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

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Abstract

In the last century, fossil fuels have been intensively used for energy production causing a dramatic increase of CO$_2$ 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 (H$_2$) 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 H$_2$ production and electricity production in MFCs. Therefore, this thesis focuses on delineating how temperature influences biological production of H$_2$ 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 H$_2$ production from xylose at 37, 55 and 70 °C. At both 37 and 55 °C, a higher H$_2$ yield was achieved by the fresh than the digested activated sludge, whereas a very low H$_2$ 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) H$_2$ producing community. Acidic and alkaline shocks selected known H$_2$ producing microorganisms belonging to Clostridiaceae at the expenses of lactate producing bacteria, resulting in the highest H$_2$ yield at 37 and 55 °C, respectively. Although a heat shock resulted in a low H$_2$ yield in a single batch, H$_2$ production by the heat-treated fresh activated sludge was shown to increase in the experiment with four consecutive batch cycles. This suggests that H$_2$ producing microbial communities may develop in the long-term as long as culture conditions are optimized for growth of H$_2$ producers.
Heat-treated fresh activated sludge was selected as inoculum for continuous H$_2$ 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 H$_2$ yield was obtained in the thermophilic than in the mesophilic FBR. Furthermore, H$_2$ production at 70 °C, which failed in the earlier batch study, was successful in the FBR, with a stable yield of 1.2 mol H$_2$ 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 H$_2$ production from thermomechanical pulping (TMP) wastewater in a temperature gradient incubator assay, as batch cultivation at 70 °C enriched the H$_2$ 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 H$_2$ 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 H$_2$ 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


Lämpökäsiteltävä aktiivilietettä käytettiin mikrobien lähteenä myös jatkuvatoimisissa leijupetireaktoreissa. Niissä tutkittiin vednyttuotoa ksyloosia sisältävää synteeettisestä jätevedestä 37 °C:ssa sekä 55-70 °C:ssa. Termofiilisessä leijupetireaktorissa vednyttuootto oli suurempaa kuin mesofiilisessä ja suurimmat vetysaannot saavutettiin 55 ja 70 °C:ssa, joissa molemmissa saanto oli 1.2 mol H\textsubscript{2} mol\textsuperscript{-1} ksyloosi. Leijupetireaktorissa vetyy onnistuttiin tuottamaan 70 °C:ssa kasvatettaessa mikrobeja vaiheittain (5 °C) 55 °C:ssa 70 °C:en nousevassa lämpötilassa, vaikka panoskokeessa vetyy ei saatu tuottavaa 70 °C:ssä tuoreella aktiivilietellä. Erillisessä panoskokeessa havaittiin, että 70 °C oli optimaalinen lämpötila vednyttuootteelle myös termomekaanisen sellunvalmistuksen jätevedestä käytettäessä leijupetireaktorissa rikastettua mikrobiviljelmää.


Tämä tutkimus osoitti, että termofiiliset mikrobit tuottavat enemmän ja vakaammin vetyä kuin mesofiiliset mikrobit. Pimeäfermentaatio 70 °C:ssa sopii erityisesti mm. termomekaanisen sellunvalmistuksen jätevesien käsitteelyyn, koska termomekaanisessa prosessissa syntyvät jätevedet ovat lämpimiä (50-80 °C). Tällöin bioreaktorin lämmittämiseen ei tarvittaisi paljon energiata. Tämän tutkimuksen perusteella termomekaanisen sellunvalmistuksen jätevesi sovelluksena käsiteltäväksi sähköä tuottavissa mesofiilisissä mikrobipolttokennoissa. Sähköntuotto termofiilisissä mikrobipolttokennoissa oli myös mahdollista, vaikka termofiilisten eksoelektrogeenisten mikrobien rikastus vaatii pitkän aloitus-vaiheen optimoimissa 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 CO$_2$ 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 sottoprodotto 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 (H$_2$) 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 H$_2$ 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 H$_2$ 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 H$_2$ a partire da xilosio a 37, 55 e 70 °C. A 37 e 55 °C, una più alta produzione di H$_2$ è stata ottenuta dal fango attivo fresco rispetto al digestato, mentre una produzione di H$_2$ 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 H$_2$ in condizioni mesofiliche (37 °C) e termofiliche (55 °C). Gli shock acido e basico hanno selezionato con successo microorganismi noti produttori di H$_2$, 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 mesofiliche (37 °C) e termofiliche (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 elettrogenici 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 mesofiliche (37 °C) e termofiliche (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 elettrogenica termofila può essere promosso 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 diffondendosi dalla camera catodica a quella anodica, favorendo i microorganismi elettrogeni strettamente anaerobici, ma competendo con loro per il substrato. Una popolazione elettrogena mesofilica 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 termofiliche piuttosto che mesofiliche. 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 termofilici 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'engagent 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. À 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. À 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 $H_2$ plus élevé 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 verwerkingswijze 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 industriën 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 aktief 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 aktief slib, terwijl een zeer lage H₂-opbrengst werd verkregen door beide inocula bij 70 °C. Vervolgens werden vier verschillende inoculumvoorbereidingen (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 aktief 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 aktief 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. Een temperatuur 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 concurreren 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
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<th>Description</th>
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<tr>
<td>AEM</td>
<td>Anion exchange membrane</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BES</td>
<td>Bioelectrochemical system</td>
</tr>
<tr>
<td>BESA</td>
<td>2-bromoethanesulfonic acid</td>
</tr>
<tr>
<td>CE</td>
<td>Coulombic efficiency</td>
</tr>
<tr>
<td>CEM</td>
<td>Cation exchange membrane</td>
</tr>
<tr>
<td>COD</td>
<td>Chemical oxygen demand</td>
</tr>
<tr>
<td>CSTR</td>
<td>Continuous stirred tank reactor</td>
</tr>
<tr>
<td>FBR</td>
<td>Fluidized bed reactor</td>
</tr>
<tr>
<td>HPR</td>
<td>Hydrogen production rate</td>
</tr>
<tr>
<td>HRT</td>
<td>Hydraulic retention time</td>
</tr>
<tr>
<td>LAB</td>
<td>Lactic acid bacteria</td>
</tr>
<tr>
<td>MBR</td>
<td>Membrane bioreactor</td>
</tr>
<tr>
<td>MEC</td>
<td>Microbial electrolysis cell</td>
</tr>
<tr>
<td>MFC</td>
<td>Microbial fuel cell</td>
</tr>
<tr>
<td>NADH</td>
<td>Nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>OLR</td>
<td>Organic loading rate</td>
</tr>
<tr>
<td>PBR</td>
<td>Packed bed reactor</td>
</tr>
<tr>
<td>PCR-DGGE</td>
<td>Polymerase chain reaction-denaturing gradient cell electrophoresis</td>
</tr>
<tr>
<td>PEM</td>
<td>Proton exchange membrane</td>
</tr>
<tr>
<td>SHE</td>
<td>Standard hydrogen electrode</td>
</tr>
<tr>
<td>TBR</td>
<td>Trickling bed reactor</td>
</tr>
<tr>
<td>TMP</td>
<td>Thermomechanical pulping</td>
</tr>
<tr>
<td>TS</td>
<td>Total solids</td>
</tr>
<tr>
<td>UASB</td>
<td>Upflow anaerobic sludge blanket</td>
</tr>
<tr>
<td>VFA</td>
<td>Volatile fatty acid</td>
</tr>
<tr>
<td>VS</td>
<td>Volatile solids</td>
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List of Publications


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 (CO$_2$) 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 Commision 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 ($\text{H}$ 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 H₂ 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 H₂, methane (which can be combined with H₂ to produce hytane), 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).](image-url)
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 (Liobikiéné 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, H\(_2\) 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). H\(_2\) 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 H\(_2\) 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 H\(_2\) through a metalloenzyme called hydrogenase (Figure 3.1), yielding the theoretical maximum (also called Thauer limit) of 4 mol H\(_2\) mol\(^{-1}\) glucose (Eq. 1) (Thauer et al. 1977). This is also valid for pentose sugars such as xylose, which yields 3.3 mol H\(_2\) mol\(^{-1}\) xylose through the acetate pathway (Eq. 2).

\[
\text{C}_6\text{H}_{12}\text{O}_6 + 2 \text{H}_2\text{O} \rightarrow 2 \text{CH}_3\text{COOH} + 4 \text{H}_2 + 2 \text{CO}_2 \quad (1)
\]

\[
\text{C}_5\text{H}_{10}\text{O}_5 + 1.67 \text{H}_2\text{O} \rightarrow 1.67 \text{CH}_3\text{COOH} + 3.33 \text{H}_2 + 1.67 \text{CO}_2 \quad (2)
\]
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 H₂ 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 H₂ (Verhaart et al. 2010). The dark fermentative process can be inhibited by the accumulation of produced H₂ in the medium, making an efficient gas stripping necessary to enable high H₂ yield (Beckers et al. 2015). Due to those limitations, several different fermentation pathways usually occur simultaneously in H₂ 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 H₂ production (Figure 3.1). This results in H₂ yields ranging between 14 and 70% of the Thauer limit (Saady 2013).

\[
\text{C}_5\text{H}_{10}\text{O}_5 \rightarrow 0.83 \text{ CH}_3\text{CH}_2\text{CH}_2\text{COOH} + 1.67 \text{ H}_2 + 1.67 \text{ CO}_2 \quad (3)
\]

\[
\text{C}_5\text{H}_{10}\text{O}_5 + 1.67 \text{ H}_2 \rightarrow 1.67 \text{ CH}_3\text{CH}_2\text{COOH} + 1.67 \text{ H}_2\text{O} \quad (4)
\]

\[
\text{C}_5\text{H}_{10}\text{O}_5 \rightarrow 1.67 \text{ CH}_3\text{CHOHCOOH} \quad (5)
\]

\[
\text{C}_5\text{H}_{10}\text{O}_5 \rightarrow 1.67 \text{ CH}_3\text{CH}_2\text{OH} + 1.67 \text{ CO}_2 \quad (6)
\]
3.1.2 Hydrogen producing bacteria

Dark fermentative H$_2$ 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.

H$_2$ 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 H$_2$ 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 H$_2$ producing Clostridium sp., such as C. butyricum and C. acetobutylicum, produce H$_2$ through both the acetate and butyrate pathway under mildly acidic conditions, with optimum H$_2$ 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 H$_2$ yielding capabilities (Hiligsmann et al. 2011).

Microorganisms of the genus Thermoanaerobacterium, and specifically T. thermosaccharolyticum, are among the most widely studied thermophilic H$_2$ 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 H$_2$ production capabilities, many hyperthermophilic microorganisms, including Thermoanaerobacter sp. (Xue et al. 2001; Soboh et al. 2004; Sigurbjomnsdottir 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 H$_2$ 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 microorganisms 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 synthetizing 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 \( \text{H}_2 \) producing microorganisms by repressing \( \text{H}_2 \) consumers. Many of the pretreatment processes rely on the fact that some \( \text{H}_2 \) 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 \( \text{H}_2 \) 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 \( \text{H}_2 \) 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 \( \text{H}_2 \) producers, while sporulating \( \text{H}_2 \) 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 \( \text{H}_2 \) consumption in the long term, making it necessary to repeat periodically the pretreatment, which is not a sustainable approach.

The growth of a \( \text{H}_2 \) 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 H$_2$ yield from various substrates, showing pH, temperature of incubation and microbial community analysis applied. The pretreatment resulting in the highest H$_2$ yield in the specific study is underlined and written in bold.

