530*	0.04084	0.000	0.6255	0.8851
	55 (M)	0.20993*	0.04084	0.001	0.0801	0.3397
	55 (T)	0.02841	0.04084	0.980	-0.1014	0.1582
	60 (T)	0.39173*	0.04084	0.000	0.2620	0.5215
	65 (T)	0.39415*	0.04084	0.000	0.2644	0.5239

*. The mean difference is significant at the 0.05 level.

** . M = mesophilic FBR. T = thermophilic FBR.

Homogeneous subset

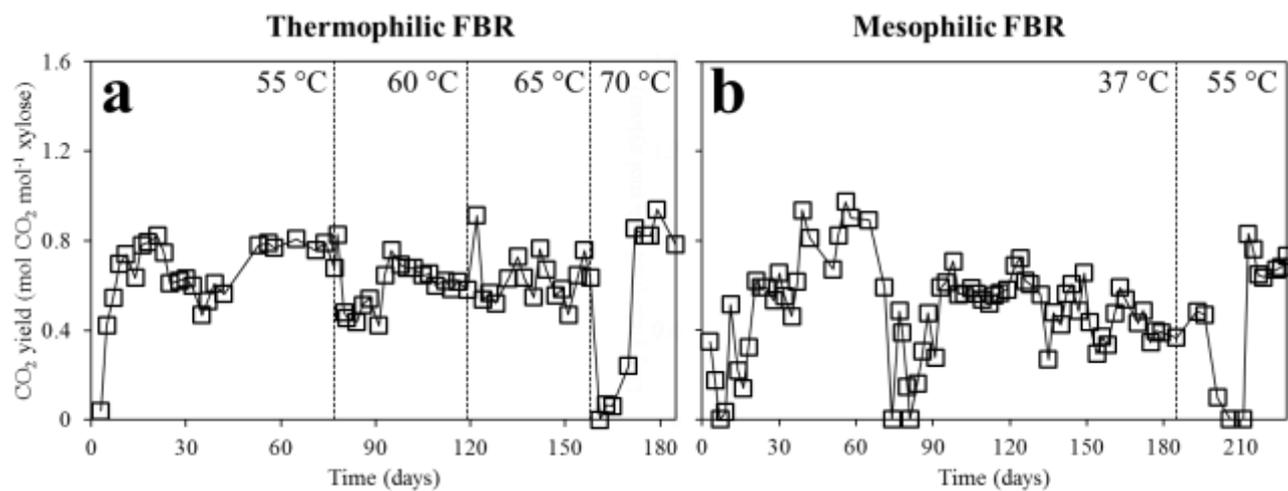
Tukey HSD*

Temperature	N	Subset for alpha = 0.05			
		1	2	3	4
37 (M) **	4	0.4586			
65 (T)	4		0.8197		
60 (T)	4		0.8221		
55 (M)	4			1.0040	
55 (T)	4				1.1855
70 (T)	4				1.2139
Sig.		1.000	1.000	1.000	0.980

Means H₂ yield for groups in homogeneous subsets are displayed.

*. Uses Harmonic Mean Sample Size = 4.000.

** . M = mesophilic FBR. T = thermophilic FBR.



File S2. CO₂ yield (mol CO₂ mol⁻¹ xylose added) in the thermophilic (a) and mesophilic (b) FBR.

The vertical dotted lines indicate a change of temperature.

IV

TEMPERATURE CONTROL AS KEY FACTOR FOR OPTIMAL BIOHYDROGEN PRODUCTION FROM THERMOMECHANICAL PULPING WASTEWATER

by

Dessi, P., Porca, E., Lakaniemi, A.-M., Collins, G., Lens, P.N.L., 2018

Biochemical Engineering Journal, *Submitted for publication*

1 Temperature control as key factor for optimal biohydrogen
2 production from thermomechanical pulping wastewater

3
4
5 *Paolo Dessì^{a,*}, Estefania Porca^b, Aino-Maija Lakaniemi^a, Gavin Collins^b, Piet N. L. Lens^{a,c}*

6
7 *^a Tampere University of Technology, Faculty of Natural Sciences, P.O. Box 541, FI-33101*

8 *Tampere, Finland*

9 *^bMicrobial Communities Laboratory, School of Natural Sciences, National University of Ireland*

10 *Galway, University Road, Galway, H91 TK33, Ireland*

11 *^cUNESCO-IHE, Institute for Water Education, Westvest 7, 2611AX Delft, The Netherlands*

12
13 Manuscript submitted to: *Biochemical Engineering Journal*

14
15 *Corresponding author: Phone: +358 417239696, e-mail: paolo.dessi@tut.fi, mail: Tampere
16 University of Technology, P.O. Box 541, FI-33101 Tampere, Finland

17 Estefania Porca: estefania.porca@gmail.com

18 Aino-Maija Lakaniemi: aino-maija.lakaniemi@tut.fi

19 Gavin Collins: gavin.collins@nuigalway.ie

20 Piet N.L. Lens: piet.lens@tut.fi

23 **Abstract**

24 This study evaluates the use of non-pretreated thermo-mechanical pulping (TMP) wastewater as a
25 potential substrate for hydrogen production by dark fermentation. Batch incubations were
26 conducted in a temperature gradient incubator at temperatures ranging from 37 to 80 °C, using an
27 inoculum from a thermophilic, xylose-fed, hydrogen-producing fluidised bed reactor. The aim was
28 to assess the short-term response of the microbial communities to the different temperatures with
29 respect to both hydrogen yield and composition of the active microbial community. High
30 throughput sequencing (MiSeq) of the reversely transcribed 16S rRNA showed that
31 *Thermoanaerobacterium* sp. dominated the active microbial community at 70 °C, resulting in the
32 highest hydrogen yield of 3.6 (\pm 0.1) mmol H₂ g⁻¹ COD_{tot} supplied. Lower hydrogen yields were
33 obtained at the temperature range 37 to 65 °C, likely due to consumption of the produced hydrogen
34 by homoacetogenesis. No hydrogen production was detected at temperatures above 70 °C. TMP
35 wastewaters are released at high temperatures (50 to 80 °C), and thus dark fermentation at 70 °C
36 could be sustained using the heat produced by the pulp and paper plant itself without any
37 requirement for external heating.

38

39 **Keywords**

40 Hydrogen, lignocellulose, MiSeq, pulp and paper mill, *Thermoanaerobacterium*, thermophilic

41

42

43

44

45

46

47

48

49 **1. Introduction**

50 Pulp and paper industry is facing an economic challenge due to globalised competition and
51 decreasing paper demand (Machani et al., 2014). The long-term success of the industry is believed
52 to be strictly linked to the ability of companies to innovate and create new value streams, which are
53 predicted to generate 40% of the companies' turnover in 2030 (Toppinen et al., 2017). A biorefinery
54 concept, in which waste from the pulp and paper making process is used as a resource to generate
55 value-added products such as biofuels and biochemicals, is a promising strategy to expand the
56 product platform, reduce waste disposal costs and fulfil the environmental policies on waste
57 emissions (Kinnunen et al., 2015; Machani et al., 2014; Moncada B. et al., 2016).

58

59 Pulping is the major source of polluted wastewaters of the whole papermaking process (Pokhrel and
60 Viraraghavan, 2004). Pulp mill wastewater is typically treated by the traditional activated sludge
61 process, but anaerobic processes have the advantages of coupling wastewater treatment to
62 renewable energy production, produce a lower amount of waste sludge and require a smaller
63 volume than aerobic processes (Ashrafi et al., 2015). Among pulping processes, thermomechanical
64 pulping (TMP) produces a wastewater more suited for anaerobic biological processes than
65 chemical-based pulping, due to the low concentrations of inhibitory compounds such as sulphate,
66 sulphite, hydrogen peroxide, resin acid and fatty acids (Ekstrand et al., 2013; Rintala and Puhakka,
67 1994).

68

69 TMP wastewater has been successfully used as a substrate for both mesophilic (Gao et al., 2016)
70 and thermophilic (Rintala and Lepistö, 1992) methane production via anaerobic digestion.
71 However, hydrogen (H₂) is a carbon free fuel expected to play a pivotal role in energy production in
72 the future (Boodhun et al., 2017). Dark fermentative H₂ production has the potential for energy
73 recovery from waste paper hydrolysate (Eker and Sarp, 2017), pulp and paper mill effluent
74 hydrolysates (Lakshmidēvi and Muthukumar, 2010) and even from untreated pulps (Nissilä et al.,

75 2012). Dark fermentative H₂ production has also been reported from carbohydrate-containing
76 wastewaters, such as starch wastewater and palm oil mill effluent (Badiei et al., 2011; Xie et al.,
77 2014). Although TMP wastewaters are characterized by a high content of carbohydrates (25 to 40%
78 of the total COD) (Rintala and Puhakka, 1994), to our knowledge it has not yet been tested as a
79 substrate for dark fermentation.

80

81 Thermophilic dark fermentation of TMP wastewater could be advantageous, as both biological
82 polysaccharide hydrolysis (Elsharnouby et al., 2013) and H₂ yielding reactions (Verhaart et al.,
83 2010) are favoured by high temperature. High temperature also limits the growth of
84 homoacetogenic bacteria and methanogenic archaea (Oh et al., 2003), which may consume the
85 produced H₂ in mixed culture systems. The main drawback of thermophilic processes is the energy
86 required to heat the reactors, but TMP wastewaters are released from the pulping process at a
87 temperature of 50 to 80 °C (Rintala and Lepistö, 1992), and could therefore be treated in
88 thermophilic bioreactors with minimal, or even without external heating.

89

90 Temperature is a key factor in dark fermentation, as even a change of a few degrees may result in
91 the development of a different microbial community and thus, affect the H₂ yield (Karadag and
92 Puhakka, 2010). Understanding of the composition of the microbial community is also crucial in
93 order to optimize the complex microbial H₂ production process, involving both hydrolytic and
94 fermentative microorganisms (Kumar et al., 2017). Microbial communities from dark fermentation
95 of lignocellulose-based waste and wastewaters have been previously studied at DNA level (Nissilä
96 et al., 2012; Xie et al., 2014), but a RNA-based approach can provide more detailed information on
97 the microorganisms that produce (and consume) H₂. Furthermore, the time response on RNA
98 changes is much faster than on DNA changes (De Vrieze et al., 2016), allowing to detect the
99 response of the microbial community to an environmental change in a relatively short time.

100

101 In a previous study, a mixed culture was successfully adapted to thermophilic (70 °C) dark
102 fermentation of xylose in a fluidised bed reactor (FBR) and the H₂ producing
103 *Thermoanaerobacterium* sp. accounted for > 99% of the active microbial community (Dessi et al.,
104 2018). In this study, the same adapted mixed culture was used to test if TMP wastewater is a
105 suitable substrate for dark fermentative H₂ production at various temperatures (37 to 80 °C), and
106 describe how the active microbial community responds to the different temperatures.

107

108 **2. Materials and methods**

109 ***2.1 Source of microorganisms***

110 The inoculum used in this study was biofilm-coated activated carbon originating from a
111 thermophilic fluidised bed reactor (FBR) used to study H₂ production from xylose via dark
112 fermentation by gradually increasing the temperature of the reactor from 55 to 70 °C (Dessi et al.,
113 2018). The FBR was initially inoculated with heat-treated (90 °C, 15 min) activated sludge
114 originating from a municipal wastewater treatment plant (Viinikanlahti, Tampere, Finland). The
115 biofilm-coated activated carbon granules were sampled after 185 days of reactor operation, at that
116 point the FBR had been operated at 70 °C for 27 days. No xylose was present in the FBR medium at
117 the sampling time. The granules were stored at 4 °C for one week prior to utilisation. This inoculum
118 was used because the microbial community was dominated by *Thermoanaerobacterium* sp. (Dessi
119 et al., 2018), which previously showed potential for hydrolysis of lignocellulosic substrates and H₂
120 production from the resulting sugars (Cao et al., 2014).

121

122 ***2.2 Wastewater characterization***

123 The wastewater was collected from a pulp and paper mill located in Finland. It was the effluent of a
124 TMP process, in which wood was exposed to a high-temperature (120 °C) steam in order to obtain
125 the pulp. The wastewater had a temperature of about 70 °C at the time of the sampling, but was

126 cooled down and stored at 4 °C to minimise biological activity that might affect its composition.
127 The wastewater had a pH of 5.0 and a composition as given in Table 1.

128

129 [Table 1 here](#)

130

131 ***2.3 Temperature-gradient batch set-up***

132 The batch cultures were conducted in anaerobic tubes with a total volume of 26 mL (17 mL
133 working volume and 9 mL headspace). The tubes were inoculated by adding 2 mL of biofilm-
134 coated activated carbon granules to 15 mL of TMP wastewater (Table 1). All the tubes were flushed
135 with N₂ for 5 min, and the internal pressure was equilibrated to atmospheric pressure by removing
136 the excess gas using a syringe and a needle before incubation. The initial pH of the batch cultures
137 (wastewater and inoculum) was adjusted to 6.3 (\pm 0.1) using 1 M NaOH, as higher pH may favour
138 the growth of methanogenic archaea (Jung-Yeol et al., 2012). The tubes were incubated at 200 rpm
139 shaking in a temperature-gradient incubator (Test Tube Oscillator, Terratec, Germany) at 37, 42, 48,
140 55, 59, 65, 70, 74 or 80°C (duplicate tubes at each temperature). The experiment was interrupted
141 after 111 hours, when no H₂ production was detected in any of the vials in two consecutive samples,
142 as long inactive periods may affect the RNA-level analysis (De Vrieze et al., 2016).

143

144 Gas samples were collected for analysis 1 to 3 times per day. End-point liquid samples were
145 collected and stored at -20 °C before analysis. Abiotic negative controls, with fresh activated carbon
146 and TMP wastewater, were prepared at 37, 55 and 70 °C. Control incubations containing 2 mL of
147 fresh activated carbon and a mix of acetate and butyrate in Milli-Q[®] water (0.86 g COD_{tot} L⁻¹ each,
148 15 mL volume) were also prepared at 42, 65 and 80 °C to assess possible adsorption of VFAs on
149 virgin activated carbon.

150

151 ***2.4 Microbial community analyses***

152 Biofilm-coated activated carbon granules and liquid medium were collected at the end of the
153 experiment and stored in 5 mL Eppendorf tubes at -80 °C. Microbial community analysis was
154 conducted separately on microbial communities growing attached to the granules and suspended in
155 the liquid medium, as the growth of suspended biomass was clearly visible in the vials after
156 incubation in the temperature range 42 to 59 °C. Nucleic acids extraction using a modified method
157 from Griffiths et al. (2000), DNA inhibition, complementary DNA (cDNA) synthesis and
158 sequencing (using an Illumina MiSeq platform) were performed as described previously (Dessi et
159 al., 2018). Sequence analysis (1,395,864 sequences in total, 1,238,862 after quality check) was also
160 performed according to Dessì et al. (2018), but using a more recent version of Mothur (v1.39.5) and
161 Silva database (v128). The Illumina sequencing data was deposited to the NCBI Sequence Read
162 Archive under BioProject Number PRJNA428338.

163

164 ***2.5 Analytical methods***

165 Gas production in the tubes was quantified by a volumetric syringe method (Owen et al., 1979), and
166 the gas composition was determined by gas chromatography-thermal conductivity detector (GC-
167 TCD) as reported previously (Dessi et al., 2017). Acetate, butyrate, ethanol, propionate, lactate, and
168 formate concentrations were measured with a high-performance liquid chromatograph (HPLC)
169 equipped with a refractive index detector (RID) (Shimadzu, Japan) and a Rezex RHM-
170 monosaccharide column (Phenomenex, USA) held at 40 °C. The mobile phase was 5 mM H₂SO₄
171 and the flow rate was 0.6 mL min⁻¹. Glucose and xylose concentrations were measured using a
172 HPLC equipped with a RID and a RPM-monosaccharide column (Phenomenex, USA) held at 85 °C
173 with Milli-Q[®] water at a flow rate of 0.6 mL min⁻¹ as the mobile phase. Furfural concentrations
174 were measured by gas chromatography-mass spectrometry (GC-MS) according to Doddapaneni et
175 al. (Doddapaneni et al., 2018). Samples for HPLC and GC-MS analysis were filtered using 0.2 µm
176 pore size filters. Total chemical oxygen demand (COD_{tot}) and COD of the soluble compounds
177 (COD_s) was measured using the dichromate method according to the Finnish standard SFS 5504.

178 Initial and final pH of the culture and the pH of the wastewater were determined using a WTW pH
179 330 meter equipped with a Hamilton® Slimtrode probe (Sigma-Aldrich, USA). Total solids,
180 volatile solids, total nitrogen and $\text{PO}_4^{3-}\text{-P}$ were determined by the APHA standard procedures
181 (APHA, 1998).

182

183 **2.6 Calculations**

184 Cumulative H_2 and CO_2 production was calculated according to Logan et al. (2002) and corrected
185 for temperature according to the Arrhenius equation. The theoretical COD_{tot} was estimated from the
186 sum of the compounds detected by HPLC, according to the following equation (Van Haandel and
187 Van der Lubbe, 2012):

188

$$189 \text{COD}_{\text{tot}} = 8 \cdot (4x + y - 2z) / (12x + y + 16z) \text{ g COD}_{\text{tot}} \text{ g}^{-1} \text{C}_x\text{H}_y\text{O}_z \quad (1)$$

190

191 where x, y and z are the number of C, H and O atoms in the organic molecule, respectively.

192

193 **2.7 Statistical analysis**

194 One-way analysis of variance (ANOVA) and the Tukey test (Box et al., 1978) at $p = 0.05$ were
195 conducted using the IBM SPSS Statistics package to assess significant differences in H_2 yield after
196 incubation at different temperatures.