<table>
<thead>
<tr>
<th>Inoculum</th>
<th>Pretreatment applied*</th>
<th>Substrate (g COD L$^{-1}$)</th>
<th>T*</th>
<th>pH*</th>
<th>H$_2$ yield (mmol H$_2$ g$^{-1}$ COD)*</th>
<th>Microbiological analysis</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sludge from soybean treatment plant</td>
<td>AS, BS, DHT, FT, HT</td>
<td>Glycerol (30.7)</td>
<td>35</td>
<td>7.0</td>
<td>n.a.</td>
<td>Plate count and 16S rRNA gene sequencing</td>
<td>Misturini Rossi et al. (2011)</td>
</tr>
<tr>
<td>Anaerobic microflora from lab-scale UASB</td>
<td>AS, AS+CT, AS+HT, AS+CT+HT, CT, CT+HT, HT</td>
<td>Dairy wastewater (10.4)</td>
<td>29</td>
<td>6.3</td>
<td>0.03</td>
<td>None</td>
<td>Venkata Mohan et al. (2008)</td>
</tr>
<tr>
<td>None (substrate was assumed to contain also the necessary microorganisms)</td>
<td>ENZ, HT, MW</td>
<td>Waste sludge (19.5)</td>
<td>35</td>
<td>7.9</td>
<td>0.29</td>
<td>PCR-DGGE and 16s rRNA gene sequencing</td>
<td>Guo et al. (2015)</td>
</tr>
<tr>
<td>Granular sludge from brewery</td>
<td>AS, BS, CT, FT, HT</td>
<td>Palm oil mill effluent (49.0)</td>
<td>35</td>
<td>5.5</td>
<td>0.44</td>
<td>None</td>
<td>Mohammadi et al. (2012)</td>
</tr>
<tr>
<td>Digested sludge from plant treating the organic fraction of municipal waste</td>
<td>AE, AS, FT HT, US</td>
<td>Rice and lettuce powder (24.8)</td>
<td>37</td>
<td>5.5</td>
<td>0.80</td>
<td>None</td>
<td>Dong et al. (2010)</td>
</tr>
<tr>
<td>Anaerobic digested sludge</td>
<td>AS, BS, CT, HT, LS</td>
<td>Sucrose (22.5)</td>
<td>60</td>
<td>5.5</td>
<td>2.14</td>
<td>PCR-DGGE, 16s rDNA sequencing, phylogenetic tree</td>
<td>O-Thong et al. (2009)</td>
</tr>
<tr>
<td>None (substrate was assumed to contain also the necessary microorganisms)</td>
<td>AS, BS, HT, US, US+AS, US+BS US+HT</td>
<td>Food waste (18.4)</td>
<td>37</td>
<td>5.5</td>
<td>2.65</td>
<td>None</td>
<td>Elbashbishy et al. (2011)</td>
</tr>
<tr>
<td>Anaerobic sludge from cassava ethanol plant</td>
<td>AS, BS, CT, HT, LS*</td>
<td>Cassava stillage (60.1)</td>
<td>60</td>
<td>6.0</td>
<td>2.91</td>
<td>None</td>
<td>Luo et al. (2010)</td>
</tr>
<tr>
<td>Sludge from cattle manure treatment plant</td>
<td>AS, CT, DHT, FT, HT, AS, BS, FT HT</td>
<td>Glucose (25.0)</td>
<td>35.5</td>
<td>7.0</td>
<td>4.40</td>
<td>None</td>
<td>Hansen (2006)</td>
</tr>
<tr>
<td>Marine intertidal sludge</td>
<td>AS, CT, DHT, FT, HT</td>
<td>Glucose (21.3)</td>
<td>37</td>
<td>7.2</td>
<td>4.48</td>
<td>PCR-DGGE and 16s rRNA gene sequencing</td>
<td>Liu et al. (2009)</td>
</tr>
<tr>
<td>Sewage sludge</td>
<td>US, UV</td>
<td>Apple pomace (n.a.)</td>
<td>37</td>
<td>5.0</td>
<td>4.77</td>
<td>None</td>
<td>Wang et al. (2010)</td>
</tr>
<tr>
<td>Suspended and granular anaerobic sludge</td>
<td>CT, HT, HT+CT</td>
<td>Wheat straw powder (20.0)</td>
<td>37</td>
<td>7.0</td>
<td>5.20</td>
<td>None</td>
<td>Argun and Kargi (2009)</td>
</tr>
<tr>
<td>Anaerobic sewage sludge and granular sludge</td>
<td>AS, CT, HT</td>
<td>Glucose (20.0)</td>
<td>35</td>
<td>7.0</td>
<td>6.08</td>
<td>None</td>
<td>Hu and Chen (2007)</td>
</tr>
<tr>
<td>Inoculum</td>
<td>Pretreatment applied&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Substrate (gCOD L&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>T&lt;sup&gt;b&lt;/sup&gt;</td>
<td>pH&lt;sup&gt;c&lt;/sup&gt;</td>
<td>H&lt;sub&gt;2&lt;/sub&gt; yield (mmol H&lt;sub&gt;2&lt;/sub&gt; g&lt;sup&gt;-1&lt;/sup&gt; COD)&lt;sup&gt;d&lt;/sup&gt;</td>
<td>Microbiological analysis</td>
<td>Reference</td>
</tr>
<tr>
<td>--------------------------------------------------------------------------</td>
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<td>-------------------------------------------------</td>
<td>---------------------------------------------</td>
<td>-----------</td>
</tr>
<tr>
<td>Sludge from secondary settler of municipal wastewater treatment plant</td>
<td>AE, AS, BS, HT,</td>
<td>Glucose (10.7)</td>
<td>35</td>
<td>6.8</td>
<td>6.26</td>
<td>PCR-DGGE and 16s rRNA gene sequencing</td>
<td>Ren et al. (2008)</td>
</tr>
<tr>
<td>Digested sewage sludge</td>
<td>EF, HT</td>
<td>Glucose (20.0)</td>
<td>37</td>
<td>8.0</td>
<td>7.44</td>
<td>RNA concentration and 16S rRNA gene sequencing</td>
<td>Jeong et al. (2013)</td>
</tr>
<tr>
<td>Digester anaerobic sludge</td>
<td>AS, BS, HT, US</td>
<td>Glucose (8.5)</td>
<td>37</td>
<td>6.5</td>
<td>8.07</td>
<td>None</td>
<td>Elbeshbishy et al. (2010)</td>
</tr>
<tr>
<td>Anaerobic sludge from plant treating soybean wastewater</td>
<td>AS, BS, HT, US</td>
<td>Glucose (10.7)</td>
<td>37</td>
<td>5.5</td>
<td>8.83</td>
<td>None</td>
<td>Mu et al. (2007)</td>
</tr>
<tr>
<td>Anaerobic sludge from ethanol manufacturing</td>
<td>AS, BS, HT, LA</td>
<td>Glucose (n.a.)</td>
<td>37</td>
<td>5.5</td>
<td>8.84</td>
<td>Plate count</td>
<td>Chaganti et al. (2012)</td>
</tr>
<tr>
<td>Activated sludge</td>
<td>AE, AS, BS, CT, HT</td>
<td>Glucose (10.7)</td>
<td>35</td>
<td>7.0</td>
<td>8.89</td>
<td>None</td>
<td>Chang et al. (2011)</td>
</tr>
<tr>
<td>Digested sewage sludge</td>
<td>AE, AS, BS, CT, HT</td>
<td>Glucose (7.1)</td>
<td>35</td>
<td>7.0</td>
<td>9.26</td>
<td>Optical density</td>
<td>Wang and Wan (2008)</td>
</tr>
<tr>
<td>Granular and flocculated anaerobic sludge from municipal wastewater treatment</td>
<td>AS, BS, CT, HT, LA, LS</td>
<td>Glucose (5.3)</td>
<td>37</td>
<td>6.0</td>
<td>9.38</td>
<td>Genomic DNA extraction, separation in polyacrylamide and sequencing</td>
<td>Pendyala et al. (2012)</td>
</tr>
<tr>
<td>Digested anaerobic sludge from municipal wastewater treatment plant</td>
<td>AS, BS, HT, II</td>
<td>Glucose (10.7)</td>
<td>36</td>
<td>7.0</td>
<td>11.20</td>
<td>None</td>
<td>Yin et al. (2014)</td>
</tr>
<tr>
<td>Anaerobic sludge from pig manure</td>
<td>AS, BS, HT</td>
<td>Glucose (21.3)</td>
<td>35</td>
<td>6.2</td>
<td>11.25</td>
<td>None</td>
<td>Wang et al. (2011)</td>
</tr>
<tr>
<td>Cow dung compost</td>
<td>AE, AE+LS, HT, IR</td>
<td>Sucrose (11.2)</td>
<td>36</td>
<td>7.0</td>
<td>11.50</td>
<td>Plate count</td>
<td>Song et al. (2012)</td>
</tr>
<tr>
<td>Fresh cattle dung</td>
<td>AS, CT, HT</td>
<td>Sago wastewater (11.6)</td>
<td>30</td>
<td>7.0</td>
<td>12.33</td>
<td>None</td>
<td>Sen and Suttar (2012)</td>
</tr>
<tr>
<td>Digested activated sludge</td>
<td>AE, AS, BS, CT, HT</td>
<td>Sucrose (11.2)</td>
<td>35</td>
<td>6.3-8.0</td>
<td>14.67</td>
<td>None</td>
<td>Zhu and Béland (2006)</td>
</tr>
</tbody>
</table>

<sup>a</sup> 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.

<sup>b</sup> Incubation temperature.

<sup>c</sup> Initial pH of incubation.

<sup>d</sup> Highest H<sub>2</sub> yield obtained per g COD added in the first batch culture of pretreated or untreated inoculum.

<sup>e</sup> The highest H<sub>2</sub> yield was obtained using the untreated inoculum.

<sup>f</sup> 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 H₂ 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 H\textsubscript{2} production rate (HPR) of 703 mL H\textsubscript{2} L\textsuperscript{-1} h\textsuperscript{-1} with a remarkably high yield of 2.4 mol H\textsubscript{2} mol\textsuperscript{-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 H\textsubscript{2} 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 N\textsubscript{2} or CO\textsubscript{2} can help decreasing the H\textsubscript{2} partial pressure inside dark fermentative bioreactors (Kim et al. 2006), but it also results in a dilution of the H\textsubscript{2} produced. To overcome this problem, two gas separation membranes were connected to a dark fermentative CSTR in order to separate the H\textsubscript{2} from the CO\textsubscript{2} (Bakonyi et al. 2017). The CO\textsubscript{2}-rich gas stream was then recirculated from the bottom of the CSTR, stripping H\textsubscript{2} 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 H\textsubscript{2} 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 H\textsubscript{2} 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 H\textsubscript{2} h\textsuperscript{-1} L\textsuperscript{-1} was obtained by dark fermentation of hydrolized 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 H\textsubscript{2} h\textsuperscript{-1} L\textsuperscript{-1}.
Table 3.2: $H_2$ 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).

<table>
<thead>
<tr>
<th>Inoculum</th>
<th>Reactor type$^a$</th>
<th>Volume (L)</th>
<th>Substrate</th>
<th>T (°C)</th>
<th>pH</th>
<th>HRT (h)</th>
<th>OLR (g COD L$^{-1}$ h$^{-1}$)</th>
<th>HPR (mL $H_2$ h$^{-1}$ L$^{-1}$)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Food waste and tap water</td>
<td>CSTR</td>
<td>200</td>
<td>Food waste</td>
<td>55</td>
<td>5-6</td>
<td>79.2</td>
<td>21.6</td>
<td>39.6</td>
<td>Cavinato et al. (2012)</td>
</tr>
<tr>
<td>Heat treated seacoast sludge</td>
<td>CSTR</td>
<td>400</td>
<td>Sucrose</td>
<td>37</td>
<td>5.6-5.8</td>
<td>8</td>
<td>2.5</td>
<td>558</td>
<td>Lin et al. (2011)</td>
</tr>
<tr>
<td>Heat treated digested sludge</td>
<td>Tank reactor</td>
<td>500</td>
<td>Liquid from food</td>
<td>33</td>
<td>5.3</td>
<td>21</td>
<td>12.3-71.3</td>
<td>0.16</td>
<td>Lee and Chung (2010)</td>
</tr>
<tr>
<td>Sludge from fructose processing</td>
<td>FBR</td>
<td>1000</td>
<td>Gluten processing wastewater</td>
<td>35</td>
<td>6.0</td>
<td>36</td>
<td>0.56</td>
<td>9.3</td>
<td>Cheng et al. (2011)</td>
</tr>
<tr>
<td>Sludge from municipal wastewater treatment plant</td>
<td>CSTR</td>
<td>1480</td>
<td>Molasses</td>
<td>35</td>
<td>7.0</td>
<td>4.2</td>
<td>2.8</td>
<td>232</td>
<td>Ren et al. (2006)</td>
</tr>
<tr>
<td>Heat treated sewage sludge</td>
<td>Baffle reactor</td>
<td>3000</td>
<td>Hydrolized corn stover</td>
<td>35</td>
<td>4.5</td>
<td>16</td>
<td>0.63</td>
<td>313</td>
<td>Zhang et al. (2018)</td>
</tr>
</tbody>
</table>

$^a$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 H$_2$ 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 H$_2$ 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 H$_2$ partial pressure). Furthermore, thermophilic conditions are not favourable for most H$_2$ 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 H$_2$ 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 H$_2$ 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 H$_2$ production. For example, H$_2$ was successfully produced from diluted sugarcane vinasse in a FBR at 55 °C, with a maximum HPR of 780 mL H$_2$ 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). H$_2$ 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 H$_2$ steadily for 150 days with an average HPR of 20 mL H$_2$ h$^{-1}$ L$^{-1}$. Continuous H$_2$ 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 H$_2$ h$^{-1}$ L$^{-1}$ (Qiu et al. 2011).
Table 3.3: Highest stable H\textsubscript{2} 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).

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

\textsuperscript{a} Continuous stirred tank reactor (CSTR), fluidized bed reactor (FBR), trickling bed reactor (TRB), upflow anaerobic sludge blanket (UASB).

\textsuperscript{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 Deferrribacteres 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 of 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% CODsoluble 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⁺, Au³⁺, Cd²⁺, Co³⁺, Co²⁺, Cr⁶⁺, Cu²⁺, Fe³⁺, Ni²⁺ and U⁶⁺) 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 (Nancharaiah 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.

<table>
<thead>
<tr>
<th>Inoculum</th>
<th>Substrate</th>
<th>Reactor type</th>
<th>T (°C)</th>
<th>Power or current density</th>
<th>CE (%)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thermophilic digester sludge</td>
<td>Acetate (32.9 mM)</td>
<td>Two-chamber</td>
<td>55</td>
<td>823 mW m⁻²</td>
<td>n.a.</td>
<td>Fu et al. (2013b)</td>
</tr>
<tr>
<td><em>Calditerrivibrio nitroreducens</em></td>
<td>Acetate (32.9 mM)</td>
<td>Two-chamber</td>
<td>55</td>
<td>272 mW m⁻²</td>
<td>n.a.</td>
<td>Fu et al. (2013b)</td>
</tr>
<tr>
<td>Thermophilic digester sludge</td>
<td>Acetate (32.9 mM)</td>
<td>Two-chamber</td>
<td>55</td>
<td>512 mW m⁻²</td>
<td>n.a.</td>
<td>Fu et al. (2013a)</td>
</tr>
<tr>
<td><em>Caloramator australicus</em></td>
<td>Yeast extract (0.2% w/v)</td>
<td>Single chamber MEC</td>
<td>55</td>
<td>3.2 mW m⁻²</td>
<td>n.a.</td>
<td>Fu et al. (2013a)</td>
</tr>
<tr>
<td>Thermophilic digester sludge</td>
<td>Acetate (3.4 mM)</td>
<td>Two-chamber</td>
<td>55</td>
<td>1030 mW m⁻²</td>
<td>80</td>
<td>Jong et al. (2006)</td>
</tr>
<tr>
<td>Thermophilic digester sludge</td>
<td>Distillery wastewater (300 mg COD L⁻¹)</td>
<td>Two-chamber MFC</td>
<td>55</td>
<td>1000 mW m⁻²</td>
<td>89</td>
<td>Ha et al. (2012)</td>
</tr>
<tr>
<td>Thermophilic digester sludge</td>
<td>Acetate (10 mM)</td>
<td>Two-chamber</td>
<td>55</td>
<td>37 mW m⁻²</td>
<td>89</td>
<td>Wrighton et al. (2008)</td>
</tr>
<tr>
<td>Thermophilic compost</td>
<td>Glucose (25 mM)</td>
<td>Two-chamber</td>
<td>57</td>
<td>4.0 mW m⁻²</td>
<td>n.a.</td>
<td>Carver et al. (2011)</td>
</tr>
<tr>
<td><em>Thermincola ferriacetica</em> DSMZ 14005</td>
<td>Acetate (35 mM)</td>
<td>Two-chamber</td>
<td>60</td>
<td>7-8 Am⁻²</td>
<td>93</td>
<td>Parameswaran et al. (2013)</td>
</tr>
<tr>
<td><em>Thermoanaerobacter pseudethanolicus</em></td>
<td>Xylose (20 mM)</td>
<td>Two-chamber</td>
<td>60</td>
<td>5.8 Am⁻²</td>
<td>35</td>
<td>Lusk et al. (2015)</td>
</tr>
<tr>
<td><em>Thermoanaerobacter pseudethanolicus</em></td>
<td>Glucose (10 mM)</td>
<td>Two-chamber</td>
<td>60</td>
<td>4.3 Am⁻²</td>
<td>65</td>
<td>Lusk et al. (2015)</td>
</tr>
<tr>
<td><em>Thermoanaerobacter pseudethanolicus</em></td>
<td>Cellobiose (7.5 mM)</td>
<td>Two-chamber</td>
<td>60</td>
<td>5.2 Am⁻²</td>
<td>28</td>
<td>Lusk et al. (2015)</td>
</tr>
<tr>
<td>Marine sediment</td>
<td>Acetate (25 mM)</td>
<td>Single chamber MFC</td>
<td>60</td>
<td>207 mW m⁻²</td>
<td>35.5</td>
<td>Mathis et al. (2008)</td>
</tr>
<tr>
<td><em>Thermincola ferriacetica</em> strain Z-0001</td>
<td>Acetate (10 mM)</td>
<td>Single chamber MFC</td>
<td>60</td>
<td>160 mW m⁻²</td>
<td>97</td>
<td>Marshall and May (2009)</td>
</tr>
<tr>
<td>Water from a petroleum reservoir</td>
<td>Glucose (11.1 mM)</td>
<td>Two-chamber</td>
<td>95</td>
<td>165 mW m⁻²</td>
<td>n.a.</td>
<td>Fu et al. (2015)</td>
</tr>
</tbody>
</table>
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 H$_2$ 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 H$_2$ 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 \( \text{H}_2 \) 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 \( \text{H}_2 \) 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 \( \text{H}_2 \) 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 \( \text{H}_2 \) 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.

<table>
<thead>
<tr>
<th>Objective</th>
<th>Experiment type</th>
<th>Temperatures tested</th>
<th>Paper</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inoculum selection</td>
<td>Batch</td>
<td>37, 55, 70</td>
<td>I</td>
</tr>
<tr>
<td>Inoculum pretreatment selection</td>
<td>Batch</td>
<td>37, 55, 70</td>
<td>II</td>
</tr>
<tr>
<td>H₂ production from xylose-containing synthetic wastewater</td>
<td>Continuous</td>
<td>37, 55, 60, 65, 70</td>
<td>III</td>
</tr>
<tr>
<td>H₂ production from TMP wastewater</td>
<td>Batch</td>
<td>37, 42, 48, 55, 59, 65, 70, 74, 80</td>
<td>IV</td>
</tr>
</tbody>
</table>

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.