197

198 **3. Results**

199 **3.1 H_2 production from TMP wastewater at the various temperatures**

200 Batch incubations with TMP wastewater resulted in a different net H_2 yield at different
201 temperatures (Figure 1; Table 2). The highest final H_2 yield of $3.6 (\pm 0.1) \text{ mmol H}_2 \text{ g}^{-1} \text{COD}_{\text{tot}}$ was
202 obtained in the batch cultures at 70°C , in which H_2 production started within 24 h of incubation and
203 remained stable after reaching the maximum (Figure 1). The maximum H_2 yield obtained at 65°C

204 was comparable to the one obtained at 70 °C, but the produced H₂ started to be consumed within 36
205 h resulting in a 51% lower final yield (Figure 1; Table 2). In the batch cultures at temperatures
206 lower than 70 °C, the H₂ produced was always partially (at 37, 42, 59 and 65 °C) or totally (at 48
207 and 55 °C) consumed. A negligible H₂ production was obtained at both 74 and 80 °C (Figure 1), as
208 well as in the negative controls (see Additional file 1).

209

210 Figure 1 here

211

212 Table 2 here

213

214 ***3.2 COD_{tot} removal and metabolite production at the various temperatures***

215 Similarly to H₂ production yields, dark fermentation of TMP wastewater at the various temperatures
216 resulted in a different composition of the liquid phase (Figure 2). Acetate was the most abundant
217 metabolite detected in the temperature range 37 to 70 °C. The final acetate concentration increased
218 with temperature from 0.34 (± 0.04) g COD_{tot} L⁻¹ at 37 °C to 0.75 (± 0.18) g COD_{tot} L⁻¹ at 55 °C,
219 and then decreased stepwise to 0.07 (± 0.00) and 0.08 (± 0.01) g COD_{tot} L⁻¹ at 74 and 80 °C,
220 respectively (Figure 2). Butyrate was found regardless of the incubation temperature, with a final
221 concentration ranging from 0.06 (± 0.00) g COD_{tot} L⁻¹ at 70 °C to 0.19 (± 0.00) g COD_{tot} L⁻¹ at 59
222 °C. Ethanol was produced at 37, 42, 59, 65 and 70 °C, with a maximum of 0.14 (± 0.02) g COD_{tot} L⁻¹
223 at 65 °C (Figure 2). Dark fermentation of TMP wastewater caused a pH decrease from the initial
224 value of 6.3: the final pH was in the range 5.7 to 6.1 after incubation at 42, 48, 55, 59, 74 and 80 °C,
225 but was only 5.5 (± 0.1) after incubation at 37 °C, 5.2 (± 0.1) at 65 °C and 5.3 (± 0.0) at 70 °C
226 (Figure 2).

227

228 Figure 2 here

229

230 In the batch incubations at various temperatures, the COD_{tot} removal efficiency ranged from 69.4%
231 at 74 °C to 79.7% at 42 °C, resulting in a decrease from the initial concentration of 2.86 (± 0.00) g
232 COD_{tot} L⁻¹ to a final concentration ranging from 0.58 (± 0.23) g COD_{tot} L⁻¹ at 42 °C and 0.88 (±
233 0.06) g COD_{tot} L⁻¹ at 74 °C (Table 3). The COD_{tot} removal efficiency was likely overestimated due
234 to the adsorption of VFAs on the activated carbon: in the adsorption experiment (see Additional file
235 2), up to 27% of the acetate and 90% of the butyrate was, in fact, adsorbed on the fresh activated
236 carbon after 111 h of incubation. The COD_{tot} measured was comparable to the COD_{tot} estimated
237 (using Eq. 1) by the sum of sugars and volatile fatty acids in the liquid phase after incubation in the
238 temperature range 42 to 65 °C (Table 3) . However, the difference between measured and estimated
239 COD_{tot} was about 0.20 g COD_{tot} L⁻¹ at 37, 70 and 80 °C, and even higher at 74 °C (0.51 g COD_{tot} L⁻¹).
240 ¹).

241

242 [Table 3 here](#)

243

244 **3.3 Effect of temperature on the active microbial community**

245 Incubation temperature clearly impacted the composition of the active microbial communities
246 growing for 111 h on TMP wastewater (Figure 3, Table 4). At 37 °C, *Clostridium* sp. accounted for
247 84 and 90% of the attached and suspended active microbial community, respectively. Higher
248 temperature resulted in a gradual decrease of the relative abundance of *Clostridium* sp., being 54%
249 of the attached active microbial community and < 2% of the suspended active microbial community
250 after incubation at 55 °C (Figure 3). *Clostridium* sp. was not detected either in the attached or
251 suspended active community after incubation at temperatures ≥ 59 °C (Figure 3). A bacterium
252 belonging to the order of *Bacillales* closely related to *B. coagulans* (Table 4) was detected in the
253 active attached and suspended microbial communities after incubation at 42 °C, with a relative
254 abundance of 14 and 10%, respectively, and only in suspended form after incubation at 48 °C, with
255 a relative abundance of 50% (Figure 3).

256

257 The relative abundance of *Thermoanaerobacterium* sp. (99% similarity to *T.*
258 *thermosaccharolyticum*) among the attached active microorganisms gradually increased with
259 temperature, being only 2% after incubation at 37 °C and 87% at 59 °C (Figure 3, Table 4).
260 *Thermoanaerobacterium* sp. was also the most common suspended active microorganism after
261 incubation at 55 and 59 °C, with a relative abundance of 96 and 83%, respectively. After incubation
262 at 65 °C, the relative abundance of *Thermoanaerobacterium* sp. in the attached and suspended
263 active microbial community decreased to 57 and 25%, respectively, whereas unclassified
264 *Firmicutes*, with 92% similarity to *Calditerricola* sp. (Table 4) were found with a relative
265 abundance of 30 and 28%, respectively. After incubation at 70 °C, *Thermoanaerobacterium* sp. was
266 again the dominant active microorganism in both attached and suspended form, with a relative
267 abundance of 88 to 89%. After incubation at 59 and 70 °C, *Caldanaerobius* sp. was also found in
268 both attached and suspended form with relative abundance > 10% (Figure 3). After incubation at
269 both 74 and 80 °C, the RNA concentration was not high enough to perform the analysis due to poor
270 microbial growth, and thus microbial communities from 74 and 80 °C could not be analysed.

271

272 Figure 3 here

273

274 Table 4 here

275

276 **4. Discussion**

277 ***4.1 Fermentation of TMP wastewater at different temperatures***

278 H₂ production from TMP wastewater inoculated with biofilm-coated activated carbon granules was
279 observed at a wide temperature range of 37 to 70 °C (Figure 1). The highest final H₂ yield of 3.6 (±
280 0.1) mmol H₂ g⁻¹ COD_{tot} supplied, or 4.9 mmol H₂ g⁻¹ COD_{tot} consumed, was obtained at 70 °C
281 (Table 2), which could be expected as the inoculum was collected from an FBR operated at 70 °C

282 (Dessì et al., 2018). Such H₂ yield is of the same order of magnitude compared to previous studies
283 on thermophilic direct dark fermentation of industrial, sugar-containing wastewaters. Xie et al.
284 (2014) obtained 5.8 mmol H₂ g⁻¹ COD_{tot} from starch wastewater at 55°C by a mixed culture
285 dominated by *T. thermosaccharolyticum*, whereas Khongkliang et al. (2017) obtained 11.4 mmol
286 H₂ g⁻¹ COD_{tot} from starch wastewater by a pure *T. thermosaccharolyticum* culture.

287

288 The thermophilic active mixed microbial community previously enriched on xylose in the FBR was
289 dominated by microorganisms closely related to *Thermoanaerobacterium thermosaccharolyticum*
290 (Dessì et al., 2018). Changing of the substrate from xylose to TMP wastewater marginally impacted
291 the active microbial community in the temperature range 59 to 70 °C, as most of the sequences
292 obtained from the RNA samples matched *T. thermosaccharolyticum* (Table 4). A mixed culture
293 dominated by *T. thermosaccharolyticum* has been shown to produce 7 mmol H₂ g⁻¹ cellulose at 70
294 °C (Gadow et al., 2013), showing potential for the one-step conversion of lignocellulosic materials
295 to H₂, avoiding a costly hydrolysis step. In fact, the genus *Thermoanaerobacterium* includes strains
296 of cellulolytic microorganisms, such as some strains of *T. thermosaccharolyticum*, able to hydrolyse
297 both cellulose and hemicellulose, and produce H₂ from the resulting monosaccharides (Cao et al.,
298 2014). In this study, however, the microbial community analysis conducted at genus level does not
299 allow to assess possible cellulolytic capabilities of the detected *Thermoanaerobacterium* sp.

300

301 Although the inoculum was enriched for dark fermentation at 70 °C, H₂ production occurred only
302 after 24 h of incubation (Figure 1). This is probably due to the handling of the inoculum, which was
303 stored at 4 °C for one week prior to being used for this experiment. Changes in gene expression and
304 DNA replication were shown to occur in *Thermoanaerobacter tengcongensis* as response to a cold
305 shock (Liu et al., 2014), as could be the case for the *Thermoanaerobacterium* sp. dominating the
306 active microbial community of the inoculum used in this study. Although *Thermoanaerobacterium*
307 sp. was the most abundant microorganism (relative abundance close to 90%) in both the attached

308 and suspended microbial community at both 59 and 70 °C, its relative abundance was lower at 65
309 °C (Figure 3). The same phenomenon was observed in the FBR from where the inoculum originated
310 (Dessi et al., 2018), and attributed to either the decreased activity of *Thermoanaerobacterium* sp. or
311 to the increased activity of competing microorganisms at 65 °C.

312

313 Despite the inoculum was enriched for thermophilic dark fermentation, H₂ was already produced
314 after 12 h of incubation at 37 °C, reaching a maximum yield of 3.2 (± 0.1) mmol H₂ g⁻¹ COD_{tot}
315 supplied within 24 h (Figure 1). A maximum yield of only 0.9 mmol H₂ g⁻¹ COD_{tot} supplied was
316 previously obtained at 37 °C from a paper mill wastewater using heat treated digested sludge as
317 inoculum (Marone et al., 2017). The H₂ yields obtained in this study are also higher than those
318 reported by Lucas et al. (2015) by mesophilic (37 °C) dark fermentation of cassava, dairy and citrus
319 wastewater, which produced 1.4, 1.7 and 1.3 mmol H₂ g⁻¹ COD_{tot} supplied, respectively. This
320 confirms the high potential of TMP wastewater for dark fermentation.

321

322 *Clostridium* sp. proliferated at 37 °C accounting for more than 80% of both the attached and
323 suspended active microbial community at the end of the batch incubation (Figure 3). It is plausible
324 that *Clostridium* sp. were present in the parent activated sludge but inactive in the FBR operated at
325 70 °C (Dessi et al., 2018). In fact, *Clostridium* sp. produce spores to survive harsh conditions, and
326 are able to restore their metabolic activity after desporulation as soon as the environmental
327 conditions become more favourable (Li and Fang, 2007). *Clostridium* sp. cells might also have been
328 present in the TMP wastewater, which was not sterilised. However, the absence of H₂ and CO₂ in
329 the abiotic negative control at 37 °C (see Additional file 1) suggests that *Clostridium* sp. did not
330 proliferate in the absence of the inoculum.

331

332 In this study, no H₂ was produced at 74 or 80 °C (Figure 1) and the RNA concentration was too low
333 to allow sequencing analysis, suggesting a lack of active species. This was attributed to the source

334 of inoculum used, as species within the *Thermoanaerobacterium* genus, such as *T.*
335 *thermosaccharolyticum*, may be inhibited by temperatures higher than 70 °C (Ren et al., 2008).
336 Gadow et al. (2013) obtained H₂ production from cellulose by a mixed microflora from a sewage
337 sludge digester even at 75 and 80 °C. However, H₂ production at such high temperatures was
338 attributed to *Thermoanaerobacter tengcongensins* (Gadow et al., 2013), which was not part of the
339 active microbial community in this study. Some degradation products of hemicellulose such as
340 furfural or hydromethylfurfural may inhibit fermentative microorganisms (Jönsson et al., 2013),
341 including *Thermoanaerobacterium*, at a concentration over 1 g L⁻¹ (Cao et al., 2010). However, the
342 TMP process is conducted at temperatures below 120 °C, which is too low to produce such high
343 concentrations of these inhibitory compounds (Baêta et al., 2017). In fact, the concentration of
344 furfural in the TMP wastewater used in this study was below the detection limit of the GC-MS
345 (Table 1).

346

347 A decrease in the cumulative H₂ production occurred in all the incubations at temperatures lower
348 than 70 °C (Figure 1), probably due to the activity of homoacetogenic bacteria. Homoacetogenesis,
349 in which 4 moles of H₂ and 2 mol of CO₂ are consumed per mol of acetate produced, often occurs
350 in batch H₂ production experiments within the first 80 h of incubation, especially under mesophilic
351 conditions (for a review, see Saady, 2013). However, in this study, H₂ seems to be consumed faster
352 under thermophilic (from 48 to 65 °C) as compared to mesophilic (37 °C) conditions (Figure 1),
353 suggesting that homoacetogenic microorganisms were mainly thermophiles or moderate
354 thermophiles. The CO₂ concentration in the batch incubations did not decrease as expected in case
355 of homoacetogenesis (see Additional file 3). However, this could be explained considering that CO₂
356 production may occur also through non-hydrogenic pathways, mainly the ethanol production
357 pathway (Figure 2). In the abiotic negative control, CO₂ was also detected, together with acetate, at
358 both 55 and 70 °C, where H₂ production was not observed (see Additional file 1), suggesting that

359 other non-hydrogenic, CO₂ producing pathways other than ethanol production could have occurred
360 as well.

361

362 Homoacetogens are among the most phylogenetically diverse functional groups of bacteria (Drake
363 et al., 2006). Among the thermophiles, *Moorella thermoacetica*, which accounted for 5% of the
364 suspended active community at 55 °C and 6% of the attached active community at 65 °C (Figure 3),
365 is a known homoacetogenic bacterium with an optimum growth temperature of 55 to 60 °C (Drake
366 et al., 2006). Also *Clostridium* sp. have been previously found in thermophilic fermentative reactors
367 and associated with homoacetogenesis (Ryan et al., 2008). It is plausible that the shift to autotrophic
368 metabolism (e.g. homoacetogenesis) occurred after substrate depletion, as suggested by Oh et al.
369 (2003).

370

371 **4.2 COD_{tot} balance and metabolite production**

372 The COD_{tot} measured in the beginning of the incubations (Table 3) was 15% lower than the value
373 obtained while characterizing the TMP wastewater (Table 1). Apparently, some biological or non-
374 biological reaction occurred while storing the TMP wastewater at 4 °C before the experiment,
375 resulting in a slight COD_{tot} concentration decrease. The COD_{tot} removal efficiency during the
376 incubations was 69 to 80% regardless the incubation temperature (Table 3). It is in line with the
377 COD_{tot} removal from anaerobic digestion of pulp and paper wastewater reported in the literature
378 (Meyer and Edwards, 2014), but higher than expected for dark fermentation which usually removes
379 only 30 to 40% of the COD_{tot} (Sharma and Li, 2010). This was due to the adsorption of VFAs on
380 the activated carbon (see Additional file 2), which caused an overestimation of the COD_{tot} removal.
381 However, it should be noted that the adsorption experiment (see Additional file 2) was performed
382 with fresh activated carbon, whereas the main experiment was conducted with biofilm-covered
383 activated carbon. The latter could have been partially saturated with VFAs at the moment of

384 inoculation, as VFAs were produced in the FBR from where the inoculum originated (Dessi et al.,
385 2018).

386

387 In the temperature range 42 to 65 °C, more than 85% of the residual COD_{tot} was detected as acetate,
388 butyrate or ethanol by HPLC analysis (Table 3). However, 30 to 37% of the residual COD_{tot} was
389 not detected as compounds identified by HPLC analysis after incubation at 37, 70 and 80 °C, and
390 even 58% of the residual COD_{tot} was not identified after incubation at 74 °C. At 74 and 80 °C, most
391 of the undetected COD_{tot} is likely constituted by polysaccharides such as cellulose, which were not
392 degraded due to the lack of bacterial activity at such high temperatures. At 74 and 80 °C, CO₂ was
393 also not produced (see Additional file 3), supporting this conclusion. Lignocellulosic materials can
394 release VFAs at temperatures around 80 °C (Veluchamy and Kalamdhad, 2017), suggesting that the
395 acetate and butyrate detected at 74 and 80 °C (Figure 2) were produced physically rather than
396 biologically.

397

398 The simultaneous production of acetate and butyrate suggests that H₂ was produced via both the
399 acetate and butyrate pathway in the temperature range 37 to 70 °C. Acetate was the main metabolite
400 found in the liquid phase at all temperatures tested, excluding 74 and 80 °C (Figure 2), and was
401 associated either to H₂ production through the acetate dark fermentative pathway or H₂
402 consumption by homoacetogenesis. Interestingly, acetate production increased with temperature in
403 the range of 37 to 55 °C, and then decreased stepwise for temperatures above 55 °C (Figure 2). In
404 particular, the high (> 0.7 g COD_{tot} L⁻¹) acetate (Figure 2) and concomitant low (< 0.5 mmol g⁻¹
405 COD_{tot}) cumulative H₂ yield (Figure 1) suggest that the optimum growth temperature for
406 homoacetogenic bacteria was about 55 °C in this study. At 70 °C, however, the H₂ produced was
407 not consumed during the incubation (Figure 1), suggesting inhibition of homoacetogenic
408 microorganisms.

409

410 Solventogenesis occurred both in mesophilic (37 and 42 °C) and thermophilic (59, 65, and 70 °C)
411 batch cultures, resulting in ethanol production (Figure 2). *Clostridium* sp., which dominated the
412 active microbial communities under mesophilic conditions (Figure 3), may shift its metabolism
413 from acidogenesis to solventogenesis as response to a change of pH or volatile fatty acids
414 concentration, but the mechanism which triggers solventogenesis is not well understood (Kumar et
415 al., 2013). A pure culture of *T. thermosaccharolyticum* has been reported to produce ethanol
416 together with acetate and butyrate by dark fermentation of cellulose and complex lignocellulosic
417 substrates such as corn cob, corn straw and wheat straw (Cao et al., 2014). Similarly, in this study,
418 acetate, butyrate and ethanol were the main metabolites (Figure 2) of the dark fermentation of TMP
419 wastewater at 65 and 70 °C by a mixed culture dominated by *T. thermosaccharolyticum* (Figure 3;
420 Table 4).

421

422 **4.3 Practical implications**

423 Hydraulic retention times lower than 24 hours are typically used for dark fermentation of
424 wastewater (Lin et al., 2012). Therefore, based on the results obtained (Figure 1), dark fermentation
425 of TMP wastewater at 37 and 65 °C appears favourable if suspended biomass bioreactors are used,
426 as homoacetogenic bacteria would be flushed out (Figure 1). However, due to the high dilution of
427 TMP wastewater, bioreactors retaining high active biomass content, such as FBRs or upflow
428 anaerobic sludge bioreactors (UASBs), would enable higher organic loading and conversion rates
429 than suspended biomass bioreactors (Koskinen et al., 2006). Therefore, dark fermentation of TMP
430 in attached biomass bioreactors at 70 °C is recommended (Figure 1). A proper insulation and
431 temperature control are nevertheless necessary to keep accurately 70 °C in the bioreactor, as a
432 decrease of 5 °C may already result in a decreased efficiency due to H₂ consumption by
433 homoacetogenic bacteria. However, H₂ production at 70 °C can be quickly restored in case of
434 failure of the temperature control. In fact, H₂ production was detected at 70 °C within only 24 h
435 (Figure 1) with a thermophilic inoculum previously stored at 4 °C for one week.

436

437 Despite the surprisingly high COD_{tot} removal efficiency of 69 to 80 % obtained in this study (Table
438 3), dark fermentation of TMP wastewater resulted in the generation of an effluent containing 0.5 –
439 1.0 g COD_{tot} L⁻¹ (Table 3), mainly in the form of VFAs, thus requiring further treatment prior to be
440 discharged. Such effluent can be either treated by a traditional activated sludge plant, or further
441 valorised by producing energy or high value chemicals. Promising strategies for the valorisation of
442 dark fermentation effluents include further H₂ production by photofermentation or microbial
443 electrolysis cells, methane production by anaerobic digestion, and bioplastics or electricity
444 production using microbial fuel cells (for reviews, see Ghimire et al., 2015 and Bundhoo, 2017).

445

446 **5. Conclusions**

447 Hydrogen was produced by dark fermentation from TMP wastewater at a wide range of
448 temperatures (37 to 70 °C) using a mixed microbial community enriched on xylose at thermophilic
449 conditions. An operation temperature of 70 °C was the most favourable for dark fermentative H₂
450 production and effectively repressed the activity of homoacetogenic bacteria. Therefore,
451 considering that TMP wastewater is produced at elevated temperature, dark fermentation at 70 °C
452 may be a cost-effective approach for the treatment and valorisation of this wastewater. Temperature
453 must be efficiently controlled, as a shift of only a few degrees may decrease the H₂ yield.

454

455 **Acknowledgements**

456 This work was supported by the Marie Skłodowska-Curie European Joint Doctorate (EJD) in
457 Advanced Biological Waste-To-Energy Technologies (ABWET) funded from Horizon 2020 under
458 grant agreement no. 643071.

459

460 **References**

461 APHA, 1998. Standard Methods for the Examination of Water and Wastewater, twentieth ed.

462 American Public Health Association/American Water Works Association/Water Environment
463 Federation, Washington DC.

464 Ashrafi, O., Yerushalmi, L., Haghghat, F., 2015. Wastewater treatment in the pulp-and-paper
465 industry: A review of treatment processes and the associated greenhouse gas emission. J.
466 Environ. Manage. 158, 146–157.

467 Badiei, M., Jahim, J.M., Anuar, N., Abdullah, S.R.S., 2011. Effect of hydraulic retention time on
468 biohydrogen production from palm oil mill effluent in anaerobic sequencing batch reactor. Int.
469 J. Hydrogen Energy 36, 5912–5919.

470 Baêta, B.E.L., Cordeiro, P.H. de M., Passos, F., Gurgel, V.A.L., de Aquino, S.F., Fdz-Polanco, F.,
471 2017. Steam explosion pretreatment improved the biomethanization of coffee husks.
472 Bioresour. Technol. 245, 66–72.

473 Boodhun, B.S.F., Mudhoo, A., Kumar, G., Kim, S.-H., Lin, C.-Y., 2017. Research perspectives on
474 constraints, prospects and opportunities in biohydrogen production. Int. J. Hydrogen Energy
475 42, 27471–27481.

476 Box, G.E.P., Hunter, W.G., Hunter, J.S., 1978. Statistics for experimenters: An introduction to
477 design, data analysis, and model building. John Wiley and sons.

478 Bundhoo, Z.M.A., 2017. Coupling dark fermentation with biochemical or bioelectrochemical
479 systems for enhanced bio-energy production: A review. Int. J. Hydrogen Energy 42, 26667–
480 26686.

481 Cao, G.-L., Zhao, L., Wang, A.-J., Wang, Z.-Y., Ren, N.-Q., 2014. Single-step bioconversion of
482 lignocellulose to hydrogen using novel moderately thermophilic bacteria. Biotechnol. Biofuels
483 7, 82.

484 Cao, G., Ren, N., Wang, A., Guo, W., Xu, J., Liu, B., 2010. Effect of lignocellulose-derived
485 inhibitors on growth and hydrogen production by *Thermoanaerobacterium*
486 *thermosaccharolyticum* W16. Int. J. Hydrogen Energy 35, 13475–13480.

487 De Vrieze, J., Regueiro, L., Props, R., Vilchez-Vargas, R., Jáuregui, R., Pieper, D.H., Lema, J.M.,

488 Carballa, M., 2016. Presence does not imply activity: DNA and RNA patterns differ in
489 response to salt perturbation in anaerobic digestion. *Biotechnol. Biofuels* 9, 244.

490 Dessì, P., Lakaniemi, A.-M., Lens, P.N.L., 2017. Biohydrogen production from xylose by fresh and
491 digested activated sludge at 37, 55 and 70 °C. *Water Res.* 115, 120–129.

492 Dessì, P., Porca, E., Waters, N.R., Lakaniemi, A.-M., Collins, G., Lens, P.N.L., 2018. Thermophilic
493 versus mesophilic dark fermentation in xylose-fed fluidised bed reactors: Biohydrogen
494 production and active microbial community. *Int. J. Hydrogen Energy* 43, 5473–5485.

495 Doddapaneni, T.R.K.C., Jain, R., Praveenkumar, R., Rintala, J., Romar, H., Kontinen, J., 2018.
496 Adsorption of furfural from torrefaction condensate using torrefied biomass. *Chem. Eng. J.*
497 334, 558–568.

498 Drake, H.L., Küsel, K., Matthies, C., 2006. Acetogenic Prokaryotes, in: Springer (Ed.), *The*
499 *Prokaryotes*. New York, pp. 354–420.

500 Eker, S., Sarp, M., 2017. Hydrogen gas production from waste paper by dark fermentation: Effects
501 of initial substrate and biomass concentrations. *Int. J. Hydrogen Energy* 42, 2562–2568.

502 Ekstrand, E.-M., Larsson, M., Truong, X.-B., Cardell, L., Borgström, Y., Björn, A., Ejlertsson, J.,
503 Svensson, B.H., Nilsson, F., Karlsson, A., 2013. Methane potentials of the Swedish pulp and
504 paper industry – A screening of wastewater effluents. *Appl. Energy* 112, 507–517.

505 Elsharnouby, O., Hafez, H., Nakhla, G., El Naggar, M.H., 2013. A critical literature review on
506 biohydrogen production by pure cultures. *Int. J. Hydrogen Energy* 38, 4945–4966.

507 Gadow, S.I., Jiang, H., Hojo, T., Li, Y.-Y., 2013. Cellulosic hydrogen production and microbial
508 community characterization in hyper-thermophilic continuous bioreactor. *Int. J. Hydrogen*
509 *Energy* 38, 7259–7267.

510 Gao, W.J., Han, M.N., Xu, C.C., Liao, B.Q., Hong, Y., Cumin, J., Dagnew, M., 2016. Performance
511 of submerged anaerobic membrane bioreactor for thermomechanical pulping wastewater
512 treatment. *J. Water Process Eng.* 13, 70–78.

513 Ghimire, A., Frunzo, L., Pirozzi, F., Trably, E., Escudie, R., Lens, P.N.L., Esposito, G., 2015. A

514 review on dark fermentative biohydrogen production from organic biomass: Process
515 parameters and use of by-products. *Appl. Energy* 144, 73–95.

516 Griffiths, R.I., Whiteley, A.S., O'Donnell, A.G., Bailey, M.J., 2000. Rapid method for coextraction
517 of DNA and RNA from natural environments for analysis of ribosomal DNA- and rRNA-
518 based microbial community composition. *Appl. Environ. Microbiol.* 66, 5488–5491.

519 Jung-Yeol, L., Chen, X.-J., Lee, E.-J., Min, K.-S., 2012. Effects of pH and carbon sources on
520 biohydrogen production by co-culture of *Clostridium butyricum* and *Rhodobacter sphaeroides*.
521 *J. Microbiol. Biotechnol.* 22, 400–406.

522 Jönsson, L.J., Aliksson, B., Nilvebrant, N.-O., 2013. Bioconversion of lignocellulose: inhibitors
523 and detoxification. *Biotechnol. Biofuels* 6, 16.

524 Karadag, D., Puhakka, J.A., 2010. Effect of changing temperature on anaerobic hydrogen
525 production and microbial community composition in an open-mixed culture bioreactor. *Int. J.*
526 *Hydrogen Energy* 35, 10954–10959.

527 Khongkliang, P., Kongjan, P., Utarapichat, B., Reungsang, A., O-Thong, S., 2017. Continuous
528 hydrogen production from cassava starch processing wastewater by two-stage thermophilic
529 dark fermentation and microbial electrolysis. *Int. J. Hydrogen Energy* 42, 27584–27592.

530 Kinnunen, V., Ylä-Outinen, A., Rintala, J., 2015. Mesophilic anaerobic digestion of pulp and paper
531 industry biosludge—long-term reactor performance and effects of thermal pretreatment. *Water*
532 *Res.* 87, 105–111.

533 Koskinen, P.E.P., Kaksonen, A.H., Puhakka, J.A., 2006. The Relationship Between Instability of H₂
534 Production and Compositions of Bacterial Communities Within a Dark Fermentation
535 Fluidized-Bed Bioreactor. *Biotechnol. Bioeng.* 97, 742–758.

536 Kumar, G., Sivagurunathan, P., Sen, B., Mudhoo, A., Davila-Vazquez, G., Wang, G., Kim, S.-H.,
537 2017. Research and development perspectives of lignocellulose-based biohydrogen production.
538 *Int. Biodeterior. Biodegradation* 119, 225–238.

539 Kumar, M., Gayen, K., Saini, S., 2013. Role of extracellular cues to trigger the metabolic phase

540 shifting from acidogenesis to solventogenesis in *Clostridium acetobutylicum*. *Bioresour.*
541 *Technol.* 138, 55–62.

542 Lakshmidevi, R., Muthukumar, K., 2010. Enzymatic saccharification and fermentation of paper and
543 pulp industry effluent for biohydrogen production. *Int. J. Hydrogen Energy* 35, 3389–3400.

544 Li, C., Fang, H.H.P., 2007. Fermentative hydrogen production from wastewater and solid wastes by
545 mixed cultures. *Crit. Rev. Environ. Sci. Technol.* 37, 1–39.

546 Lin, C.-Y., Lay, C.-H., Sen, B., Chu, C.-Y., Kumar, G., Chen, C.-C., Chang, J.-S., 2012.
547 Fermentative hydrogen production from wastewaters: A review and prognosis. *Int. J.*
548 *Hydrogen Energy* 37, 15632–15642.

549 Liu, B., Zhang, Y., Zhang, W., 2014. RNA-seq-based analysis of cold shock response in
550 *Thermoanaerobacter tengcongensis*, a bacterium harboring a single cold shock protein
551 encoding gene. *PLoS One* 9, 3.

552 Logan, B.E., Oh, S.-E., Kim, I.S., Van Ginkel, S., 2002. Biological hydrogen production measured
553 in batch anaerobic respirometers. *Environ. Sci. Technol.* 36, 2530–2535.

554 Lucas, S.D.M., Peixoto, G., Mockaitis, G., Zaiat, M., Gomes, S.D., 2015. Energy recovery from
555 agro-industrial wastewaters through biohydrogen production: Kinetic evaluation and
556 technological feasibility. *Renew. Energy* 75, 496–504.

557 Machani, M., Nourelfath, M., D'Amours, S., 2014. A mathematically-based framework for
558 evaluating the technical and economic potential of integrating bioenergy production within
559 pulp and paper mills. *Biomass and Bioenergy* 63, 126–139.

560 Marone, A., Ayala-Campos, O.R., Trably, E., Carmona-Martínez, A.A., Moscoviz, R., Latrille, E.,
561 Steyer, J.-P., Alcaraz-Gonzalez, V., Bernet, N., 2017. Coupling dark fermentation and
562 microbial electrolysis to enhance bio-hydrogen production from agro-industrial wastewaters
563 and by-products in a bio-refinery framework. *Int. J. Hydrogen Energy* 42, 1609–1621.

564 Meyer, T., Edwards, E.A., 2014. Anaerobic digestion of pulp and paper mill wastewater and sludge.
565 *Water Res.* 65, 321–349.

- 566 Moncada B., J., Aristizábal M., V., Cardona A., C.A., 2016. Design strategies for sustainable
567 biorefineries. *Biochem. Eng. J.* 116, 122–134.
- 568 Nissilä, M.E., Li, Y.-C., Wu, S.-Y., Lin, C.-Y., Puhakka, J.A., 2012. Hydrogenic and methanogenic
569 fermentation of birch and conifer pulps. *Appl. Energy* 100, 58–65.
- 570 Oh, S.-E., Van Ginkel, S., Logan, B.E., 2003. The relative effectiveness of pH control and heat
571 treatment for enhancing biohydrogen gas production. *Environ. Sci. Technol.* 37, 5186–90.
- 572 Owen, W.F., Stuckey, D.C., Healy Jr., J.B., Young, L.Y., McCarty, P.L., 1979. Bioassay for
573 monitoring biochemical methane potential and anaerobic toxicity. *Water Res.* 13, 485–492.
- 574 Pokhrel, D., Viraraghavan, T., 2004. Treatment of pulp and paper mill wastewater—A review. *Sci.*
575 *Total Environ.* 333, 37–58.
- 576 Ren, N., Cao, G., Wang, A., Lee, D., Guo, W., Zhu, Y., 2008. Dark fermentation of xylose and
577 glucose mix using isolated *Thermoanaerobacterium thermosaccharolyticum* W16. *Int. J.*
578 *Hydrogen Energy* 33, 6124–6132.
- 579 Rintala, J.A., Lepistö, S.S., 1992. Anaerobic treatment of thermomechanical pulping whitewater at
580 35-70°C. *Water Res.* 26, 1297–1305.
- 581 Rintala, J.A., Puhakka, J.A., 1994. Anaerobic treatment in pulp- and paper-mill waste management:
582 A review. *Bioresour. Technol.* 47, 1–18.
- 583 Ryan, P., Forbes, C., Colleran, E., 2008. Investigation of the diversity of homoacetogenic bacteria
584 in mesophilic and thermophilic anaerobic sludges using the formyltetrahydrofolate synthetase
585 gene. *Water Sci. Technol.* 57, 675–680.
- 586 Saady, N.M.C., 2013. Homoacetogenesis during hydrogen production by mixed cultures dark
587 fermentation: Unresolved challenge. *Int. J. Hydrogen Energy* 38, 13172–13191.
- 588 Sharma, Y., Li, B., 2010. Optimizing energy harvest in wastewater treatment by combining
589 anaerobic hydrogen producing biofermentor (HPB) and microbial fuel cell (MFC). *Int. J.*
590 *Hydrogen Energy* 35, 3789–3797.
- 591 Toppinen, A., Pätäri, S., Tuppurä, A., Jantunen, A., 2017. The European pulp and paper industry in

592 transition to a bio-economy: A Delphi study. *Futures* 88, 1–14.

593 Van Haandel, A., Van der Lubbe, J., 2012. *Handbook of biological wastewater treatment: Design*
594 *and Optimisation of Activated Sludge Systems*. Quist Publishing, Leidschendam, The
595 Netherlands.

596 Veluchamy, C., Kalamdhad, A.S., 2017. Enhancement of hydrolysis of lignocellulose waste pulp
597 and paper mill sludge through different heating processes on thermal pretreatment. *J. Clean.*
598 *Prod.* 168, 219–226.

599 Verhaart, M.R.A., Bielen, A.A.M., Van der Oost, J., Stams, A.J.M., Kengen, S.W.M., 2010.
600 Hydrogen production by hyperthermophilic and extremely thermophilic bacteria and archaea:
601 Mechanisms for reductant disposal. *Environ. Technol.* 31, 993–1003.

602 Xie, L., Dong, N., Wang, L., Zhou, Q., 2014. Thermophilic hydrogen production from starch
603 wastewater using two-phase sequencing batch fermentation coupled with UASB methanogenic
604 effluent recycling. *Int. J. Hydrogen Energy* 39, 20942–20949.