<table>
<thead>
<tr>
<th>Inoculum</th>
<th>Total solids (g L(^{-1}))</th>
<th>Volatile solids (g L(^{-1}))</th>
<th>Paper</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh activated sludge (Viinikanlahti)</td>
<td>17.3 (± 0.1)</td>
<td>8.8 (± 0.1)</td>
<td>I, III</td>
</tr>
<tr>
<td>Digested activated sludge (Viinikanlahti)</td>
<td>46.5 (± 0.4)</td>
<td>24.0 (± 0.1)</td>
<td>I</td>
</tr>
<tr>
<td>Fresh activated sludge (Mutton island)</td>
<td>28.1 (± 2.4)</td>
<td>22.7 (± 2.0)</td>
<td>II</td>
</tr>
<tr>
<td>Biofilm coated activated carbon from FBR</td>
<td>558 (± 14)</td>
<td>529 (± 14)</td>
<td>IV</td>
</tr>
</tbody>
</table>

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 H\(_2\) 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 H\(_2\) 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 H\(_2\) 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 N\textsubscript{2} 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 N\textsubscript{2} for 5 min after inoculation. The initial pH was adjusted to 6.3 (± 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>
<thead>
<tr>
<th>Parameter</th>
<th>Concentration (mg L\textsuperscript{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total solids (TS)</td>
<td>3771 ± 10</td>
</tr>
<tr>
<td>Volatile solids (VS)</td>
<td>2452 ± 8</td>
</tr>
<tr>
<td>Total COD</td>
<td>3352 ± 82</td>
</tr>
<tr>
<td>Soluble COD</td>
<td>3289 ± 54</td>
</tr>
<tr>
<td>Total nitrogen</td>
<td>&lt; 10</td>
</tr>
<tr>
<td>Total PO\textsubscript{4}-P</td>
<td>2.8</td>
</tr>
<tr>
<td>Acetate</td>
<td>&lt; 30</td>
</tr>
<tr>
<td>Furfural</td>
<td>&lt; 10</td>
</tr>
<tr>
<td>Glucose</td>
<td>43 (± 2)</td>
</tr>
<tr>
<td>Xylose</td>
<td>38 (± 0)</td>
</tr>
</tbody>
</table>

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

### 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\textsuperscript{-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 KH\textsubscript{2}PO\textsubscript{4}, K\textsubscript{2}HPO\textsubscript{4} and yeast extract
was reduced 10 times. The pH inside the reactor was kept at 5.0 (± 0.1) by automatic titration (Metrohm, Switzerland).

The FBRs were inoculated with 50 mL of heat-treated (90 °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 (± 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 and kept at 70 °C until day 185. The mesophilic FBR was operated at 37 (± 1) °C for 185 days, and then at 55 (± 1) °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 (→), gas path (↑ ). 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.

<table>
<thead>
<tr>
<th>Objective</th>
<th>Experiment type</th>
<th>Temperatures tested</th>
<th>Paper</th>
</tr>
</thead>
<tbody>
<tr>
<td>Study of the microbial communities</td>
<td>Batch</td>
<td>37, 55</td>
<td>V</td>
</tr>
<tr>
<td>Optimize thermophilic electricity production</td>
<td>Batch</td>
<td>55</td>
<td>Unpublished</td>
</tr>
<tr>
<td>Electricity production from TMP wastewater</td>
<td>Continuous</td>
<td>37</td>
<td>Unpublished</td>
</tr>
</tbody>
</table>
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\textsubscript{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 (± 0.2) and 22.1 (± 0.4) g L\textsuperscript{-1}, respectively, whereas the VS content was 10.6 (± 0.2) and 8.4 (± 0.5) g L\textsuperscript{-1}, respectively. The mixture of the two inocula, in 1/1 volume ratio, was flushed with N\textsubscript{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 (Memmert, Germany) in order to keep the temperature at 37 (± 2) °C or 55 (± 2) °C, respectively. The pH of the anolyte was adjusted to 7.0 with NaOH and kept at 7.0 (± 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 kΩ 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.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Analytical technique, instrument model</th>
<th>Paper</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gas volume</td>
<td>Syringe method (Owen et al. 1979)</td>
<td>I, II, IV</td>
</tr>
<tr>
<td></td>
<td>Gas meter, Ritter</td>
<td>III</td>
</tr>
<tr>
<td>Gas composition</td>
<td>Gas chromatography, Shimadzu GC-2014 thermal conductivity detector (TCD) and Porapak N column</td>
<td>I, II, III, IV</td>
</tr>
<tr>
<td>Sugar concentration</td>
<td>Colorimetric method (Dubois et al. 1956), Shimadzu Ordior UV-VIS spectrophotometer</td>
<td>I</td>
</tr>
<tr>
<td></td>
<td>Liquid chromatography, Shimadzu HPLC with refractive index detector (RID) and Phenomenex RHM-monosaccharide column</td>
<td>II, III</td>
</tr>
<tr>
<td></td>
<td>Liquid chromatography, Shimadzu HPLC with Phenomenex RPM-monosaccharide column</td>
<td>IV</td>
</tr>
<tr>
<td>VFA and alcohol concentration</td>
<td>Gas chromatography, Shimadzu GC-210 with flame ionization detector (FID) and Porapak N column</td>
<td>I, III</td>
</tr>
<tr>
<td></td>
<td>Liquid chromatography, Shimadzu HPLC with RID and Phenomenex RHM-monosaccharide column</td>
<td>I, III, IV, V</td>
</tr>
<tr>
<td></td>
<td>Liquid chromatography, Shimadzu HPLC with RID and Phenomenex ROA-Organic Acid H+ column</td>
<td>II</td>
</tr>
<tr>
<td>pH</td>
<td>WTW 330 pH meter with Hamilton slim electrode</td>
<td>I, II, III, IV</td>
</tr>
<tr>
<td>COD, sCOD</td>
<td>Dichromate methods, Finnish standard SFS 5504</td>
<td>IV, V</td>
</tr>
<tr>
<td>TS, VS, Total N, PO$_3^-$-P</td>
<td>Standard procedures (APHA 1998)</td>
<td>IV</td>
</tr>
<tr>
<td>Furfural</td>
<td>Gas chromatography, Agilent GC with MS detector and Agilent HP-5MS capillary column</td>
<td>IV</td>
</tr>
<tr>
<td>Voltage</td>
<td>Data logger, Agilent 34970A</td>
<td>V</td>
</tr>
<tr>
<td>Conductivity</td>
<td>Conductivity meter, WTW inoLab</td>
<td>V</td>
</tr>
<tr>
<td>Dissolved oxygen</td>
<td>Muti-parameter meter, HQ40d meter with IntelliCAL optical probe</td>
<td>V</td>
</tr>
</tbody>
</table>

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.

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

*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):

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

(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):

Net energy gain: \( NE_G = E_G - E_L \)  

(8)

Energy loss: \( E_L = C_W \cdot (T_F - T_I) \cdot \rho_W \)  

(9)

Energy gain: \( E_G = Y_H \cdot MV_H \cdot C_X \cdot \rho_H \cdot LHV_H \)  

(10)

where \( Y_H \) is the H₂ yield, \( MV_H \) is the molar volume of H₂, \( C_X \) is the influent xylose concentration, \( \rho_H \) 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 \( \rho_W \) is the density of water.

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

\[ COD_{tot} = 8 \cdot (4x + y - 2z)/(12x + y + 16z) g \] CODtot g⁻¹ CₓHᵧOᵦ  

(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:

\[ C₅H₁₀O₅ + 5 H₂O \rightarrow 5 CO₂ + 20 H^+ + 20 e^- \]  

(12)

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

\[ I = U / (R \cdot V) \]  

(13)
\[ P = \frac{U^2}{R \cdot V} \] (14)

where \( U \) is the voltage recorded in the data logger, \( R \) is the external resistance, and \( V \) is the anolyte volume.

The IBM SPSS Statistics package was used to perform the statistical analyses to assess significant differences in the \( \text{H}_2 \) 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

$\text{H}_2$ 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 $\text{H}_2$ 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 $\text{H}_2$ yield obtained with the digested activated sludge was likely due to the presence of microorganisms competing with $\text{H}_2$ 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).

$\text{H}_2$ 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 $\text{H}_2$ 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 $\text{H}_2$ production from xylose at 70 °C, but at a higher initial pH (6.5-7.5) and using an inoculum previously adapted for $\text{H}_2$ 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 $\text{H}_2$ 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.

<table>
<thead>
<tr>
<th>Inoculum</th>
<th>Pretreatment</th>
<th>T (°C)</th>
<th>Initial pH</th>
<th>Operation mode</th>
<th>H₂ yield (mol H₂ mol⁻¹ xylose added)</th>
<th>Xylose removal efficiency (%)</th>
<th>Final pH</th>
<th>Main metabolites (mM)a</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Digested activated sludge</td>
<td>Heat shock</td>
<td>37</td>
<td>5.5</td>
<td>Fed-batch (I)</td>
<td>0.9 (± 0.1)</td>
<td>98 (± 0)</td>
<td>4.7 (± 0.0)</td>
<td>Butyrate (30), Acetate (22)</td>
<td>Paper I</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(II)</td>
<td>0.5 (± 0.0)</td>
<td>98 (± 0)</td>
<td>4.2 (± 0.1)</td>
<td>Acetate (18), Butyrate (16)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(III)</td>
<td>1.0 (± 0.2)</td>
<td>69 (± 7)</td>
<td>3.5 (± 0.1)</td>
<td>Butyrate (25), Acetate (16)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(IV)</td>
<td>0.1 (± 0.2)</td>
<td>20 (± 5)</td>
<td>3.6 (± 0.5)</td>
<td>Butyrate (5), Acetate (4)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>55</td>
<td></td>
<td></td>
<td>Fed-batch (I)</td>
<td>0.2 (± 0.0)</td>
<td>93 (± 1)</td>
<td>4.1 (± 0.1)</td>
<td>Ethanol (20), Acetate (8), Butyrate (4)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(II)</td>
<td></td>
<td></td>
<td>(II)</td>
<td>0.8 (± 0.2)</td>
<td>72 (± 5)</td>
<td>4.0 (± 0.2)</td>
<td>Butyrate (17), Acetate (8), Ethanol (4)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(III)</td>
<td></td>
<td></td>
<td>(III)</td>
<td>0.6 (± 0.1)</td>
<td>51 (± 15)</td>
<td>3.5 (± 0.1)</td>
<td>Butyrate (14), Acetate (12)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(IV)</td>
<td></td>
<td></td>
<td>(IV)</td>
<td>0.4 (± 0.0)</td>
<td>28 (± 5)</td>
<td>3.5 (± 0.1)</td>
<td>Acetate (8), Butyrate (8)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>70</td>
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<td>Fed-batch (I)</td>
<td>0.2 (± 0.1)</td>
<td>71 (± 4)</td>
<td>4.5 (± 0.0)</td>
<td>Ethanol (12), Acetate (11)</td>
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<td>(II)</td>
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<td></td>
<td>(II)</td>
<td>0.2 (± 0.1)</td>
<td>59 (± 16)</td>
<td>4.2 (± 0.0)</td>
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<tr>
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<td>(III)</td>
<td></td>
<td></td>
<td>(III)</td>
<td>0.1 (± 0.0)</td>
<td>15 (± 5)</td>
<td>4.1 (± 0.1)</td>
<td>Acetate (9), Ethanol (8)</td>
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</tr>
<tr>
<td></td>
<td>(IV)</td>
<td></td>
<td></td>
<td>(IV)</td>
<td>0.1 (± 0.0)</td>
<td>12 (± 2)</td>
<td>4.1 (± 0.1)</td>
<td>Acetate (5)</td>
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<tr>
<td>Fresh activated sludge</td>
<td>37</td>
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<td>Fed-batch (I)</td>
<td>0.3 (± 0.0)</td>
<td>98 (± 0)</td>
<td>4.9 (± 0.0)</td>
<td>Butyrate (30), Acetate (21)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(II)</td>
<td></td>
<td></td>
<td>(II)</td>
<td>0.6 (± 0.0)</td>
<td>99 (± 0)</td>
<td>4.5 (± 0.1)</td>
<td>Butyrate (19), Acetate (17)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(III)</td>
<td></td>
<td></td>
<td>(III)</td>
<td>1.2 (± 0.1)</td>
<td>97 (± 2)</td>
<td>4.1 (± 0.1)</td>
<td>Butyrate (28), Acetate (19)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(IV)</td>
<td></td>
<td></td>
<td>(IV)</td>
<td>1.2 (± 0.1)</td>
<td>99 (± 0)</td>
<td>3.6 (± 0.1)</td>
<td>Butyrate (31), Acetate (17)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>55</td>
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<td></td>
<td>Fed-batch (I)</td>
<td>0.6 (± 0.1)</td>
<td>98 (± 0)</td>
<td>5.3 (± 0.1)</td>
<td>Ethanol (37), Butyrate (15), Acetate (9)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(II)</td>
<td></td>
<td></td>
<td>(II)</td>
<td>0.9 (± 0.1)</td>
<td>98 (± 0)</td>
<td>4.3 (± 0.0)</td>
<td>Acetate (24), Butyrate (23), Ethanol (13), Propionate (3)</td>
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<tr>
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<td></td>
<td>(III)</td>
<td>1.3 (± 0.1)</td>
<td>81 (± 14)</td>
<td>4.0 (± 0.0)</td>
<td>Butyrate (29), Acetate (19)</td>
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<tr>
<td></td>
<td>(IV)</td>
<td></td>
<td></td>
<td>(IV)</td>
<td>1.1 (± 0.1)</td>
<td>67 (± 9)</td>
<td>3.6 (± 0.0)</td>
<td>Butyrate (23), Acetate (18)</td>
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</tr>
</tbody>
</table>
Table 7.1: Continued.

<table>
<thead>
<tr>
<th>Inoculum</th>
<th>Pretreatment</th>
<th>T (°C)</th>
<th>Initial pH</th>
<th>Operation mode</th>
<th>H₂ yield (mol H₂ mol⁻¹ xylose added)</th>
<th>Xylose removal efficiency (%)</th>
<th>Final pH</th>
<th>Main metabolites (mM)³</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh activated sludge</td>
<td>Heat shock</td>
<td>70</td>
<td>5.5</td>
<td>Fed-batch (I)</td>
<td>0.2 (± 0.0)</td>
<td>85 (± 5)</td>
<td>4.9 (± 0.2)</td>
<td>Ethanol (31), Acetate (10)</td>
<td>Paper I</td>
</tr>
<tr>
<td></td>
<td>(II)</td>
<td></td>
<td></td>
<td></td>
<td>0.1 (± 0.0)</td>
<td>48 (± 4)</td>
<td>4.3 (± 0.1)</td>
<td>Ethanol (15), Acetate (10)</td>
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<tr>
<td></td>
<td>(III)</td>
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<td>0.1 (± 0.0)</td>
<td>14 (± 6)</td>
<td>4.2 (± 0.0)</td>
<td>Ethanol (10), Acetate (8)</td>
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<tr>
<td></td>
<td>(IV)</td>
<td></td>
<td></td>
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<td>0.1 (± 0.0)</td>
<td>21 (± 11)</td>
<td>3.9 (± 0.0)</td>
<td>Acetate (6), Ethanol (6), Lactate (3)</td>
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<tr>
<td>Untreated</td>
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<td>37</td>
<td>7.0</td>
<td>Batch</td>
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<td>4.0 (± 0.0)</td>
<td>Acetate (15), Lactate (10), Propionate (8), Ethanol (7), Butyrate (3)</td>
<td>Paper II</td>
</tr>
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<td>Acidic shock</td>
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<td>0.8 (± 0.0)</td>
<td>92 (± 2)</td>
<td>4.4 (± 0.1)</td>
<td>Butyrate (15), Acetate (14), Ethanol (13), Propionate (3)</td>
<td></td>
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<tr>
<td>Alkaline shock</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.5 (± 0.1)</td>
<td>94 (± 1)</td>
<td>4.0 (± 0.0)</td>
<td>Acetate (18), Lactate (10), Ethanol (8), Butyrate (6)</td>
<td></td>
</tr>
<tr>
<td>Heat shock</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.6 (± 0.1)</td>
<td>94 (± 1)</td>
<td>4.3 (± 0.1)</td>
<td>Ethanol (16), Acetate (13), Butyrate (9), Lactate (3)</td>
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<td>Freezing and thawing</td>
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<td>0.2 (± 0.0)</td>
<td>96 (± 1)</td>
<td>4.4 (± 0.0)</td>
<td>Ethanol (13), Acetate (10), Propionate (8), Butyrate (5)</td>
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<tr>
<td>Untreated</td>
<td></td>
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<td>0.5 (± 0.1)</td>
<td>90 (± 1)</td>
<td>4.0 (± 0.1)</td>
<td>Acetate (15), Lactate (14), Butyrate (6)</td>
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<tr>
<td>Acidic shock</td>
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<td>0.3 (± 0.0)</td>
<td>75 (± 5)</td>
<td>4.3 (± 0.0)</td>
<td>Lactate (8), Butyrate (4), Ethanol (3)</td>
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<tr>
<td>Alkaline shock</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1.2 (± 0.1)</td>
<td>96 (± 0)</td>
<td>4.5 (± 0.0)</td>
<td>Butyrate (13), Acetate (6)</td>
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</tr>
<tr>
<td>Heat shock</td>
<td></td>
<td></td>
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<td>73 (± 3)</td>
<td>4.2 (± 0.0)</td>
<td>Lactate (8)</td>
<td></td>
</tr>
<tr>
<td>Freezing and thawing</td>
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<td></td>
<td>0.6 (± 0.1)</td>
<td>96 (± 1)</td>
<td>4.2 (± 0.1)</td>
<td>Acetate (7), Butyrate (5), Ethanol (5), Lactate (5)</td>
<td></td>
</tr>
<tr>
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<td>Acetate (7), Lactate (6), Ethanol (3)</td>
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<td>54 (± 20)</td>
<td>6.0 (± 0.0)</td>
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</tr>
<tr>
<td>Inoculum</td>
<td>Pretreatment</td>
<td>T (°C)</td>
<td>Initial pH</td>
<td>Operation mode</td>
<td>H₂ yield (mol H₂ mol⁻¹ xylose added)</td>
<td>Xylose removal efficiency (%)</td>
<td>Final pH</td>
<td>Main metabolites (mM)ᵃ</td>
<td>Reference</td>
</tr>
<tr>
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<td>----------------------</td>
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<td>------------</td>
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<td>-------------------------------------</td>
<td>------------------------------</td>
<td>----------</td>
<td>-----------------------</td>
<td>-----------------------------</td>
</tr>
<tr>
<td>Fresh activated sludge</td>
<td>Alkaline shock</td>
<td>70</td>
<td>7.0</td>
<td>Batch</td>
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<td>19 (± 11)</td>
<td>6.1 (± 0.1)</td>
<td>Acetate (3)</td>
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<td></td>
<td>Heat shock</td>
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<td>29 (± 7)</td>
<td>5.3 (± 0.6)</td>
<td>Acetate (10), Lactate (7), Ethanol (6)</td>
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</tr>
<tr>
<td></td>
<td>Freezing and thawing</td>
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<td></td>
<td></td>
<td>0.1 (± 0.0)</td>
<td>33 (± 17)</td>
<td>5.9 (± 0.2)</td>
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</tr>
<tr>
<td>Heat shock</td>
<td>37ᵇ</td>
<td>5.0</td>
<td>Continuous (FBR)</td>
<td>0.5 (± 0.0)</td>
<td>100 (± 0)</td>
<td>5.0ᵇ</td>
<td>Acetate (32), Butyrate (13)</td>
<td>Paper III</td>
<td></td>
</tr>
<tr>
<td></td>
<td>55ᵇ</td>
<td></td>
<td>Continuous (FBR)</td>
<td>1.0 (± 0.0)</td>
<td>100 (± 0)</td>
<td>5.0ᵇ</td>
<td>Butyrate (22), Acetate (19)</td>
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<tr>
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<td>55ᶜ</td>
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<td>1.2 (± 0.0)</td>
<td>99 (± 2)</td>
<td>5.0ᶜ</td>
<td>Butyrate (25), Acetate (24)</td>
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<td>60ᶜ</td>
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<td>100 (± 0)</td>
<td>5.0ᶜ</td>
<td>Acetate (42), Butyrate (29)</td>
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<tr>
<td></td>
<td>65ᶜ</td>
<td></td>
<td>Continuous (FBR)</td>
<td>0.8 (± 0.1)</td>
<td>100 (± 0)</td>
<td>5.0ᶜ</td>
<td>Acetate (34), Butyrate (33)</td>
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<tr>
<td></td>
<td>70ᶜ</td>
<td></td>
<td>Continuous (FBR)</td>
<td>1.2 (± 0.0)</td>
<td>99 (± 0)</td>
<td>5.0ᶜ</td>
<td>Acetate (31), Butyrate (22)</td>
<td></td>
<td></td>
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</tbody>
</table>

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 H\(_2\) yield of 0.8 mol H\(_2\) mol\(^{-1}\) xylose\(_{\text{added}}\) (Table 7.1). Chang et al. (2011) also reported a higher H\(_2\) 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 H\(_2\) (Ren et al. 2008b).

At 55 °C, the alkaline shock was the most effective pretreatment, resulting in a H\(_2\) yield of 1.2 mol H\(_2\) mol\(^{-1}\) xylose\(_{\text{added}}\), 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 H\(_2\) 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 H\(_2\) 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\(_{\text{COD}}\) L\(^{-1}\) sucrose for two days), as the most effective pretreatment, whereas Luo et al. (2010) obtained higher H\(_2\) yields using the untreated rather than the pretreated inoculum.

At 70 °C, H\(_2\) 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 H\(_2\) yield compared to other pretreatments, but competitive pathways such as lactate and ethanol production limited the H\(_2\) 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 H\(_2\) 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 H\(_2\) 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: \( \text{H}_2 \) 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 \( \text{H}_2 \) production from thermomechanical pulping (TMP) wastewater at 37-80 °C in a batch experiment (Paper IV). \( \text{H}_2 \) was successfully produced from TMP wastewater in the temperature range 37-70 °C (Paper IV), with a maximum \( \text{H}_2 \) yield of 3.6 mmol \( \text{H}_2 \) g\(^{-1}\) COD\(_{\text{added}}\) obtained at 70 °C (Table 7.2). This yield is about two times lower than the maximum yields of 1.2-1.3 mol \( \text{H}_2 \) mol\(^{-1}\) xylose\(_{\text{added}}\) (7.5-8.1 mmol \( \text{H}_2 \) g\(^{-1}\) COD\(_{\text{added}}\)) obtained in batch and continuous dark fermentation of xylose (Table 7.1), which is, nevertheless, a more easily degradable substrate than TMP wastewater. The \( \text{H}_2 \) 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 \( \text{H}_2 \) 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 H\textsubscript{2} yields (after 111 hours of incubation). H\textsubscript{2} 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 H\textsubscript{2} producing microbial community dominated by \textit{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).

\textbf{Table 7.2:} H\textsubscript{2} 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.

<table>
<thead>
<tr>
<th>T (°C)</th>
<th>H\textsubscript{2} yield (mmol H\textsubscript{2} g\textsuperscript{-1} COD)</th>
<th>COD removal (%)</th>
<th>Final pH\textsuperscript{a}</th>
<th>Main metabolites (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>37</td>
<td>1.4 (± 0.1)</td>
<td>73 (± 0)</td>
<td>5.5 (± 0.1)</td>
<td>Acetate (4), Butyrate (1), Ethanol (1)</td>
</tr>
<tr>
<td>42\textsuperscript{b}</td>
<td>0.6</td>
<td>80 (± 8)</td>
<td>5.7 (± 0.1)</td>
<td>Acetate (5), Butyrate (1), Ethanol (1)</td>
</tr>
<tr>
<td>48</td>
<td>0.1 (± 0.0)</td>
<td>76 (± 1)</td>
<td>6.0 (± 0.0)</td>
<td>Acetate (7), Butyrate (1)</td>
</tr>
<tr>
<td>55</td>
<td>0.0 (± 0.0)</td>
<td>71 (± 5)</td>
<td>6.0 (± 0.1)</td>
<td>Acetate (9), Butyrate (1)</td>
</tr>
<tr>
<td>59</td>
<td>0.6 (± 0.3)</td>
<td>71 (± 1)</td>
<td>5.9 (± 0.0)</td>
<td>Acetate (8), Butyrate (1)</td>
</tr>
<tr>
<td>65</td>
<td>1.8 (± 0.2)</td>
<td>72 (± 1)</td>
<td>5.2 (± 0.1)</td>
<td>Acetate (5), Butyrate (1)</td>
</tr>
<tr>
<td>70</td>
<td>3.6 (± 0.1)</td>
<td>74 (± 4)</td>
<td>5.3 (± 0.0)</td>
<td>Acetate (4)</td>
</tr>
<tr>
<td>74</td>
<td>0.1 (± 0.0)</td>
<td>70 (± 2)</td>
<td>6.1 (± 0.0)</td>
<td>Acetate (1), Butyrate (1)</td>
</tr>
<tr>
<td>80</td>
<td>0.0 (± 0.0)</td>
<td>79 (± 2)</td>
<td>6.0 (± 0.0)</td>
<td>Acetate (1), Butyrate (1)</td>
</tr>
</tbody>
</table>

\textsuperscript{a} The initial pH of all batch cultures was 6.3. \textsuperscript{b} H\textsubscript{2} was produced only in one of the duplicate batch cultures.

7.1.5 \textbf{Microbial community composition}

The family of \textit{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 \textit{Clostridiaceae} includes many H\textsubscript{2} producing species widely reported to dominate mesophilic dark fermentative bioreactors (Liu et al. 2009; Si et al. 2015; Sivagurunathan et al. 2016b). \textit{Clostridium} sp. dominated the microbial communities also after four consecutive batch incubations with xylose conducted to enrich H\textsubscript{2} producers from heat-treated activated sludge (Paper I) and during the FBR operation (Paper III). \textit{Clostridium} sp. was the prevalent active microorganism also in the mesophilic batch cultures with TMP wastewater (Paper IV).

At 37 °C, the H\textsubscript{2} yield was often lowered by the proliferation of lactate producers such as \textit{Sporolactobacillus} sp. (Paper I) and \textit{Lactobacillus} sp. (Paper II), which produce lactate
at the expenses of $H_2$, and may also inhibit *Clostridium* sp. growth by excreting toxins (Noike et al. 2002). An acidic shock was the most effective pretreatment to enrich $H_2$ 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 $H_2$ 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 $H_2$ producing microorganisms (Paper II). The family of *Clostridiaceae* includes several $H_2$ 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 $H_2$ 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, $H_2$ 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 H$_2$ producing pathways, resulting in the highest H$_2$ 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). H$_2$ 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 H$_2$ yields (Table 7.1).

H$_2$ 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 H$_2$ 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 H$_2$ 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 H$_2$ 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 H$_2$ production in a glucose-fed FBR to the adhesion of homoacetogenic microorganisms to the carrier material. The more stable H$_2$ 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 H$_2$ production through the acetate pathway, or H$_2$ 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 \( \text{H}_2 \) yield may be underestimated. \( \text{H}_2 \) 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 \( \text{H}_2 \) 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 \( \text{H}_2 \) 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.

<table>
<thead>
<tr>
<th>Inoculum</th>
<th>MFC design and membrane</th>
<th>Cathode</th>
<th>Operation mode</th>
<th>T (°C)</th>
<th>Substrate</th>
<th>Power (W m⁻³)</th>
<th>CE (%)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Activated sludge + MFC effluent</td>
<td>h-type, AEM</td>
<td>Air</td>
<td>Fed-batch</td>
<td>37</td>
<td>Xylose</td>
<td>1.0</td>
<td>12</td>
<td>Paper V</td>
</tr>
<tr>
<td>MFC effluent</td>
<td>Upflow, AEM</td>
<td>Ferricyanide</td>
<td>Continuous</td>
<td>37</td>
<td>TMP wastewater</td>
<td>0.07</td>
<td>2</td>
<td>Unpublished</td>
</tr>
<tr>
<td></td>
<td>Upflow, CEM</td>
<td></td>
<td>Continuous</td>
<td>37</td>
<td></td>
<td>0.2</td>
<td>3</td>
<td>Unpublished</td>
</tr>
<tr>
<td>Activated sludge + MFC effluent</td>
<td>h-type, AEM</td>
<td>Air</td>
<td>Fed-batch</td>
<td>55</td>
<td>Xylose</td>
<td>0.03</td>
<td>3</td>
<td>Paper V</td>
</tr>
<tr>
<td>Compost and digestate</td>
<td>Upflow, AEM</td>
<td>Ferricyanide</td>
<td>Fed-batch</td>
<td>55</td>
<td>Acetate</td>
<td>0.2</td>
<td>15</td>
<td>Unpublished</td>
</tr>
<tr>
<td>Effluent from thermophilic upflow MFC</td>
<td>Cuboidal, AEM</td>
<td>Ferricyanide</td>
<td>Fed-batch</td>
<td>55</td>
<td>Acetate</td>
<td>0.8</td>
<td>9.6</td>
<td>Unpublished</td>
</tr>
</tbody>
</table>
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 *Thermiscola 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 anolyte 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 $\Omega$ 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 CO$_2$ and H$_2$O 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 Thermodesulfobacteria, 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 Methanothermobacteraeae (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). Methanobacteriaceae 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 H$_2$ 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 H\textsubscript{2} yield than heat treatment for dark fermentation of xylose at 37 and 55 °C, respectively (Paper II). However, at both 37 and 55 °C, H\textsubscript{2} 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 H\textsubscript{2} production.

Hyperthermophilic (70 °C) H\textsubscript{2} 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 H\textsubscript{2} mol\textsuperscript{-1} xylose\textsubscript{added} was obtained, resulting in a H\textsubscript{2} production rate of 282.1 mL H\textsubscript{2} h\textsuperscript{-1} L\textsuperscript{-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 H\textsubscript{2} at lower temperatures, resulting in low H\textsubscript{2} 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, Thermanaerobacterium 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 Thermonaerobacterium 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 xyloseadded) and from thermomechanical pulping (TMP) wastewater (3.6 mmol H₂ g⁻¹ CODadded) 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 H$_2$ overproduction, i.e. H$_2$ 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 H$_2$ 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.
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BIOHYDROGEN PRODUCTION FROM XYLOSE BY FRESH AND DIGESTED ACTIVATED SLUDGE AT 37, 55 AND 70 °C

by

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

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A B S T R A C T
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 (R² = 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. Thermotoga aeraerobacter sp., detected only in the fresh activated sludge cultures, may have contributed to the high H2 yield obtained with such an inoculum.