605

606

607

608

609

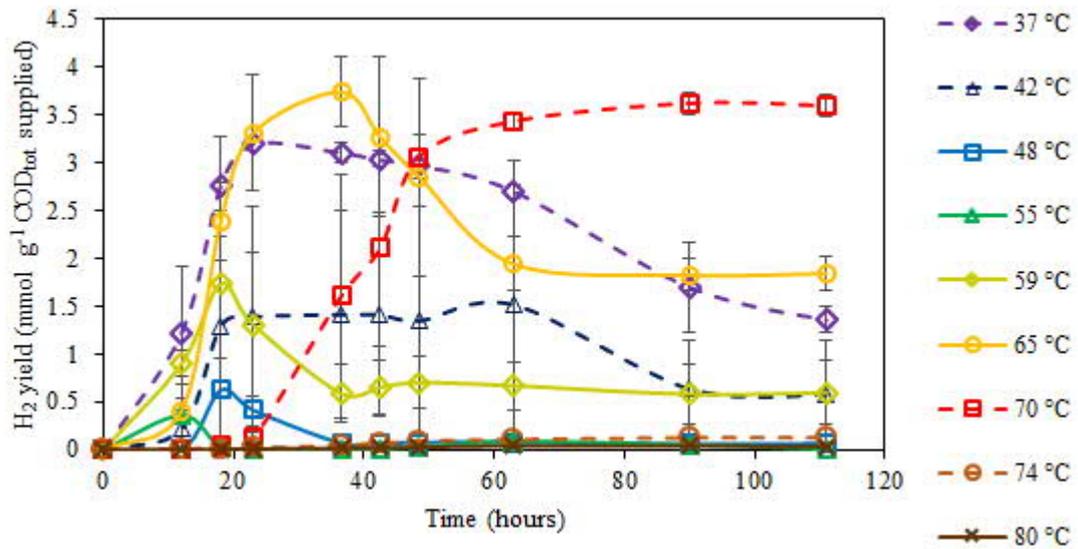
610

611

612

613 **Figures**

614 **Figure 1** – Hydrogen yield from batch incubation of thermomechanical pulping wastewater at
615 various temperatures (37 to 80 °C) using thermophilic biofilm-containing activated carbon as
616 inoculum. Error bars refer to the standard deviations of the duplicates.



617

618

619

620

621

622

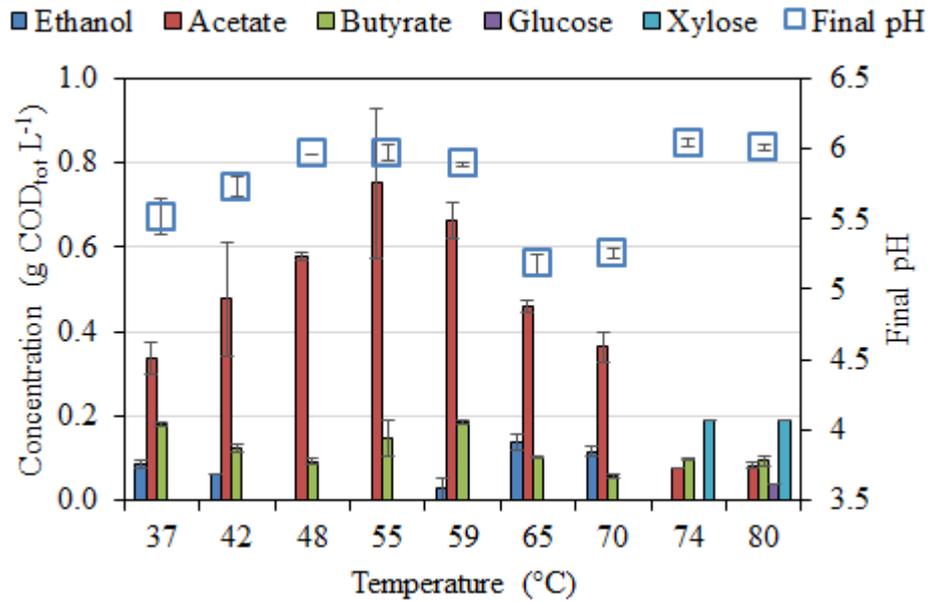
623

624

625

626 **Figure 2** – Composition and pH of the liquid phase after 111 h of incubation of thermomechanical
 627 pulping wastewater at various temperatures (37 to 80 °C) using thermophilic biofilm-containing
 628 activated carbon as inoculum. Error bars refer to the standard deviations of the duplicates.

629



630

631

632

633

634

635

636

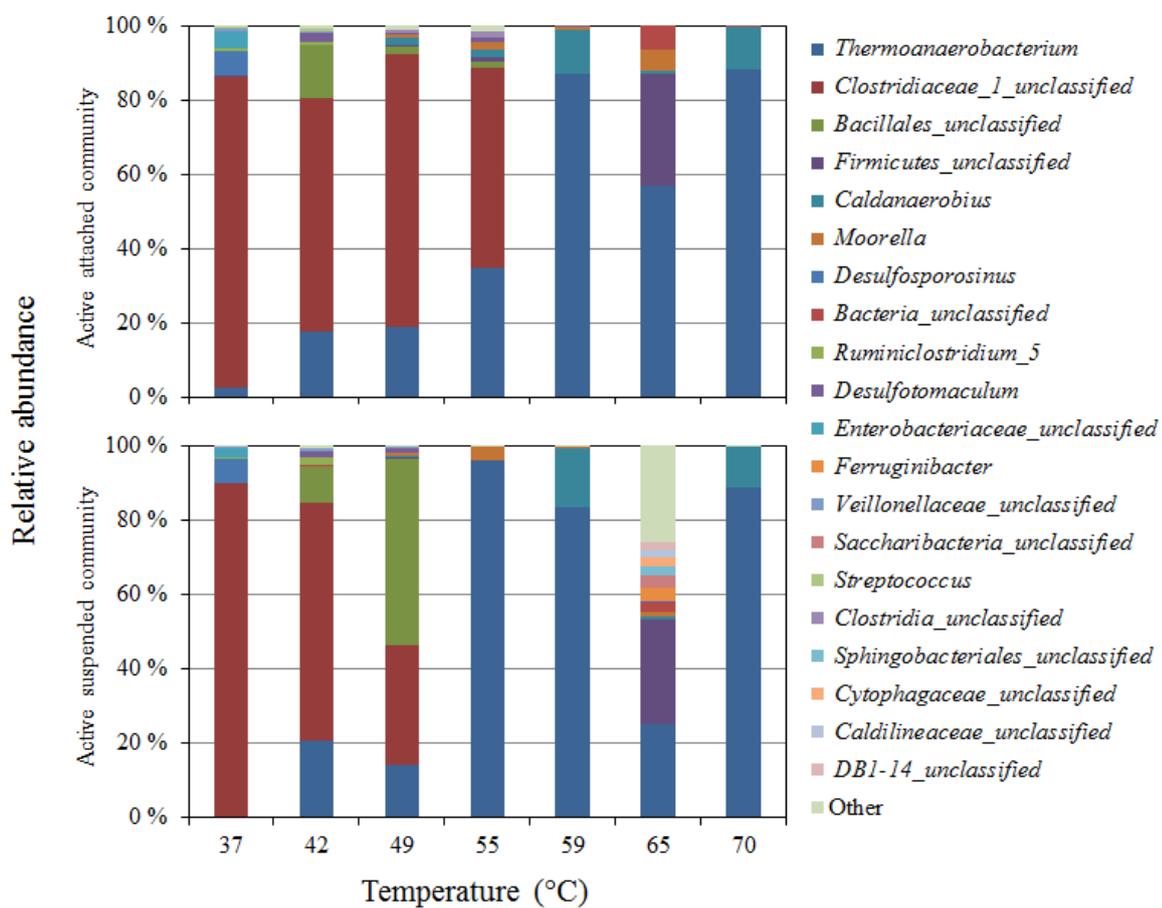
637

638

639

640 **Figure 3** – Relative abundance of the active genera resulting from MiSeq sequencing of the partial
 641 16S rRNA (transcribed to 16S cDNA) on microbiological samples obtained from the biofilm-
 642 containing activated carbon (attached) and from the liquid medium (suspended) after batch
 643 incubation with thermomechanical pulping wastewater at various temperatures (37 to 70 °C). The
 644 microbial genera are listed in order of relative abundance. Samples at 74 and 80 °C could not be
 645 analysed due to the low RNA concentration present in the samples.

646



647

648

649

650

651

652 **Table 1** - Composition of the thermomechanical pulping wastewater used in this study

Parameter	Concentration (mg L⁻¹)
Total solids	3771 ± 10
Volatile solids	2452 ± 8
Total COD	3352 ± 82
Soluble COD	3289 ± 54
Total nitrogen	< 10
Total PO ₄ ³⁻ -P	2.8
Acetate	< 30
Furfural	< 10
Glucose	43 (± 2)
Xylose	38 (± 0)

653

654

655

656

657

658

659

660

661

662 **Table 2** - Maximum and final hydrogen yield obtained from batch incubation of thermomechanical
 663 pulping wastewater at various temperatures (37 to 80 °C) using thermophilic biofilm-containing
 664 activated carbon as inoculum

Temperature (°C)	H ₂ yield (mmol H ₂ g ⁻¹ COD _{tot} supplied)		H ₂ yield (mmol H ₂ g ⁻¹ COD _{tot} consumed)	Lag time ^a (h)
	Maximum	Final	Final	
37	3.2 (± 0.1)	1.4 (± 0.1)	1.9 (± 0.2)	23
42^b	1.5	0.6	1.3	63
48	0.6 (± 0.1)	0.1 (± 0.0)	0.1 (± 0.0)	18
55	0.4 (± 0.1)	0.0 (± 0.0)	0.0 (± 0.0)	12
59	1.7 (± 0.8)	0.6 (± 0.3)	0.9 (± 0.5)	18
65	3.7 (± 0.4)	1.8 (± 0.2)	2.6 (± 0.3)	36
70	3.6 (± 0.1)	3.6 (± 0.1)	4.9 (± 0.4)	90
74	0.1 (± 0.0)	0.1 (± 0.0)	0.2 (± 0.0)	n.a. ^c
80	0.0 (± 0.0)	0.0 (± 0.0)	0.0 (± 0.0)	n.a.

665 ^a Time required to reach the maximum H₂ yield;

666 ^b H₂ was produced only in one of the duplicate tubes;

667 ^c Not applicable.

668

669

670

671

672

673

674

675 **Table 3** - COD_{tot} balances after incubation of thermomechanical pulping wastewater at various
 676 temperatures (37 to 80 °C) using thermophilic biofilm-containing activated carbon as inoculum

Temperature (°C)	Final COD_{tot} measured^a (g L ⁻¹)	Final COD_{tot} estimated^b (g L ⁻¹)	Difference (measured – estimated)	COD_{tot} removal (%)^c
37	0.79 (± 0.00)	0.60 (± 0.04)	0.19 (± 0.04)	72.5
42	0.58 (± 0.23)	0.66 (± 0.12)	-0.08 (± 0.11)	79.7
48	0.70 (± 0.01)	0.67 (± 0.00)	0.03 (± 0.02)	75.7
55	0.82 (± 0.14)	0.90 (± 0.22)	-0.07 (± 0.08)	71.2
59	0.84 (± 0.03)	0.88 (± 0.01)	-0.04 (± 0.04)	70.7
65	0.80 (± 0.04)	0.70 (± 0.03)	0.10 (± 0.00)	72.0
70	0.73 (± 0.10)	0.54 (± 0.03)	0.20 (± 0.07)	74.3
74	0.88 (± 0.06)	0.37 (± 0.00)	0.51 (± 0.07)	69.4
80	0.62 (± 0.06)	0.41 (± 0.02)	0.21 (± 0.05)	78.4

677 ^a Data obtained by measurement according to the standard procedure; the initial COD_{tot} was 2.86 g L⁻¹;

678 ^b Data obtained by the sum of the COD_{tot} equivalents (Eq. 1) of organic compounds measured in the liquid
 679 phase;

680 ^c Calculated from COD_{tot} measured.

681

682

683

684

685

686 **Table 4** - Association of the six most abundant 16S rRNA gene sequences to species collected in
 687 the GenBank

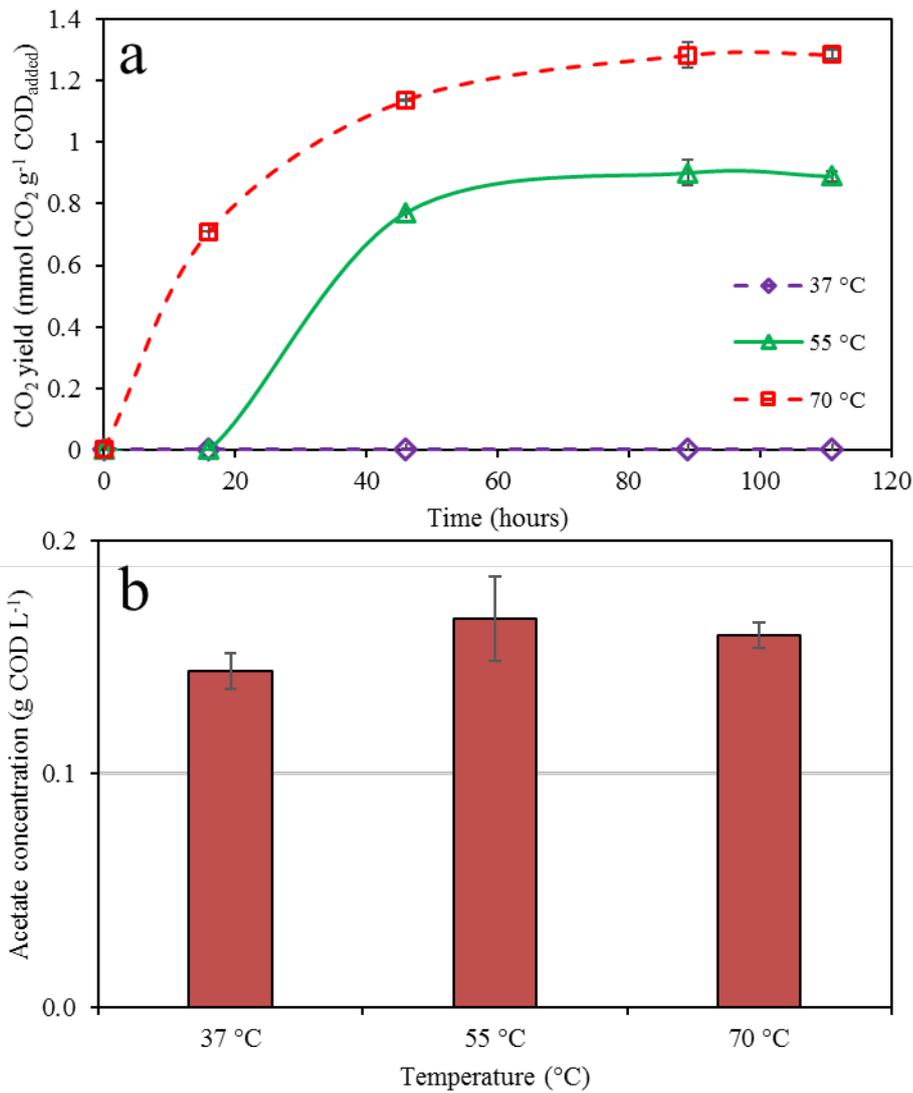
Family	Genus and species^a	Accession number	Matching sequence^b	Similarity (%)^c
<i>Thermoanaerobacteraceae</i>	<i>Thermoanaerobacterium thermosaccharolyticum</i>	JX984971	474-765	99
<i>Clostridiaceae</i>	<i>Clostridium sp.</i>	AY548785	450-741	99
<i>Bacillaceae</i>	<i>Bacillus coagulans</i>	MF373392	512-803	100
<i>Bacillaceae</i>	<i>Calditerricola yamamurae</i>	NR_112684	529-820	92
<i>Thermoanaerobacteraceae</i>	<i>Caldanaerobius sp.</i>	LC127102	482-773	99
<i>Thermoanaerobacteraceae</i>	<i>Moorella thermoacetica</i>	CP017237	145404-145695	100

688 ^a Closest cultured species in GenBank;

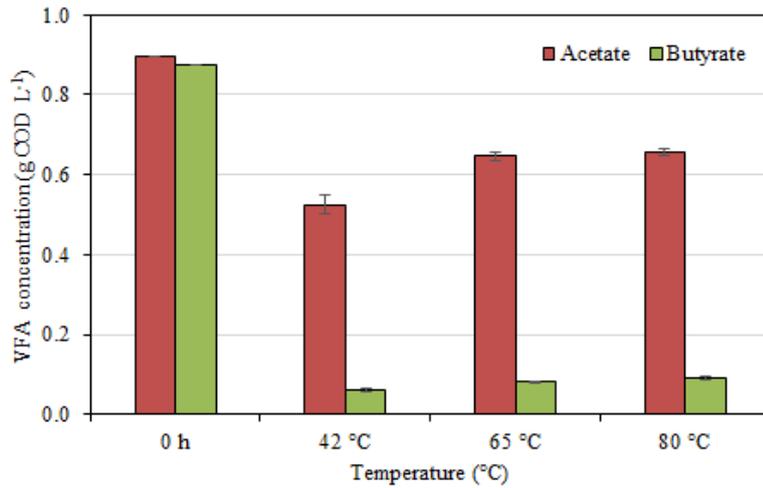
689 ^b Section of the 16S rRNA gene (in bp) matching the sequence obtained by MiSeq analysis;

690 ^c Percentage of identical nucleotide pairs between the 16S rRNA gene sequence and the closest cultured
 691 species in GenBank.