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...
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 biogas hydrogen 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 the possibility to reuse 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-decarboxylators, and even sulfate and nitrate reducing bacteria consume H₂ as a part of their metabolism (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 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 biogas hydrogen 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 biomass from H₂ producing reactor

<table>
<thead>
<tr>
<th>Inoculum</th>
<th>Pre-treatment</th>
<th>T (°C)</th>
<th>Initial pH</th>
<th>Initial xylose (mM)</th>
<th>H₂ yield (mol per mol xylose)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Activated sludge</td>
<td>Heat treatment</td>
<td>35</td>
<td>6.5</td>
<td>124.9</td>
<td>1.30</td>
<td>Lin et al. (2006)</td>
</tr>
<tr>
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<td>Heat treatment</td>
<td>35</td>
<td>5.5</td>
<td>66.6</td>
<td>1.88</td>
<td>De Sá et al. (2013)</td>
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<tr>
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<td>Heat treatment</td>
<td>35</td>
<td>6.5</td>
<td>124.9</td>
<td>2.25</td>
<td>Lin and Cheng (2006)</td>
</tr>
<tr>
<td>Clostridium butyricum</td>
<td>–</td>
<td>37</td>
<td>7.5</td>
<td>124.9</td>
<td>0.73</td>
<td>Lo et al. (2008)</td>
</tr>
<tr>
<td>Granulated sludge</td>
<td>Heat treatment</td>
<td>37</td>
<td>5.5</td>
<td>23.9</td>
<td>0.80</td>
<td>Maitinguer et al. (2011)</td>
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<tr>
<td>Digested activated sludge</td>
<td>–</td>
<td>37</td>
<td>6.7</td>
<td>33.1</td>
<td>2.64</td>
<td>Chaganti et al. (2012)</td>
</tr>
<tr>
<td>Clostridium beijerinckii</td>
<td>–</td>
<td>40</td>
<td>8</td>
<td>66.6</td>
<td>2.31</td>
<td>An et al. (2014)</td>
</tr>
<tr>
<td>Activated sludge</td>
<td>Heat treatment</td>
<td>40</td>
<td>7.1</td>
<td>124.9</td>
<td>1.30</td>
<td>Lin et al. (2008)</td>
</tr>
<tr>
<td>Mixed culture compost</td>
<td>–</td>
<td>55</td>
<td>5</td>
<td>13.3</td>
<td>1.70</td>
<td>Cali et al. (2008)</td>
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<td>Thermoaerobacter thermosaccharolyticum</td>
<td>–</td>
<td>60</td>
<td>6.7</td>
<td>33.3</td>
<td>2.07</td>
<td>Zhang et al. (2011)</td>
</tr>
<tr>
<td>Thermoaerobacter thermosaccharolyticum</td>
<td>–</td>
<td>60</td>
<td>6.5</td>
<td>66.6</td>
<td>2.09</td>
<td>Khanth and Reungsang (2012)</td>
</tr>
<tr>
<td>Thermoaerobacter thermosaccharolyticum</td>
<td>–</td>
<td>60</td>
<td>6.5</td>
<td>66.6</td>
<td>2.19</td>
<td>Ren et al. (2008)</td>
</tr>
<tr>
<td>Geothermal spring</td>
<td>–</td>
<td>60</td>
<td>7.9</td>
<td>66.6</td>
<td>1.65</td>
<td>Zeidan and Van Niel (2009)</td>
</tr>
<tr>
<td>Biomass from H₂ producing reactor</td>
<td>–</td>
<td>70</td>
<td>7.0–8.0</td>
<td>3.3</td>
<td>1.62</td>
<td>Kongjan et al. (2009)</td>
</tr>
<tr>
<td>Biomass from H₂ producing reactor</td>
<td>–</td>
<td>70</td>
<td>7.0</td>
<td>13.3</td>
<td>1.84</td>
<td>Zhao et al. (2010)</td>
</tr>
</tbody>
</table>

* 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 Porapak 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):

\[
\text{Cumulative Production} = \frac{V}{V_0} \times M \times n
\]

where \(V\) is the volume of gas produced, \(V_0\) is the volume of gas at standard temperature, \(M\) is the molecular weight of the gas, and \(n\) is the number of moles.
\[ V_{H_2} = V_{H_2-i} + C_{H_2}(V_{G_1} - V_{G_1-i}) + V(C_{H_2} - C_{H_2-i}) \] (1)

where \( V_C, V_H \) 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–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 chromatography (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\(_2\)SO\(_4\) 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. 1.a), 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. 1.b). 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. 1.a 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 digest sludge inoculum (Fig. 1.a 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. 1.c). 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. 1.c). 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. 1.d). 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. 1.f 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.](image-url)
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. (98% 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 (R² = 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.

<table>
<thead>
<tr>
<th>BM</th>
<th>Microorganism</th>
<th>Access number</th>
<th>Matching sequence length</th>
<th>Similarity (%)</th>
<th>Activated sludge</th>
<th>Digester sludge</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Clostridium sp.</td>
<td>FJ61757</td>
<td>477</td>
<td>99</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>B</td>
<td>Clostridium acetobutylicum</td>
<td>KP410577</td>
<td>457–515</td>
<td>99</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>C</td>
<td><em>Clostridium butyricum</em></td>
<td>CP013352</td>
<td>418–492</td>
<td>98–100</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>D</td>
<td>Clostridum sp.</td>
<td>KT072767</td>
<td>418–492</td>
<td>98–100</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>E</td>
<td><em>Thermoanaerobacter thermosaccharoliticum</em></td>
<td>KT274717</td>
<td>426</td>
<td>98</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>F</td>
<td>Caloramator australicus</td>
<td>HM228391</td>
<td>385–449</td>
<td>97–99</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>G</td>
<td><em>Thermoanaerobacter sp.</em></td>
<td>KR007668</td>
<td>452</td>
<td>100</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>H</td>
<td><em>Sporolactobacillus putidus</em></td>
<td>NR_112774</td>
<td>486</td>
<td>92</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>I</td>
<td>Clostridum sp.</td>
<td>ABS40378</td>
<td>433–451</td>
<td>91–98</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>J</td>
<td><em>Clostridium thermopalmarium</em></td>
<td>KM036191</td>
<td>428</td>
<td>98</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>K</td>
<td><em>Clostridium isotis</em></td>
<td>NR_026347</td>
<td>425</td>
<td>93</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>L</td>
<td><em>Caldanaerobius sp.</em></td>
<td>JK894966</td>
<td>429</td>
<td>99</td>
<td>−</td>
<td>−</td>
</tr>
</tbody>
</table>

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.
species which might have 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 both inocula. Excretion of the protons outside the cell 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 (Grupe 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 mesophlic 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.,
Cultures with the digester inoculum at 55 °C were obtained in this study during the fourth batch culture of activated sludge at 55 °C (Table 2) and associated with H2 production via the acetate and Clostridium thermopalmarium butyrate pathway. The linear regression between the H2 and butyrate yield at 55 °C in this study was previously enriched for H2 production at 70 °C (data not shown), probably due to a more diverse microbial community and thus, a wider variety of metabolic pathways.

4.4. 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). Thermoaerobacter butyrovorans thermosaccharolyticum, 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. thermosaccharolyticum 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 pretreated, 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 (Zéidan et al., 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. thermosaccharolyticum (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.

4.5. 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).

\[
\text{C}_5\text{H}_9\text{O}_5 \rightarrow 0.83 \text{CH}_3\text{CH}_2\text{CH}_2\text{COOH} + 1.67 \text{H}_2 + 1.67 \text{CO}_2 \tag{2}
\]

\[
\text{C}_5\text{H}_9\text{O}_5 + 1.67 \text{H}_2\text{O} \rightarrow 1.67 \text{CH}_3\text{COOH} + 3.33 \text{H}_2 + 1.67 \text{CO}_2 \tag{3}
\]

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. 5 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-Vázquez 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. 5 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.

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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

**Figure S1.** Evolution of H\(_2\) partial pressure with time, assuming equilibrium conditions between the liquid and gas phase. The two points represented for every sampling time refer to the H\(_2\) 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.

<table>
<thead>
<tr>
<th>Inoculum</th>
<th>T (°C)</th>
<th>Batch culture</th>
<th>Concentration (mM)</th>
<th>Acetate</th>
<th>Butyrate</th>
<th>Ethanol</th>
<th>Lactate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Activated sludge</td>
<td>37</td>
<td>I</td>
<td>21.4 (± 2.2)</td>
<td>30.2 (± 5.2)</td>
<td>1.6 (± 0.3)</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>II</td>
<td>16.8 (± 4.1)</td>
<td>18.6 (± 3.4)</td>
<td>2.0 (± 0.0)</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>III</td>
<td>19.5 (± 5.0)</td>
<td>28.0 (± 7.2)</td>
<td>2.1 (± 0.1)</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>IV</td>
<td>16.8 (± 2.1)</td>
<td>31.0 (± 4.9)</td>
<td>1.6 (± 0.0)</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>55</td>
<td>I</td>
<td>9.3 (± 4.7)</td>
<td>15.0 (± 4.0)</td>
<td>36.6 (± 8.1)</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>II</td>
<td>24.2 (± 1.8)</td>
<td>23.0 (± 1.6)</td>
<td>12.8 (± 1.4)</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>III</td>
<td>27.0 (± 3.4)</td>
<td>29.2 (± 1.9)</td>
<td>3.9 (± 0.3)</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>IV</td>
<td>17.6 (± 5.6)</td>
<td>22.5 (± 7.3)</td>
<td>1.3 (± 0.3)</td>
<td>-</td>
<td></td>
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<tr>
<td></td>
<td>70</td>
<td>I</td>
<td>9.7 (± 0.9)</td>
<td>-</td>
<td>30.8 (± 11.3)</td>
<td>15.8 (± 1.2)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>II</td>
<td>9.5 (± 1.4)</td>
<td>-</td>
<td>14.5 (± 4.7)</td>
<td>7.3 (± 0.8)</td>
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<tr>
<td></td>
<td></td>
<td>III</td>
<td>8.2 (± 0.4)</td>
<td>0.5 (± 0.2)</td>
<td>10.2 (± 1.3)</td>
<td>4.3 (± 0.4)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>IV</td>
<td>6.3 (± 0.1)</td>
<td>-</td>
<td>6.3 (± 1.2)</td>
<td>3.2 (± 0.0)</td>
<td></td>
</tr>
<tr>
<td>Digester sludge</td>
<td>37</td>
<td>I</td>
<td>21.6 (± 4.0)</td>
<td>29.5 (± 4.0)</td>
<td>2.2 (± 0.3)</td>
<td>-</td>
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INOCULUM PRETREATMENT DIFFERENTIALLY AFFECTS THE ACTIVE MICROBIAL COMMUNITY PERFORMING MESOPHILIC AND THERMOPHILIC DARK FERMENTATION OF XYLOSE

by

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

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A B S T R A C T
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 Thermoaerobacterium 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.

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I N T R O D U C T I O N
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 Poullikkas [2]), biological methods have the advantage of coupling H₂ generation with the treatment of organic carbon-containing wastes and wastewaters. Biological H₂ production
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 hydrolysate 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 regerminate 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, 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 x g, 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 chromatography (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 ΔG° values (Eqs. (1)–(4)) [37]:

Acetate fermentation (ΔG° = −250.8 kJ):

\[
\text{C}_5\text{H}_10\text{O}_5 + 1.67 \text{H}_2\text{O} \rightarrow 1.67 \text{CH}_3\text{COOH} + 3.33 \text{H}_2 + 1.67 \text{CO}_2 \tag{1}
\]

Butyrate fermentation (ΔG° = −307.9 kJ):

\[
\text{C}_6\text{H}_{12}\text{O}_6 \rightarrow 0.83 \text{CH}_3\text{CH}_2\text{CH}_2\text{COOH} + 1.67 \text{H}_2 + 1.67 \text{CO}_2 \tag{2}
\]

Propionate fermentation (ΔG° = −373.7 kJ):

\[
\text{C}_6\text{H}_{12}\text{O}_6 + 1.67 \text{H}_2 \rightarrow 1.67 \text{CH}_3\text{CH}_2\text{COOH} + 1.67 \text{H}_2\text{O} \tag{3}
\]

Homoacetogenesis (ΔG° = −75.5 kJ):

\[
4 \text{H}_2 + 2 \text{CO}_2 \rightarrow \text{CH}_3\text{CH}_2\text{COOH} + 2 \text{H}_2\text{O} \tag{4}
\]

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]:

\[
\text{HY}_\text{T} = 2\text{AY} + 2\text{BY} - \text{PY} \tag{5}
\]

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)):

\[
\text{D} = \text{HY}_\text{M} - \text{HY}_\text{T} \tag{6}
\]

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)):

\[
\text{D} = \text{HY}_\text{M} - (2\text{AY}_\text{H} + 2\text{BY} - \text{PY} - 4 \text{AY}_\text{A}) \tag{7}
\]

**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 ΔG° values [37], the most thermodynamically favourable pathway for biological ethanol production (Eq. (8), ΔG° = –286.7 kJ) does not involve directly H2, but it decreases the amount of substrate (e.g. xylose) available for H2 production:

$$C_6H_{12}O_6 \rightarrow 1.67 \text{CH}_3\text{CH}_2\text{OH} + 1.67 \text{CO}_2 \quad (8)$$

The low pH was likely associated to lactate production. In fact, the highest lactate concentration of 14 and 10 mM was
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), ΔG° = -56.2 kJ), but it decreases the substrate available for H₂ production:

\[ \text{C}_5\text{H}_10\text{O}_5 \rightarrow 1.67 \text{CH}_3\text{CHOHCOOH} \] (9)

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 aceticogenic 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,
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 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.

<table>
<thead>
<tr>
<th>Family</th>
<th>Genus and speciesb</th>
<th>Accession number</th>
<th>Matching sequencec</th>
<th>Similarity (%)d</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clostridiaceae</td>
<td>Clostridium sp.</td>
<td>HF566199</td>
<td>458–749</td>
<td>99</td>
</tr>
<tr>
<td>Lactobacillaceae</td>
<td>Lactobacillus mucosae</td>
<td>MF425117</td>
<td>490–781</td>
<td>99</td>
</tr>
<tr>
<td>Peptostreptococcaceae</td>
<td>Romboutsia ilealis</td>
<td>LNN55523</td>
<td>2,071,187–2,071,477</td>
<td>99</td>
</tr>
<tr>
<td>Thermoanaerobacterales Family III</td>
<td>Thermoanaerobacterium thermosacharolyticum</td>
<td>MF405082</td>
<td>414–705</td>
<td>99</td>
</tr>
<tr>
<td>Moraxellaceae</td>
<td>Acinetobacter sp.</td>
<td>KY818302</td>
<td>465–756</td>
<td>99</td>
</tr>
<tr>
<td>Comamonadaceae</td>
<td>Acidovorax sp.</td>
<td>LC279183</td>
<td>425–716</td>
<td>99</td>
</tr>
<tr>
<td>Veillonellaceae</td>
<td>Megamonas sp.</td>
<td>LT628480</td>
<td>522–812</td>
<td>99</td>
</tr>
<tr>
<td>Acidimicrobials Incertae Sedis</td>
<td>Candidatus Microthrix parvicella</td>
<td>KM052469</td>
<td>91–383</td>
<td>99</td>
</tr>
<tr>
<td>Rhodocyclaceae</td>
<td>Dechloromonas sp.</td>
<td>KY029047</td>
<td>486–777</td>
<td>99</td>
</tr>
<tr>
<td>Myxococcales unclassified</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Aeromonadaceae</td>
<td>Aeromonas sp.</td>
<td>MF461171</td>
<td>478–769</td>
<td>100</td>
</tr>
<tr>
<td>Chitinophagaceae</td>
<td>Terrimonas arctica</td>
<td>NR_134213</td>
<td>454–745</td>
<td>99</td>
</tr>
</tbody>
</table>

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 Azonexusaceae in the GenBank.
| g No match at family, genus and species level.
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.

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

* 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 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 digestor 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. 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.*
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 cultures receiving acidic shock, where the pH decrease was less pronounced compared to the untreated activated sludge.
cultures of alkaline shock-pretreated activated sludge, in which the measured yield of 1.3 mol H\textsubscript{2} mol\textsuperscript{-1} xylose\textsubscript{consumed} was 30% higher than the estimated yield of 0.8 mol H\textsubscript{2} mol\textsuperscript{-1} xylose\textsubscript{consumed} (Table 3). H\textsubscript{2} overproduction has been previously reported in thermophilic dark fermentation [56], and attributed to an unusual H\textsubscript{2} 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 H\textsubscript{2} 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% H\textsubscript{2} overproduction from glucose by anaerobic sludge and excluded the acetate oxidation pathway by use of an isotope feeding assay with \textsuperscript{13}C-labeled acetate. Zheng et al. [57] concluded that an unknown H\textsubscript{2} 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 H\textsubscript{2} 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 H\textsubscript{2} 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 Clostriaceae 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 Thermobacillaceae (Fig. 1), closely related to T. thermosaccharolyticum (Fig. 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 Clostriaceae includes thermophilic species, such as C. thermosaccharolyticum [60] and C. thermopalmarium [61], able to convert sugars to H\textsubscript{2}, acetate and butyrate. T. thermosaccharolyticum effectively produces H\textsubscript{2} 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 H\textsubscript{2} 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 H\textsubscript{2} production even for the long-term operation of continuous dark fermentative
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, Dessi 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.

### Table 3

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>T (°C)</th>
<th>Volatile fatty acid yield (mol H₂ mol⁻¹ xylose consumed)</th>
<th>H₂ yield (mol H₂ mol⁻¹ xylose consumed)</th>
<th>Measured H₂ yield</th>
<th>Estimated H₂ yield</th>
<th>Discrepancy</th>
<th>Estimated contribution of different pathways on total acetate production</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Acetate</td>
<td>Butyrate</td>
<td>Propionate</td>
<td>Acetate fermentation</td>
<td>Homoacetogenesis</td>
<td>Linked to acetate</td>
</tr>
<tr>
<td></td>
<td>37</td>
<td>0.32</td>
<td>0.31</td>
<td>0.14</td>
<td>0.07</td>
<td>0.16</td>
<td>0.14</td>
</tr>
<tr>
<td></td>
<td>55</td>
<td>0.22</td>
<td>0.12</td>
<td>0.01</td>
<td>0.05</td>
<td>0.06</td>
<td>0.05</td>
</tr>
</tbody>
</table>

### 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

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### 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.jhydene.2018.03.117.

REFERENCES


**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**

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

**ANOVA**

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<tr>
<th>Sum of Squares</th>
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<th>Mean Square</th>
<th>F</th>
<th>Sig.</th>
</tr>
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<tr>
<td>Between Groups</td>
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<td>0.275</td>
<td>92.195</td>
</tr>
<tr>
<td>Within Groups</td>
<td>0.030</td>
<td>10</td>
<td>0.003</td>
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<td>Total</td>
<td>1.131</td>
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</table>

**Multiple Comparisons**

Dependent Variable: H$_2$ yield 37 °C

Tukey HSD

<table>
<thead>
<tr>
<th>(I) Pretreat</th>
<th>(J) Pretreat</th>
<th>Mean Difference (I-J)</th>
<th>Std. Error</th>
<th>p-value</th>
<th>95% Confidence Interval (p=0.05)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>Acidic shock</td>
<td>-0.73000*</td>
<td>0.04462</td>
<td>0.000</td>
<td>-0.8769 - -0.5831</td>
</tr>
<tr>
<td></td>
<td>Alkaline shock</td>
<td>-0.42333*</td>
<td>0.04462</td>
<td>0.000</td>
<td>-0.5702 - -0.2765</td>
</tr>
<tr>
<td></td>
<td>Heat shock</td>
<td>-0.48000*</td>
<td>0.04462</td>
<td>0.000</td>
<td>-0.6269 - -0.3331</td>
</tr>
<tr>
<td></td>
<td>Freezing and thawing</td>
<td>-0.07000</td>
<td>0.04462</td>
<td>0.546</td>
<td>-0.2169 - 0.0769</td>
</tr>
<tr>
<td>Pretreat</td>
<td>N</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>--------------------------</td>
<td>----</td>
<td>----------</td>
<td>----------</td>
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<tr>
<td>Untreated</td>
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<td>0.1100</td>
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</tr>
<tr>
<td>Freezing and thawing</td>
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<td>0.1800</td>
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<td></td>
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<tr>
<td>Alkaline shock</td>
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<tr>
<td>Heat shock</td>
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<td>Acidic shock</td>
<td>3</td>
<td>0.546</td>
<td>0.714</td>
<td>1.000</td>
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</table>

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

b. Threshold value.

**Acidic shock > Heat shock ~ Alkaline shock > Freezing and thawing ~ Untreated**
**Batch cultures 55 °C**

### Descriptives

<table>
<thead>
<tr>
<th></th>
<th>N</th>
<th>Mean</th>
<th>Std. Deviation</th>
<th>Std. Error</th>
<th>95% Confidence Interval for Mean (p=0.05)</th>
<th>Minimum</th>
<th>Maximum</th>
</tr>
</thead>
<tbody>
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<td>0.07371</td>
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<td>0.3136 - 0.6798</td>
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<td>0.58</td>
</tr>
<tr>
<td>Acidic shock</td>
<td>3</td>
<td>0.2767</td>
<td>0.02309</td>
<td>0.01333</td>
<td>0.2193 - 0.3340</td>
<td>0.25</td>
<td>0.29</td>
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### ANOVA

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### Multiple Comparisons

**Dependent Variable:** H₂ yield 55 °C

**Tukey HSD**

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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

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

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A B S T R A C T

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.

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I N T R O D U C T I O N

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,
and the substrate used. Glycolysis is the most common route for degradation of hexoses by Clostridium [5] and most thermophiles, including Thermanaerobacter [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 Thermanaerobacterium 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 Thermanaerobacterium 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 Thermanaerobacter 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 75 °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). An additional sample of carrier material was collected from the thermophilic FBR on day 119 (60 °C), 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), 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 N2 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 H2, CH4, and CO2 with a Shimadzu gas chromatograph GC-2014 operated at 37 (±1) °C, followed by incubation at 37 °C for 30 min. DNase was then inactivated by addition of 2.5 μL DNase inactivator (Invitrogen). After centrifugation (10000 × 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, chimera 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 NEg (kJ L−1) (Equation (1)) was estimated by the difference between the energy EG recovered by combustion of the H2 produced per L of wastewater treated (Equation (2)) and the energy ET required to heat the FBR to the desired temperature (Equation (3)) [41]:

Net energy gain: NEg = EG − EL

Energy gain: EG = (YH_MVH · CX · pH · LHVH)

Energy loss: EL = Cw · (TF − Ti) · ρw

where YH is the H2 yield (mol H2 mol−1 xylose), MVH is the molar volume of H2 (22.414 L mol−1), CX is the influent xylose concentration (50 mol L−1), ρH is the density of gaseous H2 (8.9 × 10−5 kg L−1), LHVH is the lower heating value of H2 (120 × 103 kJ kg−1), Cw is the specific heat of water (4.2 kJ kg−1 K−1), TF and Ti (K) is the temperature of the wastewater after and before heating, respectively, and ρw is the density of water (1 kg L−1).

Statistical analysis

To assess significant differences in H2 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 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](image-url)  
**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.
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 thermonerolitycum (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 Thermoclostridium 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 Ruminoclostridium 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 Thermoclostridium genus (>90% of the relative abundance).

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 thermonerolitycum (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).

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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.
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

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<td><em>Clostridium acetobutylicum</em></td>
<td>KP410577</td>
<td>446–472</td>
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</tr>
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<td></td>
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<td>96–97</td>
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<tr>
<td>C</td>
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<td>98–99</td>
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<tr>
<td>D</td>
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<td>EU887966</td>
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Table 1 – Association of 16S rRNA gene sequences of DGGE bands to those collected in the GenBank.

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 xyllose at various temperatures, obtaining a maximum yield of about 2.2 mol H2 mol⁻¹ xyllose at 60 °C [59,60]. However, in this study, the highest H2 yield was only about 1.2 mol H2 mol⁻¹ xyllose 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 ºC, *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. thermoacetica*, may oxidize sugars through competitive pathways to H2 production or can even carry out homoacetogenesis [61]. The role of *Clostridium* in the xylene degradation at 65 °C could be further detailed by a proteomics analysis. At 65 °C, a bacterium closely related to *Alicyclacabacillus* sp. appeared in the DGGE profile (Fig. 3). Genera with high similarity (99%) to *Alicyclacabacillus* 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 ºC, as well as the decreased xyllose 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 *Thermoaerobacter tengcongensis*, cultured at 55, 75 and 80 ºC, and showed the existence of temperature-dependent protein complexes, which may affect the H2 yield. The stable H2 yield obtained at 70 ºC 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 xyllose was obtained at both 55 and 70 °C, whereas the H2 yield was significantly lower (0.8 mol H2 mol⁻¹ xyllose) 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 *Clostridiales* 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).

<table>
<thead>
<tr>
<th>Inoculum</th>
<th>Pre-treatment</th>
<th>Reactor type</th>
<th>Substrate (g COD L⁻¹)</th>
<th>T (°C)</th>
<th>pHᵇ</th>
<th>HRT (h)</th>
<th>HPR (mL H₂ h⁻¹ L⁻¹)</th>
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<td>–</td>
<td>UASB</td>
<td>Sucrose (11.2)</td>
<td>55</td>
<td>4.5–5.0 (nc)</td>
<td>3</td>
<td>112.5</td>
<td>[33]</td>
</tr>
<tr>
<td>Sludge from anaerobic digester immobilised on ceramic rings</td>
<td>Heat treatment (105 °C, 5 min)</td>
<td>UASB</td>
<td>Sucrose (11.2)</td>
<td>55</td>
<td>5.0–5.5 (nc)</td>
<td>1.5</td>
<td>124.2</td>
<td>[24]</td>
</tr>
<tr>
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<td>Heat treatment (80 °C, 60 min)</td>
<td>FBR</td>
<td>Sucrose (5.0)</td>
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<td>5.5 (i)</td>
<td>12</td>
<td>60.5</td>
<td>[34]</td>
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<tr>
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<td>Heat treatment (121 °C, 30 min)</td>
<td>TBR</td>
<td>Glucose (7.3)</td>
<td>60</td>
<td>5.5 (c)</td>
<td>2</td>
<td>980.6</td>
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<tr>
<td>Thermoanaerobacterium thermosaccharolyticum immobilised on methanogenic granules</td>
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<td>UASB</td>
<td>Sucrose (22.5)</td>
<td>60</td>
<td>5.0–5.5 (nc)</td>
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<td>CSTR</td>
<td>Xylose (1.1)</td>
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<td>6.7 (nc)</td>
<td>72</td>
<td>2.6</td>
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<tr>
<td>Biomass from H₂ producing CSTR</td>
<td>–</td>
<td>UASB</td>
<td>Glucose (2.1)</td>
<td>70</td>
<td>7.0 (i)</td>
<td>24</td>
<td>12.7</td>
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</tr>
<tr>
<td>Methanogenic biomass from CSTR</td>
<td>Chemical treatment (BES)</td>
<td>UASB</td>
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<td>7.2 (i)</td>
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<td>47.3</td>
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<tr>
<td>Biomass from H₂ producing CSTR</td>
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<td>6.5 (c)</td>
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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 or alcohols or for further energy harvesting (e.g., H₂ production through photofermentation 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**

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**Acknowledgements**

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**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**


File S1. Statistical analysis (ANOVA and Tukey test) conducted at p = 0.05 to assess significant differences in H₂ yield at the various temperatures investigated in the mesophilic (37 °C) and thermophilic (55 °C) FBR.

### Descriptives

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<th>Std. Error</th>
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### ANOVA

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### Multiple Comparisons

**Dependent Variable: Yield**

**Multiple Comparisons**

Tukey HSD

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* The mean difference is significant at the 0.05 level.

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

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Means H$_2$ yield for groups in homogeneous subsets are displayed.

* Uses Harmonic Mean Sample Size = 4.000.

** M = mesophilic FBR. T = thermophilic FBR.
File S2. CO\textsubscript{2} yield (mol CO\textsubscript{2} mol\textsuperscript{-1} xylose added) in the thermophilic (a) and mesophilic (b) FBR.

The vertical dotted lines indicate a change of temperature.
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
Temperature control as key factor for optimal biohydrogen production from thermomechanical pulping wastewater

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Abstract

This study evaluates the use of non-pretreated thermo-mechanical pulping (TMP) wastewater as a potential substrate for hydrogen production by dark fermentation. Batch incubations were conducted in a temperature gradient incubator at temperatures ranging from 37 to 80 °C, using an inoculum from a thermophilic, xylose-fed, hydrogen-producing fluidised bed reactor. The aim was to assess the short-term response of the microbial communities to the different temperatures with respect to both hydrogen yield and composition of the active microbial community. High throughput sequencing (MiSeq) of the reversely transcribed 16S rRNA showed that Thermoanaerobacterium sp. dominated the active microbial community at 70 °C, resulting in the highest hydrogen yield of 3.6 (± 0.1) mmol H$_2$ g$^{-1}$ COD$_{tot}$ supplied. Lower hydrogen yields were obtained at the temperature range 37 to 65 °C, likely due to consumption of the produced hydrogen by homoacetogenesis. No hydrogen production was detected at temperatures above 70 °C. TMP wastewaters are released at high temperatures (50 to 80 °C), and thus dark fermentation at 70 °C could be sustained using the heat produced by the pulp and paper plant itself without any requirement for external heating.

Keywords

Hydrogen, lignocellulose, MiSeq, pulp and paper mill, Thermoanaerobacterium, thermophilic
1. Introduction

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

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

TMP wastewater has been successfully used as a substrate for both mesophilic (Gao et al., 2016) and thermophilic (Rintala and Lepistö, 1992) methane production via anaerobic digestion. However, hydrogen (H₂) is a carbon free fuel expected to play a pivotal role in energy production in the future (Boodhun et al., 2017). Dark fermentative H₂ production has the potential for energy recovery from waste paper hydrolysate (Eker and Sarp, 2017), pulp and paper mill effluent hydrolysates (Lakshmidevi and Muthukumar, 2010) and even from untreated pulps (Nissilä et al.,...
Dark fermentative $\text{H}_2$ production has also been reported from carbohydrate-containing wastewaters, such as starch wastewater and palm oil mill effluent (Badiei et al., 2011; Xie et al., 2014). Although TMP wastewaters are characterized by a high content of carbohydrates (25 to 40% of the total COD) (Rintala and Puhakka, 1994), to our knowledge it has not yet been tested as a substrate for dark fermentation.

Thermophilic dark fermentation of TMP wastewater could be advantageous, as both biological polysaccharide hydrolysis (Elsharnouby et al., 2013) and $\text{H}_2$ yielding reactions (Verhaart et al., 2010) are favoured by high temperature. High temperature also limits the growth of homoacetogenic bacteria and methanogenic archaea (Oh et al., 2003), which may consume the produced $\text{H}_2$ in mixed culture systems. The main drawback of thermophilic processes is the energy required to heat the reactors, but TMP wastewaters are released from the pulping process at a temperature of 50 to 80 °C (Rintala and Lepistö, 1992), and could therefore be treated in thermophilic bioreactors with minimal, or even without external heating.

Temperature is a key factor in dark fermentation, as even a change of a few degrees may result in the development of a different microbial community and thus, affect the $\text{H}_2$ yield (Karadag and Puhakka, 2010). Understanding of the composition of the microbial community is also crucial in order to optimize the complex microbial $\text{H}_2$ production process, involving both hydrolytic and fermentative microorganisms (Kumar et al., 2017). Microbial communities from dark fermentation of lignocellulose-based waste and wastewaters have been previously studied at DNA level (Nissilä et al., 2012; Xie et al., 2014), but a RNA-based approach can provide more detailed information on the microorganisms that produce (and consume) $\text{H}_2$. Furthermore, the time response on RNA changes is much faster than on DNA changes (De Vrieze et al., 2016), allowing to detect the response of the microbial community to an environmental change in a relatively short time.
In a previous study, a mixed culture was successfully adapted to thermophilic (70 °C) dark fermentation of xylose in a fluidised bed reactor (FBR) and the H₂ producing *Thermoanaerobacterium* sp. accounted for > 99% of the active microbial community (Dessì et al., 2018). In this study, the same adapted mixed culture was used to test if TMP wastewater is a suitable substrate for dark fermentative H₂ production at various temperatures (37 to 80 °C), and describe how the active microbial community responds to the different temperatures.