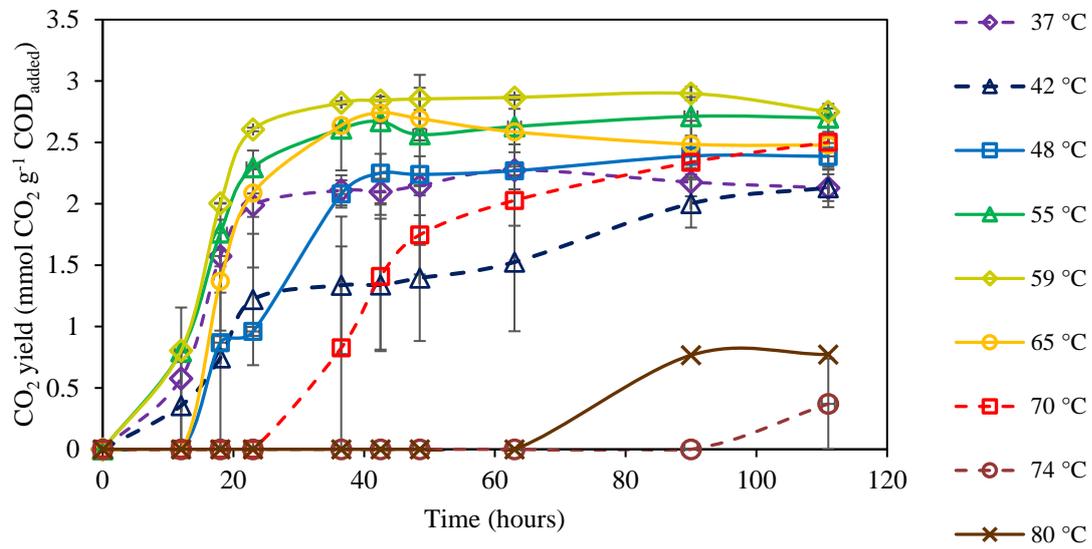
Additional file 1 –CO₂ yield profiles (a) and acetate yield after 111 h of incubation (b) obtained in the abiotic batch incubation of thermomechanical pulping wastewater at 37, 55 and 70 °C. H₂ was not detected at any of the temperatures tested. Error bars refer to the standard deviations of the duplicates.



Additional file 2 – VFA adsorption on activated carbon. Acetate and butyrate concentration before and after 111 h of incubation with fresh activated carbon at 42, 65 and 80 °C. The initial concentration of VFAs was chosen hypothesizing that only 40% of the 2.86 g COD_{tot} L⁻¹ was removed through dark fermentation, and equally distributing the remaining 1.71 g COD_{tot} L⁻¹ between acetate and butyrate. Error bars refer to the standard deviations of the duplicates.



Additional file 3 – CO₂ yield from batch incubation of thermomechanical pulping wastewater at various temperatures (37 to 80 °C) using thermophilic biofilm-containing activated carbon as inoculum. Error bars refer to the standard deviations of the duplicates.



V

**COMPOSITION AND ROLE OF THE ATTACHED AND
PLANKTONIC MICROBIAL COMMUNITIES IN MESOPHILIC AND
THERMOPHILIC XYLOSE-FED MICROBIAL FUEL CELLS**

by

Dessì, P., Porca, E., Haavisto, J., Lakaniemi, A.-M., Collins, G., Lens, P.N.L.,
2018

RSC Advances 8:3069-3080

This article is licensed under a Creative Commons Attribution-NonCommercial
3.0 Unported License


 Cite this: *RSC Adv.*, 2018, 8, 3069

Composition and role of the attached and planktonic microbial communities in mesophilic and thermophilic xylose-fed microbial fuel cells†

 Paolo Dessi,^a Estefania Porca,^b Johanna Haavisto,^a Aino-Maija Lakaniemi,^a Gavin Collins^b and Piet N. L. Lens^{ac}

A mesophilic (37 °C) and a thermophilic (55 °C) two-chamber microbial fuel cell (MFC) were studied and compared for their power production from xylose and the microbial communities involved. The anode-attached, membrane-attached, and planktonic microbial communities, and their respective active subpopulations, were determined by next generation sequencing (Illumina MiSeq), based on the presence and expression of the 16S rRNA gene. *Geobacteraceae* accounted for 65% of the anode-attached active microbial community in the mesophilic MFC, and were associated to electricity generation likely through direct electron transfer, resulting in the highest power production of 1.1 W m⁻³. A lower maximum power was generated in the thermophilic MFC (0.2 W m⁻³), likely due to limited acetate oxidation and the competition for electrons by hydrogen oxidizing bacteria and hydrogenotrophic methanogenic archaea. Aerobic microorganisms, detected among the membrane-attached active community in both the mesophilic and thermophilic MFC, likely acted as a barrier for oxygen flowing from the cathodic chamber through the membrane, favoring the strictly anaerobic exoelectrogenic microorganisms, but competing with them for xylose and its degradation products. This study provides novel information on the active microbial communities populating the anodic chamber of mesophilic and thermophilic xylose-fed MFCs, which may help in developing strategies to favor exoelectrogenic microorganisms at the expenses of competing microorganisms.

Received 10th November 2017

Accepted 8th January 2018

DOI: 10.1039/c7ra12316g

rsc.li/rsc-advances

1. Introduction

The microbial fuel cell (MFC) is an emerging technology for the direct bioconversion of chemical energy of organic substrates to electrical energy. MFCs consist of two electrodes (anode and cathode) connected through an external electrical circuit. The anode acts as electron acceptor in the bioelectrochemical redox reactions of microbial metabolism, whereas the cathode acts as electron donor for biotic or abiotic reactions. The combination of anodic and cathodic reactions creates a potential difference between the electrodes which drives the electrons to migrate from the anode to the cathode, thus generating electrical current (for a review, see Butti *et al.*¹).

Biological electricity production in MFCs requires microorganisms capable to oxidize the substrates and transfer the

electrons exogenously to the solid anode electrode. Electrons can be transferred to the anode essentially through three mechanisms: short range, long range, and mediated electron transfer (for reviews, see Kumar *et al.*² and Kalathil *et al.*³). Some microorganisms, such as *Geobacter sulfurreducens*, can transfer electrons to a surface directly *via* redox-active proteins present on the outer surface of their cell membrane, such as c-type cytochromes, or *via* conductive pili called nanowires.^{4,5} *G. sulfurreducens* develops multi-layer structured biofilms, in which nanowires connect the different cells, enabling the electron transfer to the anode.⁶ Mediators, in their oxidized form, penetrate the microbial cell and become reduced during cellular metabolism. They then diffuse out of the cell and release the electrons at the anode, becoming oxidized again and thus reusable.⁵ Some species, such as *Pseudomonas*, produce mediators such as pyocyanin endogenously.⁷ Once mediators are produced, also other microorganisms present in the mixed culture system can use them to transfer the electrons to the anode.⁸

Pure cultures of electrochemically active microorganisms, such as *Geobacter* sp.⁹⁻¹¹ and *Shewanella* sp.,^{12,13} have shown power production from simple substrates such as volatile fatty acids and sugars at mesophilic conditions (25–37 °C) and neutral pH (6.8–7.3). Mixed cultures are more practical for

^aLaboratory of Chemistry and Bioengineering, Tampere University of Technology, P.O. Box 541, FI-33101 Tampere, Finland. E-mail: paolo.dessi@tut.fi; Tel: +358 417239696

^bMicrobial Communities Laboratory, School of Natural Sciences, National University of Ireland Galway, University Road, Galway, H91 TK33, Ireland

^cUNESCO-IHE, Institute for Water Education, Westvest 7, 2611AX Delft, The Netherlands

† Electronic supplementary information (ESI) available. See DOI: 10.1039/c7ra12316g



wastewater treatment, as they contain a consortium of hydrolytic, fermentative and electroactive microorganisms able to produce electricity from complex substrates.⁹ However, due to the competition for electron donor with non-exoelectrogenic microorganisms such as methanogenic archaea,¹⁴ power production can remain low, and operational conditions must be optimized to favor exoelectrogenic microorganisms. Catal *et al.*¹⁵ compared electricity production from 12 monosaccharides present in lignocellulosic biomass, including pentoses and hexoses, in a mesophilic (30 °C) MFC inoculated with a mixed culture adapted to acetate. Xylose resulted in the highest potential for electricity production over the other hexoses and pentoses tested.

Thermophilic electricity production could be advantageous because of the high rate of biochemical reactions, and thus high electron production rates, of thermophilic microorganisms.¹⁶ MFCs have been operated at temperatures up to 98 °C.¹⁷ However, although over 20 species of microorganisms, mainly belonging to the *Proteobacteria* phylum, have been reported to produce electricity under mesophilic conditions, the number of known thermophilic exoelectrogenic microorganisms is much lower.¹⁸ To date, only few species have been reported to produce electricity at thermophilic conditions, including *Firmicutes* such as *Caloramator australicus*,¹⁸ *Thermincola potens*,¹⁹ *Thermincola ferriacetica*,²⁰ and *Thermoanaerobacter pseudethanolicus*,²¹ as well as *Deferribacteres* such as *Calditerrivibrio nitroreducens*.²²

Investigating the composition of the active subpopulation, rather than the whole microbial community, is crucial in understanding the role of microorganisms in MFCs. DNA-based methods may drive to erroneous conclusions in the detection of the key species in bioreactors.²³ Previously performed microbial community analyses have, nevertheless, mainly targeted the presence of the 16S rRNA gene (DNA) whereas, to our knowledge, only one study¹⁹ has also focused on 16S rRNA gene expression (RNA), which is an indicator of the microbial activity.²³ Furthermore, especially in studies on thermophilic MFCs, microbial community analyses have mainly focused on the anode-attached microbial community, lacking information on the planktonic microbial community. The latter community could be involved in electricity generation as well, either directly, by performing mediated electron transfer to the anode²⁴ or indirectly, by converting the substrates to compounds readily available for the exoelectrogenic microorganisms.

In addition, the membrane is a suitable surface for the establishment of a biofilm. Although biofouling of the membrane has been reported in MFC studies,^{14,25} only Lu *et al.*²⁶ have reported the composition of a membrane-attached microbial community in two brewery wastewater-fed MFCs operated in series at ambient temperature (20–22 °C). However, the microbial community analysis was performed only at DNA level, and the role of the membrane-attached microorganisms detected on the MFC performance was not discussed.²⁶ Although likely not directly involved in electricity generation, membrane-attached microorganisms may have a role in the functioning of MFCs, which must be elucidated. Therefore, the aim of this study was to investigate the microbial communities

growing (i) as anodic biofilm, (ii) in suspended form in the anodic solution (planktonic), and (iii) as biofilm on the membrane of a mesophilic (37 °C) and a thermophilic (55 °C) xylose-fed MFC. Both presence and expression of the 16S rRNA gene were determined with the aim to investigate both the composition of the overall microbial community and the active subpopulation. Power production, as well as xylose and metabolite concentration profiles were also analyzed to determine the possible differences in the electricity production pathways at 37 and 55 °C.

2. Experimental

2.1 Source of anodic microorganisms

In order to ensure a large variety of microbial species capable of living under a broad temperature range and degrading xylose, two inocula (15 mL each) were mixed and provided to each MFC. The first one was activated sludge from a municipal wastewater treatment plant (Viinikanlahti, Tampere, Finland), which has shown potential for anaerobic energy production in the form of dark fermentative hydrogen production at temperatures up to 55 °C.²⁷ The second one was anolyte from a xylose-fed MFC operating at 37 °C.²⁸ The volatile solids content was 10.6 (±0.2) and 8.4 (±0.5) g L⁻¹ for the activated sludge and the anolyte, respectively. The mixture of the two inocula was flushed with N₂ for 10 min before introducing into the anode chambers of the MFCs.

2.2 Anolyte composition

The anolyte was prepared according to Mäkinen *et al.*,²⁹ but EDTA, yeast extract, and resazurin were not added. The substrate was xylose (0.3 or 1 g L⁻¹, as specified in Section 2.4). The pH was kept at 7 (±0.2) using phosphate buffer. The anolyte conductivity was 14.6 mS cm⁻¹. The composition of the feeding solution was the same as the anolyte, but with a 10-times higher xylose concentration.

2.3 MFC configuration

The h-type two-chamber MFCs were constructed by connecting two glass bottles (Adams & Chittenden Scientific Glass, USA) separated by an anion exchange membrane (AMI-7001, Membranes International Inc., USA) with a diameter of 5.2 cm. The total volume of the anodic and the cathodic chamber was 350 mL each. The anode was a carbon brush (5 cm length and 1.5 cm diameter), while the cathode was a carbon cloth (5 × 4 cm) coated with approximately 20 mg of a Pt-based catalyst (20% platinum on Vulcan XC-72R carbon, E-TEK, USA). The two electrodes were connected through an external resistance of 100 Ω. A reference electrode (BASi RE-5B Ag/AgCl) was inserted into the anodic chamber, close to the anode. The anodic chamber was filled with the anolyte (270 mL) and inoculum (30 mL), and its content was continuously mixed by magnetic stirring. The cathodic chamber was filled with milliQ water (300 mL), and oxygen was provided as the terminal electron acceptor by pumping air from outside using an aquarium air pump (Marina 50) at a flow rate of 130 mL min⁻¹. In the



mesophilic MFC, the water lost by evaporation (circa 15 mL per day) was replaced manually every 1–2 days. In the thermophilic MFC, due to the faster evaporation (circa 50 mL per day), the water was replaced daily through a pump connected to a timer. Temperature of the mesophilic and thermophilic MFC was kept at 37 (± 2) °C and 55 (± 2) °C, respectively, by using two incubators (Memmert, Germany).

2.4 MFC operation

Both MFCs were operated in fed-batch mode. The feeding steps were done by replacing 30 mL of anolyte (10% of the total volume) with 30 mL of the feeding solution. In order to avoid substrate overload in the start-up phase, the first six fed-batch cycles were conducted with an initial xylose concentration of 0.3 g L⁻¹. The initial xylose concentration was then increased to 1.0 g L⁻¹ for the following eleven fed-batch cycles, referred to as I–XI. In the results section, day 0 refers to the first day of operation with a xylose concentration of 1 g L⁻¹. The MFCs were fed every 5–6 days when the initial xylose concentration was 0.3 g L⁻¹, and every 7–8 days when initial xylose concentration was 1.0 g L⁻¹.

2.5 Sampling

Anolyte samples were collected at the beginning and at the end of every feeding step. During the feeding step “IX” (from day 55 to day 61) anolyte samples were collected at various time points to determine the COD, xylose and volatile fatty acids concentration profiles. Biomass samples from both MFCs were collected at the end of the experiment (day 72) from three different sampling points: anodic electrode (anode-attached), membrane (membrane-attached) and anolyte (planktonic). The anodic electrode and the membranes were taken out of the MFC, put into 50 mL sterile Falcon tubes with about 30 mL of autoclaved 0.9% NaCl solution, sonicated for 2 minutes at 50–60 Hz (Finnsonic, Finland) and strongly shaken in order to detach as much biofilm as possible. The two resulting samples, as well as a sample of the anolyte, were concentrated by consecutive centrifugation cycles (10 min, 5000 rpm) in 5 mL sterile Eppendorf tubes using a Sigma 4k145 centrifuge. The resulting pellets were re-suspended in 5 mL autoclaved 0.9% NaCl solution and stored at –85 °C until carrying out the microbial community analyses.

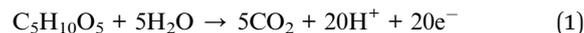
2.6 Analytical methods

Voltage and anodic potential were measured with a data logger (Agilent 34970A, Agilent technologies, Canada) at 2 minutes intervals. The anodic potential was measured against the Ag/AgCl reference electrode. Soluble COD was measured using the dichromate method according to the Finnish standard SFS 5504. Anolyte conductivity and pH were measured with a conductivity meter (WTW inoLab, Germany) and a pH meter (WTW pH 330 meter with Hamilton Slimtrode probe), respectively. Dissolved oxygen in the cathodic chamber was measured by a multi-parameter meter (HQ40d) with a standard luminescent/optical dissolved oxygen probe (IntelliCAL). Xylose, volatile fatty acids, and alcohols were measured by a high performance liquid chromatography (HPLC) system equipped with a Rezex RHM-

monosaccharide column (Phenomenex, USA) as described earlier by Dessi *et al.*²⁷ Some of the chromatograms obtained are provided as an example in the ESI (Fig. S1†).

2.7 Coulombic efficiency, power and polarization curves

Coulombic efficiency (CE) was calculated according to Oh *et al.*,³⁰ accounting 20 mol electrons exchanged per mol of xylose, according to the following equation:



Power and polarization curves were obtained on day 64 and 71 from the mesophilic and thermophilic MFC, respectively. The electrical circuit was kept open for 30 minutes before the analysis to obtain the open circuit voltage (OCV). The circuit was then closed through a resistor box (TENMA 72-7270, Taiwan) and the resistance was decreased stepwise from 15 k Ω to 5 Ω at 30 minute intervals. Voltage was recorded just before switching the resistance. Power density and current density were calculated as $P = U^2/(R \cdot V)$ and $I = U/(R \cdot V)$, respectively, where U is the voltage recorded in the data logger, R is the external resistance, and V is the anolyte volume (300 mL).

2.8 Microbial community analyses

Nucleic acids were co-extracted from the biomass samples using the method from Griffiths *et al.*,³¹ with the following modifications: 3 M sodium acetate (1/10 of sample volume) and cold (–20 °C) 100% isopropanol (1 sample volume) were added for precipitation instead of polyethelene glycol and nucleic acids were re-suspended in sterile water instead of tris–EDTA buffer. DNA and RNA were quantified by a Nanodrop spectrophotometer (NanoDrop Technologies, Wilmington, USA), and their quality was assessed by measuring the absorbance ratio at 260/280 nm and 260/230 nm wavelength. No further treatment was performed on nucleic acid samples for DNA level analysis. For RNA level analysis, nucleic acid samples were diluted to a final concentration of 25 ng mL⁻¹. DNA was removed by the addition of 1 μ L turbo DNase and 2.5 μ L turbo DNase buffer (Invitrogen, Thermo Fisher, USA), followed by incubation at 37 °C for 30 minutes. DNase was then inactivated by addition of 2.5 μ L DNase inactivator (Invitrogen) and separated from the RNA containing liquid by centrifugation (10 000 $\times g$, 1.5 minutes). The absence of DNA was confirmed by bacterial 16S rRNA gene PCR (primers 338f and 805r) followed by electrophoresis in 1% agarose gel (no bands obtained). Complementary DNA (cDNA) was obtained from RNA using M-MuLV Reverse Transcriptase (New England BioLabs, USA), according to the instructions provided by the supplier. Bacterial 16S rRNA gene PCR was then applied to confirm the success of the reverse transcription (bands appeared).

Samples of both DNA and cDNA (12 μ L) were collected in a 96-well plate and sent to FISABIO (Valencia, Spain) for partial 16S rRNA genes (DNA) or 16S rRNA (cDNA) high-throughput sequencing on an Illumina MiSeq platform. Forward and reverse primers for PCR were 515f and 806r, respectively.³² Sequence screening, alignment to the Silva (v128) database, clustering, chimeras removal and taxonomic classification (97%



cutoff) were performed using Mothur v1.39.3,³³ following the procedure described by Kozich *et al.*³⁴ A total of 1,130,353 raw sequences was obtained from 12 samples, and 1,058,675 passed the quality filters. Relative abundance and diversity analyses were performed using the *R* software.³⁵ The Illumina sequencing data was deposited to the NCBI Sequence Read Archive under BioProject Number PRJNA428321.

3. Results

3.1 Power production in the mesophilic and thermophilic MFC

After the start-up period, the anodic potential in the mesophilic MFC was reproducible in all of the eleven fed-batch cycles with a xylose concentration of 1.0 g L⁻¹ (Fig. 1a). It ranged between -450 mV and -520 mV (*vs.* Ag/AgCl). The anode potential of the thermophilic MFC was stable during the first 20 days of operation, with a minimum of about -100 mV. It then started to decrease, reaching a minimum of -230 mV in cycle IX, but increased again in cycles X and XI (Fig. 1b). A higher power density was obtained from the mesophilic MFC compared to the thermophilic MFC in the eleven fed-batch cycles (Fig. 1c *vs.* 1d). The maximum power density in the mesophilic MFC ranged between 0.55 W m⁻³ in cycle IX and 1.0 W m⁻³ in cycles X and XI, with a maximum CE of about 12%, while the power density in the thermophilic MFC rarely exceeded 0.03 W m⁻³ with a maximum CE of about 3%.

3.2 Substrate degradation

At the end of all the eleven fed-batch cycles, the concentrations of xylose and organic metabolites in the anolyte of the

mesophilic MFC were below the detection limit of the HPLC. However, acetate was found in the anolyte of the thermophilic MFC with a maximum concentration of 0.1 g L⁻¹. Substrate consumption and metabolite production were monitored more closely in the fed-batch cycle IX, and compared to the obtained power density (Fig. 2). In the mesophilic MFC, xylose (1.0 g L⁻¹) was consumed within 4 hours after its addition. Acetate and butyrate (0.2 and 0.1 g L⁻¹, respectively) were detected after xylose consumption, but were then consumed within 48 hours. The soluble COD concentration decreased from 1.3 g L⁻¹ to a final concentration of 0.25 g L⁻¹. The power density increased until xylose was completely depleted, reaching a maximum of about 0.50 W m⁻³, then stabilized for about 80 hours before dropping to <0.05 W m⁻³ about 30 hours after the complete depletion of acetate and butyrate (Fig. 2).

In the thermophilic MFC, xylose was depleted within 12 hours, and the resulting acetate produced reached a maximum of 0.2 g L⁻¹ after 22 hours. The acetate concentration remained stable for about 45 hours after xylose depletion, before starting to be slowly consumed to a final concentration of 0.1 g L⁻¹ after 143 hours (Fig. 2). The soluble COD concentration also slowly decreased to a final concentration of 0.5 g L⁻¹. A power density peak of 0.03 W m⁻³ was obtained immediately after xylose depletion but, unlike the mesophilic MFC, no sudden power density drop occurred (Fig. 2).

3.3 Power and polarization curve

Based on the polarization data (Fig. 3), the power density was higher in the mesophilic MFC than in the thermophilic MFC regardless of the resistance applied. The maximum power densities of 1.1 and 0.2 W m⁻³ were obtained applying

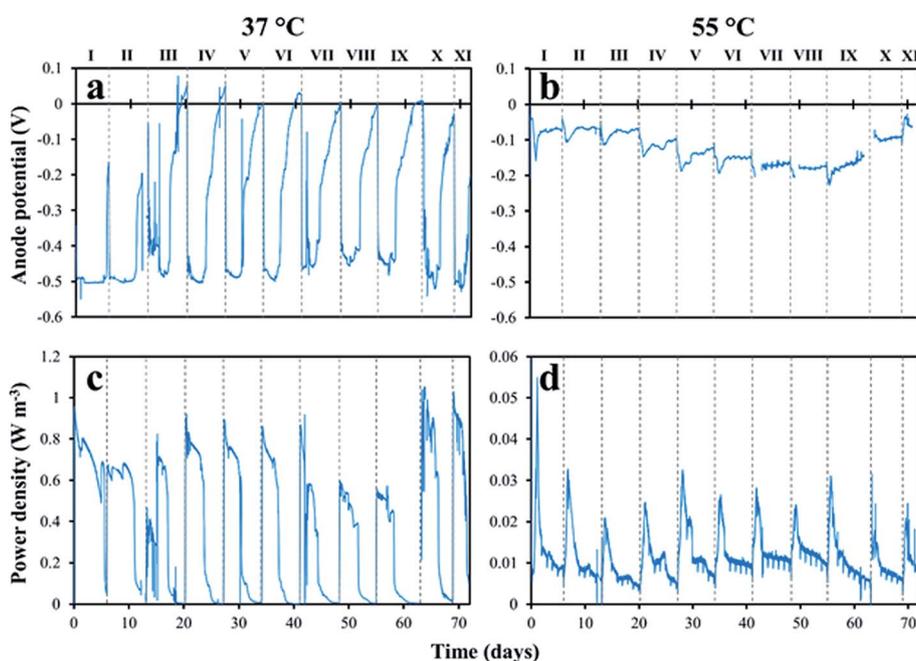


Fig. 1 Anode potential (a, b) and power density (c, d) obtained in the mesophilic (37 °C) and thermophilic (55 °C) xylose-fed MFCs. Note the different Y-axis scale in the power density graphs (c, d). Roman numerals represent the fed-batch cycles with 1.0 g L⁻¹ xylose as the substrate. The MFCs were previously operated for 33 days (six fed-batch cycles) with a xylose concentration of 0.3 g L⁻¹ (not shown).



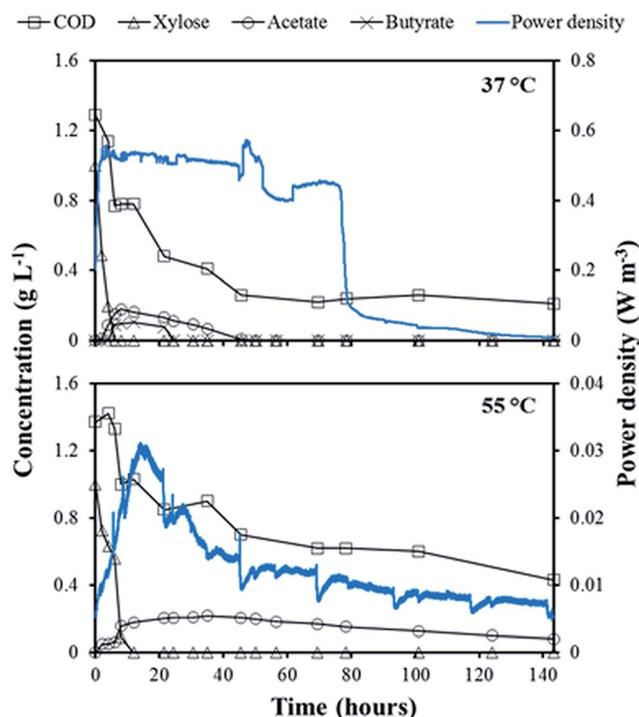


Fig. 2 Power density and concentration of soluble COD, xylose, acetate, and butyrate present in the anolyte of the mesophilic (37 °C) and thermophilic (55 °C) MFC during fed-batch cycle IX.

a resistance of 250 and 1000 Ω to the mesophilic and thermophilic MFC, respectively (Fig. 3a). The open circuit voltage (OCV) was approximately 0.7 and 0.4 V for the mesophilic and thermophilic MFC, respectively (Fig. 3b). Power overshoot (drastic drop of power and current density) occurred in the thermophilic MFC, when a resistance lower than 500 Ω was applied. The internal resistance, measured as the slope of the linear part of the polarization curve, was 270 Ω and 560 Ω for the mesophilic and the thermophilic MFC, respectively.

3.4 Microbial community analysis

High-throughput Illumina MiSeq sequencing showed a clear difference in the composition of the microbial community not only among the mesophilic and thermophilic MFC, but also among the different sampling points in the same MFC (Fig. 4 and 5). A higher diversity was obtained in the DNA than in the cDNA samples, and in the mesophilic than in the thermophilic MFC, based on the diversity and evenness indexes (Table 1).

In the mesophilic MFC, the active anode-attached subpopulation was mainly composed of *Proteobacteria* belonging to the family of *Geobacteraceae* (65% of the total relative abundance), but *Sphingobacteriales* (14%) were also found (Fig. 4 and 5). The planktonic active subpopulation was more diverse, with the families of *Porphyromonadaceae*, *Rikenellaceae*, and *Sphingobacteriales* WCHB1-69 all above 10% of the relative abundance. *Proteobacteria* also dominated the membrane-attached active microbial community in the mesophilic MFC. *Comamonadaceae* was the most abundant family (20%), followed by a variety of

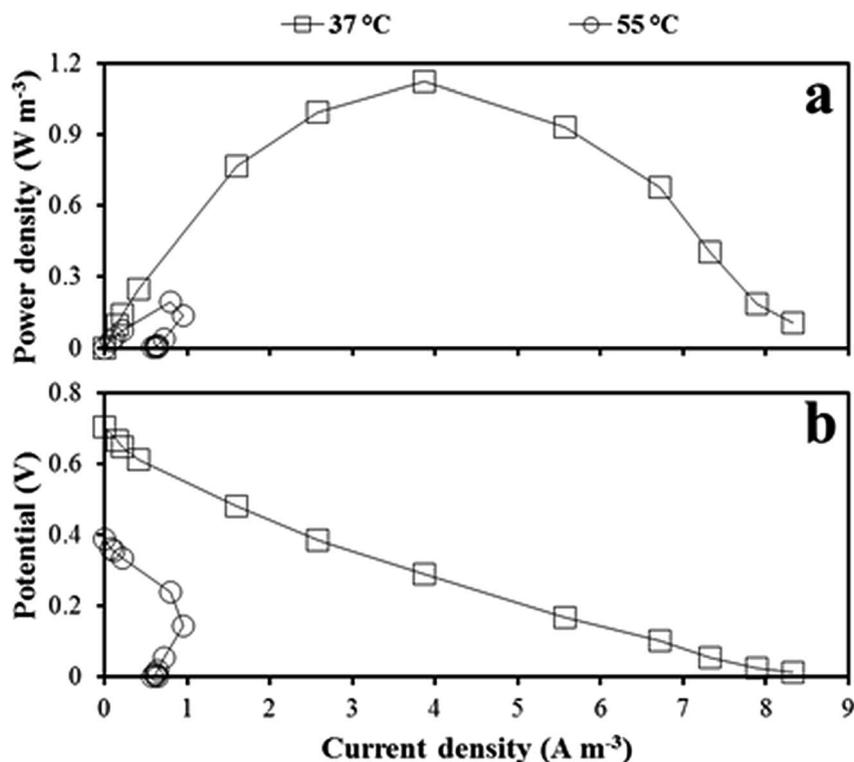


Fig. 3 Power (a) and polarization (b) curve obtained from the mesophilic MFC in cycle X, and from the thermophilic MFC in cycle XI. Current and power density were normalized to the anolyte volume.



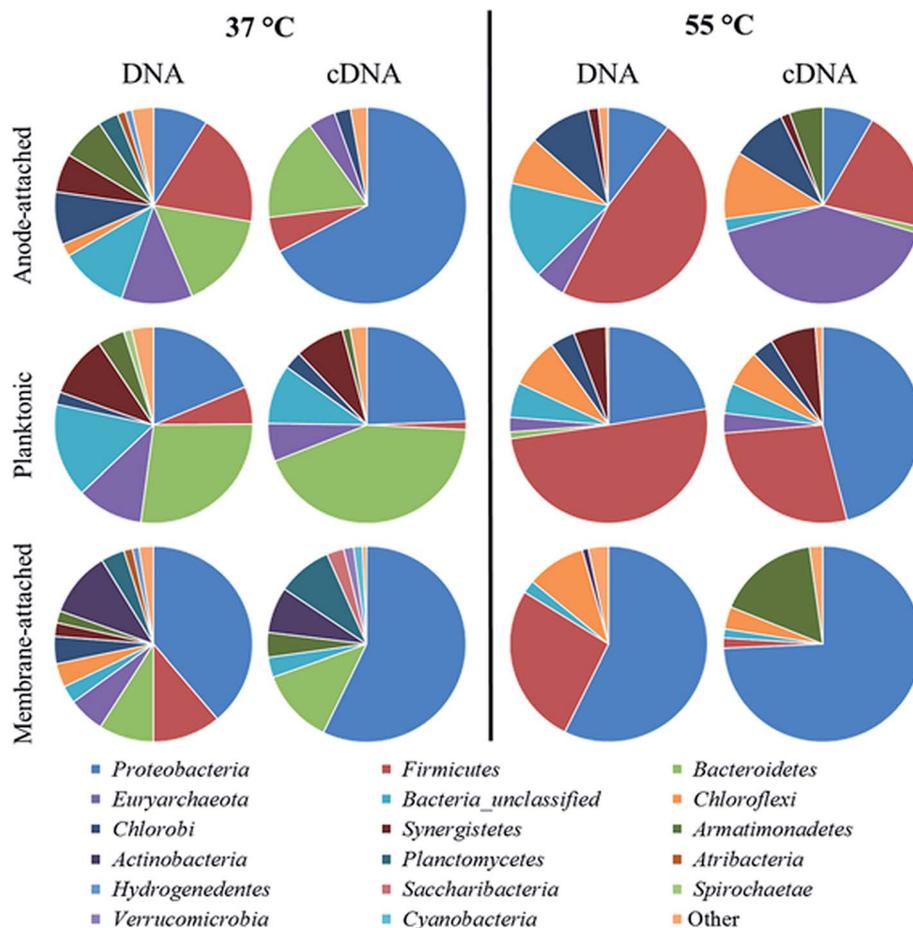


Fig. 4 Phylum-level taxonomic classification and relative abundance of the 16S rRNA gene (DNA) or reversely transcribed 16S rRNA (cDNA) obtained from Illumina MiSeq sequencing of nucleic acids from microbiological samples collected in different sampling points of the mesophilic (37 °C) and thermophilic (55 °C) xylose-fed MFC. "Other" represents the sum of the phyla with a relative abundance <1%.

families contributing to <10% of the total relative abundance (Fig. 4 and 5).

In the thermophilic MFC, the anode-attached active subpopulation was composed by *Euryarchaeota* (Fig. 4 and 5), mainly *Methanobacteriaceae* (38% of the total relative abundance), *Firmicutes* such as *Thermodesulfobiaceae* (13%), and *Chloroflexi* such as *Anaerolineaceae* (11%). *Thermodesulfobiaceae* were found also in the planktonic active subpopulation (23%), together with *Hydrogenophylaceae* (46%), and other less abundant families. *Comamonadaceae* was the most abundant active membrane-attached family (53%) of the thermophilic MFC, which included also *Hydrogenophylaceae* (18%), and an unclassified family belonging to the order of *Armatimonadetes* (17%) (Fig. 4 and 5).

4. Discussion

4.1 Bioelectricity production and microbial dynamics in the mesophilic MFC

An active microbial community mainly composed of *Proteobacteria* (Fig. 4) generated a relatively high power density in the mesophilic xylose-fed MFC (Fig. 1c). Indeed, most of the known

mesophilic exoelectrogens belong to the phylum *Proteobacteria*.³⁶ The diversity of the active anode-attached subpopulation (cDNA) was remarkably lower than the diversity of the whole community (DNA) (Table 1), confirming that the presence of microorganisms in a bioreactor does not relate to their activity.²³ In particular, *Geobacteraceae* accounted only for 2% of the anode-attached microbial community, but was the prevalent (65%) active family (Fig. 5), and likely played a major role in power production. In fact, the *Geobacteraceae* family includes known exoelectrogenic microorganisms which have been widely reported to dominate the anodic microbial community in mesophilic MFCs, regardless of the inoculum source, substrate, and the MFC set-up.^{28,37–39} For example, Mei *et al.*⁴⁰ showed that different microbial communities could develop in mesophilic (30 °C) MFCs started-up with different inocula, but *Geobacter* was found regardless of the inoculum.