2. Materials and methods

2.1 Source of microorganisms

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

2.2 Wastewater characterization

The wastewater was collected from a pulp and paper mill located in Finland. It was the effluent of a TMP process, in which wood was exposed to a high-temperature (120 °C) steam in order to obtain the pulp. The wastewater had a temperature of about 70 °C at the time of the sampling, but was
cooled down and stored at 4 °C to minimise biological activity that might affect its composition. The wastewater had a pH of 5.0 and a composition as given in Table 1.

Table 1 here

2.3 Temperature-gradient batch set-up

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

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

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

2.5 Analytical methods

Gas production in the tubes was quantified by a volumetric syringe method (Owen et al., 1979), and the gas composition was determined by gas chromatography-thermal conductivity detector (GC-TCD) as reported previously (Dessì et al., 2017). Acetate, butyrate, ethanol, propionate, lactate, and formate concentrations were measured with a high-performance liquid chromatograph (HPLC) equipped with a refractive index detector (RID) (Shimadzu, Japan) and a Rezex RHM-monosaccharide column (Phenomenex, USA) held at 40 °C. The mobile phase was 5 mM H₂SO₄ and the flow rate was 0.6 mL min⁻¹. Glucose and xylose concentrations were measured using a HPLC equipped with a RID and a RPM-monosaccharide column (Phenomenex, USA) held at 85 °C with Milli-Q® water at a flow rate of 0.6 mL min⁻¹ as the mobile phase. Furfural concentrations were measured by gas chromatography-mass spectrometry (GC-MS) according to Doddapaneni et al. (Doddapaneni et al., 2018). Samples for HPLC and GC-MS analysis were filtered using 0.2 µm pore size filters. Total chemical oxygen demand (CODₜₜ) and COD of the soluble compounds (CODₙ) was measured using the dichromate method according to the Finnish standard SFS 5504.
Initial and final pH of the culture and the pH of the wastewater were determined using a WTW pH 330 meter equipped with a Hamilton® Slimtrode probe (Sigma-Aldrich, USA). Total solids, volatile solids, total nitrogen and PO₄³⁻-P were determined by the APHA standard procedures (APHA, 1998).

2.6 Calculations
Cumulative H₂ and CO₂ production was calculated according to Logan et al. (2002) and corrected for temperature according to the Arrhenius equation. The theoretical CODₜot was estimated from the sum of the compounds detected by HPLC, according to the following equation (Van Haandel and Van der Lubbe, 2012):

\[
COD_{tot} = \frac{8(4x+y-2z)}{(12x+y+16z)} \text{ g COD}_{tot} \text{ g}^{-1} \text{ C}_x\text{H}_y\text{O}_z
\]

(1)

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

2.7 Statistical analysis
One-way analysis of variance (ANOVA) and the Tukey test (Box et al., 1978) at p = 0.05 were conducted using the IBM SPSS Statistics package to assess significant differences in H₂ yield after incubation at different temperatures.

3. Results
3.1 H₂ production from TMP wastewater at the various temperatures
Batch incubations with TMP wastewater resulted in a different net H₂ yield at different temperatures (Figure 1; Table 2). The highest final H₂ yield of 3.6 (± 0.1) mmol H₂ g⁻¹ CODₜot was obtained in the batch cultures at 70 °C, in which H₂ production started within 24 h of incubation and remained stable after reaching the maximum (Figure 1). The maximum H₂ yield obtained at 65 °C
was comparable to the one obtained at 70 ºC, but the produced H₂ started to be consumed within 36 h resulting in a 51% lower final yield (Figure 1; Table 2). In the batch cultures at temperatures lower than 70 ºC, the H₂ produced was always partially (at 37, 42, 59 and 65 ºC) or totally (at 48 and 55 ºC) consumed. A negligible H₂ production was obtained at both 74 and 80 ºC (Figure 1), as well as in the negative controls (see Additional file 1).

Figure 1 here

Table 2 here

3.2 COD\textsubscript{tot} removal and metabolite production at the various temperatures

Similarly to H₂ production yields, dark fermentation of TMP wastewater at the various temperatures resulted in a different composition of the liquid phase (Figure 2). Acetate was the most abundant metabolite detected in the temperature range 37 to 70 ºC. The final acetate concentration increased with temperature from 0.34 (± 0.04) g COD\textsubscript{tot} L\textsuperscript{-1} at 37 ºC to 0.75 (± 0.18) g COD\textsubscript{tot} L\textsuperscript{-1} at 55 ºC, and then decreased stepwise to 0.07 (± 0.00) and 0.08 (± 0.01) g COD\textsubscript{tot} L\textsuperscript{-1} at 74 and 80 ºC, respectively (Figure 2). Butyrate was found regardless of the incubation temperature, with a final concentration ranging from 0.06 (± 0.00) g COD\textsubscript{tot} L\textsuperscript{-1} at 70 ºC to 0.19 (± 0.00) g COD\textsubscript{tot} L\textsuperscript{-1} at 59 ºC. Ethanol was produced at 37, 42, 59, 65 and 70 ºC, with a maximum of 0.14 (± 0.02) g COD\textsubscript{tot} L\textsuperscript{-1} at 65 ºC (Figure 2). Dark fermentation of TMP wastewater caused a pH decrease from the initial 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 ºC, but was only 5.5 (± 0.1) after incubation at 37 ºC, 5.2 (± 0.1) at 65 ºC and 5.3 (± 0.0) at 70 ºC (Figure 2).

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

Table 3 here

3.3 Effect of temperature on the active microbial community

Incubation temperature clearly impacted the composition of the active microbial communities growing for 111 h on TMP wastewater (Figure 3, Table 4). At 37 °C, Clostridium sp. accounted for 84 and 90% of the attached and suspended active microbial community, respectively. Higher temperature resulted in a gradual decrease of the relative abundance of Clostridium sp., being 54% of the attached active microbial community and < 2% of the suspended active microbial community after incubation at 55 °C (Figure 3). Clostridium sp. was not detected either in the attached or suspended active community after incubation at temperatures ≥ 59 °C (Figure 3). A bacterium belonging to the order of Bacillales closely related to B. coagulans (Table 4) was detected in the active attached and suspended microbial communities after incubation at 42 °C, with a relative abundance of 14 and 10%, respectively, and only in suspended form after incubation at 48 °C, with a relative abundance of 50% (Figure 3).
The relative abundance of *Thermoanaerobacterium* sp. (99% similarity to *T. thermosaccharolyticum*) among the attached active microorganisms gradually increased with temperature, being only 2% after incubation at 37 °C and 87% at 59 °C (Figure 3, Table 4). *Thermoanaerobacterium* sp. was also the most common suspended active microorganism after incubation at 55 and 59 °C, with a relative abundance of 96 and 83%, respectively. After incubation at 65 °C, the relative abundance of *Thermoanaerobacterium* sp. in the attached and suspended active microbial community decreased to 57 and 25%, respectively, whereas unclassified *Firmicutes*, with 92% similarity to *Calditerricola* sp. (Table 4) were found with a relative abundance of 30 and 28%, respectively. After incubation at 70 °C, *Thermoanaerobacterium* sp. was again the dominant active microorganism in both attached and suspended form, with a relative abundance of 88 to 89%. After incubation at 59 and 70 °C, *Caldanaerobius* sp. was also found in both attached and suspended form with relative abundance > 10% (Figure 3). After incubation at both 74 and 80 °C, the RNA concentration was not high enough to perform the analysis due to poor microbial growth, and thus microbial communities from 74 and 80 °C could not be analysed.

4. Discussion

4.1 Fermentation of TMP wastewater at different temperatures

H₂ production from TMP wastewater inoculated with biofilm-coated activated carbon granules was observed at a wide temperature range of 37 to 70 °C (Figure 1). The highest final H₂ yield of 3.6 (± 0.1) mmol H₂ g⁻¹ CODₜot supplied, or 4.9 mmol H₂ g⁻¹ CODₜot consumed, was obtained at 70 °C (Table 2), which could be expected as the inoculum was collected from an FBR operated at 70 °C.
Such H\textsubscript{2} yield is of the same order of magnitude compared to previous studies on thermophilic direct dark fermentation of industrial, sugar-containing wastewaters. Xie et al. (2014) obtained 5.8 mmol H\textsubscript{2} g\textsuperscript{-1} COD\textsubscript{tot} from starch wastewater at 55°C by a mixed culture dominated by \textit{T. thermosaccharolyticum}, whereas Khongkliang et al. (2017) obtained 11.4 mmol H\textsubscript{2} g\textsuperscript{-1} COD\textsubscript{tot} from starch wastewater by a pure \textit{T. thermosaccharolyticum} culture.

The thermophilic active mixed microbial community previously enriched on xylose in the FBR was dominated by microorganisms closely related to \textit{Thermoanaerobacterium thermosaccharolyticum} (Dessì et al., 2018). Changing of the substrate from xylose to TMP wastewater marginally impacted the active microbial community in the temperature range 59 to 70 °C, as most of the sequences obtained from the RNA samples matched \textit{T. thermosaccharolyticum} (Table 4). A mixed culture dominated by \textit{T. thermosaccharolyticum} has been shown to produce 7 mmol H\textsubscript{2} g\textsuperscript{-1} cellulose at 70 °C (Gadow et al., 2013), showing potential for the one-step conversion of lignocellulosic materials to H\textsubscript{2}, avoiding a costly hydrolysis step. In fact, the genus \textit{Thermoanaerobacterium} includes strains of cellulolytic microorganisms, such as some strains of \textit{T. thermosaccharolyticum}, able to hydrolyse both cellulose and hemicellulose, and produce H\textsubscript{2} from the resulting monosaccharides (Cao et al., 2014). In this study, however, the microbial community analysis conducted at genus level does not allow to assess possible cellulolytic capabilities of the detected \textit{Thermoanaerobacterium} sp.

Although the inoculum was enriched for dark fermentation at 70 °C, H\textsubscript{2} production occurred only after 24 h of incubation (Figure 1). This is probably due to the handling of the inoculum, which was stored at 4 °C for one week prior to being used for this experiment. Changes in gene expression and DNA replication were shown to occur in \textit{Thermoanaerobacter tengcongensis} as response to a cold shock (Liu et al., 2014), as could be the case for the \textit{Thermoanaerobacterium} sp. dominating the active microbial community of the inoculum used in this study. Although \textit{Thermoanaerobacterium} sp. was the most abundant microorganism (relative abundance close to 90%) in both the attached
and suspended microbial community at both 59 and 70 °C, its relative abundance was lower at 65 °C (Figure 3). The same phenomenon was observed in the FBR from where the inoculum originated (Dessì et al., 2018), and attributed to either the decreased activity of *Thermoanaerobacterium* sp. or to the increased activity of competing microorganisms at 65 °C.

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

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

In this study, no H₂ was produced at 74 or 80 °C (Figure 1) and the RNA concentration was too low to allow sequencing analysis, suggesting a lack of active species. This was attributed to the source
of inoculum used, as species within the *Thermoanaerobacterium* genus, such as *T. thermosaccharolyticum*, may be inhibited by temperatures higher than 70 °C (Ren et al., 2008). Gadow et al. (2013) obtained H₂ production from cellulose by a mixed microflora from a sewage sludge digester even at 75 and 80 °C. However, H₂ production at such high temperatures was attributed to *Thermoanaerobacter tengcongensis* (Gadow et al., 2013), which was not part of the active microbial community in this study. Some degradation products of hemicellulose such as furfural or hydroxymethylfurfural may inhibit fermentative microorganisms (Jönsson et al., 2013), including *Thermoanaerobacterium*, at a concentration over 1 g L⁻¹ (Cao et al., 2010). However, the TMP process is conducted at temperatures below 120 °C, which is too low to produce such high concentrations of these inhibitory compounds (Baêta et al., 2017). In fact, the concentration of furfural in the TMP wastewater used in this study was below the detection limit of the GC-MS (Table 1).

A decrease in the cumulative H₂ production occurred in all the incubations at temperatures lower than 70 °C (Figure 1), probably due to the activity of homoacetogenic bacteria. Homoacetogenesis, in which 4 moles of H₂ and 2 mol of CO₂ are consumed per mol of acetate produced, often occurs in batch H₂ production experiments within the first 80 h of incubation, especially under mesophilic conditions (for a review, see Saady, 2013). However, in this study, H₂ seems to be consumed faster under thermophilic (from 48 to 65 °C) as compared to mesophilic (37 °C) conditions (Figure 1), suggesting that homoacetogenic microorganisms were mainly thermophiles or moderate thermophiles. The CO₂ concentration in the batch incubations did not decrease as expected in case of homoacetogenesis (see Additional file 3). However, this could be explained considering that CO₂ production may occur also through non-hydrogenic pathways, mainly the ethanol production pathway (Figure 2). In the abiotic negative control, CO₂ was also detected, together with acetate, at both 55 and 70 °C, where H₂ production was not observed (see Additional file 1), suggesting that
other non-hydrogenic, CO$_2$ producing pathways other than ethanol production could have occurred as well.

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

### 4.2 COD$_{\text{tot}}$ balance and metabolite production

The COD$_{\text{tot}}$ measured in the beginning of the incubations (Table 3) was 15% lower than the value obtained while characterizing the TMP wastewater (Table 1). Apparently, some biological or non-biological reaction occurred while storing the TMP wastewater at 4 °C before the experiment, resulting in a slight COD$_{\text{tot}}$ concentration decrease. The COD$_{\text{tot}}$ removal efficiency during the incubations was 69 to 80% regardless the incubation temperature (Table 3). It is in line with the COD$_{\text{tot}}$ removal from anaerobic digestion of pulp and paper wastewater reported in the literature (Meyer and Edwards, 2014), but higher than expected for dark fermentation which usually removes only 30 to 40% of the COD$_{\text{tot}}$ (Sharma and Li, 2010). This was due to the adsorption of VFAs on the activated carbon (see Additional file 2), which caused an overestimation of the COD$_{\text{tot}}$ removal. However, it should be noted that the adsorption experiment (see Additional file 2) was performed with fresh activated carbon, whereas the main experiment was conducted with biofilm-covered activated carbon. The latter could have been partially saturated with VFAs at the moment of
inoculation, as VFAs were produced in the FBR from where the inoculum originated (Dessì et al., 2018).

In the temperature range 42 to 65 °C, more than 85% of the residual COD$_{\text{tot}}$ was detected as acetate, butyrate or ethanol by HPLC analysis (Table 3). However, 30 to 37% of the residual COD$_{\text{tot}}$ was not detected as compounds identified by HPLC analysis after incubation at 37, 70 and 80 °C, and even 58% of the residual COD$_{\text{tot}}$ was not identified after incubation at 74 °C. At 74 and 80 °C, most of the undetected COD$_{\text{tot}}$ is likely constituted by polysaccharides such as cellulose, which were not degraded due to the lack of bacterial activity at such high temperatures. At 74 and 80 °C, CO$_2$ was also not produced (see Additional file 3), supporting this conclusion. Lignocellulosic materials can release VFAs at temperatures around 80 °C (Veluchamy and Kalamdhad, 2017), suggesting that the acetate and butyrate detected at 74 and 80 °C (Figure 2) were produced physically rather than biologically.