In this study, the remarkably higher diversity of the anode-attached community (DNA) than the active subpopulation (cDNA) (Table 1) suggests the presence of inactive or dead microorganisms, which could have hampered the activity of the *Geobacteraceae*, thus lowering power production.⁴¹ The relative abundance of active planktonic *Geobacteraceae* was only 3%



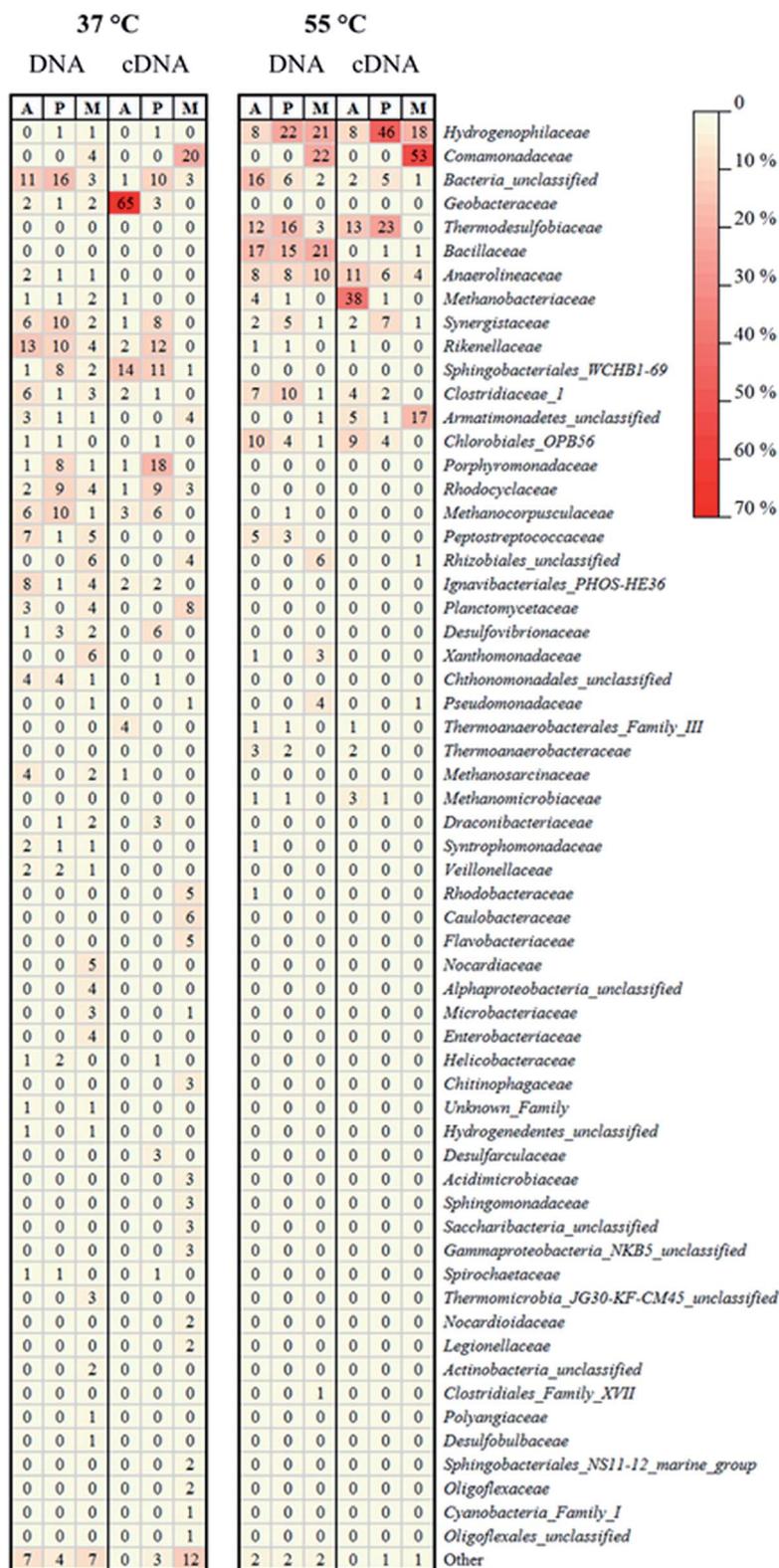


Fig. 5 Heat-map representing the relative abundance of the 16S rRNA gene (DNA) or reversely transcribed 16S rRNA (cDNA) obtained from MiSeq sequencing of nucleic acids from microbiological samples collected in different sampling points of the mesophilic (37 °C) and thermophilic (55 °C) MFC. A, P, and M refer to the anode-attached, planktonic, and membrane-attached microbial community, respectively. The taxonomic classification was conducted on family level. "Other" represents the sum of the families with relative abundance <1%.



Table 1 Diversity of the microbial community (DNA) and active microbial community (cDNA) in three different sampling points of the mesophilic (37 °C) and thermophilic (55 °C) xylose-fed MFC, measured by the Shannon, Simpson and Pielou's J' index

Temperature (°C)	Sample type	Microbial community	No. of sequences ^a	No. of families	Shannon diversity	Simpson diversity	J' evenness
37	DNA	Anode-attached	197,036	617	3.22	0.94	0.71
		Planktonic	100,125	391	2.95	0.92	0.68
		Membrane-attached	136,388	798	3.72	0.97	0.83
	cDNA	Anode-attached	28,349	161	1.45	0.55	0.36
		Planktonic	108,967	326	2.74	0.91	0.64
		Membrane-attached	68,638	341	3.26	0.93	0.80
55	DNA	Anode-attached	96,743	237	2.59	0.90	0.63
		Planktonic	55,907	136	2.24	0.85	0.58
		Membrane-attached	68,558	141	2.40	0.87	0.56
	cDNA	Anode-attached	103,439	72	2.06	0.80	0.56
		Planktonic	33,729	98	1.75	0.72	0.46
		Membrane-attached	60,796	80	1.48	0.65	0.41

^a Refers to sequences which passed the quality check. All sample sizes were normalized according to the lowest number of sequences (28,349) prior to perform relative abundance and diversity analysis.

(Fig. 5), suggesting that they were mainly growing attached to the anode. In fact, *Geobacter* sp. transfers electrons to the anode by direct contact transfer, but is unable to conduct long-range electron transfer.⁴² This is confirmed by the prompt power increase after the addition of xylose at the beginning of each fed-batch cycle (Fig. 1c), which is common in MFCs dominated by microorganisms performing direct electron transfer.²⁹ *Sphingobacteriales*, found among both the active anode-attached and planktonic subpopulations in the mesophilic MFC (14 and 11% relative abundance, respectively), have been previously reported as part of the anodic microbial community,^{39,43} but further studies are required to assess their role in electricity generation.

No dominant family was detected in the active mesophilic planktonic subpopulation, but instead 6–7 families were present with a similar relative abundance (Fig. 5). Among them, both *Desulfovibrionaceae*⁴⁴ and *Rikenellaceae*⁴⁵ have been reported to produce electricity as pure cultures in MFCs. *Rikenella* sp. can perform glycolysis and mediated electron transfer to the anode,⁴⁵ which likely explains its presence among the active mesophilic planktonic microbial community in this study (Fig. 5). The *Rhodocyclus* family includes Fe(III) reducers, such as *Ferribacterium*, which can be involved in bioelectricity production⁴⁶ and has also been found in an anodic biofilm of an acetate-fed MFC.⁴⁷ *Porphyromonadaceae*, which accounted for 18% of the active mesophilic planktonic subpopulation, have been previously detected both in the anode-attached and planktonic population in a mesophilic MFC treating starch, peptone, and fish extract.⁴⁸ Although likely not directly involved in bioelectricity production, other microorganisms may also have contributed to the overall performance of the MFC. For example, *Synergistaceae* (8% of the relative abundance in the mesophilic active planktonic community) may be involved in the recycling of nutrients by quickly digesting the proteins of dead microorganisms.³⁸

The membrane-attached active microbial community in the mesophilic MFC was highly diverse (Table 1). *Comamonadaceae*, which accounted for 20% of the active population, include

facultative anaerobic microorganisms capable of using short chain volatile fatty acids as a source of carbon for their metabolism.⁴⁹ Species belonging to the *Comamonadaceae* family, such as *Comamonas denitrificans*, have been previously found in the anodic biofilm of MFCs, and even shown to produce electricity in the absence of oxygen.⁵⁰ However, *Comamonadaceae* were found in this study exclusively on the membrane, suggesting that they had a minor role in bioelectricity generation. Oxygen can flow from the cathodic to the anodic chamber through the AMI-7001 anion exchange membrane with a diffusivity coefficient of $4.3 \times 10^{-6} \text{ cm}^2 \text{ s}^{-1}$,⁵¹ thus exposing the anodic microorganisms to oxygen. The aerobic or facultative membrane-attached microorganisms may consume the oxygen crossing the membrane, favoring the strictly anaerobic exoelectrogens, but also competing with them for the substrates. Kim *et al.*⁵¹ estimated that, due to the higher biomass yield of aerobes compared to anaerobes, about 10% of the substrate was consumed through aerobic metabolism, reducing the CE of their acetate-fed (1.2 g L^{-1}) MFCs. However, they did not perform microbial community analysis to confirm their hypothesis. Besides, membrane-attached microorganisms may reduce power output also by forming a thick biofilm which limits proton transfer from the anodic to the cathodic chamber.²⁵

4.2 Bioelectricity production and microbial dynamics in the thermophilic MFC

In the thermophilic MFC, the relatively low number of active anode-attached microbial families (Table 1) suggests the scarcity of thermophilic exoelectrogenic species. The inoculum selected for the experiment, which was not previously enriched for thermophilic electricity production, can be one of the causes hindering the establishment of an active exoelectrogenic community. However, the same activated sludge was successfully used to enrich dark fermentative hydrogen producers at 55 °C in a previous study.²⁷ In addition, 20% of the anode-attached active subpopulation was composed by *Firmicutes*,



which have been previously reported to generate electricity in thermophilic, acetate-fed MFCs.¹⁹ About 66% of *Firmicutes* found in the thermophilic anode-attached community belonged to the family *Thermodesulfobiaceae*, which includes *Coprothermobacter* sp., a proteolytic microorganism involved in the fermentation of organic substrates, with production of pyruvate, formate and acetate, and also in syntrophic acetate oxidation (for a review, see Gagliano *et al.*⁵²). The activity of *Coprothermobacter* is enhanced by establishing a syntrophy with hydrogenotrophic methanogenic archaea such as *Methanothermobacter*.⁵³ *Methanothermobacter* belongs to the family of *Methanobacteriaceae*, which was indeed among the most abundant active anode-attached families in the thermophilic MFC in this study (Fig. 5). Although *Coprothermobacter* was previously found among the anode-attached microbial community of thermophilic acetate-fed MFCs,^{19,54} and is thus a possible acetate-utilizing anode respiring bacterium, its electrochemical activity as a pure culture has not yet been investigated. Also microorganisms belonging to the order of *Chlorobiales*, despite being mainly phototrophs, can perform heterotrophic anaerobic respiration, and have been reported as part of the anodic biofilm in MFCs.^{46,55} *Anaerolineaceae*, also found among the thermophilic anode-attached microbial community, is a family of filamentous bacteria involved in the fermentation of various sugars.⁵⁶ They are also involved in the syntrophic oxidation of butyrate, and, similarly to *Coprothermobacter*, grow better in the presence of H₂-consuming microorganisms, such as methanogenic archaea.⁵⁷

The lower power production in the thermophilic MFC is likely due to the lack of effective exoelectrogens and to the consequent high activity of non-exoelectrogenic microorganisms, which consumed part of the electrons through pathways competitive to electricity generation. In fact, the methanogenic archaeal family of *Methanobacteriaceae*, belonging to the order of *Methanobacteriales*, accounted for 38% of the active anode-attached community in the thermophilic MFC. *Methanobacteriales* lack cytochromes and methanophenazine, and are thus able to perform hydrogenotrophic, but not acetoclastic, methanogenesis.⁵⁸ Therefore, *Methanobacteriaceae* cannot compete for the substrate with exoelectrogenic microorganisms, but their metabolism decreases the availability of electrons for electricity production. *Methanobacteriaceae* have been previously found in a glucose-fed (1.8 g L⁻¹) MFC operated at room temperature, and indicated as one of the causes for low bioelectricity production, as about 16% of the electrons were directed to methane production.¹⁴ Rismani-Yazdi *et al.*⁵⁹ reported methane production by *Methanobacteriaceae* in a mesophilic (39 °C) cellulose-fed MFC only at the beginning of the operation, whereas Hussain *et al.*⁶⁰ reported *Methanobacteriaceae* in a thermophilic (50 °C) syngas-fed MFC. Such microorganisms likely decreased the efficiency of their MFC by performing hydrogenotrophic methanogenesis.

The family of *Hydrogenophilaceae*, which accounted for 46% of the active planktonic community in the thermophilic MFC, includes the thermophilic *Hydrogenophylus* sp., which could have consumed a share of electrons by H₂ oxidation,⁶¹ lowering power production in the thermophilic MFC.

Thermodesulfobiaceae, found among the anode-attached families, were also found among the planktonic community (Fig. 5). *Coprothermobacter* is able to perform extracellular electron transfer,⁵² but further studies are required to understand its possible involvement in long-range electron transfer to the anode.

In the thermophilic MFC, the family of *Comamonadaceae* was the most abundant membrane-attached family and, similarly to the mesophilic MFC, it was likely related to aerobic metabolism and thus, oxygen consumption. *Armatimonadetes*, which accounted for 17% of the active membrane-attached community, is also an order of aerobic microorganisms.⁶²

4.3 Xylose degradation pathways

In the mesophilic MFC, the xylose consumption and metabolite production profiles (Fig. 2) suggest that xylose was firstly converted to volatile fatty acids, which were subsequently oxidized to CO₂ and H₂O likely mainly by *Geobacteraceae*, which dominated the anode-attached active community. Interestingly, the power density remained stable for about 30 hours after the depletion of acetate and butyrate. A possible explanation is that acetate and butyrate were accumulated and oxidized intracellularly, thus not detectable in the anolyte and resulting in a flow of electrons directed outside the cell to the anode.⁴ In fact, after substrate depletion, the soluble COD remained stable (Fig. 2), suggesting that the electron donor was not in the anolyte but likely inside the cells. Also Marshall and May²⁰ observed the same phenomenon and decided to starve a pure culture of *Thermincola* for two cycles before electrochemical measurements to avoid interferences from the intracellularly accumulated acetate, and its associated storage products.

In the thermophilic MFC, xylose was consumed relatively fast, but acetate, the only metabolite found in the anolyte, was not fully consumed even after 144 hours. The power density peak obtained just after the xylose depletion suggests that exoelectrogenic thermophiles were growing on xylose, but the microbial community was lacking effective acetate-utilizing microorganisms. However, it should be noted that the profiles in Fig. 2 were obtained in the feeding cycle "IX", whereas the samples for microbial community analysis were collected at the end of cycle "XI". The anodic potential, which increased from cycle IX to cycle XI in the thermophilic MFC (Fig. 1b), suggests a possible shift in the microbial community.

4.4 Performance of the MFCs

In the mesophilic MFC, the shape of the polarization curve (the stable slope in the last part of the curve) suggests low mass transfer limitation, as expected in MFCs using soluble sugars as the substrate. The low CE (12 and 3% for the mesophilic and thermophilic MFC, respectively) was attributed to the MFC design, which was not optimized for power production. The slow rate of oxygen reduction in the cathodic surface and the low proton conduction through the membrane are often the main causes of low power production in air-cathode MFCs.⁶³ In fact, a CE up to 82% was obtained in a xylose-fed, two-chamber MFC (75 mL anodic chamber volume) using 50 mM ferricyanide



for the cathodic reaction and a cation exchange membrane.⁶⁴ Haavisto *et al.*,²⁸ with a similar inoculum and substrate, obtained an 18% higher CE than the one obtained in this study operating a mesophilic (37 °C) upflow microbial fuel cell in continuous mode using ferricyanide at the cathode. Huang and Logan⁶⁵ obtained a power production of 13 W m⁻³ (61% CE) using a xylose-fed air cathode MFC, against the 1.1 W m⁻³ (12% CE) obtained in this study. However, the anodic chamber of their MFC was equipped with four carbon brushes (6 cm diameter and 7 cm length each), against the single carbon brush (1.5 cm diameter and 5 cm length) used in this study, and their xylose load was three times higher.

The structure of the active microbial community in the thermophilic MFC, lacking a known effective exoelectrogen such as *Geobacter* and including competitors such as methanogenic archaea, was likely the main cause for the lower power produced from the thermophilic MFC in comparison to the mesophilic MFC (Fig. 3a). In fact, the non-exoelectrogenic anode-attached microbial community in the thermophilic MFC likely caused a high internal resistance (560 Ω). Temperature also affects oxygen solubility in water, resulting in a decreased availability of oxygen at high temperature. In fact, the oxygen concentration at the cathode was about 7.0 and 5.6 g L⁻¹ in the mesophilic and thermophilic MFC, respectively. In the thermophilic MFC, the power overshoot curve (Fig. 3b), previously reported in MFCs,^{66,67} prevented the detection of possible mass transfer limitations. A multiple-cycle method, consisting in running the MFC at a fixed resistance for one entire batch cycle, can be applied to avoid overshoot.⁶⁸

5. Conclusions

The composition of the anode-attached, planktonic and membrane-attached microbial community, and the active subpopulation, was evaluated in a mesophilic (37 °C) and a thermophilic (55 °C) xylose-fed MFC. This study contributes in understanding of the microbial communities directly and indirectly involved in mesophilic and thermophilic electricity generation. An active microbial community dominated by *Geobacteraceae* was enriched and shown to sustain power production in mesophilic (37 °C) MFCs, whereas thermophilic (55 °C) power production was hampered by the development of competitors such as hydrogenotrophic methanogens and hydrogen oxidizers. A RNA-based analysis is required to understand the role of the microorganisms in MFCs, as a DNA-based analysis may lead to overestimation or underestimation of the contribution of certain species on power production.

A different inoculum source, possibly from thermophilic anaerobic processes, and a different start-up strategy, for example by using a poised anode potential or by suppressing the methanogenic archaea *e.g.* by addition of bromoethanesulphonic acid (BESA), could be viable alternatives to facilitate the establishment of an efficient thermophilic exoelectrogenic biofilm in future studies. The power production from pure cultures of potentially exoelectrogenic thermophilic microorganisms, for example species of the *Thermodesulfobiaceae* family detected from the thermophilic anodes in this study,

must also be evaluated to confirm their role in electricity production.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

This work was supported by the Marie Skłodowska-Curie European Joint Doctorate (EJD) in Advanced Biological Waste-To-Energy Technologies (ABWET) funded from Horizon 2020 under grant agreement no. 643071. JH and AML would also like to acknowledge the Academy of Finland (New Indigo ERA-Net Energy 2014; Project no. 283013) for financial support. The authors gratefully thank Mira Sulonen (TUT, Finland) for helping with the performance analysis, Antti Nuottajärvi (TUT, Finland) for helping with the experimental set-up, and the Viinikanlahti municipal wastewater treatment plant (Tampere, Finland) for providing the activated sludge used as inoculum.