The simultaneous production of acetate and butyrate suggests that H$_2$ was produced via both the acetate and butyrate pathway in the temperature range 37 to 70 °C. Acetate was the main metabolite found in the liquid phase at all temperatures tested, excluding 74 and 80 °C (Figure 2), and was associated either to H$_2$ production through the acetate dark fermentative pathway or H$_2$ consumption by homoacetogenesis. Interestingly, acetate production increased with temperature in the range of 37 to 55 °C, and then decreased stepwise for temperatures above 55 °C (Figure 2). In particular, the high (> 0.7 g COD$_{\text{tot}}$ L$^{-1}$) acetate (Figure 2) and concomitant low (< 0.5 mmol g$^{-1}$ COD$_{\text{tot}}$) cumulative H$_2$ yield (Figure 1) suggest that the optimum growth temperature for homoacetogenic bacteria was about 55 °C in this study. At 70 °C, however, the H$_2$ produced was not consumed during the incubation (Figure 1), suggesting inhibition of homoacetogenic microorganisms.
Solventogenesis occurred both in mesophilic (37 and 42 °C) and thermophilic (59, 65, and 70 °C) batch cultures, resulting in ethanol production (Figure 2). *Clostridium* sp., which dominated the active microbial communities under mesophilic conditions (Figure 3), may shift its metabolism from acidogenesis to solventogenesis as response to a change of pH or volatile fatty acids concentration, but the mechanism which triggers solventogenesis is not well understood (Kumar et al., 2013). A pure culture of *T. thermosaccharolyticum* has been reported to produce ethanol together with acetate and butyrate by dark fermentation of cellulose and complex lignocellulosic substrates such as corn cob, corn straw and wheat straw (Cao et al., 2014). Similarly, in this study, acetate, butyrate and ethanol were the main metabolites (Figure 2) of the dark fermentation of TMP wastewater at 65 and 70 °C by a mixed culture dominated by *T. thermosaccharolyticum* (Figure 3; Table 4).

### 4.3 Practical implications

Hydraulic retention times lower than 24 hours are typically used for dark fermentation of wastewater (Lin et al., 2012). Therefore, based on the results obtained (Figure 1), dark fermentation of TMP wastewater at 37 and 65 °C appears favourable if suspended biomass bioreactors are used, as homoacetogenic bacteria would be flushed out (Figure 1). However, due to the high dilution of TMP wastewater, bioreactors retaining high active biomass content, such as FBRs or upflow anaerobic sludge bioreactors (UASBs), would enable higher organic loading and conversion rates than suspended biomass bioreactors (Koskinen et al., 2006). Therefore, dark fermentation of TMP in attached biomass bioreactors at 70 °C is recommended (Figure 1). A proper insulation and temperature control are nevertheless necessary to keep accurately 70 °C in the bioreactor, as a decrease of 5 °C may already result in a decreased efficiency due to H₂ consumption by homoacetogenic bacteria. However, H₂ production at 70 °C can be quickly restored in case of failure of the temperature control. In fact, H₂ production was detected at 70 °C within only 24 h (Figure 1) with a thermophilic inoculum previously stored at 4 °C for one week.
Despite the surprisingly high COD\textsubscript{tot} removal efficiency of 69 to 80% obtained in this study (Table 3), dark fermentation of TMP wastewater resulted in the generation of an effluent containing 0.5 – 1.0 g COD\textsubscript{tot} L\textsuperscript{-1} (Table 3), mainly in the form of VFAs, thus requiring further treatment prior to be discharged. Such effluent can be either treated by a traditional activated sludge plant, or further valorised by producing energy or high value chemicals. Promising strategies for the valorisation of dark fermentation effluents include further H\textsubscript{2} production by photofermentation or microbial electrolysis cells, methane production by anaerobic digestion, and bioplastics or electricity production using microbial fuel cells (for reviews, see Ghimire et al., 2015 and Bundhoo, 2017).

5. Conclusions

Hydrogen was produced by dark fermentation from TMP wastewater at a wide range of temperatures (37 to 70 °C) using a mixed microbial community enriched on xylose at thermophilic conditions. An operation temperature of 70 °C was the most favourable for dark fermentative H\textsubscript{2} production and effectively repressed the activity of homoacetogenic bacteria. Therefore, considering that TMP wastewater is produced at elevated temperature, dark fermentation at 70 °C may be a cost-effective approach for the treatment and valorisation of this wastewater. Temperature must be efficiently controlled, as a shift of only a few degrees may decrease the H\textsubscript{2} yield.

Acknowledgements

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Figures

Figure 1 – Hydrogen 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.
Figure 2 – Composition and pH of the liquid phase after 111 h of 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.
Figure 3 – Relative abundance of the active genera resulting from MiSeq sequencing of the partial 16S rRNA (transcribed to 16S cDNA) on microbiological samples obtained from the biofilm-containing activated carbon (attached) and from the liquid medium (suspended) after batch incubation with thermomechanical pulping wastewater at various temperatures (37 to 70 °C). The microbial genera are listed in order of relative abundance. Samples at 74 and 80 °C could not be analysed due to the low RNA concentration present in the samples.
Table 1 - Composition of the thermomechanical pulping wastewater used in this study

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Concentration (mg L$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total solids</td>
<td>3771 ± 10</td>
</tr>
<tr>
<td>Volatile solids</td>
<td>2452 ± 8</td>
</tr>
<tr>
<td>Total COD</td>
<td>3352 ± 82</td>
</tr>
<tr>
<td>Soluble COD</td>
<td>3289 ± 54</td>
</tr>
<tr>
<td>Total nitrogen</td>
<td>&lt; 10</td>
</tr>
<tr>
<td>Total PO$_4^{3-}$-P</td>
<td>2.8</td>
</tr>
<tr>
<td>Acetate</td>
<td>&lt; 30</td>
</tr>
<tr>
<td>Furfural</td>
<td>&lt; 10</td>
</tr>
<tr>
<td>Glucose</td>
<td>43 (± 2)</td>
</tr>
<tr>
<td>Xylose</td>
<td>38 (± 0)</td>
</tr>
</tbody>
</table>
Table 2 - Maximum and final hydrogen yield obtained from batch incubation of thermomechanical pulping wastewater at various temperatures (37 to 80 °C) using thermophilic biofilm-containing activated carbon as inoculum

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>H$_2$ yield (mmol H$<em>2$ g$^{-1}$ COD$</em>{tot}$ supplied)</th>
<th>H$_2$ yield (mmol H$<em>2$ g$^{-1}$ COD$</em>{tot}$ consumed)</th>
<th>Lag time$^a$ (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Maximum Final</td>
<td>Final</td>
<td></td>
</tr>
<tr>
<td>37</td>
<td>3.2 (± 0.1) 1.4 (± 0.1)</td>
<td>1.9 (± 0.2)</td>
<td>23</td>
</tr>
<tr>
<td>42$^b$</td>
<td>1.5</td>
<td>0.6</td>
<td>63</td>
</tr>
<tr>
<td>48</td>
<td>0.6 (± 0.1) 0.1 (± 0.0)</td>
<td>0.1 (± 0.0)</td>
<td>18</td>
</tr>
<tr>
<td>55</td>
<td>0.4 (± 0.1) 0.0 (± 0.0)</td>
<td>0.0 (± 0.0)</td>
<td>12</td>
</tr>
<tr>
<td>59</td>
<td>1.7 (± 0.8) 0.6 (± 0.3)</td>
<td>0.9 (± 0.5)</td>
<td>18</td>
</tr>
<tr>
<td>65</td>
<td>3.7 (± 0.4) 1.8 (± 0.2)</td>
<td>2.6 (± 0.3)</td>
<td>36</td>
</tr>
<tr>
<td>70</td>
<td>3.6 (± 0.1) 3.6 (± 0.1)</td>
<td>4.9 (± 0.4)</td>
<td>90</td>
</tr>
<tr>
<td>74</td>
<td>0.1 (± 0.0) 0.1 (± 0.0)</td>
<td>0.2 (± 0.0)</td>
<td>n.a.$^c$</td>
</tr>
<tr>
<td>80</td>
<td>0.0 (± 0.0) 0.0 (± 0.0)</td>
<td>0.0 (± 0.0)</td>
<td>n.a.</td>
</tr>
</tbody>
</table>

$^a$ Time required to reach the maximum H$_2$ yield;

$^b$ H$_2$ was produced only in one of the duplicate tubes;

$^c$ Not applicable.
Table 3 - COD$_{tot}$ balances after incubation of thermomechanical pulping wastewater at various temperatures (37 to 80 °C) using thermophilic biofilm-containing activated carbon as inoculum

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

$^a$ Data obtained by measurement according to the standard procedure; the initial COD$_{tot}$ was 2.86 g L$^{-1}$;

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

$^c$ Calculated from COD$_{tot}$ measured.
Table 4 - Association of the six most abundant 16S rRNA gene sequences to species collected in the GenBank

<table>
<thead>
<tr>
<th>Family</th>
<th>Genus and species*</th>
<th>Accession number</th>
<th>Matching sequenceb</th>
<th>Similarity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thermoanaerobacteraceae</td>
<td>Thermoanaerobacterium thermosaccharolyticum</td>
<td>JX984971</td>
<td>474-765</td>
<td>99</td>
</tr>
<tr>
<td>Clostridiaceae</td>
<td>Clostridium sp.</td>
<td>AY548785</td>
<td>450-741</td>
<td>99</td>
</tr>
<tr>
<td>Bacillaceae</td>
<td>Bacillus coagulans</td>
<td>MF373392</td>
<td>512-803</td>
<td>100</td>
</tr>
<tr>
<td>Bacillaceae</td>
<td>Calditerricola yamamurae</td>
<td>NR_112684</td>
<td>529-820</td>
<td>92</td>
</tr>
<tr>
<td>Thermoanaerobacteraceae</td>
<td>Caldanaerobius sp.</td>
<td>LC127102</td>
<td>482-773</td>
<td>99</td>
</tr>
<tr>
<td>Thermoanaerobacteraceae</td>
<td>Moorella thermoacetica</td>
<td>CP017237</td>
<td>145404-145695</td>
<td>100</td>
</tr>
</tbody>
</table>

* Closest cultured species in GenBank;

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

c Percentage of identical nucleotide pairs between the 16S rRNA gene sequence and the closest cultured 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.
COMPOSITION AND ROLE OF THE ATTACHED AND PLANKTONIC MICROBIAL COMMUNITIES IN MESOPHILIC AND THERMOPHILIC XYLOSE-FED MICROBIAL FUEL CELLS

by

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

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

Paolo Dessi, a, b Estefania Porca, a Johanna Haavisto, a Aino-Maija Lakaniami, b and Piet N. L. Lens b 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.

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.7 Some species, such as Pseudomonas, produce mediators such as pyocyanin endogenously.8 Once mediators are produced, also other microorganisms present in the mixed culture system can use them to transfer the electrons to the anode.9

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...
wastewater treatment, as they contain a consortium of hydrolytic, fermentative and electroactive microorganisms able to produce electricity from complex substrates. However, due to the competition for electron donor with non-exoelectrogenic microorganisms such as methanogenic archaea, power production can remain low, and operational conditions must be optimized to favor exoelectrogenic microorganisms. Catal et al. compared electricity production from 12 mono-saccharides 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. MFCs have been operated at temperatures up to 98 °C. 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. To date, only few species have been reported to produce electricity at thermophilic conditions, including Firmicutes such as Caloramator australicus, Thermiscina potens, Thermiscina ferricatetica, and Thermoaerobacter pseudethanolicus, as well as Deferrirabacteres such as Calditerrivibrio nitroreducens.

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. Previously performed microbial community analyses have, nevertheless, mainly targeted the presence of the 16S rRNA gene (DNA) whereas, to our knowledge, only one study has also focused on 16S rRNA gene expression (RNA), which is an indicator of the microbial activity. 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 anode 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, only Lu et al. 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. 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. The second one was anolyte from a xylose-fed MFC operating at 37 °C. 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., 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 anolye (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 precipitation instead of polyethelene glycol and nucleic acids were re-suspended in sterile water instead of tris-EDTA buffer.

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 multiparameter 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.,²⁰ accounting 20 mol electrons exchanged per mol of xylose, according to the following equation:

\[
C_6H_{12}O_6 + 5H_2O \rightarrow 5CO_2 + 20H^+ + 20e^-
\]  

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 kΩ to 5 Ω at 30 minute intervals. Voltage was recorded just before switching the resistance. Power density and current density were calculated as \( P = \frac{U^2}{(R \cdot V)} \) and \( I = \frac{U}{(R \cdot V)} \), 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.,²⁻ 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 μL turbo DNase and 2.5 μL 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 μL DNase inactivator (Invitrogen) and separated from the RNA containing liquid by centrifugation (1 000 × 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 μL) 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, chimera 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

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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 numbers 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).
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
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. 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. 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. For example, Mei et al. 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. 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%.
(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. 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.\(^{46}\) 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.\(^{45}\) However, a planktonic subpopulation, but instead 6\(^{44}\) families were previously detected both in the anode-attached and planktonic subpopulations in the mesophilic MFC, and even shown to produce electricity in the absence of oxygen.\(^{30}\) 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 \(\times 10^{-6}\) cm\(^2\) s\(^{-1}\),\(^{31}\) 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.\(^{44}\) 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.\(^{25}\)

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Sample type</th>
<th>Microbial community</th>
<th>No. of sequences(^a)</th>
<th>No. of families</th>
<th>Shannon diversity</th>
<th>Simpson diversity</th>
<th>J’ evenness</th>
</tr>
</thead>
<tbody>
<tr>
<td>37</td>
<td>DNA</td>
<td>Anode-attached</td>
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<td>3.22</td>
<td>0.94</td>
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<td>2.95</td>
<td>0.92</td>
<td>0.68</td>
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<tr>
<td></td>
<td></td>
<td>Membrane-attached</td>
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<td>798</td>
<td>3.72</td>
<td>0.97</td>
<td>0.83</td>
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<tr>
<td></td>
<td>cDNA</td>
<td>Anode-attached</td>
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<td>161</td>
<td>1.45</td>
<td>0.55</td>
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<td></td>
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<td>Anode-attached</td>
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<td>72</td>
<td>2.06</td>
<td>0.80</td>
<td>0.56</td>
</tr>
</tbody>
</table>

\(^{a}\) 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.

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. About 66% of *Firmicutes* found in the thermophilic anode-attached community belonged to the family *Thermodesulfobiales*, 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.*]. The activity of *Coprothermobacter* is enhanced by establishing a syntrophy with hydrogenotrophic methanogenic archaea such as *Methanothermobacter*. *Methanothermobacter* belongs to the family of *Methanobacteriales*, 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, 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 *Chlororibiales*, despite being mainly phototrophs, can perform heterotrophic anaerobic respiration, and have been reported as part of the anodic biofilm in MFCs. *Anaerolineaceae*, also found among the thermophilic anode-attached microbial community, is a family of filamentous bacteria involved in the fermentation of various sugars. 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.

The lower power production in the thermophilic MFC is likely due to the lack of effective exoelectricans and to the consequent high activity of non-exoelectricans microorganisms, which consumed part of the electrons through pathways competitive to electricity generation. In fact, the methanogenic archaeal family of *Methanobacteriales*, 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. Therefore, *Methanobacteriales* cannot compete for the substrate with exoelectricans microorganisms, but their metabolism decreases the availability of electrons for electricity production. *Methanobacteriales* 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. Rismani-Yazdi *et al.* reported methane production by *Methanobacteriales* in a mesophilic (39 °C) cellulose-fed MFC only at the beginning of the operation, whereas Hussain *et al.* reported *Methanobacteriales* 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 *Hydrogenophilus* sp., which could have consumed a share of electrons by H2 oxidation, lowering power production in the thermophilic MFC. *Thermodesulfobiales*, found among the anode-attached families, were also found among the planktonic community (Fig. 5). *Coprothermobacter* is able to perform extracellular electron transfer, 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.

### 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. 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 May 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 exoelectrican 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. 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.,65 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 Logan66 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 bromoethane-sulphonic 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 Thermodesulfojobiaceae 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.

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References

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

Supporting information

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Figure S1. Examples of chromatograms obtained with the HPLC when performing the substrate degradation test (Figure 2 in the manuscript).