References

- 1 S. K. Butti, G. Velvizhi, M. L. K. Sulonen, J. M. Haavisto, E. Oguz Koroglu, A. Yusuf Cetinkaya, S. Singh, D. Arya, J. Annie Modestra, K. Vamsi Krishna, A. Verma, B. Ozkaya, A.-M. Lakaniemi, J. A. Puhakka and S. Venkata Mohan, *Renewable Sustainable Energy Rev.*, 2016, **53**, 462–476.
- 2 R. Kumar, L. Singh, Z. A. Wahid and M. F. Din, *Int. J. Energy Res.*, 2015, **39**, 1048–1067.
- 3 S. Kalathil and D. Pant, *RSC Adv.*, 2016, **6**, 30582–30597.
- 4 R. M. Snider, S. M. Strycharz-glaven, S. D. Tsoi, J. S. Erickson and L. M. Tender, *Proc. Natl. Acad. Sci. U. S. A.*, 2012, **109**, 15467–15472.
- 5 R. Kumar, L. Singh and A. W. Zularisam, *Renewable Sustainable Energy Rev.*, 2016, **56**, 1322–1336.
- 6 S. P. Bonanni, G. D. Schrott and J. P. Busalmen, *Biochem. Soc. Trans.*, 2012, **40**, 1274–1279.
- 7 K. Rabaey, N. Boon, M. Höfte and W. Verstraete, *Environ. Sci. Technol.*, 2005, **39**, 3401–3408.
- 8 T. H. Pham, N. Boon, P. Aelterman, P. Clauwaert, L. De Schamphelaire, L. Vanhaecke, K. De Mayer, M. Höfte, W. Verstraete and K. Rabaey, *Appl. Microbiol. Biotechnol.*, 2008, **77**, 1119–1129.
- 9 K. P. Nevin, H. Richter, S. F. Covalla, J. P. Johnson, T. L. Woodard, A. L. Orloff, H. Jia, M. Zhang and D. R. Lovley, *Environ. Microbiol.*, 2008, **10**, 2505–2514.
- 10 M.-S. Kim and Y.-J. Lee, *Int. J. Hydrogen Energy*, 2010, **35**, 13028–13034.
- 11 D. Sun, J. Chen, H. Huang, W. Liu, Y. Ye and S. Cheng, *Int. J. Hydrogen Energy*, 2016, **41**, 16523–16528.
- 12 M. A. Rosenbaum, H. Y. Bar, Q. K. Beg, D. Segrè, J. Booth, M. A. Cotta and L. T. Angenent, *Bioresour. Technol.*, 2011, **102**, 2623–2628.
- 13 D. Wu, D. Xing, X. Mei, B. Liu, C. Guo and N. Ren, *Int. J. Hydrogen Energy*, 2013, **38**, 15568–15573.



- 14 K. Chung and S. Okabe, *Appl. Microbiol. Biotechnol.*, 2009, **83**, 965–977.
- 15 T. Catal, K. Li, H. Bermek and H. Liu, *J. Power Sources*, 2008, **175**, 196–200.
- 16 Z. Du, H. Li and T. Gu, *Biotechnol. Adv.*, 2007, **25**, 464–482.
- 17 Q. Fu, N. Fukushima, H. Maeda, K. Sato and H. Kobayashi, *Biosci., Biotechnol., Biochem.*, 2015, **79**, 1200–1206.
- 18 Q. Fu, H. Kobayashi, H. Kawaguchi, J. Vilcaez, T. Wakayama, H. Maeda and K. Sato, *J. Biosci. Bioeng.*, 2013, **115**, 268–271.
- 19 K. C. Wrighton, P. Agbo, F. Warnecke, K. A. Weber, E. L. Brodie, T. Z. DeSantis, P. Hugenholtz, G. L. Andersen and J. D. Coates, *ISME J.*, 2008, **2**, 1146–1156.
- 20 C. W. Marshall and H. D. May, *Energy Environ. Sci.*, 2009, **2**, 699–705.
- 21 B. G. Lusk, Q. F. Khan, P. Parameswaran, A. Hameed, N. Ali, B. E. Rittmann and C. I. Torres, *Environ. Sci. Technol.*, 2015, **49**, 14725–14731.
- 22 Q. Fu, H. Kobayashi, H. Kawaguchi, T. Wakayama, H. Maeda and K. Sato, *Environ. Sci. Technol.*, 2013, **47**, 12583–12590.
- 23 J. De Vrieze, L. Regueiro, R. Props, R. Vilchez-Vargas, R. Jáuregui, D. H. Pieper, J. M. Lema and M. Carballa, *Biotechnol. Biofuels*, 2016, **9**, 244.
- 24 D. K. Newman and R. Kolter, *Nature*, 2000, **405**, 94–97.
- 25 M. Miskan, M. Ismail, M. Ghasemi, J. Md Jahim, D. Nordin and M. H. Abu Bakar, *Int. J. Hydrogen Energy*, 2016, **41**, 543–552.
- 26 M. Lu, S. Chen, S. Babanova, S. Phadke, M. Salvacion, A. Mirhosseini, S. Chan, K. Carpenter, R. Cortese and O. Bretschger, *J. Power Sources*, 2017, **356**, 274–287.
- 27 P. Dessì, A.-M. Lakaniemi and P. N. L. Lens, *Water Res.*, 2017, **115**, 120–129.
- 28 J. M. Haavisto, M. E. Kokko, C.-H. Lay and J. A. Puhakka, *Int. J. Hydrogen Energy*, 2017, **42**, 27494–27501.
- 29 A. E. Mäkinen, M. E. Nissilä and J. A. Puhakka, *Int. J. Hydrogen Energy*, 2012, **37**, 12234–12240.
- 30 S.-E. Oh, B. Min and B. E. Logan, *Environ. Sci. Technol.*, 2004, **38**, 4900–4904.
- 31 R. I. Griffiths, A. S. Whiteley, A. G. O'Donnell and M. J. Bailey, *Appl. Environ. Microbiol.*, 2000, **66**, 5488–5491.
- 32 J. G. Caporaso, C. L. Lauber, W. A. Walters, D. Berg-Lyons, C. A. Lozupone, P. J. Turnbaugh, N. Fierer and R. Knight, *Proc. Natl. Acad. Sci. U. S. A.*, 2011, **108**, 4516–4522.
- 33 P. D. Schloss, S. L. Westcott, T. Ryabin, J. R. Hall, M. Hartmann, E. B. Hollister, R. A. Lesniewski, B. B. Oakley, D. H. Parks, C. J. Robinson, J. W. Sahl, B. Stres, G. G. Thallinger, D. J. Van Horn and C. F. Weber, *Appl. Environ. Microbiol.*, 2009, **75**, 7537–7541.
- 34 J. J. Koziach, S. L. Westcott, N. T. Baxter, S. K. Highlander and P. D. Schloss, *Appl. Environ. Microbiol.*, 2013, **79**, 5112–5120.
- 35 R Development Core Team, *R Found. Stat. Comput. Vienna*, Austria, ISBN 3-900051-07-0, <http://www.R-project.org>.
- 36 W. Zhi, Z. Ge, Z. He and H. Zhang, *Bioresour. Technol.*, 2014, **171**, 461–468.
- 37 C. Gao, A. Wang, W.-M. Wu, Y. Yin and Y.-G. Zhao, *Bioresour. Technol.*, 2014, **167**, 124–132.
- 38 K. L. Lesnik and H. Liu, *Appl. Microbiol. Biotechnol.*, 2014, **98**, 4187–4196.
- 39 Q. Jiang, D. Xing, R. Sun, L. Zhang, Y. Feng and N. Ren, *RSC Adv.*, 2016, **6**, 85149–85155.
- 40 X. Mei, C. Guo, B. Liu, Y. Tang and D. Xing, *RSC Adv.*, 2015, **5**, 78136–78141.
- 41 S. Saheb Alam, F. Persson, B. Wilén, M. Hermansson and O. Modin, *Sci. Rep.*, 2015, **5**, 18433.
- 42 A. Kumar, L. H.-H. Hsu, P. Kavanagh, F. Barrière, P. N. L. Lens, L. Lapinonnière, J. H. Lienhard V, U. Schröder, X. Jiang and D. Leech, *Nat. Rev. Chem.*, 2017, **1**, 24.
- 43 T. P. Sciarria, A. Tenca, A. D'Epifanio, B. Mecheri, G. Merlino, M. Barbato, S. Borin, S. Licocchia, V. Garavaglia and F. Adani, *Bioresour. Technol.*, 2013, **147**, 246–253.
- 44 C. S. Kang, N. Eaktasang, D.-Y. Kwon and H. S. Kim, *Bioresour. Technol.*, 2014, **165**, 27–30.
- 45 M. Grattieri, K. Hasan, R. D. Milton, S. Abdellaoui, M. Suvira, B. Alkotaini and S. D. Minter, *Sustainable Energy Fuels*, 2017, **1**, 1568–1572.
- 46 G. Zhang, Q. Zhao, Y. Jiao, K. Wang, D.-J. Lee and N. Ren, *Water Res.*, 2012, **46**, 43–52.
- 47 Y. Song, L. Xiao, I. Jayamani, Z. He and A. M. Cupples, *J. Microbiol. Methods*, 2015, **108**, 4–11.
- 48 T. Shimoyama, A. Yamazawa, Y. Ueno and K. Watanabe, *Microbes Environ.*, 2009, **24**, 188–192.
- 49 R. A. Timmers, M. Rothballer, D. P. B. T. B. Strik, M. Engel, S. Schulz, M. Schloter, A. Hartmann, B. Hamelers and C. Buisman, *Appl. Microbiol. Biotechnol.*, 2012, **94**, 537–548.
- 50 D. Xing, S. Cheng, B. E. Logan and J. M. Regan, *Appl. Microbiol. Biotechnol.*, 2010, **85**, 1575–1587.
- 51 J. R. Kim, S. Cheng, S.-E. Oh and B. E. Logan, *Environ. Sci. Technol.*, 2007, **41**, 1004–1009.
- 52 M. C. Gagliano, C. M. Braguglia, M. Petruccioli and S. Rossetti, *FEMS Microbiol. Ecol.*, 2015, **91**, 1–12.
- 53 K. Sasaki, M. Morita, D. Sasaki, J. Nagaoka, N. Matsumoto, N. Ohmura and H. Shinozaki, *J. Biosci. Bioeng.*, 2011, **112**, 469–472.
- 54 B. C. Jong, B. H. Kim, I. S. Chang, P. W. Y. Liew, Y. F. Choo and G. S. Kang, *Environ. Sci. Technol.*, 2006, **40**, 6449–6454.
- 55 M. Wei, F. Harnisch, C. Vogt, J. Ahlheim, T. R. Neu and H. H. Richnow, *RSC Adv.*, 2015, **5**, 5321–5330.
- 56 T. Yamada, T. Yamauchi, K. Shiraishi, P. Hugenholtz, A. Ohashi, H. Harada, Y. Kamagata, K. Nakamura and Y. Sekiguchi, *ISME J.*, 2007, **1**, 246–255.
- 57 P. Liu, Q. Qiu and Y. Lu, *Appl. Environ. Microbiol.*, 2011, **77**, 3884–3887.
- 58 R. K. Thauer, A.-K. Kaster, H. Seedorf, W. Buckel and R. Hedderich, *Nat. Rev. Microbiol.*, 2008, **6**, 579–591.
- 59 H. Rismani-Yazdi, S. M. Carver, A. D. Christy, Z. Yu, K. Bibby, J. Peccia and O. H. Tuovinen, *Bioresour. Technol.*, 2013, **129**, 281–288.
- 60 A. Hussain, P. Mehta, V. Raghavan, H. Wang, S. R. Guiot and B. Tartakovsky, *Enzyme Microb. Technol.*, 2012, **51**, 163–170.
- 61 N. R. Hayashi, T. Ishida, A. Yokota, T. Kodama and Y. Igarashi, *Int. J. Syst. Bacteriol.*, 1999, **49**, 783–786.
- 62 K. C. Y. Lee, P. F. Dunfield and M. B. Stott, in *The Prokaryotes: Other Major Lineages of Bacteria and The Archaea*, ed. E. Rosenberg, E. F. DeLong, S. Lory, E. Stackebrandt and F.



- Thompson, Springer Berlin Heidelberg, Berlin, Heidelberg, 2014, pp. 447–458.
- 63 H. Rismani-Yazdi, S. M. Carver, A. D. Christy and O. H. Tuovinen, *J. Power Sources*, 2008, **180**, 683–694.
- 64 A. E. Mäkinen, C. H. Lay, M. E. Nissilä and J. a. Puhakka, *Int. J. Hydrogen Energy*, 2013, **38**, 15606–15612.
- 65 L. Huang and B. E. Logan, *Appl. Microbiol. Biotechnol.*, 2008, **80**, 655–664.
- 66 S. M. Carver, P. Vuoriranta and O. H. Tuovinen, *J. Power Sources*, 2011, **196**, 3757–3760.
- 67 P.-C. Nien, C.-Y. Lee, K.-C. Ho, S. S. Adav, L. Liu, A. Wang, N. Ren and D.-J. Lee, *Bioresour. Technol.*, 2011, **102**, 4742–4746.
- 68 V. J. Watson and B. E. Logan, *Electrochem. Commun.*, 2011, **13**, 54–56.



Composition and role of the attached and planktonic microbial
communities in mesophilic and thermophilic xylose-fed microbial
fuel cells

Supporting information

Paolo Dessì^{a,}, Estefania Porca^b, Johanna Haavisto^a, Aino–Maija Lakaniemi^a, Gavin Collins^b, Piet N. L.
Lens^{a,c}*

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

*^bMicrobial Communities Laboratory, School of Natural Sciences, National University of Ireland Galway,
University Road, Galway, H91 TK33, Ireland*

^cUNESCO–IHE, Institute for Water Education, Westvest 7, 2611AX Delft, The Netherlands

*Corresponding author:

Phone: +358 417239696, e-mail: paolo.dessi@tut.fi, mail: Tampere University of Technology, P.O. Box
541, FI-33101 Tampere, Finland, ORCID: 0000-0002-9935-3038

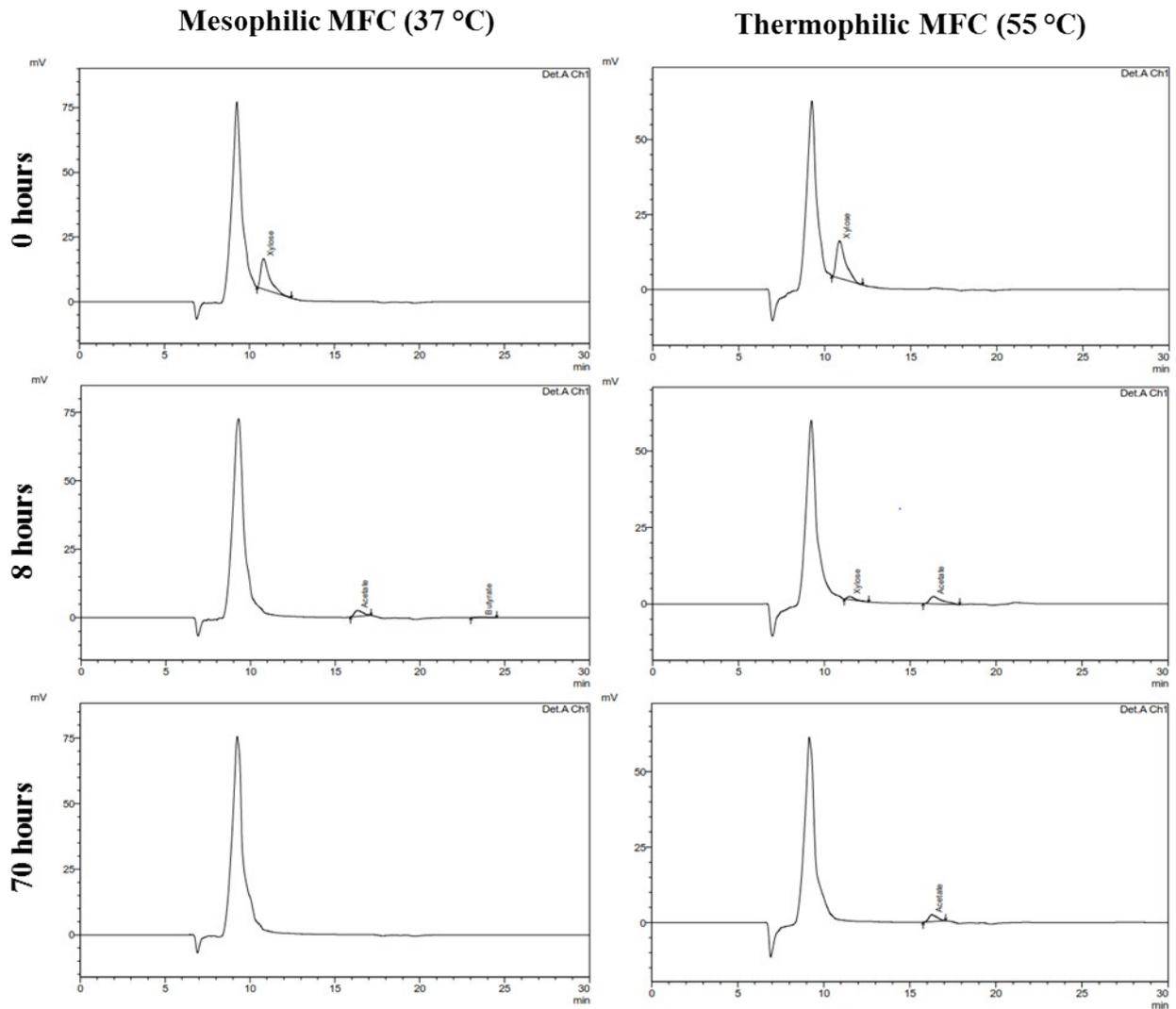


Figure S1. Examples of chromatograms obtained with the HPLC when performing the substrate degradation test (Figure 2 in the manuscript).

